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THE ISOLATION OF PREY IDENTIFICATION SUBSTANCES THAT ARE UTILIZED BY GARTER SNAKES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Scot E Goss

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DEDICATION

The author wishes to dedicate this work to his wife, Ruth, for her love, encouragement, and help during his graduate studies.

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ABSTRACT

Garter snakes (Thamnophis sirtalis) identify food sources by chemical compounds obtained from prey. Analysis of garter snake prey chemicals was conducted on earthworms (Lumbricus terrestris), fathead minnows (Pimephales promelas), and mosquito fish (Gambusia affina) in order to isolate and analyze these chemical substances. The substances were isolated from the prey by a variety of extraction techniques and chromatography methods in conjunction with a snake bioassay procedure. The snake bioassay procedure consisted of presenting prey derived chemicals to garter snakes on cotton swabs and observing tongue flicking and attacks. Sugar analysis was conducted by gas chromatography and gas chromatography mass spectrometry. Proton and carbon nuclear magnetic resonance, amino acid analysis, and molecular weight estimates were also implemented to analyze these chemical substances.

There were numerous similarities among the different substances isolated from each prey. The carbon and proton nuclear magnetic resonance spectra obtained from the isolated substances of the earthworm and fathead minnow resulted in very similar spectra. The earthworm and fathead minnow substances also resulted in similar sugar and amino acid contents with approximately the same molecular weight components. It was concluded that the active substances isolated from the fathead minnow and the earthworm had very similar chemical and physical properties even though the substances were isolated from different phyla found in different environments.

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LIST OF CODES FOR ISOLATED ANIMAL FRACTIONS

Code	Description
AQ	Aqueous prey extract
AQd	Dialyzable portion of aqueous prey extract
AQm_{insol}	Materials insoluble in the methanol extracts from the aqueous prey extract
AQm_{sol}	Materials soluble in the methanol extracts from the aqueous prey extract
$AQmc_{insol}$	Material insoluble in the methanol:chloroform extracts from the aqueous prey extract
$AQmc_{sol}$	Material soluble in the methanol:chloroform extracts from the aqueous prey extracts
AQnd	Nondialyzable portion of aqueous prey extract
AQs_1 or AQs_2	First or second soxhlet extraction from the aqueous prey extract
AQs_r	Materials soluble in methanol which were refluxed with the aqueous prey extract
CM	Chloroform:methanol extraction of the prey
CMo	Organic layer obtained from the chloroform:methanol extraction of the prey
CMw	Water layer obtained from the chloroform:methanol extraction of the prey

LIST OF CODES (continued)

Code	Description
CUT_{a1} or CUT_{a2}	First or second acid extraction from the earthworm cuticle
CUT_{cd}	Materials obtained from collagenase degregation of earthworm cuticles
CUT_{s1} or CUT_{s2}	First or second salt extraction from the earthworm cuticles
EW	Obtained from earthworm
FM	Obtained from fathead minnow
HMW_{P10}	High molecular weight fraction from P-10 column
LMW_{P10}	Low molecular weight fraction from P-10 column
MF	Obtained from mosquito fish
PC	Purified collagen
$Silica_1$ or $Silica_2$	First or second active fraction obtained from silica gel chromatography
void vol_{A25}	Materials obtained from void volume of A25 column

CHAPTER I

INTRODUCTION

A. CHEMICAL SIGNALS FOUND IN NATURE

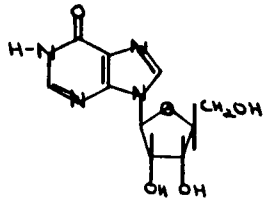
Communication is well known and utilized by society today. Radio, television, and literature are all forms of communication between different people all over the world. Body language, even though it is a nonverbal form, is considered as a major form of communication.

Communication can also take the form of chemical cues. The importance of chemical cues as a means of communication is well known in the insect and animal kingdoms. Most of the research in chemical communication of insects, has been conducted in the area of sex attractants[1,2,3].

Considerable research has been conducted on chemical signals in the animal kingdom. The compound 3-methylbutyl methyl sulfide is believed to be used as a chemical marker by the red fox[4] and is found in its urine. The fox has been observed marking spoiled food sources thus warning other foxes that the food is nonpalatable. It also has been noted that the concentration of this compound in the urine increases during the fox's mating season, and it is believed that the compound could be associated with mating behavior.

Chemical signals have been also studied in relation to finding and identifying food. For example research has been conducted in identifying feeding stimulants for different types of fish. A feeding stimulant is a compound found on a food item that allows the animal to identify it as food. The presence of these compounds on unfamiliar items can initiate feeding behavior on that item since

the animal correlates the feeding stimulant to a potential food source. Inosine, shown below, is a feeding stimulant for the rainbow trout[5]. Likewise, betaine is a feeding stimulant for the pigfish[6].



Inosine

$(CH_3)_2$-N-CH_2COOH

Betaine

B. CHEMICAL SIGNALS FOUND IN SNAKE BEHAVIOR

Snakes also use chemical stimuli in their environment. King snakes prey on other snakes, and it has been shown that crotalines can recognize the odor of king snakes[7,8,9]. When a crotaline is presented with skin extracts of a king snake, it assumes a body bridging position in which the medial portion of the snake is arched from the substrate. This body bridging is assumed to be a defensive behavior for the crotaline snake. Garter snakes also recognize the scent of predator snakes[10].

Snakes use their tongue-vomeronasal organ system to locate and identify different items in their environment[10,11,12,13]. If an item that is of interest is in close proximity to the snake, the snake tries to identify and locate it by tongue

flicking. Presumably the tongue collects identifying compounds. When a garter snake is exposed to extracts of a king snake, the tongue flicking rate for the garter snake increases[10]; apparently in an attempt to locate and identify the snake for self-preservation.

C. GARTER SNAKE PREY RECOGNITION

Garter snakes can identify their prey by chemical stimuli[12,13,14,15]. Extracts can be made of garter snake prey by placing the prey for two minutes in 60°C water[12]. The resulting solution is centrifuged. The supernatent is the aqueous extract of the prey. When the extract is presented to a garter snake on a cotton swab, there is an increase in tongue flicking and possibly an attack at the swab that may be followed by an attempt to ingest it. Swabs soaked in distilled water or nonprey extracts do not elicit the same response. Since the swab is a different color and form than the prey, one would therefore assume the snake identifies the swab by chemical and not visual cues.

Burghardt[14,16] conducted research with naive newborn garter snakes (Thamnophis sirtalis) and other species that had never been fed. The advantages of using naive snakes for testing is that they have not been conditioned by their prior diet. The snake's knowledge of food sources is thus innate. Burghardt also studied other species that had different feeding habits. For example, some species feed primarily on fish, and others on earthworms, whereas other snakes feed on both. The innate responses of the newborn snakes to chemical cues followed these differences in food habits. He also found differences in chemical prey preferences of the same species of garter snakes from different geographic

locations[17]. Even in garter snakes from the same litter, some may show reliable preferences to earthworm over fish, and some have the opposite preference[18,19].

Burghardt developed a bioassay procedure to monitor the newborn snakes' responsiveness to different prey stimuli and compounds[15] based on the much earlier work of Wilde[20]. In this bioassay procedure, for example each snake might be presented, for thirty seconds, with cotton swabs soaked in a test solution. During each testing period, the amount of tongue flicking that the snake exhibits toward and away from the swab is recorded. The score for each sample tested is based on the tongue flicking rate unless an attack occurs, in which case the score is based on the following equation:

$$\text{Score} = \text{Base Score} + (30 - \text{Latency}). \qquad (1\text{-}1)$$

The base score is the maximum number of tongue flicks recorded from any snakes to any of the stimuli tested during the bioassay. The latency is the time, in seconds, required for a strike to occur in the thirty second test. The data obtained for each compound tested is compared to the test run of the cotton swabs soaked in water and of an extract of the prey item which was also included in the same test.

Reformato, Kirschenbaum, and Halpern has also developed a bioassay procedure to monitor the snakes response effectiveness to different stimuli[21]. In this bioassay procedure, the snake is presented with two coplin jar covers. One jar cover contains water and the other contains the specific compound to be studied. The tongue flicking that occurs to each jar cover is then recorded . The tongue flicking for the compound is compared to a test done with the aqueous prey extract versus water to determine the activity of that compound.

Halpern's bioassay method differs from Burghardt's method in that Halpern's bioassays only uses tongue flick data while Burghardt's uses tongue flick and attack data. This additional attack information utilized in Burghardt's bioassays may lead to different results concerning activity.

D. VOMERONASAL SYSTEM FOR CHEMICAL CUE RECOGNITION

The vomeronasal organ is a sensory organ, connected to the nasal cavity, that opens to the anterior roof of the mouth. Since the taste buds of the snake are located at the back of the snake's mouth and none are found on the tongue, it can be surmised that gustation via the tongue flicking is not a form of chemical sensing for identifying prey[22].

Scientists have determined that the tongue-vomeronasal system is the primary system that allowing garter snakes to identify their prey. Gillingham and Clark have suggested that pads are located on the floor of the snake's mouth. These pads may serve to push chemical stimulants on the tips of the snake's tongue into the vomeronasal organ[23,24]. Halpern and Kubie also determined that the tips of the snake's tongue delivers materials to the vomernasal organ by tracing radioactive H^3-proline from the tongue to the vomeronasal organ of the snake[25].

Research conducted by Burghardt and Hess also concluded that the vomeronasal system is the primary organ responsible for identifying prey by garter snakes[22]. Four groups of five snakes were tested for their discrimination ability between water and an earthworm extract. Olfaction and vision were eliminated in some of the snakes by painting a black collodion solution over the

eyes and nostrils respectively. This collodion layer could be removed with forceps after softening the material with a dilute aqueous ethanol solution[22]. In one group the olfaction of the snakes was eliminated. In another group, the vision of the snakes was eliminated. In the third group, vision and olfaction were eliminated, and the fourth served as the control group.

On day one, all the snakes were tested. On day two, the procedure was repeated except that the collodion layers were removed from the first three groups of snakes. The result of the experiment was that there were no significant differences among the four groups. It was concluded that vision was not a critical cue for eliciting prey attack response; olfaction was also eliminated as not being essential in identifying prey, but Burghardt and Hess believed that it might serve as a distant receptor to alert the snake to the possible presence of food.

Another test to support the theory that the vomeronasal organ is the primary organ for chemical sensing was done by Burghardt and Denny[13]. In this experiment, two garter snakes (Thamnophis sirtalis) were tested using moving earthworm sections. One of the snakes had its tongue surgically removed while the other served as the control. The control snake, when presented with earthworm sections, could readily locate the prey item by tongue flicking. When the tongueless snake was presented with the prey, the feeding strategy of the snake changed. Instead of tongue flicking to locate the prey, the snake, in a frantic manner, tried to randomly cover the most territory with its mouth open in an attempt to contact the prey item. The tongueless snake was alerted to the presence of the prey without tongue flicking but was unable to locate it even though it was clearly visible. It is believed that olfaction alerted the snake to the

presence of the prey[13]. Once the snake was stimulated, the tongue-vomeronasal system is normally utilized by the snake to collect airborne and surface material to locate food items. In the case of the tongueless snake, the vomeronasal organ was stimulated but the prey could not be easily located because of the absence of the tongue. Burghardt and Pruitt also showed when a newborn snake's tongue was completely removed, the snake stopped feeding. If the snake's tongue was partially severed, which would allow a small exposure of the tongue to the outside of the snake's mouth, the snake was able to continue to feed[26]. It seems that the tongue is essential for the feeding ability of the snake.

Sheffield, Law, and Burghardt conducted an experiment to determine if the garter snakes tongue had to make contact with the prey before striking behavior was exhibited[12]. In the experiment an electric circuit was constructed in which one electrode of the circuit was a metal plate covered with a wet paper towel. A snake was placed on the paper towel. The other electrode of the circuit consisted of a metal wire inserted in an earthworm. A small voltage was applied to the circuit which the snake could not sense. When the snake made contact with the earthworm section, a small current could be monitored with a voltmeter. In this study, Sheffield, Law, and Burghardt concluded that the young snake must make contact with the prey using its tongue before striking occurred.

Halpern and Kubie measured the effectiveness of snakes with sensory deficits in following the trails of earthworm extracts in a maze[11]. Some snakes trained to follow earthworm extract paths then had the vomeronasal nerve severed; others had the olfactory nerve severed. The results proved that snakes with the vomeronasal nerve completely severed could not follow the extract trail

through the maze above chance levels, while snakes with the olfactory nerve cut could follow the extract path. Halpern and Kubie also sutured the vomeronasal duct closed on the snakes that had the olfactory nerve severed. These snakes could not follow the extract trail. The snakes did regain their ability to follow the paths when the sutures were removed.

Other studies have been conducted by Halpern and Kubie which monitor the effectiveness of garter snakes following the trails of earthworm extracts[11]. In this study the garter snakes could follow trails of earthworm extracts through a four choice maze as long as their tongues could contact the extract trail. Kubie and Halpern noticed that the snakes lost their ability to follow the extract trail if the trail was marked below a perforated false floor[11]. It was concluded that the snake must make direct contact with its tongue to the earthworm trail, for tracking to occur. Thus, the vomeronasal and not the olfactory system is shown to be the essential system used by garter snakes for prey tracking and identification.

E. PREVIOUS WORK ON THE CHEMICAL IDENTIFICATION OF PREY

It is believed that recognition of prey by garter snakes may be a multicomponent system. One component of the system may consist of nonvolatile compounds. The work conducted by Sheffield, Law, and Burghardt suggests that contact by the snake's tongue must be made with the prey before any chemically elicited prey attacks can occur[12]. This observation can lead one to conclude that the snakes are sampling the prey with its tongue for nonvolatile compounds.

Another component of the mixture may be volatile. Compounds are being registered by the snake without tongue flicking. In the study of the tongueless snake mentioned previously, the snake did seem to preceive that food was present since the snake did search for the prey[13].

There have been studies conducted in which air has been passed over a prey item and is then introduced into a chamber containing the snake, as in an olfactometer[27]. An olfactometer is a holding chamber for animals in which air enters which has been routed over different substances. The response of different animals is monitored when the air has been routed over the different stimuli. It has been noticed when air passed over prey items is introduced into the chamber of an olfactometer containing a garter snake, that there is an increase in tongue flicking and activity of the snake[27]. It was noted that some snakes even gasped at the air which had been passed over the prey and was entering the holding chamber of the snake.

