TOXIC MATERIAL FROM THE PUSS CATERPILLAR, *LAGOA CRISPATA*

By

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TOXIC MATERIAL FROM THE PUSS CATERPILLAR,

LAGOA CRISPATA

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LIST OF SYMBOLS AND ABBREVIATIONS

A_{280}/A_{260}	- absorbance at 280 and 260 nm.
ATP	- adenosine 5'-triphosphate
BSA	- bovine serum albumin
DNP	- dinitrophenyl
GABA	- γ-amino butyric acid
GTP	- guanosine 5'-triphosphate
TLC	- thin layer chromatography

CHAPTER I

INTRODUCTION

It is well accepted that caterpillars often defoliate and devour tremendous amounts of vegetation. However, Gilmer (1) notes that some species possess another deleterious feature--urticating spines--which may, on occasion, constitute a health menace to man.

Although urticating caterpillars are distributed worldwide, according to Foot (2) the most offensive species inhabit South America. McMillan and Purcell (3) state that at least eight species of urticating caterpillars native to North America are relative common--among these are the puss caterpillars, Megalopygidae opercularis (Sm. & Abb.) and Lagoa crispata (Packard).

In general appearance these two caterpillars are practically indistinguishable to an inexperienced observer. Figure 1 shows a typical mature larva which is approximately 3 cm. in length and is covered with tufts of brownish-orange hair. Davidson (4) mentions that M. opercularis inflicts the severest sting of any lepidopterous larva indigenous to the United States, and although slightly less pronounced, the same symptoms are elicited by the sting of L. crispata, the crinkled flannel moth. In this country

M. opercularis occurs abundantly only in Texas, although it is found casually in the Southern states according to Foot (2). McMillan and Purcell (3) report a wider area of relative abundance for L. crispata, extending into the Northeast as well as the South.

Normally the population of puss caterpillars in any one area is not sufficiently large to constitute a serious danger, but when the occasional rangeland outbreaks of these larvae occur, urban areas may also become infested. Heavy infestations have forced the closing of public schools in San Antonio, Texas and reached near epidemic proportions in North Carolina (3). The larvae feed on shin oak--however, during high population years, hardwood trees or ornamental host types may be infested. The presence of the caterpillars on these plants contributes to the medical problem due to the greater number of human-caterpillar contacts made in populated areas. McMillan and Purcell (3) report that most of the patients in North Carolina receiving medical treatment for caterpillar stings have been observed upon the opening of school in September, when the concentration of unsuspecting tree-climbing children and completion of larval development in puss caterpillars may combine to create a serious health problem.

The ornamental hairs and urticating spines of L. crispata are arranged in clusters attached to ridges called verrucae. These cover the entire dorsal and lateral surfaces of the caterpillar. When alarmed, the larvae curl

into a ball leaving only spine-covered surfaces exposed. A study of the histology and cytology of the urticating structures of L. crispata by Lamdin (5) reveals the morphology of the poison apparatus. The hollow, chitinous spines (1 mm. or less in length) are connected to a bulb-like structure. A tube proceeds from this bulb, through the cuticle and and epidermis, and extends to numerous unicellular glands located within the body of the caterpillar. This region also contains nerve and muscle tissue which appears to function with the poison apparatus. These glands appear to be the site of toxin synthesis. Lamdin (5) reports that the cytoplasm of the glands contains large amounts of granular endoplasmic reticulum and large golgi regions indicative of protein secretion.

When a foreign body makes contact with the caterpillar the spines penetrate and break off, and it has been speculated by Lamdin (5) that the hydrostatic pressure resulting from the larva curling itself up forces the toxic material up through the tube and the broken spine tip into the victim. The severity of the sting varies with its location and the thickness of the skin affected. The urticating spines are unable to penetrate areas of relatively tough skin, such as calloused fingertips, but have little trouble penetrating such relatively thin-skinned areas as the flexor surface of the forearm. There is at first a localized painful area which stings from the initial contact with the spines, then burns rather intensely. Symptoms

vary somewhat depending upon the susceptibility of the individual to insect bites and/or stings in general, but in most cases, small vesicles then appear--whitish spots on a reddish area with slight swelling. Foot (2) reports that there may be generalized symptoms of numbness in the area affected. In children (or with multiple contacts in adults) there may be 1-5^{o} of fever, throbbing pain, extreme restlessness, and mild spasms. Most of these symptoms dissipate within a few hours, but an intense itching in the affected area may persist periodically for several days.

McMillan and Purcell (3) indicate the usual treatment for puss caterpillar stings to be a combination of analgesics and diphenhydramine hydrochloride (Benadryl), and Arena (6) reports some success with intravenous administration of calcium gluconate.

Attempts have been made to resolve the question of whether a toxin is produced by urticating caterpillars or are the observed symptoms induced by the mechanical action of spine penetration into the victim. In his preliminary investigation of M. operacularis Foot (2) demonstrates that boiling the caterpillar, after killing it, inactivates the ability of the spines to elicit the irritating symptoms. He also reports success in obtaining a distilled water extract from the hair and spines which, upon inoculation into human skin, elicits the same responses as the live caterpillar. Although the small amount of material obtained in this manner limited his investigation somewhat,

he concludes the existence of a venom, probably proteinaceous in nature. Valle, et al. (7) have speculated that the cutaneous reactions following contact with urticating caterpillars might be due to a combination of acetylcholine and histamine. In the genus Megalopyge, however, they were unable to detect acetylcholine and found very low levels of histamine.

Although Packard (8) and Gilmer (1) conducted histological studies on L. crispata, very little has been published on this species in the last 50 years. L. crispata is found in limited numbers throughout Oklahoma, and particularly large populations are found on the shinnery oak of the Francis Davidson ranch in Ellis County. During a trip to this area hundreds of caterpillars were seen in a stand of shinnery oak no more than 50 feet in diameter.

