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THE VENOM DUCT OF *CONUS GEOGRAPHUS*: SOME BIOCHEMICAL AND HISTOLOGIC STUDIES

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ABSTRACT

The toxicity pattern and biochemical properties of the contents of successive sections of the venom ducts of *Conus geographus* were determined. Both lethal and paralytic activities were observed to increase towards the posterior duct region. In all segments, toxicity was found to be mainly associated with the soluble rather than the granular fraction. Data from SDS gel electrophoresis and thin layer gel chromatography suggest that the lethal activity of the venom is due mainly to the low molecular weight components found in the soluble fraction of the posterior segments.

Histologic examination of the *C. geographus* venom duct reveals two types of granules: the smaller anterior granules which are eosin staining and the larger hematoxylin staining granules which occupy the middle to the posterior sections. Both types of granules are ellipsoidal in shape and appear to be enveloped by eosin staining sheath or membrane.

Proteolytic activity was found to be higher in the soluble fraction, with the activity being highest in the anterior region and decreasing toward the posterior end. Although the proteolytic activity of the granule extract from the anterior region is just slightly lower than that of the corresponding soluble fraction, no activity was found in granule extracts from the middle and posterior regions.

Treatment of the residue (obtained after extraction of the venom with buffer and 10% acetic acid) with elastase released toxic peptides from the granules. These peptides exhibited chemical and symptomatic properties similar to those exhibited by Conotoxins GI and GII.

Based on the biochemical and histologic data presented, the possible role of venom granules as storage packets for zymogens of digestive enzymes and precursors of the lethal components is discussed.

INTRODUCTION

The marine snails belonging to the genus *Conus* are predators with a highly developed venom apparatus. One fish hunting species, *Conus geographus*, the geographer cone, has a venom sufficiently lethal to kill man. There are at least a dozen known cases of human fatalities from *C. geographus* stings.

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Recently, two groups (1,2) have investigated the nature of the toxic factor in *C. geographus* venom. A series of toxic peptides have been isolated, and these appear to account for most of the toxicity to vertebrates. Nevertheless, the venom is not simply a fluid containing these toxic peptides.

The venom is produced by a long tube-like structure called the venom duct, which connects into the pharynx of the mollusc (3). It is extruded through the pharynx and the proboscis, and is injected into the prey by the animal by means of a disposable harpoon-like tooth which is hollow and allows passage of the venom. Distal from the pharynx end of the venom duct is a muscular sac-like structure called the venom bulb. The venom itself is a milky white fluid, and upon microscopic examination many discretely formed granules are seen. Such granules contain much of the protein in the venom.

Toxicity studies on venom from several *Conus* species have indicated that venom extracted from portions proximal to the pharynx are less potent in terms of paralyzing and lethal activity than from portions of the duct proximal to the venom bulb (3). This was an unexpected toxicity distribution, since if the venom is to be injected into prey, the fluid closest to the pharynx would have been expected to be most lethal.

In this report, we have examined the distribution of toxic activity as a function of position in the venom duct, and differences in composition and toxicity between granular proteins and soluble proteins in these venoms were also analyzed.

In addition, we have also studied the toxicity of the insoluble components present in these venoms. Because there is some evidence for proteins as possible precursors to the toxic peptides in the venom, these studies were of particular interest.

MATERIALS AND METHODS

Specimen

The specimens of *Conus geographus* used were collected from the sea waters of Marinduque and airlifted alive to Manila.

Fish used for bioassays were fresh water carps *(Cyprinus carpio)* and gurami (*Helostoma tenminki*) maintained in an acquarium at room temperature. Mice used for the activation experiment were of the Swiss Webster strain.

Reagents

Acrylamide and methylene bisacrylamide (Bis) were obtained from Eastman, Kodak. N, N, N^1,N^1 -tetramethyl ethylenediamine (TEMED) was from

Canalco Industrial Corporation, Rockville, Maryland. Standard proteins and azocasein were from Sigma Chemicals. Sephadex /-50 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of reagent grade.

Equipment

Centrifugations were done in an automatic refrigerated Sorvall Centrifuge (Model RC2-B) using SS-34 and SM-24 rotors. All color absorbances were measured by a Spectronic 20 spectrophotometer using either macro or micro cuvettes. Gel electrophoresis was done on a Canalco unit complete with destainer. Thin layer gel filtration was done on a Pharmacia TLG apparatus. An American Optical teaching microscope was used to study and photograph slides.

Venom Extraction

Specimens were immobilized by burying in ice or chilling inside the freezer for 30 minutes. They were then removed from their shells with the aid of strong curved forceps. The venom apparatus was carefully dissected out of the body cavity and washed with cold distilled water. Extraneous connective tissues and membranes were removed. The duct was placed on an ice cold stainless steel spatula and the contents were expressed with a scalpel. Total duct extracts are those stripped from the whole duct starting from the junction with the pharynx to that of the venom bulb. Extracts from segments were taken after cutting successive 2-cm sections of the duct. Extracts from portions proximal to the venom bulb are referred to as "posterior duct extracts'. For a standard expression of relative distances of sections along the duct, the length of the entire duct is considered as one, taking the junction with the prepharynx as zero and the junction with the bulb as one. Relative locations of subsequent sections are expressed as fractions of the entire length of the duct. Hence, 0.5 would represent the midpoint of one duct.