F. SEPARATIONS OF PREY IDENTIFICATION SUBSTANCES

Several attempts have been made to isolate chemical components from earthworm extracts. The basic procedure for making prey extract is to extract 6 grams of the prey animal for two minutes in 20 mL of 60°C water[12]. The solution is decanted and centrifuged for ten minutes at 2,500 rpm. The resulting water extract can be lyophilized or frozen to preserve activity. The lyophilized powder can be reconstituted with water to provide a solution which elicits a prey-attacking response from garter snakes[12].

Dialysis was conducted on the lyophilized water extract to determine if any small molecules could be associated with attack eliciting responses. After 48 hours, the dialysis solution in the tube remained fully active, while the dialysate accumulated no activity. It was concluded that the activity was not associated with small molecules; or that an active small molecule was tightly bound to a macromolecule or to the dialysis tubing[12].

Another experiment done by Sheffield, Law, and Burghardt tried to dissociate an active small molecule by altering the pH of the solution[12]. Extracts remained active when adjusted from a pH of 2 to 11. Solutions of 0.01M NaOH, 0.1M NaOH, and 0.01M HCl containing the earthworm extract were allowed to equilibrate for one hour. After the extract solutions were adjusted back to a pH of seven, the activity still remained. No activity could be extracted with ether or pentane after the extracts were treated with an acid or a base.

Sheffield, Law, and Burghardt also conducted a gel filtration experiment with Sephedex G-200 gel to see if an active component could be purified by column chromatography[12]. Sephedex G-200 separates globular proteins with molecular weights in the range of 5,000 to 250,000 daltons[28]. Three active regions were obtained from the column chromatography. One fraction was of high molecular weight since it had about the same retention volume as the void volume of the column. The second and third fractions eluted just before the gel filtration marker of Vitamin B-12 (molecular weight of 1,350 daltons) and was estimated to be greater than 5,000 daltons.

Other experiments have been done by Sheffield, Law, and Burghardt to purify the earthworm water extract. The columns that were used for separation

were carboxymethyl Sephedex (cation exchanger), diethylaminoethyl Sephedex (anion exchanger) and Dowex 1 (anion exchanger)[12]. The active components were not retained on any of the columns. On Dowex 50, eluting with 0.5M ammonium hydroxide solution, the active components were retained by the column. Dowex resins have ion exchange and gel filtration properties; thus, one could suspect that the active material could contain a net positive charge and/or it is a small molecule.

The early work has been followed by ongoing studies in Halpern's laboratory[21,29]. In one study, the lyophilized earthworm extract was purified with an "AcA44 column" eluting with a 0.15M NaCl solution[29]. According to their results, the only active fraction contained material with a molecular weight in excess of 67,000 daltons. This 67,000 molecular weight material was identified as a protein containing elements of carbohydrates as determined by a colorimetic test. Halpern also stated that the active compound(s) could be precipitated from the water extract with 90% and 100% saturation of ammonium sulfate in water. This precipitation technique is used to purify proteins and polysaccharides.

Halpern boiled an earthworm water extract for two hours and noticed that the first significant decline in activity occurred in the first fifteen to twenty minutes of boiling[29]. However, even after one to two hours of boiling, the snakes could distinguish the boiled extract from water. After two hours of boiling, the carbohydrate content of the extract diminished rapidly which was paralleled by a decreased ability of the snakes to distinguish the boiled extract from water.

Attempts have been made to isolate volatile components from the earthworm water extract. Sheffield, Law, and Burghardt heated the lyophilized

extract to 100°C at 1 torr[12]. Volatile components were collected on a cold finger cooled with dry ice. The materials that were condensed on the cold finger were inactive, whereas the lyophilized material remained fully active.

Singer and Burghardt stirred 70 mL of earthworm aqueous extract in a 100 mL flask heated to 53°C[16]. A nitrogen stream (30 mL/min) was passed over the extract and into a 15 cm long reflux condensor at 10°C, and then through a tenax resin column. After five hours, the tenax column was heated for four minutes, at 220°C, in a 5 mL/min, nitrogen stream. The volatile components obtained from the tenax packing were condensed in a capillary tube surrounded by dry ice. The capillary tube was rinsed with 750 uL of propylene glycol or 1 mL of water for testing purposes. The snake bioassay procedure using tongue flicking and prey attack data showed some marginal activity in the distillate.

G. STATEMENT OF THE PROBLEM

The purpose of this project was to isolate the chemical component or components from the earthworm (Lumbricus terrestris), fathead minnow (Pimephales promelas), and the mosquito fish (Gambusia affina), that allow garter snakes (e.g. Thamnophis sirtalis) to identify each animal species as prey. Earthworms were chosen since preliminary work had been conducted by Burghardt, Halpern, and others, as stated previously. The commercial availability of the earthworms and the fathead minnows was another reason for choosing these prey. The mosquito fish was available from local ponds. Only preliminary research was conducted since large quantities of the relatively small mosquito fish were difficult to obtain. Another reason for choosing these prey was that a

known method of extraction for the earthworm, fathead minnow, and mosquito fish had been developed by Burghardt. This extraction method consisted of extracting every 6 g of prey with 20 mL of 60°C water for two minutes. This aqueous extraction procedure obtained the active material directly from the prey for bioassays and chemical separations.

Burghardt's bioassay method was utilized to help identify the active components. The genera of garter snakes that was used in the bioassays were Thamnophis sirtalis. Thamnophis sirtalis preys on earthworms as well as fish. This species will serve as a discriminating test animal since it feeds on all three types of prey.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. ANALYSIS OF EARTHWORM CUTICLE COLLAGEN

Earthworm cuticles were removed for analysis by freezing the worms in a dry ice:methanol bath and thawing the worms slowly at 4°C[30]. The earthworm cuticles were removed from the earthworm and rinsed with water until they were opaque. The cuticles were then lyophilized.

The cuticles were sectioned into pieces and ground with a mortar and pestle in a small volume of 0.5M NaCl solution. An extraction was conducted for 24 hours at 4°C with a concentration of 100 earthworm cuticles per 100 mL of salt solution. The processed cuticles were separated from the extract by centrifugation. This extraction procedure was repeated two additional times with the cuticles being ground between each extraction. The cuticles were extracted two more times with 0.1N acetic acid in water by the same method.

All the materials obtained from the salt extractions of the cuticles were labeled CUT_s-EW, and the acetic acid extractions were labeled CUT_a-EW. A number following the "s" and "a" in the code indicates from which extraction the materials were obtained. For example, the CUT_{s1}-EW is the material obtained from the first salt extraction of the earthworm cuticle, and CUT_{a2}-EW is the material obtained from the second acid extraction of the earthworm cuticle.

The collagen obtained from CUT_{s1}-EW was purified by two 20% ammonium sulfate precipitations in water[30]. The purified collagen obtained from CUT_{s1}-EW

was labeled CUT_{sl}-EW-PC, where "PC" is an abbreviation for purified collagen in the code.

An enzyme degregation was conducted on the earthworm cuticle collagen[30]. The earthworm cuticles (0.5 g) were allowed to be digested for 72 hours at 43°C, with 1,750 units of collagenase. The digestion media was 100 mL of pH 7.6 autoclaved solution containing 0.05M trimethyol aminomethane hydrochloride and 0.01M $CaCl_2 \cdot 2H_20$. The mixture obtained from the collagenase digestion was labeled CUT_{cd}-EW, where "cd" is an abbreviation for collagenase digestion.

B. PREY EXTRACTION PROCEDURES

Aqueous Prey Extract (AQ)

The general procedure for obtaining the aqueous prey extract, based on Wilde[20] and Burghardt[14], was to extract prey animals for two minutes with a ratio of 6 g of prey for every 20 mL of distilled water at 60°C. The solution was decanted and centrifuged for 10 min at 2,500 rpm. The aqueous extract was then removed from the centrifuge tubes and was frozen or lyophilized to preserve activity. The amount of material obtained from all three prey ranged from two to three grams per liter.

All three prey have been extracted by this method. Future references to this aqueous extract obtained from the earthworm, fathead minnow, and mosquito fish will be referred to as AQ-EW, AQ-FM, and AQ-MF respectively. In the codes of the prey extracts, the type of prey extraction is abbreviated first with the type of prey extracted abbreviated after the dash.

Methanol:Chloroform Extractions of the Aqueous Extract (AQmc)

Another method of extraction was developed in which the lyophilized aqueous extract was extracted with methanol and chloroform solutions. First, 0.71 g of the lyophilized aqueous extract was extracted for 15 min with 100 mL of 1:4 methanol:chloroform. The solution was filtered and extracted with 100 mL of 1:1 methanol:chloroform. The methanol:chloroform extracts were combined and evaporated to dryness[31]. This extraction procedure will be referred to as the AQmc extraction. Approximately 6% and 24% by mass of the materials obtained from the AQmc extraction from the earthworm and fathead minnow, respectively, were soluble in the methanol and chloroform extracts.

From this extraction procedure there was material that was soluble and insoluble in the methanol:chloroform solutions. The compounds that were soluble from the earthworm, fathead minnow, and mosquito fish lyophilized aqueous extract were coded $AQmc_{sol}$-EW, $AQmc_{sol}$-FM, and $AQmc_{sol}$-MF respectively. The compounds which were insoluble in the methanol:chloroform solution were referred to as $AQmc_{insol}$-EW, $AQmc_{insol}$-FM, and AQ_{insol}-MF. The letters "sol" and "insol" found in the subscript of the codes are abbreviations for soluble and insoluble which characterizes the solubility of the material in the methanol:chloroform extraction solutions.

Methanol Extractions of the Aqueous Extract (AQm)

A third method of extracting the aqueous lyophilized extract was using methanol as the extracting solvent. The lyophilized aqueous extract (1.74 g) was extracted for 15 min with 75 mL of methanol. The insoluble materials were

filtered with a fine glass frit and extracted again in the same manner. The insoluble compounds were extracted four additional times for 10 min with 25 mL of methanol and filtered each time between extractions. All the methanol extracts were combined and evaporated to dryness. The materials that were soluble in the methanol extractions from the earthworm and fathead minnow will be referred to as AQm_{sol}-EW and AQm_{sol}-FM. Approximately 24% and 52% by mass of the material extracted was soluble in the methanol extractions from the earthworm and fathead minnow aqueous extracts.

Soxhlet Extraction of the Aqueous Extract (AQs)

A fourth method of extraction was developed in which the lyophilized aqueous earthworm was extracted with methanol in a soxhlet extractor. First, 7.25 g of lyophilized aqueous extract was refluxed for one hour with 138 mL of methanol. The insoluble material was filtered and rinsed with three 30 mL portions of methanol. The insoluble material was extracted in a soxhlet extractor with 200 mL of methanol at 80°C. After three days the methanol extraction solvent was changed, and new methanol was added. The soxhlet extraction was continued for an additional three days. The compounds obtained from the soxhlet extraction will be referred to as AQs_1-EW for the first three day extraction and AQs_2-EW for the second three day extraction. The methanol insoluble compounds after six days of refluxing will be referred to as AQs_r-EW.

Chloroform:Methanol Extractions of the Prey (CM)

A method was developed which directly extracted the prey with choloform and methanol. In the procedure, 1.00 kg of prey was extracted for 4 min at 23°C with a solution containing 2.00 L of methanol and 1.00 L of chloroform. The solution was decanted, and the prey was extracted again with 1.00 L of chloroform. After four additional minutes, the second solution was decanted from the prey. The solutions were combined and filtered with a coarse glass frit to remove particulate matter. The filtered solution was poured into a 5 L separatory funnel and extracted with 1.00 L of distilled water. The resulting emulsion was allowed to settle at 4°C. After two days, the biphasic solution was allowed to warm to room temperature. The water layer was removed and filtered with a medium glass frit. Both the water and organic layers were evaporated to dryness.

This extraction method will be referred to as the CM extraction. The material from the organic layer of the earthworm and the fathead minnow will be referred to as CMo-EW and CMo-FM. The compounds obtained from the water layer will be coded CMw-EW and CMw-FM. The CM extraction provided approximately three to five grams of material per kilogram of prey. Around 31% and 41% by mass of the material obtained from the CM extraction from the earthworm and fathead minnow, respectively, was isolated from the water layer.

Acid and Base Purification Method

The final extraction method was devised for purifying materials obtained from $AQmc_{sol}$-EW. This purification procedure consisted of a variety of

extractions using acids and bases as described in Figure 2.1. This procedure shown in Figure 2.1 will be referred to as the acid and base purification method.

C. CHROMATOGRAPHIC SEPARATIONS

All chromatography separations, unless otherwise stated, were conducted on a low pressure liquid chromatography system. The chromatography pump was an FMI lab pump, model RP-SV. A Laboratory Data Control refractive index detector, model 1107H, was implemented in the system, and a Scientific Manufacturing Industries, model 12050 fraction collector was used.

For the gel filtration separations, Bio-Gel P-10 and P-2, 50 to 100 mesh packing material was utilized. Sephedex G-200 filtration gel, 40 to 120 um particle size, was also used for separations. For all gel filtrations, the column packing material was swollen and eluted with 10% ethanol in distilled water. The column size used for the analytical separations was 1.1 x 30 cm, and for the preparative separation was 3 x 23 cm. The flow rates for the analytical and prepartive columns were approximately 0.4 mL/min and 1.4 mL/min, respectively. The pressure in the sytem was maintained below 4 psi in all cases. The fraction collector was adjusted to collect fractions at time intervals of five minutes for the analytical separations and 15 min for the preparative separations. The sample injections ranged from 30 to 40 mg for the analytical separations, while 0.50 g was used for the preparative separations.