This afforded the opportunity to collect sufficient numbers of the larvae to conduct an investigation. The present work was initiated to determine whether the irritation caused by contact with L. crispata is induced mechanically or chemically, and, if chemically activated, to investigate the nature of the material responsible for the observed symptoms.

CHAPTER II

METHODS AND MATERIALS

Caterpillars

During the last week of September and the first week of October, 1975, approximately 7,000 specimens of sixth instar Lagoa crispata larvae were collected on the Francis Davidson ranch in Ellis County, south of Arnett, Oklahoma. The larvae were carefully picked by hand from several small groves of shinnery oak, stored in paper cartons with a supply of oak leaves, and transported to the laboratory where they were immediately stored at 8°C. This slowed their metabolism to prevent excessive accumulation of fecal material in the cartons. Within 48 hours they were cleaned of fecal material and plant debris, sorted into groups of 75 larvae per carton, and stored at -10°C. for future use.

Extraction of the Larvae

Collection of material for extraction was accomplished by submerging the larvae into liquid nitrogen to freeze all body fluids and crispen the hair and spines which were then scraped into a tared beaker and weighed. This material was placed in a mortar, crispened once again with liquid

nitrogen, and ground to a fine powder. When this powder represented material from 75 caterpillars (the usual number extracted at one time), it was extracted three times with approximately 35 ml. of .02 M NH_4HCO_3 and vacuum filtered through Whatman no. 4 filter paper. The three extracts were pooled and filtered once more to clarify the solution. The protein content was estimated on the whole extract and column fractions by measuring the A_{280}/A_{260} on a Coleman 101 Perkin-Elmer spectrophotometer and utilizing a nomograph (9). Except where otherwise indicated, all absorbance measurements were made on this instrument. The whole extract was lyophilized and stored at -10°C. until needed.

Fractionation of the Extract by Gel Filtration

All packing materials for gel filtration columns were obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.). Sephadex G-25 (fine) was allowed to swell in water overnight at room temperature, then deaerated under a water aspirator for one hour. After silanizing the glass column (with dimethyl dichlorosilane), a 1 x 90 cm column was poured and allowed to pack by gravity flow. The column was moved to the cold room (8°C), equilibrated a flow rate of 6 ml/hr., and calibrated with a solution containing blue dextran and DNP-phenylalanine. Two ml. fractions were collected and A_{260} was determined on each fraction. A rough estimation of molecular weight range was obtained

by elution volume.

After equilibrating the G-25 column overnight with .02 M NH_4HCO_3, batches of whole extract (representing 75 caterpillars) were dissolved in 3 ml. of the .02 M NH_4HCO_3, applied to the column, and allowed to flow through at a rate of 6 ml/hr., while 2 ml. fractions were collected. The presence of material such as aromatic compounds, proteins, peptides, nucleotides, etc. in the fractions was monitored by A_{280}/A_{260} measurements and fractions comprising individual peaks in the elution profile were pooled, lyophilized and stored at -10°C until needed for further study.

Fractionation was continued by applying the material from each of the G-25 column peaks to one of another three Sephadex columns which were prepared in the same manner: a 1 x 85 cm G-15 column (flow rate--7 ml/hr.), a 1 x 80 cm G-50 column (flow rate--6.5 ml/hr.), and a 1.5 x 95 cm G-100 column (flow rate--4 ml/hr.). Again the elution profile was monitored by A_{280}/A_{260} determination and all columns were operated in the cold room to retard thermal decomposition of the extracted material. The data from the column runs was summarized as total protein and % of protein in the extract for recovery.

Protein Measurements

To obtain a more accurate determination of protein content in the whole extract and resulting fractions than

could be obtained by simple A_{280}/A_{260} measurements, the modified Lowry procedure of Hartree (10) was employed. Measurements of A_{650} for this method were made on a Beckman DU spectrophotometer. Determination of A_{280}/A_{260} were also made on all solutions analyzed by this procedure so that values obtained by the two methods could be compared.

Bioassay for Toxic Activity

Charles River CD-1 (outbred albino) mice were used to monitor the toxic activity of the caterpillars. After shaving a spot approximately three cm in diameter on the back of the mice, live caterpillars and frozen larvae were applied to the spot. In order to check the effects of the whole extract and the resulting fractions a syringe tip was scraped over the shaven spot to allow penetration of the applied material beneath the mice's epidermis. Following administration of live caterpillars, frozen caterpillars, whole extract, heated extract (100°C for 10 min.), and the various fractions of the whole extract, the animals were observed for 48 hours and the resulting symptoms were noted.

A series of mice were also injected intraperitoneally with whole extract and observed for 24 hours.

Attempts were made to utilize rabbits and Holtzman rats, but both proved to be unsuitable as test subjects since neither gave an easily observed response to the caterpillars. An LD_{50} value was not determined because,

as far as is known, the caterpillars do not use their toxin to kill other species, and injection of the toxic material from approximately 25 caterpillars was not sufficient to kill a 30g. mouse.

Enzyme Assays

The whole extract from L. crispata was assayed for enzyme activities associated with other venoms and toxins as reported by Geren (11), Jacques (12), and Goldman (13).

Hyaluronidase activity was assayed according to the method of Tolksdorf, et al. (14). This procedure is based on the fact that hyaluronic acid forms turbidity with an acid albumin solution. Since the turbidity is a function of hyaluronic acid concentration, enzyme activity was determined by the decrease in turbidity (measured as A_{540} on a Coleman 124 Hitachie Perkin-Elmer double beam spectrophotometer) of a solution of known hyaluronic acid content following incubation at 37°C with the whole extract. Hyaluronic acid from human umbilical cord (grade 1) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and hyaluronidase from Worthington Biochemical Corp. (Freehold, N.J.). Activity was measured as mg hyaluronic acid digested/mg extract protein/min.