Treatment of Extracts

All duct extracts were weighed and diluted to 10% or 20% (w/v) solutions. Samples for bioassay were diluted with 0.9% sodium chloride or normal saline solution (NSS); those for protein studies were suspended in distilled water and those for thin layer gel filtration, in 0.01M Tris-0.25 M NaCl buffer, pH 8. The suspension of duct extracts in water or sodium chloride without further treatment comprise the "whole venom preparations." The soluble fraction or supernate was obtained by centrifugation of

diluted extracts in a Sorvall refrigerated centrifuge at 10,000 rpm. Granules were also separated in this procedure as the pellet. Granule contents were extracted by sonication followed by centrifugation. The supernates from this procedure comprise the "granule extracts".

Preparation of the Standard Dose-Response Curve

Two-month old fish were obtained from the same school. A twenty percent solution of venom extracted from the total length of the duct was used at different concentrations. Five fish were injected per dose and observed for the onset of partial paralysis and death times. The time points were averaged and plotted against the reciprocal of the dose expressed in μg protein per gram of fish shown in Figure 1A. The linear relationship of dose and response time has previously been shown (4) for *C. geographus* venom using mice as an assay animal.

From the straight line drawn through the points one unit of activity (U) was arbitrarily designated as the amount of toxin injected per gram of fish which can induce partial paralysis in 1.25 minutes and death in 2.5 minutes. Based on this defined unit, the standard curve (Figure 1B) was replotted as time versus $1/\mu$. This curve was utilized for the computation of activities contained in venom preparations used throughout the study. For values beyond the range of the graph, the units of activity were estimated by extrapolation.

Protein Determination

For the determination of proteins in the crude whole venom, soluble fractions, granules and granule extracts, the procedure of Lowry *et al* (5) for difficultly soluble proteins was used, and the fractions from thin layer gel filtration were assayed using the direct micro method.

Sodium Dodecyl Sulfate Electrophoresis

This was done following the procedure by Weber and Osborn (6) using 7.5% gels run at pH 7.5 and 4.5 mamps per gel. Bacitracin, lysozyme, cytochrome c, ovalbumin and β - lactoglobulin were used as molecular weight standards.

Thin Layer Gel Filtration

This was done on a bed of Sephadex G-50, spread 0.8 mm thick over a 20 x 40 cm glass plate and developed at an angle of 45° with 0.01 M Tris-