Sephedex A-25 (diethylaminoethane anion exchange resin), 40 to 120 um particle size, was packed in an analytical column. The exchange resin was utilized in the chloride form. The column was eluted with water and a step

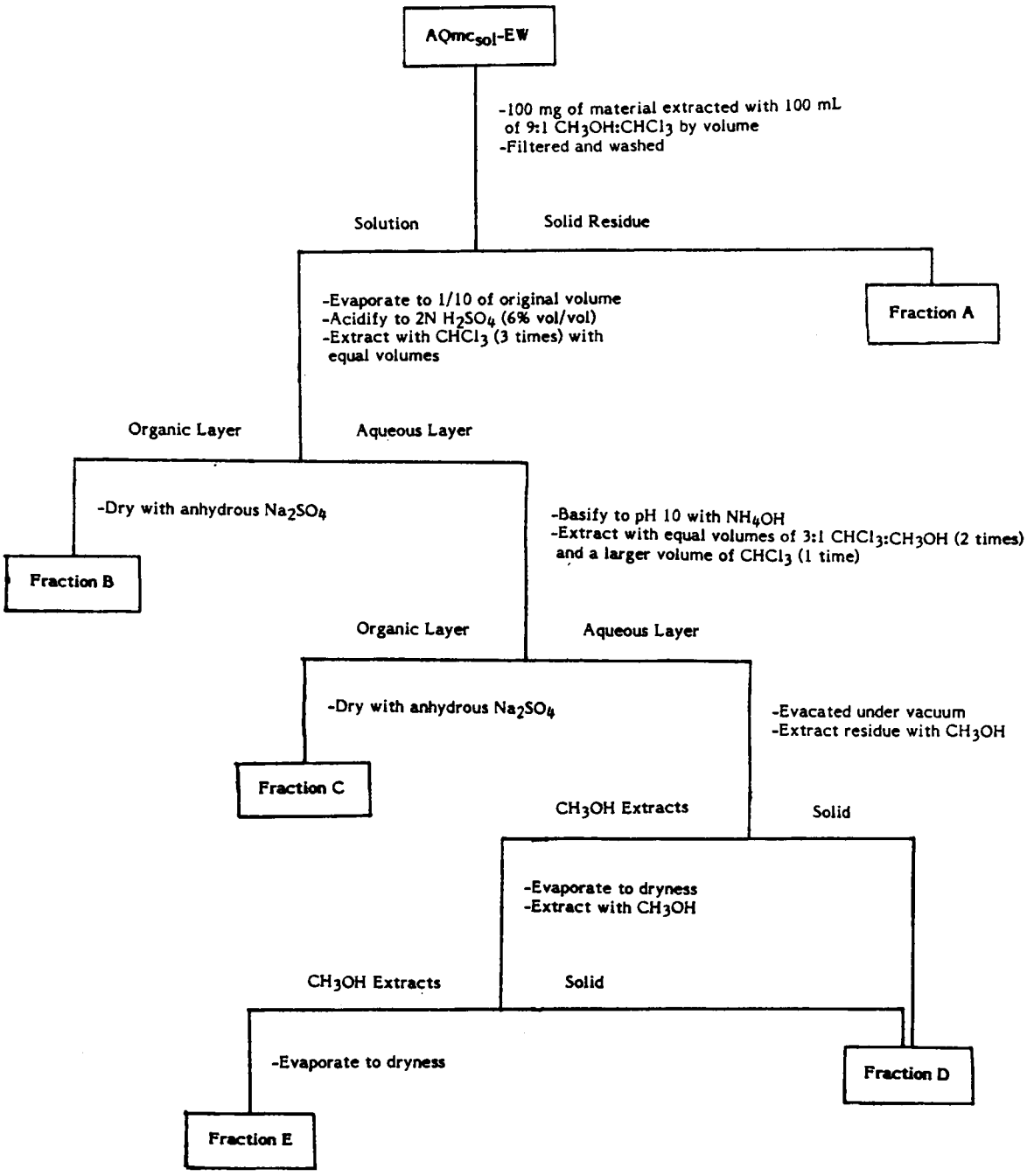


Figure 2.1. Acid and Base Purification Method[38].

gradient of NaCl solution from 0.25M to 1.0M in increments of 0.25M. The flow rate was 1.0 mL/min.

An analytical silica column was made for the low pressure liquid chromatography system. The analytical column was packed with Silica Gel 60, particle size 0.04 to 0.063 nm, from EM Laboratories Incorporated. The column was filled with 17 g of silica and vibrated with an engraver until the packing material finished settling. The column was packed and eluted with 3:2 methanol:chloroform by volume. The flow rate of the system was 0.5 mL/min.

Silica gel separations were also conducted with gravity eluted (3 x 75 cm) columns. Silica, 60 to 200 mesh, was obtained from J. T. Baker Chemical Company. Silica (60 g) was packed with 3:2 methanol:chloroform. The sample (0.30 g) was rotorary evaporated until dry with 5 g of silica in methanol. The silica containing the sample was applied to the top of the silica column and eluted with 3:2 methanol:chloroform. A second solvent was flushed on the column of the fathead minnow separations which consisted of 3:2 methanol:chloroform which was saturated with water.

Florisil chromatography was conducted on a 3 x 40 cm gravity eluted column. Florisil, 60 to 100 mesh, was obtained from the Floridine Company. The column was packed with 30 g of florisil in hexane. The sample was applied to the column as was done with the silica gravity eluted column except 0.057 g of AQs_r-EW was applied on 4.6 g of florisil. The column was flushed with solvent mixtures of: 0%, 5%, 15%, 25%, and 50% diethylether in hexane. The column was also eluted with 2% methanol in ether and 4% acetic acid in ether.

Reverse phase chromatography was conducted with a Waters Preparative Liquid Chromatography System, model 500A. The eluants for the separations were water and methanol mixtures at a flow rate of 100 mL/min. The preparative liquid chromatography system was equipped with a refractive index detector which was set at a relative response factor of twenty.

Silica thin layer chromatography (TLC) was also conducted on the extracts. Silica gel G (type 60) from EM Laboratories Incorporated was used for making the TLC plates. The silica TLC plates were eluted with 3:2 methanol:chloroform or 3:2 methanol:chloroform which was saturated with water. The plates were visualized by blowing iodine vapor on the TLC plate or by treating the plate with an α-naphthol sulfuric acid solution. The α-naphthol sulfuric acid solution was used to detect the presence of carbohydrates and was obtained by mixing 1.0 g of α-naphthol with 5 mL of concentrated sulfuric acid. After 24 hours the resulting mixture was diluted with 95 mL of ethanol. The TLC plates were sprayed with the α-naphthol sulfuric acid solution and inserted into a drying oven at 140°C for two minutes. Carbohydrates were visualized as purple spots on the TLC plate.

D. DIALYSIS PROCEDURE

Sixty-four centimeters of 6,000 to 8,000 molecular weight cutoff dialysis tubing was boiled for 10 minutes in 950 mL of 2% sodium bicarbonate with 1 mM of disodium ethylenediamine-tetraacetate in water. The dialysis tubing was cooled to room temperature in the solution.

Two knots were tied on the bottom of the dialysis tubing. Aqueous prey extract (18 mL) was poured into the dialysis tubing and then was enclosed by tying two additional knots at the upper end of the dialysis tubing. The filled dialysis membrane was inserted into a beaker containing 100 mL of distilled water and was allowed to stand at 4°C for 24 hours. The dialysis solution was changed every 24 hours for three days.

E. ANALYTICAL ANALYSIS OF ACTIVE COMPONENTS

Molecular Weight Estimates

Materials that were not retained by the Bio-Gel P-10 gel filtration column were estimated for molecular weight on a Sephedex G-200 column (1.1 x 30 cm). G-200 resin, 40 to 120 um particle size, was reconstituted and packed with water. A mixture of molecular weight standards used for calibration were obtained from Bio-Rad and are shown in Table 2.1. The molecular weight standards and samples were eluted with water at a flow rate of 3.2 mL/hr. The range of the detector was set at 8 for the 2 to 5 mg sample injections. The fractioning range of Sephedex G-200 for globular proteins is 5,000 to 600,000 daltons[28]. The upper limit for fractionation was assumed to be 600,000 daltons. The lower limit was estimated to be 17,000 daltons since the 1,350 and 17,000 molecular weight markers had the same retention volume. A graph was plotted of $\log_{10}$ of the molecular weight marker versus its Kav value. The definition of the Kav value is as follows:

$$Kav = (Ve - Vo)(Vt - Vo)^{-1} \qquad (2\text{-}1)$$

TABLE 2.1

COMPOUNDS USED AS MOLECULAR WEIGHT MARKERS[a]

Name	Molecular Weight in Daltons
Thyroglobulin (bovine)	670,000
Gamma Globulin (bovine)	158,000
Ovalbumin (chicken)	44,000
Myoglobin (horse)	17,000
Vitamin B-12	1,350

[a]Purchased from Bio-Rad Laboratories as a mixture of molecular weight markers for gel filtration.

Ve was the elution of the molecular weight marker. Vo is the void volume of the column, and Vt is the total volume of the column[32].

A least square approximation was made to obtain the best linear correlation. This linear correlation was used as a calibration plot to estimate the molecular weights of the unknown samples.

Molecular weight components that were retained by the Bio-Gel P-10 gel filtration column were calibrated for molecular weight on a Bio-Gel, 50 to 100 mesh, P-2 column (1.1 x 30 cm). The column was packed with water and the flow rate for the separation was 1.1 mL/min. The low molecular weight samples and the gel filtration standards that were used to calibrate the G-200 column were injected and eluted with water. Bio-Gel P-2 has the fractioning range of globular proteins of 100 to 1,800 daltons[32]. No calibration plot was obtained from the Bio-Gel P-2 column since sufficient standards were not available from the Bio-Rad gel filtration mixture of standards.

Sugar Analysis

Samples that were analyzed for carbohydrates were derivitized by the following method. To 30 mg of sample, 2.0 mL of aqueous 2N trifluoroacetic acid was added. The solution was heated for two hours at 120°C. The hydrolyzed solution was evaporated to dryness and washed with 3 mL of water. The water was evaporated to dryness. This washing procedure was repeated twice.

The sample was reconstituted with 3 mL of water containing 25 mg of $NaBH_4$. The solution was stirred for 16 hours. The $NaBH_4$ solution was acidified with Dowex 50W-X2 to a pH of 2. The mixture was filtered with a fine glass frit.

The water from the filtrate was co-distilled with four 5 mL portions of methanol. Next, 3 mL of a 1:1 mixture of pyridine and acetic anhydride was added to the sample and stirred for 16 hours. The pyridine, excess acetic anhydride, and other by-products of the reaction were removed from the sample by co-distilling with four 5 mL portions of toluene. With each co-distillation, the mixture's concentration was increased. With the last co-distillation the sample was evaporated to dryness. The mixture was reconstituted with 300 uL of methylene chloride for gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS) analysis[33].

Standards of sugars were derivitized in the same way as the samples analyzed for carbohydrates except that the hydrolysis with 2N trifluoroacetic acid was omitted. The following sugars were used as standards in the amounts indicated: glucose (4.8 mg), galactose (2.0 mg), mannose (1.8 mg), xylose (4.1 mg), arabinose (1.9 mg), and myo-inositol (2.2 mg). The standard was reconstituted with 900 uL of methylene chloride for GC and GC/MS analysis.

Gas chromatography was conducted with 0.4 ul sample injections using a 6 ft x 2 mm ID glass column containing 3% SP-2330 on 100/120 Supelcoport. The chromatography conditions were at an initial temperature of 180°C with an initial temperature hold of 6 min. The temperature ramp rate was 2°C/min to a final temperature of 240°C. The nitrogen carrier gas was at a flow of 20 mL/min and a flame ionization detector was used[34]. The identity and concentrations of the unknown sugars were determined by comparing retention times and peak areas to the standards of the known sugars of known concentrations.

A GC/MS analysis was implemented to confirm the identity of the acetylated sugars with a Hewlett Packard, model 5710H GC and 5980H MS. The same type of column was used for the GC/MS as for the GC separations. The same separation parameters were utilized as was done on the GC except that the carrier gas was changed to helium at a flow rate of 25 mL/min. The sample injection size was changed to 10 uL. Electron impact was used at an ionization potential of 25 eV.

Amino Acid Analysis

A sample for an amino acid anlysis was reconstituted with water at a concentration of 1.0 mg/mL. Ten nanomoles of compound from the 1.0 mg/mL sample was inserted into a small vacuum vial along with constant boiling HCl (0.4 to 1.0 mL). The resulting solution was frozen with liquid nitrogen and vacuum sealed. The vial was heated to 105°C. After 23 hours the sample was frozen. The vial was opened and dried under vacuum over KOH. The sample was submitted to the Biological Services Department at The University of Tennessee, Knoxville for analysis.

The amino acid analysis consisted of a reverse phase column with a high pressure liquid chromatograph. A precolumn derivitization method was used with phenol isothyocyanate for detection at 254 nm. A 0% to 60% acetonitrile gradient program was run at 37°C.

Nuclear Magnetic Resonance

Proton and carbon nuclear magnetic resonance (NMR) of mixtures were obtained using a NT 200-WB Nicolete NMR. All samples were submitted for analysis in D_2O with acetonitrile as the reference. Presaturation experiments were conducted with all samples to reduce the HOD peak found in all proton magnetic resonance spectra.

F. BIOASSAY PROCEDURE

Burghardt's bioassay procedure was used to determine which compounds from each prey extract were active[15]. The working definition of an active component was a compound or mixture of compounds that both 1) elicited a minimum of two prey attacks by two different garter snakes of the same species and 2) that exhibited a tongue flick-attack score statistically greater than water. Due to a shortage of garter snakes early on, the first criterion of this definition was relaxed if a replicate test series demonstrated statistical significance using the tongue flick-attack score and a subsequent bioassay based on the results led to satisfaction of the first criterion.

Burghardt's bioassay method as cited on page 4 was adapted here as follows. At the beginning of each testing period, the temperature and date were recorded on a test record sheet for each snake. The number of snakes tested in each bioassay varied between five and twelve depending on the number of extracts and the number of responsive snakes available for testing. As the number of extracts tested in the bioassay increased, more snakes were needed in order to systematically balance the orders. In some cases, fewer responsive snakes were

available for testing due to heavy recent feeding, snake illness, or skin shedding. At these times each snake was tested twice on each stimulus. The second time the snakes were tested, the stimulus was presented to the snakes in reverse order from how they were presented during the first testing.

Later in the bioassays, the snakes were screened a few hours before the bioassay was started by testing their responsiveness to aqueous prey extracts of the control stimuli being tested in the bioassay. The screening resulted in a more discriminating bioassay, since nonresponsive snakes were eliminated from the bioassay and were thus excluded from the statistical evaluation. It was also found that the snakes were more responsive if they were fed on half food rations one to two weeks before the bioassay. The food rationing technique usually resulted in a three week feeding schedule where the snakes were given all the food they could eat every third week, and their food supply was reduced by half on the first and second weeks.

Precautions were taken to prevent the person who conducted the bioassay from knowing what the stimuli represented. One person coded all the stimuli for the test; another person tested the snakes.

The number of stimuli in each bioassay ranged from three to seven; always including a distilled water control and an AQ or CM extract of the prey being tested. It had been previously found that eight or more stimuli resulted in a loss of interest by the snakes participating in the bioassay. The extracts were arranged for testing such that the order of the stimuli presented to each snake was systematically balanced. This decreased the chance of the activity of a

previously tested stimuli having an effect on the results of another stimuli tested later. It has been demonstrated by Burghardt[18] that controls following effective extracts elicit a higher rate of tongue flicking than when presented after an ineffective extract. The order that the extracts were tested were noted on each animal's test record.