Phosphohydrolase (ATPase) activity was assayed according to the method of Marsh (15). In this procedure enzyme activity was determined by measuring the increase in phosphate concentration released by hydrolytic action of the

enzyme upon the substrate, ATP. The inorganic phosphate was converted to phosphomolybdic acid, monitored by A_{310} measurements. Whole extract, heat denatured extract (100°C for 10 min.), and a blank solution were incubated at 37°C with ATP for 30 min. and 90 min. Activity is measured as moles of inorganic phosphate/mg protein in extract/min.

Protease activity in the extract was examined by a method modified by Geren (11). Following incubation of a protease with the protein substrate, the increase in A_{280} (due to release of aromatic amino acid residues) is measured after the remaining protein is precipitated with 10% trichloroacetic acid. Whole extract and trypsin (B grade, from Calbiochem, Los Angeles, Ca.) were incubated at 36°C for 30 min. and 4 hr. with two substrates, 1% bovine serum albumin and 0.5% casein in 0.1 M phosphate buffer (pH 7.6). Activity is expressed as micromoles of substrate hydrolyzed/mg of extract protein/min.

Thin-Layer Chromatography for Amino Acids

In order to examine the presence of amino acids in the extracted material, thin-layer plates of 0.5 mm thickness were prepared according to Stahl (16). Ten g. of MN 300 Cellulose (Macherey, Nagel, and Co., Germany), four g. of Silica Gel G (Brinkmann and Co., Westbury, N.Y.) and 80 ml. of distilled water were blended for 30 sec. in a Waring blender and the resulting uniform suspension was

spread over cleaned glass plates (20 x 20 cm) with a commercial TLC spreading apparatus (Brinkmann and Co.). The plates were allowed to air dry and were activated in an oven at 110°C for 2 hrs. prior to use.

Aliquots of standard amino acids and samples of caterpillar extract were applied to the plates with Pasteur pipette tips which had been drawn out to very small apertures over a Bunsen burner to prevent excessive spreading of material during the spotting of the plates.

Two solvent systems were utilized in this one-dimensional TLC work. One plate was developed in phenol: water (80:20, w/w) for four hrs. and then dried overnight at 35-40°C in insure adequate removal of phenol. A second plate was developed in the top phase of a butanol--96% acetic acid-water mixture (5:1:4, v/v/v) for three hrs. and air dried for two hours.

Amino acids were detected by spraying the plates with 0.5% ninhydrin in butanol, air drying the plates in a hood, and heating them at 110°C for 5-10 minutes.

Further Amino Acid Analysis

Dr. Ta-Hsiu Liao, adapting the basic design of Spackman, Stein, and Moore (17), constructed an automated microanalyzer that uses narrow bore columns and an expanded-scale recorder for measuring amino acids at the nanomole levels. This instrument was utilized to obtain complete analysis for free amino acids in the ninhydrin-positive

fractions of the extract.

Additional Thin-Layer Chromatography

Utilizing the same type of plates and solvent systems as were used for amino acids, the ninhydrin-positive components of the material extracted from the caterpillars were examined by thin-layer chromatography for the presence of such physiologically active compounds as histamine, tryptamine, 5-hydroxytryptamine (serotonin), and GABA. Aliquots of standard solutions and extract were spotted, developed, and detected as before.

Disc Gel Electrophoresis

In order to obtain more information concerning the protein content of the caterpillar extract, the Canalco system of disc gel electrophoresis was utilized. All reagents were obtained from Canalco (Rockville, Md.)--acrylamide, N,N'--methylenebisacrylamide (Bis), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, riboflavin, 2-amino-2 hydroxymethyl-1,3 propanediol (Tris), ammonia-free glycine, bromophenol blue (tracking dye), and column coat solution. The gels, pH 8.9, were 7% acrylamide and five mm in diameter. The stacking gels measured 1.5 cm, while the separating gels were ten cm in length. The samples contained tracking dye and 10% sucrose. The upper and lower reservoirs of the electrophoresis chamber contained Tris-glycine buffer (pH 8.3). A Heathkit Regulated

Power Supply (Model 1P-32, from Heath Co., Benton Harbor, Mich.) provided two mA/gel until the samples formed discrete discs within the stacking gels, at which time the current was increased to four mA/gel. The current remained at this level until the tracking dye was one-two cm from the bottom of the gels (approximately two hrs.) and the protein bands were visualized by staining with coomassie blue. A photograph of the gels was taken to record the number of bands and their migration distance.

CHAPTER III

RESULTS AND DISCUSSION

Investigators have employed various solvents in attempts to extract toxic material from the spines of other urticating caterpillars--water, saline, glycerin, ether, chloroform, toluol, and ethanol--but success has been reported only with water, saline, and glycerin (2, 13, 19).

The major problem has been to effectively extract as much toxic material as possible without collecting extraneous substances. Attempts were made to do this by rolling intact larvae on filter paper in hopes of adsorbing/absorbing enough of the liquid from within the spines to investigate its nature, but this technique proved to be completely ineffective.

Our method of "shaving" the caterpillars after freezing them in liquid nitrogen proved much more satisfactory. Examination of the larvae under a stereomicroscope demonstrated that virtually all of the hair and spines, and very little else, was collected in this manner.

Batches of 75 caterpillars yielded 400-500 mg. of hair and spines which gave 15-30 mg. of protein when extracted with .02 M NH_4HCO_3 and filtered through Whatman no. 4 paper. In order to determine the pH of the toxic material

one batch of caterpillars was extracted with distilled water and gave a pH of 6.8.