In the bioassay, the stimulus coated test swab was presented about 2 cm from the snake's head for thirty seconds. The response to each stimulus by each snake was recorded on the record sheet in terms of the number of tongue flicks directed towards and away from the swab. An attack latency time was recorded if the snake opened its jaw to attack or strike at the swab. If the snake did not tongue flick in ten to fifteen seconds after being presented with a stimulus, the end of the swab was brushed lightly on the snake's back to alert the snake to the swab's presence. The reason for alerting the snake to the presence of the swab is that snakes lacks eyelids and thus can sleep with their eyes open. This contact of the swab to the snake was also noted on the record sheet. During testing all of the snakes were tested with one stimulus before the second stimulus was introduced to any of the snakes. The time was recorded for each round of testing. The time lapse between tests was not less than seven minutes. An example of a test record sheet is shown in Figure 2.2.

The data from the bioassay were statistically analyzed to distinguish the active stimuli from the inactive ones. This was accomplished by comparing the data to controls of water and of the AQ or CM extract. The water control

SNAKE TEST SHEET

Series_____ Subject_____ Sheet_____

Species_____ Date born_____ Where found_____

No.	Date	Time	Temp.	Rel. H.	Stim.	Tongue flick; to	total	Latency	Remarks
1.									
2.									
3.									
4.									
5.									
6.									
7.									
8.									
9.									
10.									
11.									
12.									
13.									
14.									
15.									
16.									

FIGURE 2.2. SNAKE TEST SHEET

represented no activity whereas the extract represented strong activity. The basic data for the statistical analysis were the number of attacks, attack latency time, and the number of tongue flicks each snake exhibited toward each stimulus. The tongue flick-attack score was calculated based on Equation 1-1 (page 4).

A two-way analysis of variance (stimulus x subjects) was run on the data for all the extracts tested in the bioassay excluding data from snakes that neither attacked any stimulus and nor gave at least five tongue flicks toward at least one stimulus. If the variances were not homogeneous (Hartley's F-Max Test), a square root transformation was performed before all parametric tests. A nonparametric Friedman's two-way analysis of variance (ANOVA) that used ranks was also performed. If the overall ANOVA was significant, planned comparisons of the AQ or CM extract and other stimuli with water were determined. A nonparametric Wilcoxon Signed rank-matched pair test was run, or a dependent t-test if the number of subjects were six or fewer. All paired comparison statistic p values are presented one-tailed since in all cases only an increase in responsivity over water was being evaluated. All statistical calculations were performed using the Statgraphics (APL) package (Version 1.1) on an IBM personal computer. Statistical tables for the controls and relevant stimuli used in the bioassay includes the overall ANOVA and paired comparisons results, the tongue flick-attack scores, and the number of attacks. The cut-off for p values presented was 0.0001, although many were more significant.

CHAPTER III

THE ISOLATION OF ACTIVE COMPONENTS FROM EARTHWORMS

A. ANALYSIS OF EARTHWORM CUTICLE COLLAGEN

Work was directed toward analysis of the earthworm cuticle since it is a protective layer found on the outside of the earthworm. Earthworm cuticles consist of the collagen containing attached di and tri galactose units[30,35]. Analysis was conducted on the cuticle to determine if any substances found in the cuticle were responsible for the snakes predatory behavior.

Several people had previously conducted analysis on the earthworm cuticle[30,35,36]. Some known sugars and amino acid components of the cuticle collagen were tested for activity by the snake bioassay. None of the sugars or amino acids tested were found to be active. The compounds that were found and the ones that were tested are shown in Table 3.1.

Salt and acetic acid extractions from the earthworm cuticle were obtained by the method of Muir and Lee[30]. None of the extracts were active except the first salt extraction which was coded CUT_{s1}-EW. A trial bioassay was performed to see if the material was active. The material obtained from CUT_{s1}-EW resulted in a single prey attack and was about as active as the aqueous prey extract (AQ-EW). Purification of the collagen from the first salt extraction (CUT_{s1}- EW-PC), by two 20% ammonium sulfate precipitations, was also tested in this trial bioassay and showed a significant loss of activity when compared to

TABLE 3.1

AMINO ACID AND SUGAR DETERMINATION IN EARTHWORM CUTICLE COLLAGEN[30]

Compound	Concentration Found in Cuticle[a]
Lysine	21
Histidine	1
Arginine	23
Aspartic acid	61
Hydroxyproline	159
Threonine	52
Serine[b]	89
Glutamic acid	75
Proline	10
Glycine[b]	320
Alanine	98
Valine	25
Methionine	trace
Isoleucine	21
Leucine	30
Tyrosine	4
Phenylalianine	9
Galactose[b]	13.8%
Xylose	1.3%
Fucose	trace
Mannose	trace
Glucosamine[b]	1.3%
Galactose amine[b]	2.0%

[a]Number of amino acid residues found per 1,000 amino acids. Sugars are expressed as mass percent.

[b]Compounds tested for activity in water which were greater than 1 g/L.

CUT_{s1}-EW. A bioassay test was performed to see if the second salt extraction (CUT_{s2}-EW) or the acetic acid extraction (CUT_{a1}-EW) on the earthworm cuticle were active. None of the other extracts showed activity as seen in Table 3.2.

It was concluded that the active components from the earthworm extract was not the purified collagen since it was found to be inactive. It was also concluded that the active components may be associated with the cuticle but not attached, since the cuticle lost all of its activity after the first salt extraction.

An enzyme experiment was conducted with collagenase to degrade the cuticle and to provide smaller active compounds[30]. Collagenase cleaves the collagen molecule into large fragments. Some of these fragments consist of the triple helical structure and glycopeptides from the collagen[37]. The cuticles after collagenase degregration were labeled CUT_{cd}-EW. No activity could be associated with the collagenase degregation mixture as seen in Table 3.3. From this experiment, it was concluded that the collagenase may have destroyed the active components beyond recognition by the garter snakes.

B. SEPARATIONS OF EARTHWORM EXTRACTS

The lyophilized aqueous earthworm extract (AQ_{insol}-EW) was separated on an analytical Bio-Gel P-10 gel filtration column. Bio-Gel P-10 resin fractionates globular proteins from 1,800 to 20,000 daltons[28]. The lyophilized extract (AQ-EW) was extracted with methanol and chlorofrom to obtain $AQmc_{sol}$-EW. It was believed that the methanol:chloroform extracts would remove lipids which would normally adhere to the gel filtration column[24]. This lipid extract was

TABLE 3.2

BIOASSAY RESULTS OF THE EXTRACTION OF EARTHWORM CUTICLES[a]

	Stimuli[b]			
	Water	AQ-EW	CUT_{a1}-EW	CUT_{s2}-EW
Combined mean tongue flick-attack score	10.60	42.24	10.40	14.30
Statistical comparison to water[c]		t=6.17 $p<0.002$	t=0.05 not significant	t=1.13 not significant
Number of attacks	0	9	0	0

[a]Five Thamnophis sirtalis were tested twice on four different stimuli.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 10.56$, df = 12, $p<0.02$;
$F = 25.69$, df = 3,12, $p<0.0001$.

[c]Dependent t-test, df = 4.

TABLE 3.3

BIOASSAY RESULTS OF THE ENZYME DEGREGATION OF EARTHWORM CUTICLES[a]

	Stimuli[b]		
	Water	AQ-EW	CUT_{cd}-EW
Mean tongue flick-attack score	7.88	38.78	19.25
Statistical comparison to water[c]		t=5.91 p=0.005	t=2.93 p=0.03
Number of attacks	0	6	0

[a]Five Thamnophis sirtalis were tested twice on five different stimuli, one subject omitted.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 14.31$, df = 12, $p<0.01$;
$F = 20.45$, df = 4,12, $p<0.0001$.

[c]Dependent t-test, df = 3.

relatively active and elicited one prey attack as seen in the first test of the bioassay in Table 3.4.

The material that was not soluble in the methanol:chloroform extract ($AQmc_{insol}$-EW) was separated on the P-10 gel filtration column. Two major fractions of compounds were obtained from the separation. The first fraction was a multicomponent mixture eluting close to the void volume of the column. This indicates a molecular weight of at least 20,000 daltons or greater. The second fraction contained a mixture of compounds that eluted close to the column volume, indicating a molecular weight of approximately 1,800 daltons or less.

The high molecular weight fraction will be referred to as $AQmc_{insol}$-EW-HMW_{P10}. The low molecular weight fraction will be referred to as $AQmc_{insol}$-EW-LMW_{P10}. In this code, "HMW" is an abbreviation for high molecular weight, where as "LMW" is an abbreviation for low molecular weight. Both fractions from the P-10 separation were active, although the low molecular weight fraction was shown to be active only for the second test as exhibited in the bioassay in Table 3.5.

Since it was found that the methanol:chloroform extracts from the lyophilized aqueous extracts ($AQmc_{sol}$-EW) were active, another extraction method was developed using methanol as the extraction solvent. Methanol was chosen since it was assumed that the active components were polar because of their solubility in water. These materials extracted by the methanol extracts from the lyophilized aqueous extracts were coded AQm_{sol}-EW. The materials obtained from AQm_{sol}-EW were active as seen in Table 3.6.

TABLE 3.4

BIOASSAY RESULTS OF THE METHANOL:CHLOROFORM EXTRACTS OBTAINED FROM THE AQ-EW EXTRACT[a]

	Stimuli[b]		
	Water	AQ-EW	$AQmc_{sol}$-EW
First Test			
Mean tongue flick-attack score	5.8	38.8	20.2
Statistical comparison to water[c]		t=5.44 p<0.005	t=5.07 p<0.005
Number of attacks	0	2	1
Second Test			
Mean tongue flick-attack score	9.6	48.3	14.6
Statistical comparison to water[c]		t=6.06 p<0.002	t=0.73 not significant
Number of attacks	0	2	0

[a]Five Thamnophis sirtalis were tested twice on five different stimuli.

[b]Square root transformation on all data for parametric statistics. Overall two-way ANOVA results for stimuli:

First test - Friedman's, $X^2 = 11.20$, df = 16, $p<0.025$;
$F = 3.92$, df = 4,16, $p<0.025$.

Second test - Friedman's, $X^2 = 12.32$, df = 16, $p<0.02$;
$F = 9.68$, df = 4,16, $p<0.001$.

[c]Dependent t-test, df = 4.

TABLE 3.5

BIOASSAY RESULTS OF THE HIGH AND LOW MOLECULAR WEIGHT FRACTIONS FROM THE AQ-EW EXTRACT[a]

	Stimuli[b]			
	Water	AQ-EW	$AQmc_{insol}$-EW-HMW_{P10}	$AQmc_{insol}$ EW-LMW_{P10}
First Test				
Mean tongue flick-attack score	10.2	45.9	33.3	10.8
Statistical comparison to water[c]		t=6.30 $p<0.002$	t=5.58 $p<0.005$	t=0.19 not significant
Number of attacks	0	2	2	0
Second Test				
Mean tongue flick-attack score	9.6	49.6	36.8	25.8
Statistical comparison to water[c]		t=7.39 $p<0.001$	t=5.32 $p<0.005$	t=3.37 $p<0.02$
Number of attacks	0	3	1	1

[a]Five Thamnophis sirtalis were tested twice on five different stimuli.

[b]Square root transformation on all data for parametric statistics. Overall two-way ANOVA results for stimuli:

First test - Friedman's $X^2 = 15.31$, df = 16, $p<0.005$;
$F = 11.02$, df = 4,16, $p<0.001$.

Second test - Friedman's $X^2 = 13.54$, df = 16, $p<0.01$;
$F = 9.88$, df = 4,16, $p<0.001$.

[c]Dependent t-test, df = 4.

TABLE 3.6

BIOASSAY RESULTS OF THE METHANOL EXTRACTS OF AQ-EW[a]

	Stimuli[b]		
	Water	AQ-EW	AQm_{sol}-EW
Mean tongue flick-attack score	21.00	51.84	53.40
Statistical comparison to water[c]		t=2.46 p<0.05	t=2.90 p<0.05
Number of attacks	1[d]	4	3

[a]Six Thamnophis sirtalis were tested once each on six different stimuli. (One snake omitted.)

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 10.89$, df = 20, p = 0.05;
F = 4.00, df = 5,20, p<0.02.

[c]Dependent t-test, df = 4.

[d]Subject previously attacked the five other stimuli in the bioassay.

The materials obtained from AQm-EW were separated on the Sephedex A-25 column. The column was injected with 0.47 g of material that had been reconstituted with water. All the active compounds from the extract were inseparable from the compounds eluting at the void volume of the column. These materials were labeled AQm-EW-void vol_{A25}. The activity of the compound obtained from the separation is shown in Table 3.7. It should be noted that in the bioassay in Table 3.7, no tongue flick data were recorded, reducing the discrimination ability of the bioassay test, in spite of the large number of prey attacks. The first fraction, which included all of the compounds eluting at the void volume of the column, consisted of 71% by mass of the materials injected on the column.

A purification procedure was sought which would purify the active components directly from the earthworm extracts. A procedure was implemented with material obtained from AQm_{sol}-EW using acid and base extractions[38]. This procedure was known as the acid base purification method and is outlined in Figure 2.1 (page 20). No activity was found in fractions B, C and E and, it was concluded that the acids or bases used in the extractions could have destroyed the active materials.

The active material obtained from AQm_{sol}-EW was applied to a silica low pressure liquid chromatography column. No active fractions could be obtained when eluting with 3:2 methanol:chloroform. It was assumed that the active components from the extract were destroyed by the acidic packing material or adhered to the column.

TABLE 3.7

BIOASSAY RESULTS OF THE A-25 CHROMATOGRAPHY OF AQm-EW[a]

	Stimuli[b]		
	Water	AQ-EW	AQm-EW-void vol$_{A25}$
Mean attack score	7.25	53.12	24.81
Statistical comparison to water[c]		t=6.69 p<0.001	t=2.19 p=0.03
Number of attacks	1	8	5

[a]Eight Thamnophis sirtalis were tested once each on four different stimuli. No tongue flicks counted. Attack score based on (60 - latency); zero if no attack occurred.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 15.45$, df = 21, $p<0.005$;
$F = 16.24$, df = 3,21; $p<0.001$.

X^2 for 4 x 2 table of stimulus x attack:

$X^2 = 12.70$, df = 3, $p<0.01$.

[c]Dependent t-test, df = 7.