Observations of the effects elicited by multiple contacts with L. crispata were made during field collection of the larvae. Due to the clustered arrangement of the larvae on the shin oak and our desire to prevent spine breakage during collection, rubber examination gloves proved to be either too thin to prevent spine penetration or too bulky to be effective. Consequently, the caterpillars were picked by bare hand--as long as contact was restricted to the fingertips, no effects were felt. However, during the course of several hours in the field a half dozen or so caterpillars were brushed across the tops of the hands. Within a few minutes following contact the hands began to ache, and the pain increased with each new contact. For one person, by the end of the day both hands were throbbing, accompanied by a fever of 101^{o}F and a generalized body ache. Other than the pain in the hands, the symptoms were not unlike those experienced during a siege of influenza. Administration of analgesics and submerging the hands in cold water gave some symptomatic relief, but the effects persisted for two or three days and were followed by several days of severe itching.

Contact with one larva (live or frozen) on the flexor surface of the forearm evokes an immediate stinging sensation (due to penetration by the spines), followed by localized burning and wheal formation at the site of

contact. These symptoms persist for an hour or two. As the pain diminishes, an intense itching begins which continues periodically for several days. Contact with warm water in the area of the sting causes the itching to intensify, while cold packs give some relief.

When the whole caterpillars were tested on white mice a state of extreme restlessness and agitation was induced. The animals seemed greatly irritated and for the next 24 hours they scratched and gnawed at themselves, primarily near the site of application, until most of the animals had lesions such as that seen in Figure 2. Application of whole extract gave the same symptoms, somewhat less in intensity. This was expected since it was known that only the toxic material actually contained in the hollow spines was collected by the "shaving" procedure. Control animals which received application of only the ammonium bicarbonate solution or whole extract which had been heated to 100°C for ten minutes showed no effects.

Injection of whole extract (approximately five mg. protein in 0.5 ml ammonium bicarbonate) intraperitoneally into mice produced a roughening of the coat, profuse defecation, a swelling and watering of the eyes, and impaired use of the rear limbs. Similar effects were seen by Foot (2) when he injected mice intraperitoneally with his saline extract from M. opercularis, except that he reported death within an hour. The mice injected with extract from L. crispata recovered from their symptoms within 36 hours

and appeared to be completely normal. Control animals injected with BSA in ammonium bicarbonate showed no effects.

After demonstrating that the whole extract contained the toxic activity, fractionation of the extract on the Sephadex G-25 column gave the elution profile seen in Figure 3. In order of elution from the column, the first peak (pk. I) eluted in the same fractions as did blue dextran during the calibration run, indicating very high molecular weight material. The last peak (pk. III) eluted in the same fractions as did DNP-phenylalanine during the calibration run, indicating rather low molecular weight compounds. All fractions between the two major peaks were pooled and referred to as pk. II. On the column the brown-colored extract separated into three visually distinct bands: pk. I--brown, pk. II--orange, pk. III-yellow.

The pk. I material was run through a Sephadex G-100 column and gave the elution profile shown in Figure 4. The major peak (pk. IA) was the highest in molecular weight and was followed by four minor peaks (IB, IC, ID, and IE).

Resolution of the pk. II material on a Sephadex G-50 column gave one major and one minor peak as shown in Figure 5. Likewise, when run on a Sephadex G-15 column, the pk. III material gave one major and minor peak as shown in Figure 6.

The fractionated material from the G-25 column was tested for toxic activity by application on mice--activity was localized in pk. I. Over a period of several months,

although approximately the same amount of material was extracted, it was observed that the toxicity of the material freshly obtained from the frozen larvae diminished considerably. The loss in activity could have been due to an unfortunate loss of power (for several hours) to the freezer in which the larvae were stored, or it might be that the activity is not stable over long periods of time, even at -10°C. It was definitely noted that activity was not stable in solution at 8°C for more than a few days. These two factors made difficult the determination of which fraction(s) of pk. I was responsible for the toxicity since the two column runs necessary to obtain pk.s IA-IE required 80-90 hours for completion. When the five peaks from the G-100 column were applied to mice, activity appeared to be found only with pk. IA, although the effects elicited by this fraction were slight. When this test was repeated at a later date with material from another batch of 75 caterpillars, no response was seen with any of the fractions. After eight months storage of the larvae, even freshly prepared whole extract elicited no response in humans or mice, and application of the intact larvae gave no symptoms in humans other than the initial stinging due to spines penetrating the skin.

Demonstration of the toxicity localized in the high molecular weight fraction and the loss of toxicity after heating the whole extract at 100°C for ten minutes implied that the toxic material was proteinaceous. Although

measurement of A_{280}/A_{260} had been routinely used to obtain crude estimations of protein content, analysis by the modified Lowry procedure (10) revealed that the A_{280}/A_{260} method was subject to significant interferences due to non-protein substances in the extract. The whole extract contained six to seven times less protein than was indicated by A_{280}/A_{260} measurement and pk. I contained two or three times less.

During the extraction and fractionation of 1.813 g of hair and spines from a batch of 300 caterpillars, the protein content was monitored at each step (Table I). When the whole extract was lyophilized and redissolved in a small enough volume (3-5 ml.) for running on the G-25 column, the extract was not totally solubilized. Adjustment of pH did not increase solubility and attempts to increase the volume to a level sufficient to completely solubilize the extract resulted in a volume of sample that could not be well resolved on the column. Thus, while all of the protein eluted from the G-25 column was found in pk. I, only half of the protein in the extract was recovered. Half of the protein in pk. I was eluted in pk. IA from the G-100 column.