Since the methanol extractions of the lyophilized aqueous extract (AQm_{sol}- EW) contained active material, a soxhlet extraction extraction was attempted to obtain the quantitative yield of the active components from the aqueous extract. Methanol was used as the extraction solvent. Methanol was still extracting active material by the soxhlet method after three days of extracting. The material obtained from the soxhlet on the fourth to sixth days were coded AQ_{s2}-EW. The activity of this material is shown in Table 3.8.

After each three day extraction, a white precipitate was noted on the bottom of the soxhlet extraction flask. It was concluded that the active material may not have had a high solubility in methanol and this resulted in the long extraction times.

The active material obtained from refluxing the lyophilized aqueous extract in methanol (AQ_r-EW) was applied to a florisil column. No active fractions could be obtained from the column when eluting with different mixtures of polar and non-polar solvents. It was concluded that the active compounds from the extract adhered to the florisil column or were destroyed by reacting with the packing material or the eluent containing acetic acid.

Since it was observed that some active components from the lyophilized aqueous extract of the earthworm could be extracted with methanol, a procedure was sought which would directly extract the prey with methanol and chloroform. A procedure that had been developed by Blight and Dryer[39] fulfilled these requirements. This extraction procedure was known as the CM extraction.

TABLE 3.8

BIOASSAY RESULTS OF THE SOXHLET EXTRACTS FROM AQ-EW[a]

	Stimuli[b]		
	Water	AQ-EW	AQs_2-EW
Mean tongue flick-attack score	11.84	38.76	32.33
Statistical comparison to water[c]		Z=2.61 p<0.005 n=10	Z=2.34 p=0.01 n=9
Number of attacks	1	7	6

[a]Ten Thamnophis sirtalis were tested once each on five different stimuli.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 18.55$, df = 36, $p<0.001$;
$F = 11.54$, df = 4,36, $p<0.0001$.

[c]Wilcoxon signed rank-matched pairs test, ties omitted.

The organic layer from the chloroform:methanol extract of the prey (CMo-EW) contained no activity as seen in Table 3.9. The compounds obtained from the water layer were separated on the preparative Bio-Gel P-10 column. The P-10 resin fractionates globular proteins with molecular weights of 1,800 to 20,000 daltons[27]. The column separated the mixture into two fractions. A multicomponent high molecular weight fraction eluting at the void volume of the column, and a low molecular weight fraction eluting approximately at the volume of the column. The low molecular weight fraction was referred to as CMw-EW-LMW$_{P10}$. The high molecular weight fraction was labeled CMw-EW-HMW$_{P10}$. Both the low and high molecular weight fractions were active as seen in Tables 3.9 and 3.10.

Preparative reverse phase chromatography was implemented on the low molecular weight fraction obtained from the water layer of the earthworm CM extraction. No active fractions were obtained when 0.426 g of material was separated on a Water's Preparative Liquid Chromatography System using 15% methanol in water as the eluent. It was believed that the active components adhered to or were destroyed by the acidic silica based packing of the column.

TABLE 3.9

BIOASSAY RESULTS OF THE CM EXTRACTION OF EARTHWORMS
PART I[a]

	Stimuli[b]			
	Water	AQ-EW	CMw-EW-HMWp_{10}	CMo-EW
Mean Tongue flick-attack score	6.43	29.37	24.70	6.43
Statistical comparison to water[c]		Z=2.28 $p<0.02$	Z=2.11 $p<0.02$	Z=0.08 not significant
Number of attacks	0	6	5	0

[a]Seven Thamnophis sirtalis were tested once each on seven different stimuli.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 20.22$, df = 36, $p<0.005$;
$F = 7.39$, df = 6,36, $p<0.0001$.

[c]Wilcoxon signed rank-matched pairs test, n = 7.

TABLE 3.10

BIOASSAY RESULTS OF THE CM EXTRACTION OF EARTHWORMS
PART II[a]

	Stimuli[b]		
	Water	AQw-EW	CMw-EW-LMW$_{P10}$
Mean tongue flick-attack score	6.40	37.01	25.54
Statistical comparison to water[c]		Z=2.65 p<0.005	Z=2.29 p<0.02
Number of attacks	0	6	4

[a]Ten Thamnophis sirtalis were tested once each on five different stimuli.

[b]Square root transformation on all data for parametric statistics. Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 15.06$, df = 36, $p<0.005$;
$F = 10.13$, df = 4,36, $p<0.0001$.

[c]Wilcoxon signed rank-matched pair test, n = 10.

CHAPTER IV

THE ISOLATION OF ACTIVE COMPONENTS FROM FISH EXTRACTS

A. SEPARATIONS OF FATHEAD MINNOW EXTRACTS

The AQmc extraction was conducted on the fathead minnow lyophilized aqueous extract. The soluble ($AQmc_{sol}$-FM) compounds obtained from the chloroform:methanol extractions were active as seen in Table 4.1. Chromatography was conducted on the insoluble material in the chloroform:methanol extractions ($AQmc_{insol}$-EW) using a Bio-Gel P-10 analytical gel filtration column. Two fractions were obtained. A high molecular weight fraction eluted at the void volume of the column and was coded $AQmc_{insol}$-FM-HMW_{P10}. The low molecular weight fraction will be referred to as $AQmc_{insol}$-FM-LMW_{P10} and eluted at the column volume. Both the high and low molecular weight fractons were active as seen in Table 4.1.

The methanol extraction made from the fathead minnow lyophilized aqueous extract (AQm-FM) was also active as shown in Table 4.2. The active materials obtained from AQm-FM were separated on a Sephedex A-25 column in the same manner as the earthworm AQm-EW material. Not all of the fractions were tested for activity--only the ones that corresponded to active regions from the earthworm A-25 separation. The active compounds from the fathead minnow were eluted at the void volume of the column and labeled AQm-FM-void vol_{A25}. The relative activity of this A-25 fraction is shown in Table 4.2. The percent by mass of the compounds that were eluted at the void volume of the column to the amount injected was 64%.

TABLE 4.1

BIOASSAY RESULTS OF THE HIGH AND LOW MOLECULAR WEIGHT COMPONENTS FROM THE AQmc-FM EXTRACT[a]

	Stimuli[b]				
	Water	AQ-FM	$AQmc_{sol}$-FM	$AQmc_{insol}$-FM-HMW_{P10}	$AQmc_{insol}$-FM-LMW_{P10}
Mean tongue flick-attack score	8.88	53.00	49.35	34.98	18.63
Statistical comparison to water[c]		t=4.56 p<0.01	t=8.27 p<0.002	t=2.57 p<0.05	t=2.28 p=0.05
Number of attacks	0	6	7	3	0

[a]Five Thamnophis sirtalis were each tested twice on five different stimuli; one subject omitted. Scores on both tests were combined for each snake.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 12.60$, df = 12, p = 0.01;
F = 9.05, df = 4,12, p = 0.001.

[c]Dependent t-test, df = 3.

TABLE 4.2

BIOASSAY RESULTS OF THE A-25 CHROMATOGRAPHY OF AQm-FM[a]

	Stimuli[b]			
	Water	AQm-FM	AQm_{sol}-FM	AQm-FM-void vol_{A25}
Mean tongue flick-attack score	10.25	58.40	43.20	41.10
Statistical comparison to water[c]		t=4.84 $p<0.01$	t=2.81 $p<0.05$	t=2.28 p=0.05
Number of attacks	0	3	2	2

[a]Five Thamnophis sirtalis were tested once each on five different stimuli; one subject omitted.

[b]Square root transformation on all data for parametric statistics. Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 10.38$, df = 12, $p<0.05$;
$F = 5.26$, df = 4,12, $p = 0.01$.

[c]Dependent t-test, df = 3.

Active material obtained from the extraction (AQm_{sol}-FM) was separated on a low pressure liquid chromatography silica gel column. The extract (150 mg) was reconstituted with 4 mL of 3:2 methanol:chloroform. The material was injected on an analytical silica column and eluted with 3:2 methanol:chloroform. One active fraction was obtained that had an R_f by silica gel TLC of 0.48 when eluting with 3:2 methanol:chloroform. This fraction had a carbohydrate component which was visualized by the ∝-naphthol sulfuric acid solution. This fraction was labeled AQm_{sol}-FM-$Silica_1$. The activity of this fraction is shown in Table 4.3.

The activity isolated from AQm_{sol}-FM-$Silica_1$ was never duplicated on gravity eluted silica column separations. When the column was eluted with 3:2 methanol:chloroform, no active fractions were obtained from AQm_{sol}-FM. Next the same column that was eluted with 3:2 methanol:chloroform, was eluted with 3:2 methanol:chloroform that was saturated with water. This later eluent provided an active component labeled AQm_{sol}-FM-$Silica_2$. The activity of the fraction is shown in Table 4.4. This active component had an R_f by silica gel TLC of 0.08 when eluted with 3:2 methanol:chloroform. When the ∝-naphthol sulfuric acid solution was applied to the silica TLC plate, carbohydrate components were visualized in the region of the plate that had an R_f of 0.08. This ∝-naphthol sulfuric acid test indicated that the active components could contain carbohydrates.

The CM extraction was implemented on the fathead minnow in the same manner as with the earthworm. The organic layer from the extraction was somewhat active as seen in Table 4.5 and was labeled CMo-FM. The water layer

TABLE 4.3

BIOASSAY RESULTS OF SILICA GEL CHROMATOGRAPHY
OF THE FATHEAD MINNOW
PART I[a]

	Stimuli[b]		
	Water	AQ-FM	AQm_{sol}-FM-Silica$_1$
Mean tongue flick-attack score	12.80	51.76	30.06
Statistical comparison to water[c]		Z=1.89 p<0.05	Z=1.62 p=0.05
Number of attacks	0	4	2

[a]Five Thamnophis sirtalis were tested once each on seven different stimuli.

[b]Square root transformation on all parametric statistics. Overall two way ANOVA results for stimuli:

Friedman's, $X^2 = 17.96$, df = 24, $p<0.01$;
$F = 5.92$, df = 6,24, $p<0.001$.

[c]Wilcoxon signed rank-matched pairs tests, n = 5.

TABLE 4.4

BIOASSAY RESULTS OF SILICA GEL CHROMATOGRAPHY
OF THE FATHEAD MINNOW
PART II[a]

	Stimuli[b]		
	Water	AQ-FM	AQm_{sol}-FM-$Silica_2$
Mean tongue flick-attack score	3.20	29.96	23.54
Statistical comparison to water[c]		t=3.88 p<0.01	t=2.65 p<0.05
Number of attacks	0	3	2

[a]Five Thamnophis sirtalis were tested once each on three different stimuli.

[b]Square root transformations on all data for parametric tests. Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 6.00$, df = 8, $p<0.05$;
$F = 12.28$, df = 2,8, $p<0.005$.

[c]Dependent t-test, df = 4.

TABLE 4.5

BIOASSAY RESULTS OF THE CM EXTRACTION
OF THE FATHEAD MINNOW
PART I[a]

	Stimuli[b]		
	Water	AQ-FM	CMo-FM
Mean tongue flick-attack score	9.18	43.80	20.23
Statistical comparison to water[c]		Z=2.10 p<0.02	Z=2.10 p<0.02
Number of attacks	1	5	1

[a]Six Thamnophis sirtalis were tested once each on six different stimuli.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 13.50$, df = 25, p 0.02;
F = 5.53, df = 5,25, p 0.002.

[c]Wilcoxon signed rank-matched pairs test, n = 6.

contained much of the activity and was labeled CMw-FM. A dependent t-test was performed comparing the water layer to the organic layer. The t-test showed that the water layer was significantly more active than the organic layer ($t = 4.30$, $p < 0.01$, $df = 5$).

A Bio-Gel P-10 separation was conducted on the active water layer (CMw-FM). Two fractions were obtained from the preparative P-10 column. A high molecular weight fraction eluted at the void volume of the column, and a low molecular weight fraction eluted approximately at the volume of the column. Both fractions indicate, from the chromatogram, a multicomponent mixture. The high molecular weight fraction was labeled CMw-FM-HMW$_{P10}$, whereas the low molecular weight fraction was labeled CMw-FM-LMW$_{P10}$. Both fractions are active as indicated in Table 4.6.

Silica gel chromatography was conducted on the low molecular components from the CM fathead minnow extraction (CMw-FM-LMW$_{P10}$). No active fractions were found when a gravity eluted silica column was eluted with 3:2 methanol:chloroform and 3:2 methanol:chloroform which was saturated with water.

Preparative reverse phase chromatography was implemented on the fathead minnow low molecular weight components (CMw-FM-LMW$_{P10}$). The separation was conducted on 700 mg of material in the same manner as was done with CMw-EW-LMW$_{P10}$. The column was eluted with water and 100% methanol. No active fractions were obtained from either eluent.

TABLE 4.6

BIOASSAY RESULTS OF THE CM EXTRACTION
OF THE FATHEAD MINNOW
PART II[a]

	Stimuli[b]			
	Water	CMw-FM	CMw-FM-HMW$_{P10}$	CMw-FM LMW$_{P10}$
Mean tongue flick-attack score	10.71	52.71	50.09	40.51
Statistical comparison to water[c]		Z=2.28 p=0.01	Z=2.28 p=0.01	Z=2.28 p=0.01
Number of attacks	0	6	5	3

[a]Seven Thamnophis sirtalis were tested once each on four different stimuli.

[b]Square root transformatations on all data for parametric test. Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 13.29$, df = 18, $p<0.005$;
$F = 17.76$, df = 3,18, $p<0.001$.

[c]Wilcoxon signed rank-matched pairs test, n = 7.

B. SEPARATIONS OF MOSQUITO FISH EXTRACTS

A dialysis experiment was conducted on the mosquito fish aqueous extract that was similar to the one conducted on the earthworm aqueous extract by Sheffield, Law, and Burghardt[12]. As with the earthworm aqueous extract, all the activity remained in the dialysis tubing. Therefore, it was suspected that the compounds from the mosquito fish extract are of high molecular weight or adhered to the dialysis tubing. The dialysate of the mosquito fish was coded AQd-MF. The materials that were not dialyzable were coded AQnd-MF. The "d" in the code is an abbreviation for dialysate whereas "nd" is an abbreviation for not dialyazable. The activity of AQd-MF and AQnd-MF is shown in Table 4.7.

The AQmc extraction was conducted on the mosquito fish aqueous extract. The materials that were soluble in the methanol:chloroform solution were labeled $AQmc_{sol}$-MF, and the materials that were insoluble were labeled $AQmc_{insol}$-MF. A P-10 gel filtration separation was conducted on the materials obtained from $AQmc_{insol}$-MF. Two fractions were obtained when using the analytical column. The high molecular weight fraction eluted at the void volume of the column, and a low molecular weight fraction eluted at the column volume. The high molecular weight fraction was labeled $AQmc_{insol}$-MF-HMW_{P10}. The low molecular weight fraction was labeled $AQmc_{insol}$-MF-LMW_{P10}. The low molecular weight fraction was active. The bioassay showing the relative activity for each fraction and extract is shown in Table 4.8.