Goldman (13) claims to have detected "slight proteolytic activity" in a saline extract from the larval form of the Buck moth, Hemileuca maia (Drury), by placing a drop on a gelatin bath and observing a softening of the gel. In the case of L. crispata it is possible that

freezing and thawing denatured the enzyme or that conditions of storage and/or assay were not appropriate for the specificity of the enzyme, but no protease activity toward either casein or BSA was detected. Up to 100 ug of protein in the aliquots of whole extract were assayed and trypsin was utilized to insure that the method could measure protease activity with only a few ug of enzyme present in the incubation mixture.

Incubation of ATP alone, with whole extract, and with heat-treated extract revealed no detectable phosphohydrolase activity. Geren (1) found phosphohydrolase activity toward ATP and GTP in the venom of the brown recluse spider, Loxosceles reclusa (Gertsch & Mulaik), but there have been no reports of such activity in the toxins from urticating caterpillars.

Shanbacker, et al. (19) suggest that hyaluronidase may act as a spreading factor for other toxic components present in venoms, but no measurable amounts of hyaluronidase could be found in the whole extract of L. crispata. These results do not preclude the possibility of enzyme action as an explanation of the observed toxicity of the extract, but only serve to exclude these three enzyme activities, within the limits of our assay conditions.

Spotting aliquots of whole extract and the three fractions from the G-25 column on filter paper and spraying with ninhydrin showed that pk. II was ninhydrin-positive. Analysis with thin layer chromatography and the automated

microanalyzer indicated the presence of 13 amino acids--histidine, glutamic acid, methionine, isoleucine, threonine, alanine, valine, leucine, glycine, serine, aspartic acid, lysine, and phenylalanine--as well as other amino compounds.

Valle, et al. (7) report conspicuous amounts of histamine in the spines of caterpillars belonging to the genus Dirphia, but very little in the spines of larvae from the genus Megalopyge. They were unable to detect acetylcholine in the crude extracts from either genus. Further examination of pk. II with thin-layer chromatography to determine the nature of the other components failed to demonstrate the presence of histamine, tryptamine, serotonin, or γ-amino butyric acid.

Electrophoretic separation of the whole extract and the three peaks from the G-25 column (prepared from caterpillars which had been frozen for less than two months) indicated that at least eight different proteins were in the extract and that all of these were eluted in pk. I, as seen in Figure 7. A few months later, in hopes of determining which of the bands on the gels corresponded to pk. IA and with plans to conduct a preparative run using disc gel electrophoresis to obtain enough material to investigate which of the bands contained the toxic activity, another series of disc gel was initiated. Using the same experimental conditions as in the earlier electrophoretic work, whole extract, pk. I, and pk. IA were run. The

results indicated that rather dramatic changes in protein composition had occurred during storage. Only three protein bands were present and the stacking gel stained darkly, indicating that, in contrast to the previous gels, some of the protein had not even entered the separating gel. This correlated with the fact that, when tested on mice, the whole extract had lost its toxic activity. Either the protein components were not stable at -10°C or the power loss to the freezer allowed the caterpillars to thaw and at least some of the protein, including the one(s) responsible for the toxicity, were denatured.

Regardless of the cause, the alteration of protein composition and loss of activity in the whole extract made it impossible to determine which of the nine protein bands seen in the early disc gel study corresponded to pk. IA and which band(s) contained toxic activity. Thus, the preparative electrophoretic run was not attempted.

It may be that frozen storage of hair and spines (shaved immediately upon return to the laboratory) would be preferable to storage of intact larvae. Any further investigation to further purify and characterize the nature of the toxin of L. crispata must be conducted on freshly collected larvae which have been stored at sub-zero temperatures for as short a time period as possible.

CHAPTER IV

SUMMARY

Sixth instar larvae of L. crispata were "shaved" and the toxic material contained in the hollow spines was extracted with .02 M ammonium bicarbonate. In mice the extract elicited the same symptoms as the intact larvae, but heat-treated extract (100°C for 10 min.) gave no effects.

A Sephadex G-25 gel filtration column resolved the extract into three primary fractions: pk. I (high MW), pk. II (intermediate MW), and pk. III (low MW). Disc gel electrophoresis revealed nine protein bands in the extract and all of these were contained in pk. I. Toxicity was also localized in pk. I. A Sephadex G-100 column resolved pk. I into one major (pk. IA) and four minor components. Only pk. IA appeared to contain the toxic component(s).

Enzyme assays revealed no detectable amounts of protease, phosphohydrolase, or hyaluronidase activity in the extract. The pk. II was found to contain several free amino acids, but TLC indicated no histamine, tryptamine, serotonin, or γ-aminobutyric acid.

Even with the larvae stored at sub-zero temperatures (although a temporary power loss to the freezer may have allowed some thawing to occur), over a period of eight

months, the toxicity of freshly extracted material diminished until no activity could be observed. Concomitantly, alterations in protein composition occurred which resulted in only three protein bands appearing on disc gels.

The material responsible for the toxicity of L. crispata appears to be proteinaceous, but its mode of action remains unknown.

TABLE I

FRACTIONATION OF PROTEIN FROM THE EXTRACT OF LAGOA CRISPATA

	Total Protein Content mg	Protein obtained/ caterpillar ug	Recovery % of total protein in ext.
Whole extract	63.0	210	100
Pk. I	30.6	100	48
Pk. IA	15.0	50	24

Figure 1. A Sixth Instar Larva of the Puss Caterpillar, Lagoa crispata.