The high molecular weight fraction as well as the compounds extracted with the methanol:chloroform solutions were statistically inactive but there were

TABLE 4.7

BIOASSAY RESULTS OF THE DIALYSIS EXPERIMENT CONDUCTED ON AQ-MF[a]

	Stimuli[b]			
	Water	AQ-MF	AQnd-MF	AQd-MF
Mean tongue flick-attack score	3.70	16.88	10.70	5.60
Statistical comparison to water[c]		Z=2.55 p=0.005	Z=2.29 p=0.01	Z=1.24 p>0.10 not significant
Number of attacks	0	1	0	0

[a]Twelve Thamnophis sirtalis were tested once each on six different stimuli. Two nonresponsive animals were eliminated from statistical analysis.

[b]Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 23.35$, df = 45, $p < 0.001$;
$F = 8.96$, df = 5,45, $p < 0.0001$.

[c]Wilcoxon signed ranked-matched pairs test, n = 10.

TABLE 4.8

BIOASSAY RESULTS FOR THE AQmc EXTRACTION ON AQ-MF[a]

	Stimuli[b]				
	Water	AQ-MF	$AQmc_{sol}$-MF	$AQmc_{insol}$-MF-HMW_{P10}	$AQmc_{insol}$-MF-LMW_{P10}
Mean tongue flick-attack score	19.75	36.28	33.88	24.01	34.04
Statistical comparison to water[c]		t=3.71 p<0.02	t=1.34 not significant (p=.14)	t=0.23 not significant	t=2.56 p<0.05
Number of attacks	0	1	3	1	2

[a]Five Thamnophis sirtalis were tested twice on five different stimuli. One snake omitted from statistical analysis due to cloudy eyes. Data combined from both testings of stimuli.

[b]Square root transformation on all data for parametric test. Overall two-way ANOVA results for stimuli:

Friedman's, $X^2 = 9.80$, df = 12, $p<0.05$;
$F = 2.40$, df = 4,12, $p=0.10$.

[c]Dependent t-test, df = 3.

complications with the statistical evaluation of the data. In the statistical analysis, the methanol:chloroform extracts that elicited the most prey attacks did not result in its being significantly different from water. This was due to combining the results from both tests where water elicited a high rate of tongue flicking from one subject in each test, but no attacks. The Wilcoxon signed rank-matched pairs test gave a stronger result that showed that the high molecular weight material may be considered more effective than water ($z = 1.28$, $p = 0.10$, $n = 4$).

CHAPTER V

ANALYSIS OF ACTIVE COMPONENTS

A. AQUEOUS PREY EXTRACTS (AQ)

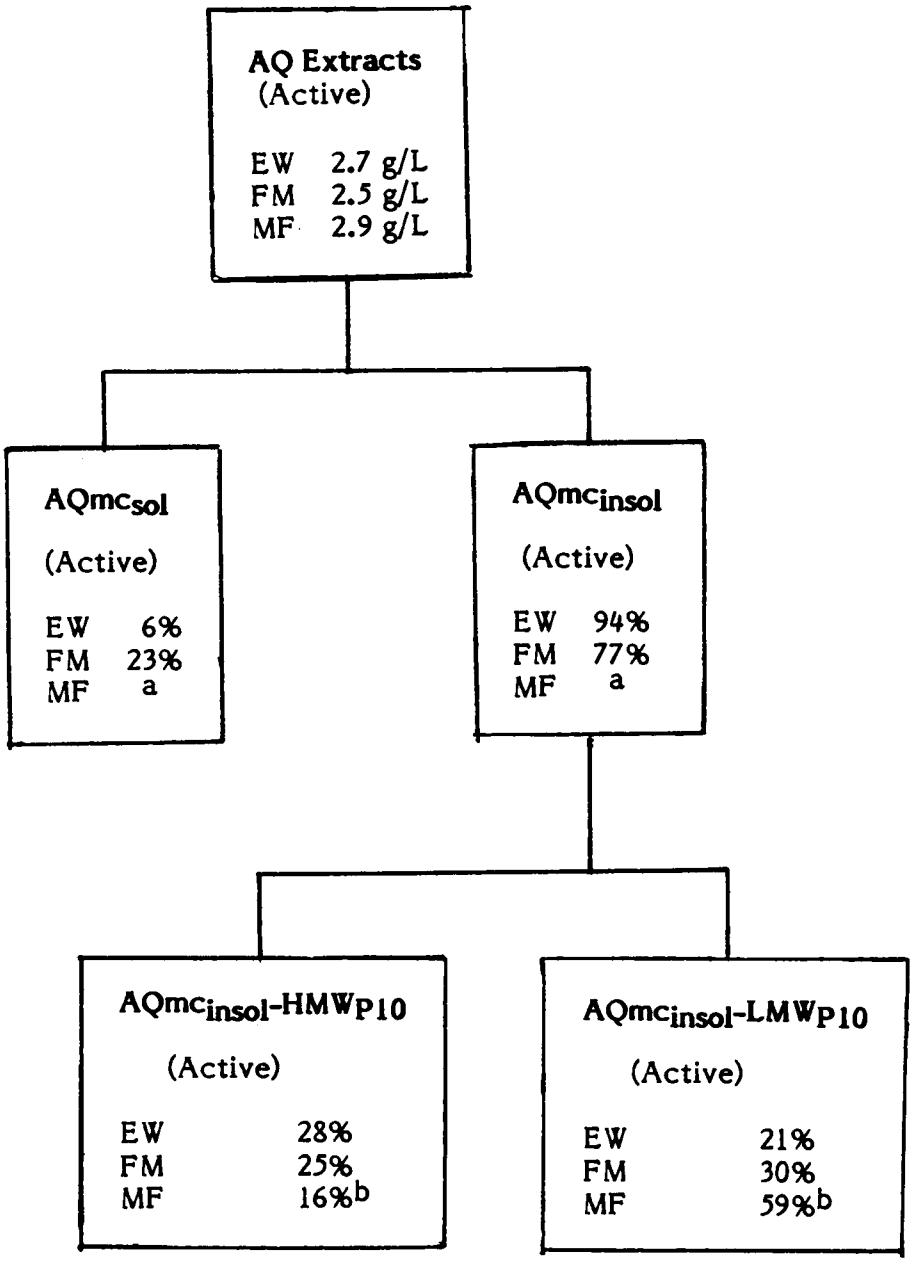
As seen in the bioassay Tables 3.4 (page 39), 4.1 (page 50), and 4.7 (page 59), the aqueous extracts of the earthworm, fathead minnow, and mosquito fish all are active. Other similarities in the prey AQ extracts are found in these bioassay tables in that the methanol:chloroform extractions from the lyophilized extract are also active. The high and low molecular weight components of the AQmc extracts from these prey also showed definite activity by an increase in the tongue flicking-attack score as exhibited in Tables 3.5 (page 40), 4.1 (page 50), and 4.8 (page 60). These similarities are noteworthy since the earthworm and fish are from different phyla and live in different environments.

The mass composition of the AQmc extraction obtained from each prey is recorded in Figure 5.1. As shown in Figure 5.1, there are similarities in the solubility properties of these prey extracts. The amount of material obtained per liter of extract is similar for each prey extracted, and the majority of the material from the earthworm and fathead minnow AQmc extraction was insoluble in the methanol:chloroform solutions.

B. CHLOROFORM:METHANOL EXTRACTS (CM)

Mass Composition

As observed with the aqueous extracts, the CM extraction also resulted in similar observations between the fathead minnow and the earthworm. Both of the



[a]Percentage not determined.

[b]Percentage of material by mass injected on column.

Figure 5.1. Percent composition of aqueous prey extract.

extracts' active components were found primarily in the water layer of the CM extraction as seen in Tables 3.6 (page 41) and 4.5 (page 55). The high and low molecular weight materials from the water layer elicited prey attacks from both the earthworm and fathead minnow as seen in Tables 3.6 (page 41), 3.7 (page 43), and 4.6 (page 57). The low molecular weight materials from each extract consisted of six times more material by mass than the high molecular weight materials. The percent composition by mass of the CM extraction for both prey is shown in Figure 5.2.

Molecular Weight Estimates

Molecular weight estimates of the high and low molecular weight materials from the CM extraction for both the earthworm and fathead minnow were determined. The high molecular weight materials were estimated on the basis of a molecular weight calibration plot obtained from Bio-Rad gel filtration standards on a Sephdex G-200 column. In the estimating of the high molecular weight materials from the CM extraction of both prey, the Sephedex G-200 column separated the material into two distinct fractions. In the case of the earthworm, the major constituent of the high molecular weight materials had a molecular weight of 432,000 daltons. The minor constituent could not be calibrated since it partially adhered to the column and eluted after the void volume. With the fathead minnow, the major constituent of the high molecular weight materials were determined to have a molecular weight of approximately 519,000 daltons. The minor constituents were estimated to be 27,000 daltons. It was interesting to note that both the earthworm and fathead minnow high molecular weight material

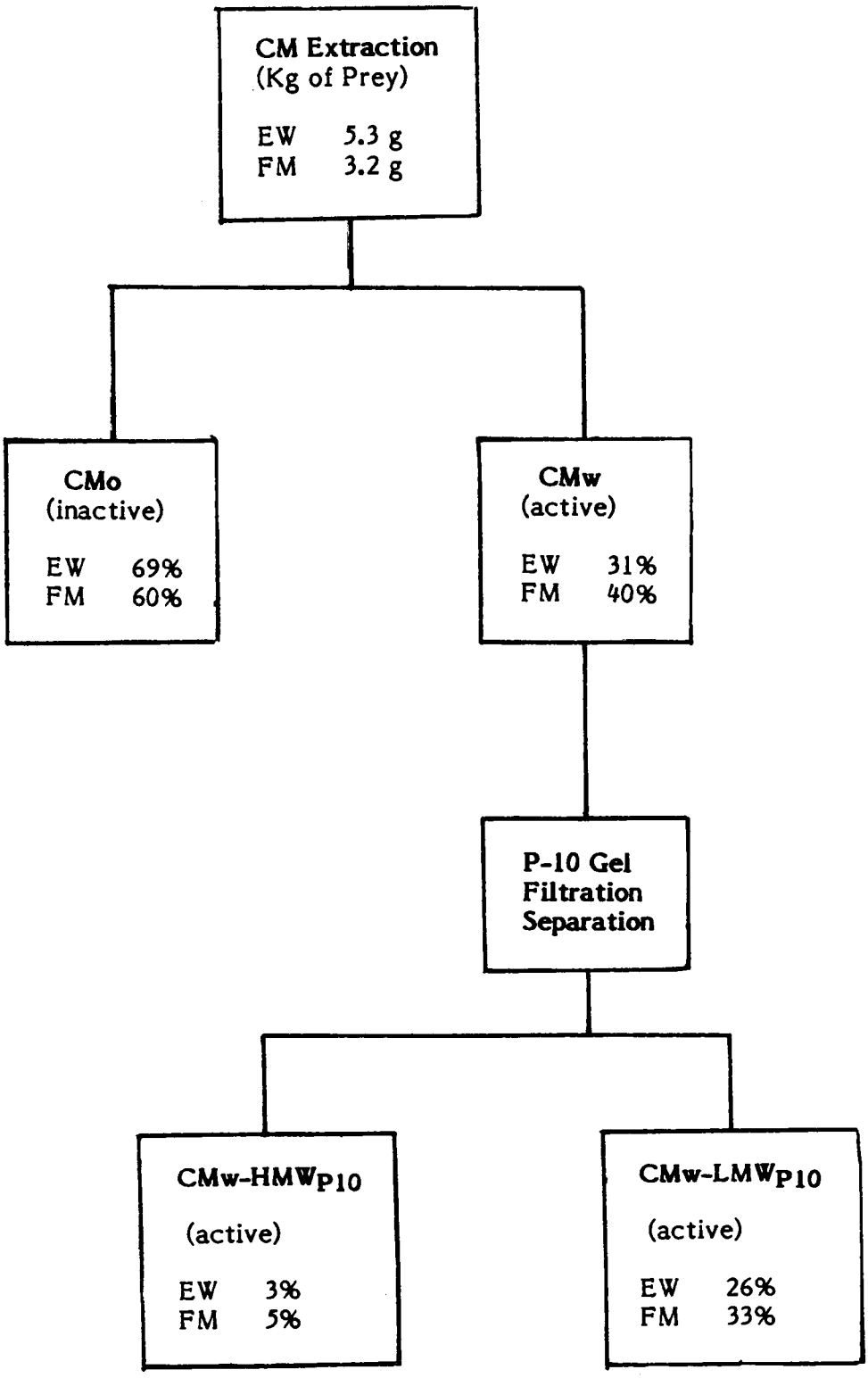


Figure 5.2. Percent composition by weight of the CM extraction.

from the CM extraction had a similar molecular size composition. The estimations of the earthworm and fathead minnow high molecular weight materials along with their Kav values are shown in Table 5.1.

The low molecular weight materials obtained from the CM extraction from both prey were not estimated for molecular weight due to the lack of an accurate calibration plot. The data obtained from the P-2 calibration column is presented in Table 5.2. The P-2 chromatograms of both prey obtained from the low molecular weight materials are shown in Figures 5.3 and 5.4.

As seen in Table 5.2, the low molecular weight materials from the earthworm and the fathead minnow are very similar. Figures 5.3 and 5.4 show very similar chromatograms for the low molecular weight materials from each prey. All of the materials from each extract eluted between the 1,350 molecular weight marker and the void volume of the column. Bio-Gel P-2 resin fractionates globular proteins of molecular weight 100 to 1,800 daltons[28]. If it is assumed that the upper limit of fractionation for the resin under these separation conditions is 1,800 daltons, the estimated molecular weight range for the low molecular weight materials from both prey is between 1,350 and 1,800 daltons.