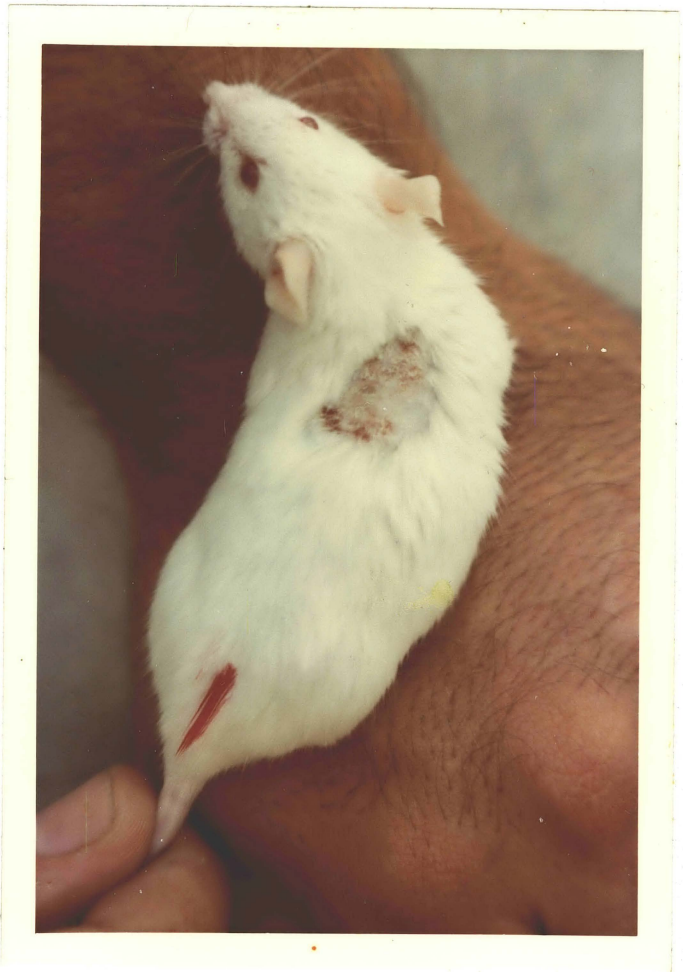


Figure 2. A White Mouse 24 Hrs. Following Administration of One Caterpillar.
Part of the lesion observed is due to the mouse chewing and scratching at the site of application.

Figure 3. Elution Profile of Whole Extract on a Sephadex G-25 Column.
The column was eluted with .02 M ammonium bicarbonate at a flow rate of 6 ml/hr. and 2 ml. fractions were collected. In order of elution, the peaks were referred to as pk. I, pk. II, and pk. III.

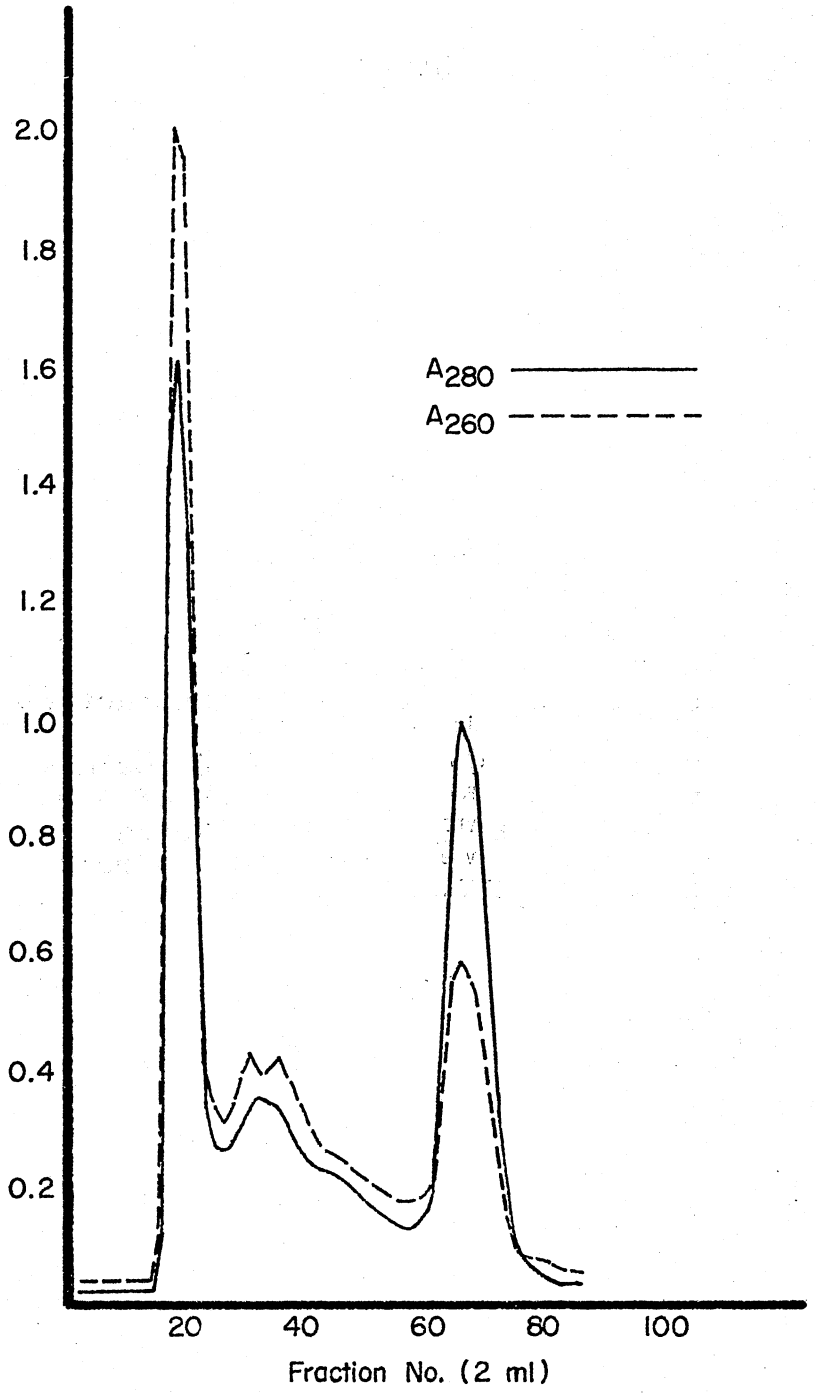

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A280
A260
20
40
60
80
100
Fraction No. (2 ml)