Sugar Analysis

Analysis was conducted for the sugar composition in the high and low molecular weight materials from both the earthworm and fathead minnow CM extractions. When silica thin layer chromatography was conducted with 3:2 methanol:chloroform on the high and low molecular weight materials, the α-naphthol sulfuric acid spray indicated the presence of sugars. The sugars in

TABLE 5.1

HIGH MOLECULAR WEIGHT CALIBRATION DATA

Sample	Total Amount Injected on Column	Peaks Obtained From Chromatogram				
Standard	4.5 mg					
Molecular Weight x 10^{-3}		670	158	44	17	1.35
Kav		0.00	0.31	0.86	0.97	
CMw-EW-HMW$_{P10}$		1.7 mg				
Molecular Weight Estimation x 10^{-3}		432				a
Kav		0.07				1.05
CMw-FM-HMW$_{P10}$		1.7 mg				
Molecular Weight Estimation x 10^{-3}		519		27		
Kav		0.02		0.90		

[a]Material partially adhered to the column which resulted in the inability to estimate molecular weight.

TABLE 5.2

LOW MOLECULAR WEIGHT CALIBRATION DATA

Sample	Total Amount Injected on Column	Peaks Obtained From Chromatogram				
Standards	4.5 mg					
Molecular Weight x 10^{-3}		670 158 44 17			1.35	
Kav		0.0			0.72	
Sodium Azide	1.7 mg					
Molecular Weight x 10^{-3}						0.65
Kav						0.83
CMw-EW-LMW$_{P10}$	3.0 mg					
Kav			0.06	0.28	0.56	
CMw-FM-LMW$_{P10}$	3.0 mg					
Kav			0.06	0.22	0.61	

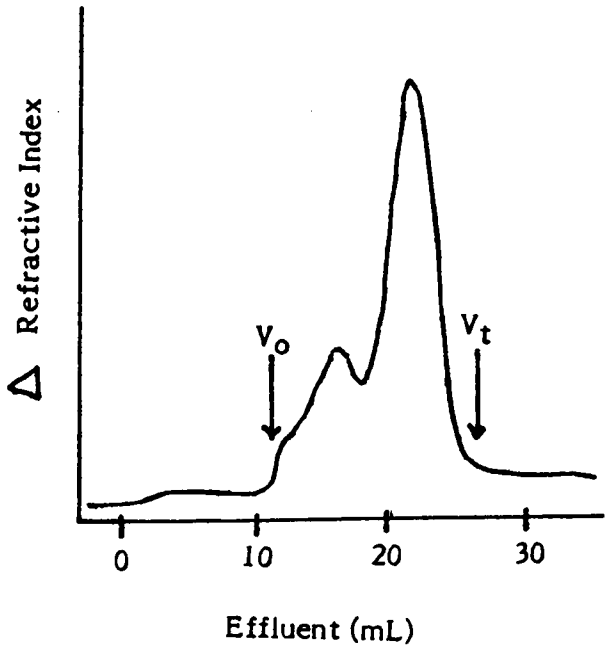


Figure 5.3. Chromatogram of CMw-EW-LMW$_{P10}$ from the P-2 column (1.0 cm x 29.2 cm) eluted with distilled water.

Abbreviations:

V_o - void volume of the column
v_t - column volume

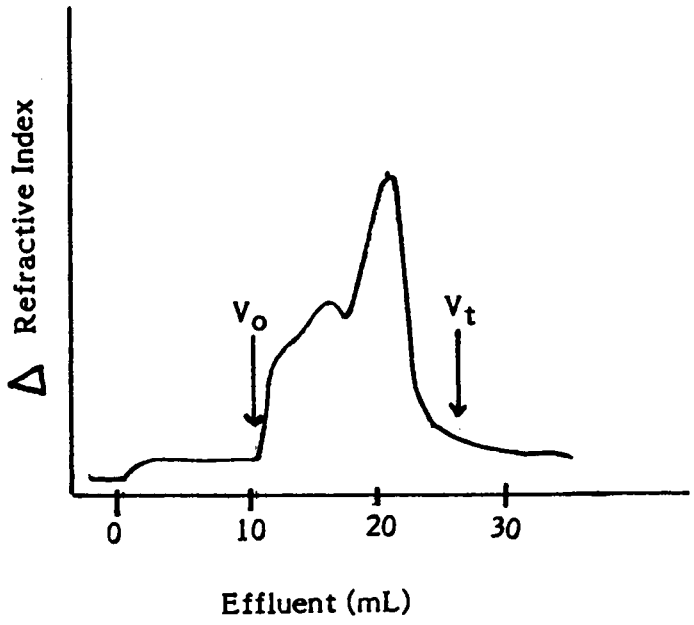


Figure 5.4. Chromatogram of CMw-FM-LMW$_{P10}$ from the P-2 column (1.0 cm x 29.2 cm) eluted with distilled H_2O.

Abbreviations:

V_o - void volume of the column
v_t - column volume

these materials were identified by comparing gas chromatography data of the acid hydrolized high and low molecular weight acetylated materials to known data of acetylated sugars. The identities of the sugars were also confirmed by mass spectroscopy data[33]. The percent sugars by mass of these materials could not be determined because the materials were very hygroscopic and could not be weighed accurately with available equipment.

The percent composition of sugars was estimated by comparing peak areas of gas chromatography data of known concentrations of acetylated sugars separated under the same conditions. Since known concentrations of derivatized fucose and arabinose were not obtained for concentration data, an integration constant was calculated from the average of (mg of an acetylated sugar)(peak area)$^{-1}$ from the acetylated sugars used as standards. This averaging was implemented since it was assumed that all acetylated sugars should result in the same relative response by the GC detector. In the case of inositol, two inositol isomers were found in the low molecular weight materials of the earthworm. Both isomers resulted in the same mass spectroscopy data but different retention times by the GC[40]. One isomer of inositol was determined to be myo-inositol since it had the same retention time as a myo-inositol standard. The integration constant was assumed to be the same for both isomers.

As seen in Table 5.3, the percent composition of sugars is shown for the high and low molecular weight materials from both prey. All four samples contain relatively high glucose, fucose, galactose and mannose concentrations. Myo-inositol was found in each sample. The low molecular weight materials of the

TABLE 5.3

PERCENT COMPOSITION OF SUGARS IN THE HIGH AND LOW MOLECULAR WEIGHT FRACTIONS FROM CMw

Sample	Arabinose	Fucose	Galactose	Glucose	Myo-Inositol	Unknown Inositol	Mannose	Ribose	Xlyose
CMw-EW-HMW$_{P10}$	2.0%	19.6%	39.2%	5.9%	1.0%	0.5%	19.6%	(a)	9.8%
CMw-EW-LMW$_{P10}$	0.9%	1.6%	6.9%	13.8%	46.2%	23.1%	4.6%	2.3%	1.4%
CMw-FM-HMW$_{P10}$	1.8%	16.4%	61.3%	12.3%	4.1%	0.8%	2.0%	2.0%	0.8%
CMw-FM-LMW$_{P10}$	2.6%	5.9%	5.3%	39.5%	19.7%	2.6%	3.3%	26.3%	2.6%

[a]The standard mass spectra for the acetalated ribose does not match this compounds mass spectra but results in a peak at the same GC retention time as other ribose derivatives.

earthworm had two isomers of inositol which comprised of a large portion of the sugar concentration.

The major sugar components of the high molecular weight materials and the minor components of the low molecular weight materials for both prey were galactose and fucose. If it is assumed that the activity of each sample is correlated to the sugar composition, galactose and fucose oligomers may be of interest to study. As noted in Table 3.9 (page 47), Table 3.10 (page 48), and Table 4.6 (page 57), the high molecular weight samples are significantly more active than the corresponding low molecular weight samples. Both galactose and fucose oligomers are present in greater amount in the high molecular weight than in the low molecular weight materials.

In comparing the sugar analysis of the CMw extraction to the sugar analysis conducted by Muir and Lee[30] of the earthworm cuticle (Table 5.3 and Table 3.1 (page 34), galactose and xylose were major sugar materials in the high molecular weight materials of the CMw extraction and the earthworm cuticle. Since many other sugars are present in the CMw extraction for the earthworm than found in the earthworm cuticle, it could be concluded that the materials obtained from the CM extraction are different than the earthworm cuticle or the CM extraction extracted other sugars from the earthworm tissue in addition to the ones found in the cuticle.

Amino Acid Analysis

Amino acid analysis was conducted on the high and low molecular weight materials from the water layer of the chloroform:methanol extractions from the

earthworm and fathead minnow. The results of the analysis are shown in Table 5.4 along with the amino acid composition of glycopeptides and collagen obtained from the earthworm cuticle determined by Muir and Lee[30]. The high and low molecular weight materials from the earthworm and fathead minnow had similar amino acid compositions when they are compared with Muir and Lee's work. Five of the eight most common amino acids found in the high and low molecular weight fractions from the earthworm and fathead minnow are also the eight most common amino acids found in the earthworm cuticle collagen and cuticle glycopeptide. These amino acids are: serine, glutamic acid, glycine, alanine and leucine. However, the earthworm collagen and glycopeptide do possess a higher content of glycine and aspartic acid and lower lysine and phenylalianine content. All of the amino acids determined in CMw-EW-HMWp_{10} fall within four percent of the concentration of the same amino acids found in CMw-FM-HMWp_{10}. It was noted that the eight most common amino acids were the same for the earthworm and fathead minnow high molecular weight materials. A difference between the earthworm and fathead minnow was that the earthworm materials contained a higher serine concentration than the fathead minnow materials. Since the earthworm and fathead minnow materials were mixtures as shown by molecular weight estimates studies, it could not be determined if the active material was a collagen or a glycopeptide.

Nuclear Magnetic Resonance

Carbon and proton magnetic resonance spectra were obtained for the low molecular weight materials obtained from the CM extraction. The carbon and

TABLE 5.4

PERCENT TOTAL OF AMINO ACID RESIDUES[a]

Amino Acid	Earthworm Cuticle Glycopeptide[30]	Earthworm Cuticle Collagen[30]	CMw-EW-HMW$_{P10}$	CMw-EW-LMW$_{P10}$	CMw-FM-HMW$_{P10}$	CMw-FM-LMW$_{P10}$
Lysine	trace	2.1	**6.8**	2.1	**10.0**	**3.2**
Histidine	0	0.1	1.1	2.3	0.8	**2.7**
Arginine	0	2.3	3.9	1.3	2.1	**59.3**
Aspartic Acid	**8.8**	**6.1**	4.3	**4.1**	3.2	0.4
Hydroxyproline	**9.7**	**15.9**	(b)	(b)	(b)	(b)
Threonine	**9.8**	**5.2**	**7.7**	**4.5**	**8.1**	1.6
Serine	**11.4**	**8.9**	**11.0**	**25.0**	**6.7**	**2.1**
Glutamic Acid	**6.0**	**7.5**	**8.2**	**9.7**	**8.5**	**6.7**
Proline	0.9	1.0	6.5	2.6	3.0	1.4
Glycine	**38.2**	**32.0**	**11.3**	**16.9**	**12.8**	**11.2**
Alanine	**12.0**	**9.8**	**11.6**	**17.6**	**12.5**	**5.0**
Valine	1.0	2.5	5.5	**3.4**	4.5	1.4
Methionine	0.0	trace	0.2	0.7	0.6	0.2
Isoleucine	1.5	2.1	4.2	2.1	5.8	0.9
Leucine	**2.3**	**3.0**	**8.8**	**3.3**	**12.9**	**2.1**
Tyrosine	0.0	0.4	2.7	2.1	1.5	0.5
Phenylalanine	trace	0.9	**5.8**	1.9	**6.8**	0.9
Cysteine	(b)	(b)	0.5	0.2	0.2	0.2

[a]The numbers in bold print represent the eight most common amino acids found in the mixture.

[b]Not determined.

proton magnetic spectra for the earthworm are shown in Figures 5.5 and 5.6. The carbon and proton magnetic spectra for the fathead minnow low molecular weight materials are shown in Figures 5.7 and 5.8. As seen from these spectra both fractions indicate a high carbohydrate content by the regions from 45 to 100 ppm and 170 to 200 ppm in the carbon spectra and the regions 2.7 and 4.3 ppm in the proton spectra. If one integrates the area of the peaks in the carbon spectra from 45 to 100 ppm and divides this area by the total area from 10 to 100 ppm, the percent carbohydrates are estimated to be 52.3% for the earthworm and 60.5% for the fathead minnow. Both of these fractions indicate a high carbohydrate content which explains the hydroscopic nature of these materials.

The high molecular weight materials from each prey did not result in a clear proton or carbon magnetic resonance spectra due to the high molecular weight nature of these materials.

C. CONCLUSION

The active material from the earthworm (Lumbricus terrestris) and fathead minnow (Pimephales promelas), have been isolated through the AQ and CM extraction procedures. The active materials isolated by these extraction procedures exhibit similar chemical properties through: NMR, amino acid analysis, sugar analysis, and gel filtration data.

The active materials isolated from the earthworm through the CM extraction method also exhibited similar chemical and physical properties to active earthworm material isolated by studies of Sheffield, Law, and Burghardt[12]

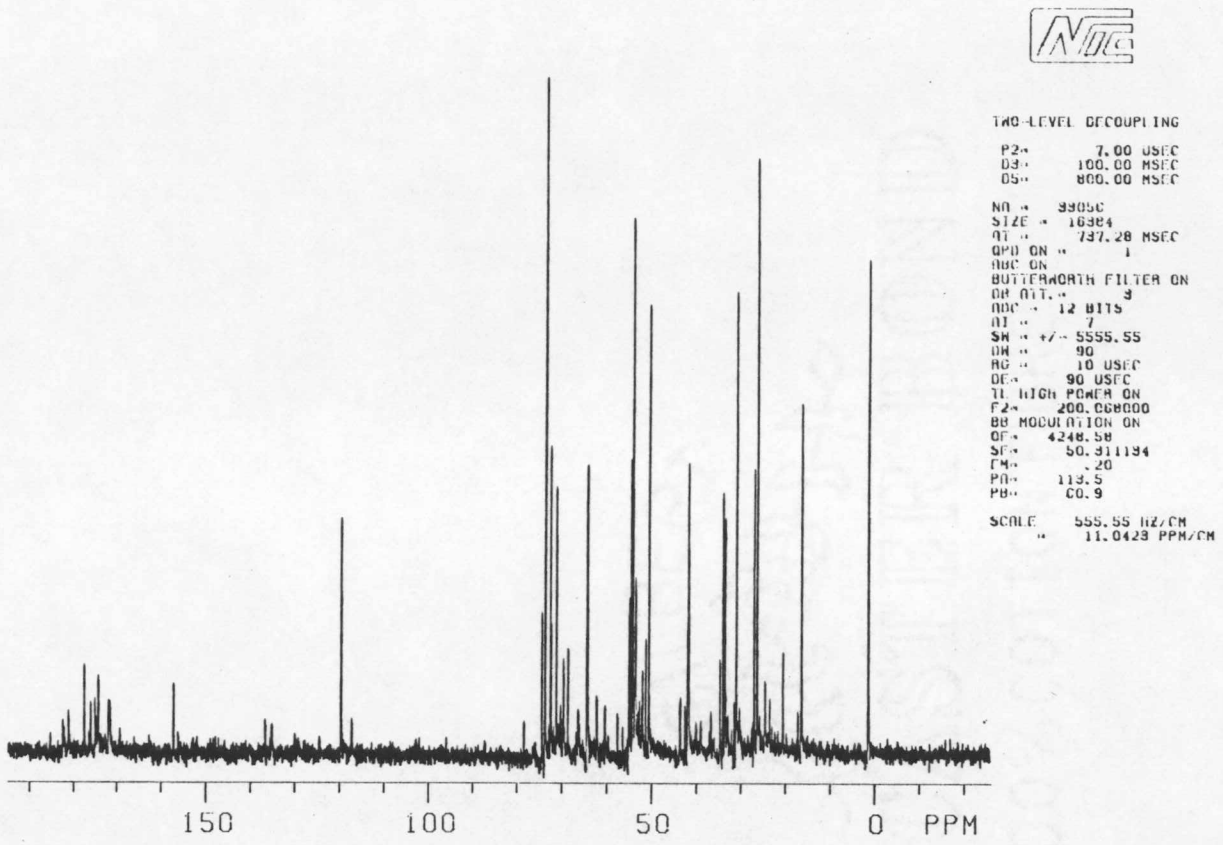


Figure 5.5. Carbon Magnetic Resonance Spectra of CMw-EW-LMW$_{P10}$.