Figure 4. Elution Profile of pk. I on a Sephadex G-100 Column.
The column was eluted with .02 M ammonium bicarbonate at a flow rate of 4 ml/hr. and 1.5 ml fractions were collected. In order of elution, the peaks were referred to as pk. IA, pk. IB, pk. IC, pk. ID, and pk. IE.

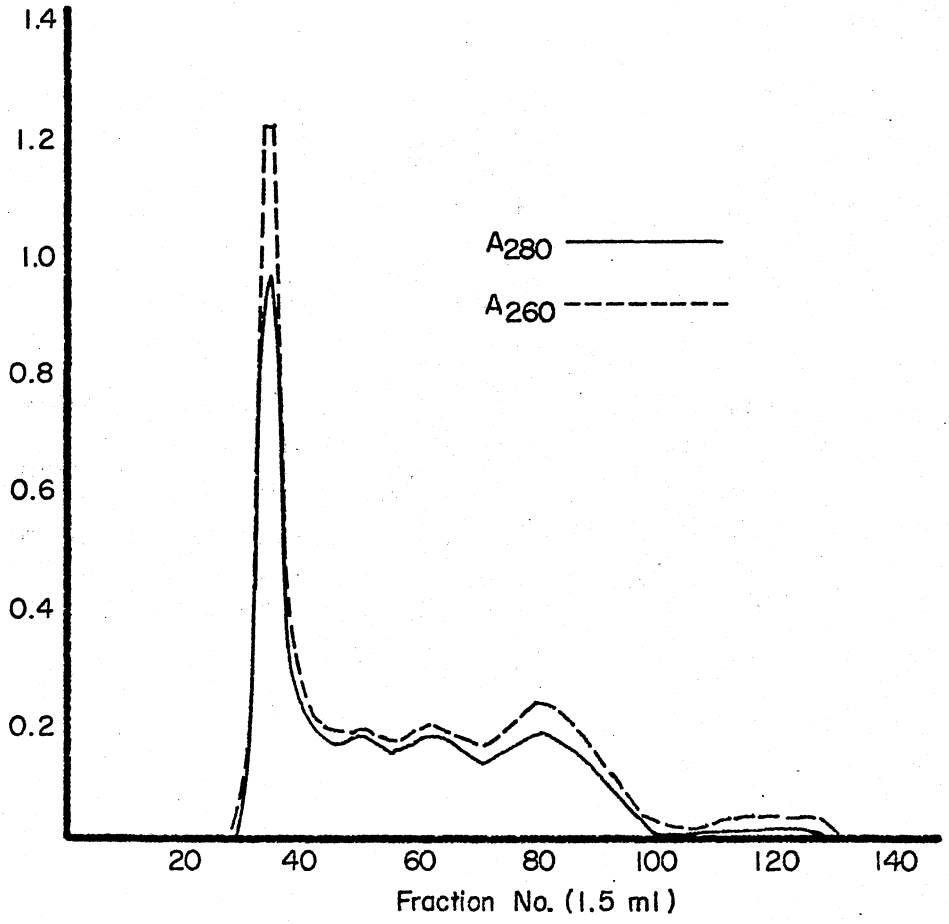

1.4
1.2
1.0
0.8
0.6
0.4
0.2
A280
A260
20
40
60
80
100
120
140
Fraction No. (1.5 ml)

Figure 5. Elution Profile of pk. II on a Sephadex G-50 Column.
The column was eluted with .02 M ammonium bicarbonate at a flow rate of 6.5 ml/hr. and 2 ml fractions were collected. In order of elution, the peaks were referred to as pk. IIA and pk. IIB.

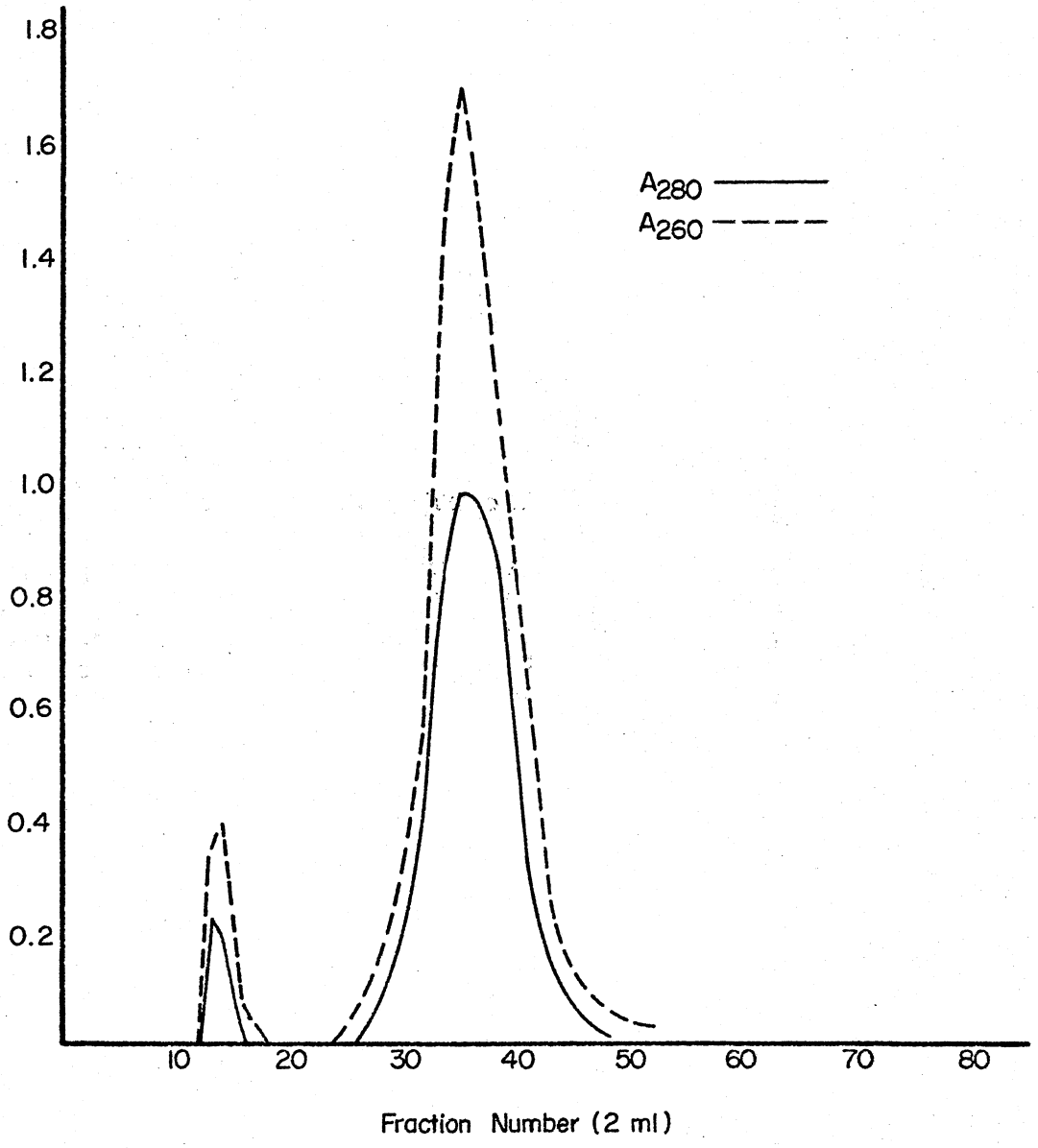
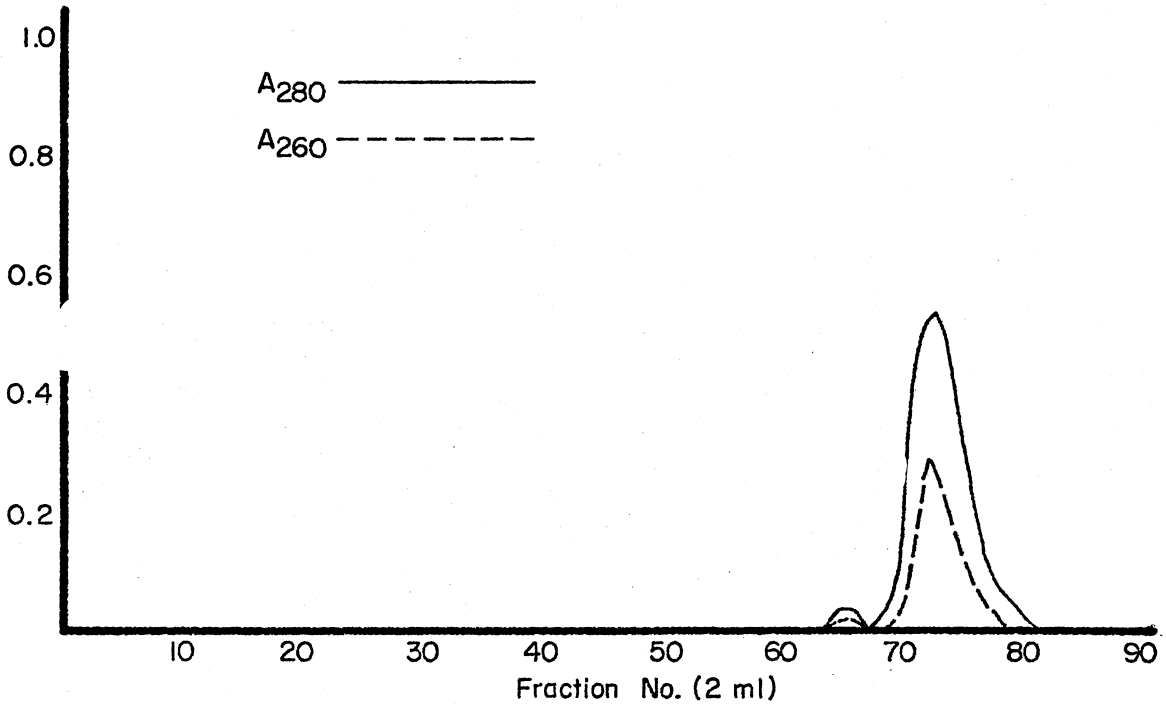

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A280
A260
10
20
30
40
50
60
70
80
Fraction Number (2 ml)

Figure 6. Elution Profile of pk. III on a Sephadex G-15 Column.
The column was eluted with .02 M ammonium bicarbonate at a flow rate of 7 ml/hr. and 2 ml fractions were collected. In order of elution, the peaks were referred to as pk. IIIA and pk. IIIB.


1.0
0.8
0.6
0.4
0.2
A_{280}
A_{260}
10
20
30
40
50
60
70
80
90
Fraction No. (2 ml)

APPENDIXES

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VITA

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