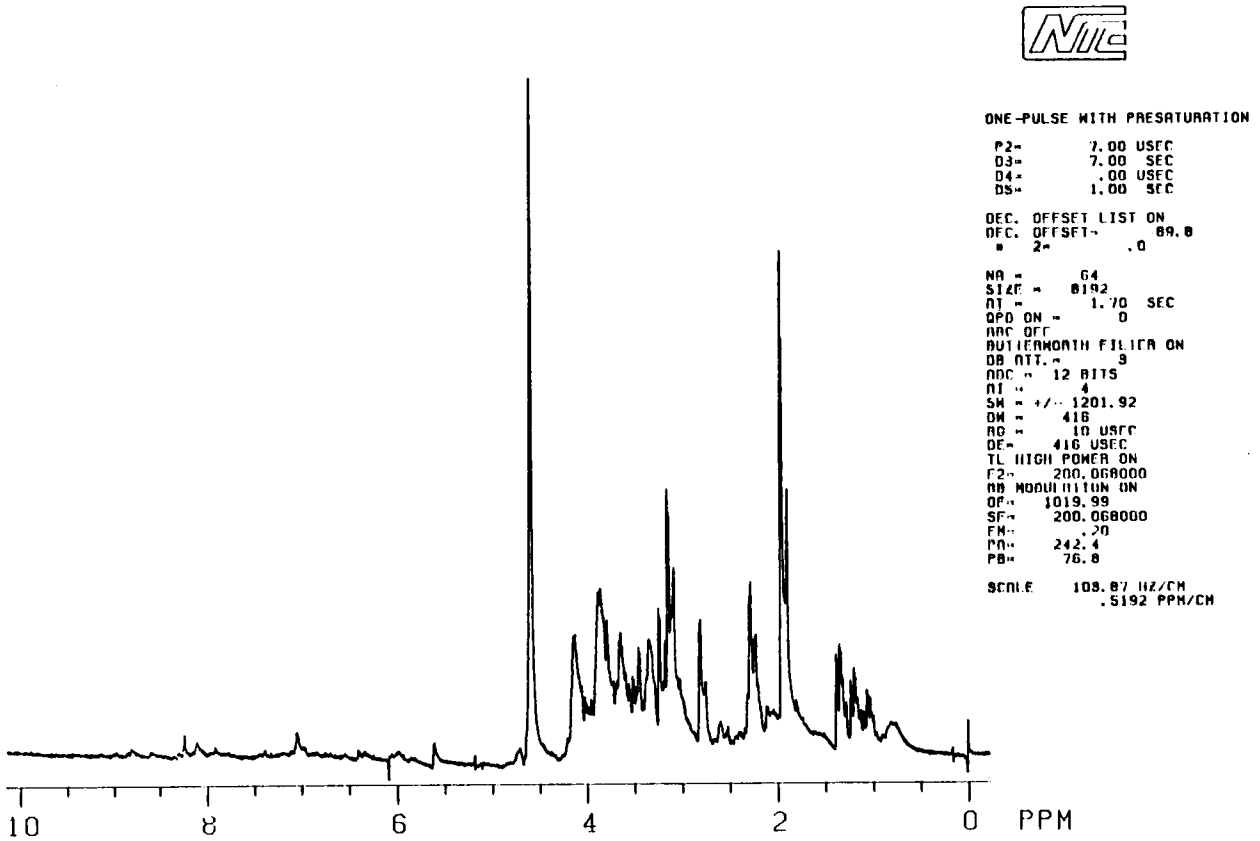


Figure 5.6. Proton Magnet Resonance Spectra of CMw-EW-LMW$_{P10}$.

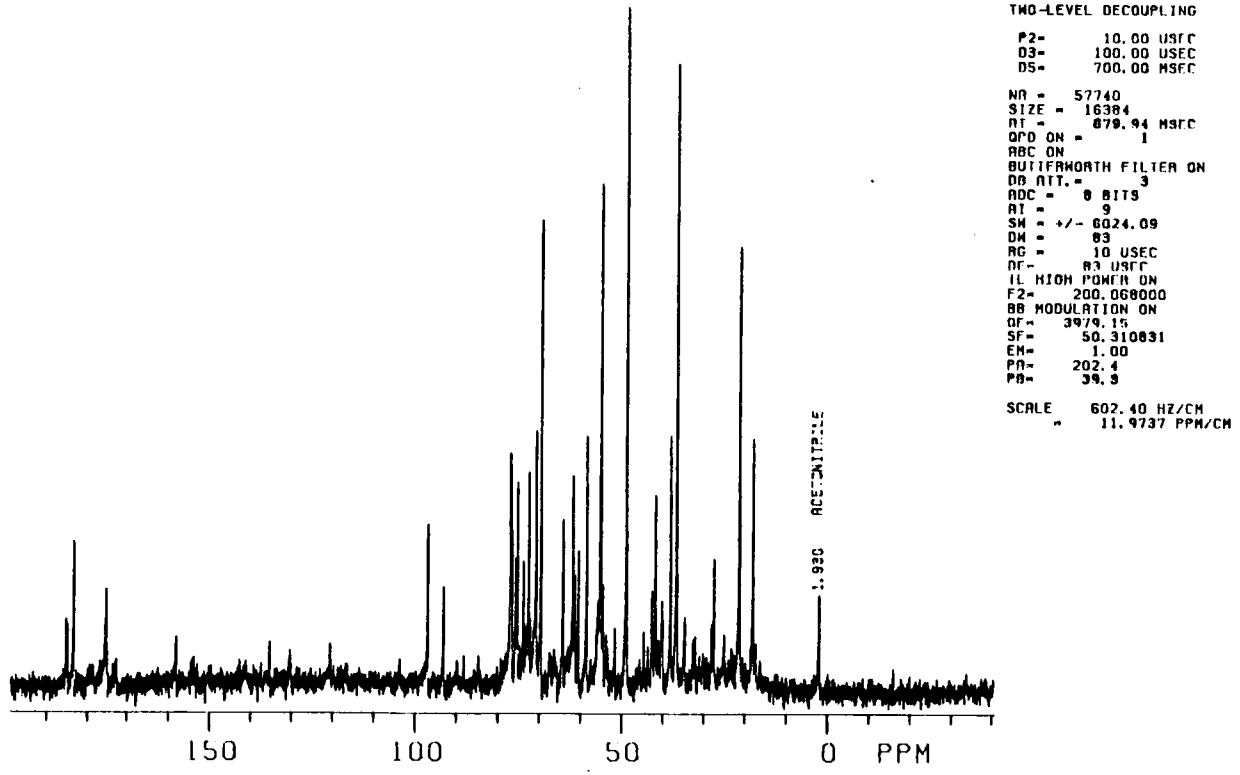


Figure 5.7. Carbon Magnetic Resonance Spectra of CMw-FM-LMW$_{P10}$.

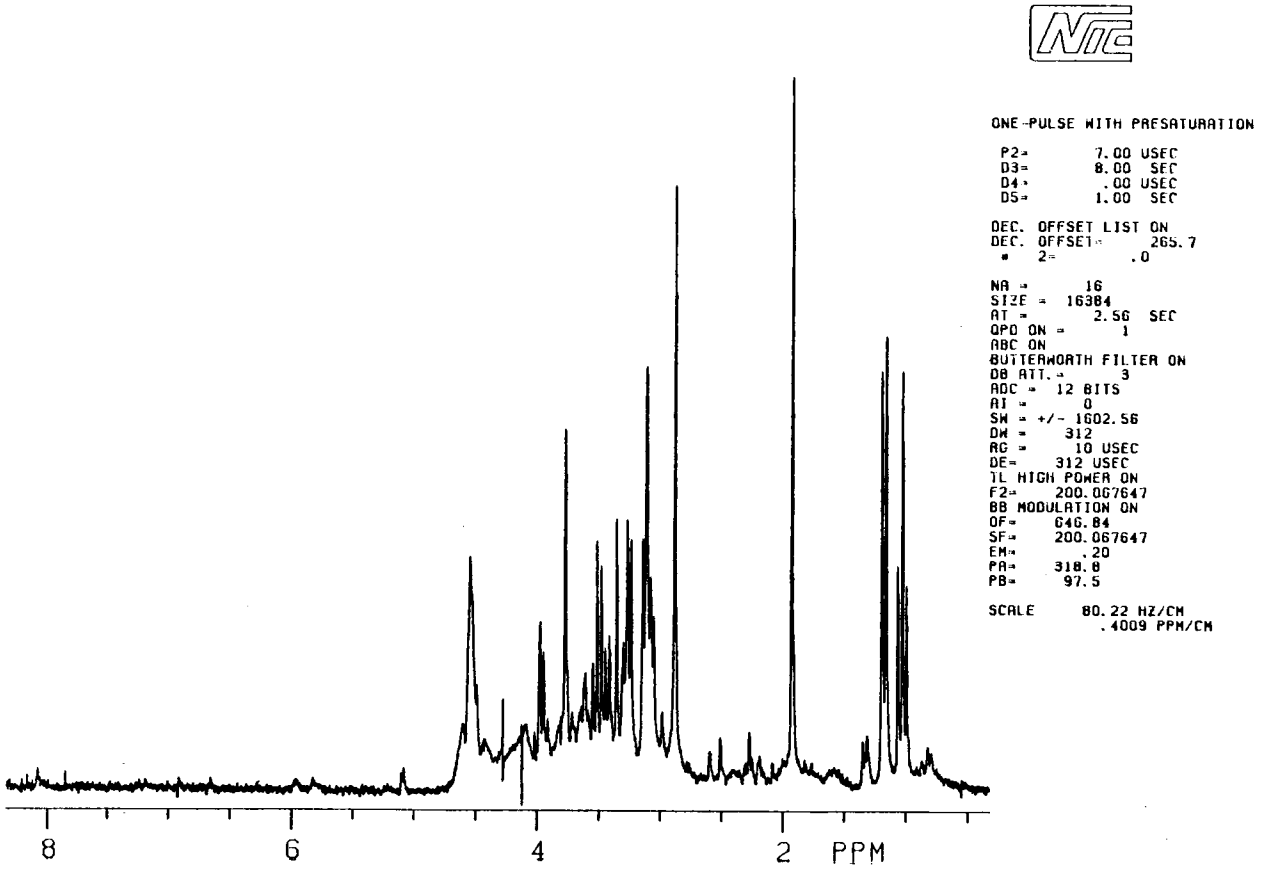


Figure 5.8. Proton Magnetic Resonance Spectra of CMw-FM-LMW$_{P10}$.

and of Halpern's laboratory[21,29]. According to Sheffield, Law, and Burghardt[12], the water extract of the prey at 60°C resulted in two active fractions when fractioned by a Sephedex G-200 column. These two fractions were estimated to have a molecular weight in excess of 5,000 daltons. Halpern's laboratory[29] also isolated an active earthworm material and determined the material to be greater than 67,000 daltons. In comparison, the work of this thesis determined two active fractions from the earthworm material. One was approximately 432,000 daltons, and one was less than 1,800 daltons. The high molecular weight material seem to be in agreement with the Sheffield, Law, and Burghardt and Halpern's laboratory studies. The low molecular weight fraction was possibly noted in the Sheffield, Law and Burghardt[12] papers but was not noted in the Halpern's laboratory studies[29].

Halpern's laboratory may not have found this active low molecular weight fraction since the bioassay method utilized by that research group deals strictly with tongue flick data. In comparison, Burghardt's bioassay method uses tongue flicking and prey attack data. Perhaps the bioassay procedure used may lead to different conclusions as to what materials are active. This would explain why Halpern's laboratory did not detect the active low molecular weight region if it was only detectable by prey attack data.

Halpern's laboratory studies noted that activity of the earthworm material seemed to diminish with the decreace of the carbohydrate content of the material[29]. It is of interest to note that both active fractions of CMw-EW-HMW$_{P10}$ and of CMw-EW-LMW$_{P10}$ contained a very high carbohydrate

content. This supports Halpern's laboratory theory that carbohydrates may be related to the activity of the material. In the same paper, Halpern noted that the active material resulted in a positive assay for proteins[29]. The studies reported in this thesis showed that both active fractions obtained from the CM extraction of the earthworm indicate the presence of proteins by the amino acid analysis.

The exact identity and total isolation of the active material from the earthworm (Lumbricus terrestris) and fathead minnow (Pimephales promelas) was not achieved. However, other methods have been discovered which extract the active material from the prey through the CM extraction, the soxhlet extraction, and the methanol extraction of the lyophilized water extract. It is the hope of the author that this study may be of help to others attempting to identify and isolate the active material of garter snake prey.

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VITA

Scot E Goss was born in Dayton, Ohio on January 16, 1961. He lived and attended school in Dayton until he graduated from Centerville High School in May 1979. The following August he enrolled at Eastern Kentucky University and graduated May 1983 with a Bachelor of Science in Chemistry. In the fall of 1983 he enrolled in the graduate chemistry program at The University of Tennessee, Knoxville. While attending, he was awarded a teaching and research assistantship with the chemistry and psychology departments. He was awarded a Master's of Science degree in Chemistry in March 1986.