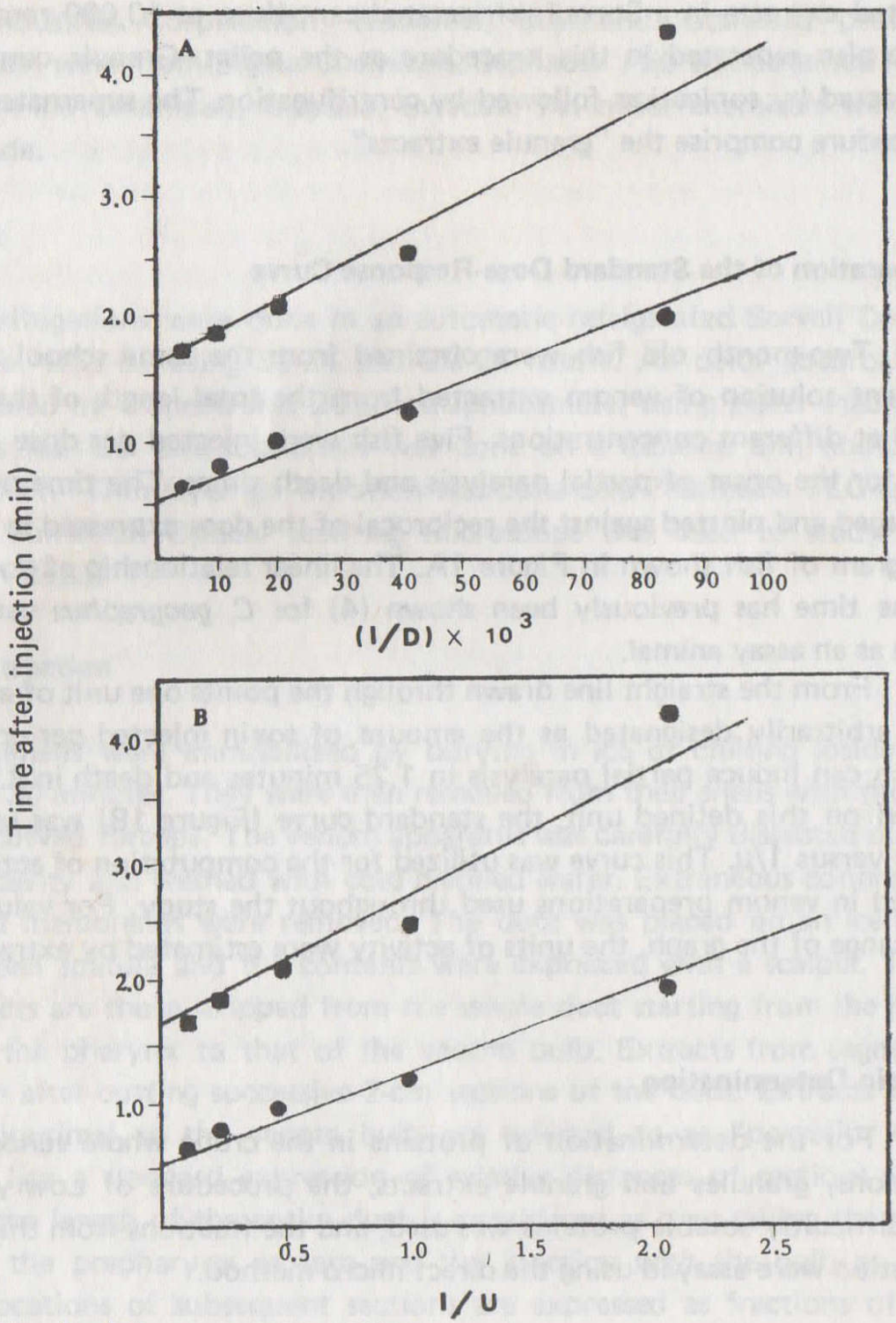

Figure 1. Standard dose-response curve

A 20% suspension of venom extracted from the entire duct was used for the assays. Different doses were injected on two-month old carps averaging one gram in weight, using 5 fish per dose. Time of onset of partial paralysis (●_____●) and death (■_____■) were noted for each. Controls consisted of fish injected with 1, 5 and 10 μl of normal saline solution and observed for 12 hours after injection.

A Plot of onset of symptom against reciprocal of dose expressed in μg venom protein per gram of fish.

B Replot of the same data presented in A but with the dose expressed as arbitrary units (U) defined under methods.

0.25 M NaCl buffer, pH 8.0, as eluant. Samples were applied together with standards for calibration. At the end of the run, the gel bed was then sectioned at 2 cm intervals, scraped and placed in individual tubes for elution with 0.2 ml of distilled water. Aliquots were then taken for protein determination and for bioassay.

Microscopic Studies

The venom apparatus was excised, cleaned and preserved in formaldehyde. After 48 hours, the tissues were dehydrated by soaking in successive changes of 80 - 100% ethanol for a total of 6 hours, and finally in chloroform. Paraffin infiltration was allowed to proceed in an oven at 40°C for 6 hours before the tissues were finally cast and chilled in paper embedding boats. Sectioning of paraffin blocks into 10 μ sections was done using a microtome. Ribbons were fixed with egg albumin and floated over a water bath at 95°C. The fixed tissues were then dried in an oven at 55°C for 3 hours.

The slides were dewaxed by dipping in xylene and washed with absolute alcohol and water before staining. Staining was done in Harris' alum hematoxylin for 10 min. After washing excess dye, the slides were dipped in 2% ammonia and counterstained with aqueous eosin for 10 min. The stained sections were again dehydrated with ethanol and cleared with xylene, then mounted in Canada balsam with a slide cover. Stained sections were viewed under a microscope at 25X magnification to compare duct size, staining of cells and granularity of sections. Using an oil immersion lens at 1000X magnification, the granules from representative sections were focused and studied.

Fresh venom stripped from anterior and posterior segments were smeared on glass slides and fixed in 95% ethanol for one min. and washed off with iodine. This was decolorized by acetone until no more blue color washed out. After washing with water, it was counterstained with dilute Safranin for one min, rinsed with water and dried. These were magnified under oil and studied.

Assays for Proteolytic Activity

Gelatin method: Preliminary assays for proteolytic activity were done using the gelatin plate assay of Grasset *et al.* (7). Twenty-five percent solutions of gelatin in 0.1 M Tris buffer were allowed to solidify in Petri dishes using three drops of tincture of thimerosal as preservative.

Azocasein method: The hydrolysis of azocasein according to the procedure of Kreger and Giffin was also used (8) as a more quantitative assay for venom proteases.

Treatment of *C. Geographus* venom Residue with Elastase

The toxicity of the insoluble components in *C. geographus* venom was studied. An attempt was made to treat the residues after sonication and extraction with different proteases. The venom was sonicated as previously described, and the soluble components after centrifugation were removed. The pellet was then re-extracted with 10% acetic acid, and the material insoluble in both buffer and acetic acid was pelleted by centrifugation. A 1.85 mg sample of such an acetic acid extracted pellet was suspended in 0.7 ml of 0.2 M Tris, pH 8.8. Elastase (Worthington Biochem. Corp., specific activity 60.6 units/mg, 3.2 mg/ml) was added; 0.012 ml of elastase solution was used. This gave a ratio of elastase protein to residue protein of 1:48. The reaction mixture was incubated at 37°C and 0.1 ml aliquots were taken over a period of 2 hr. These aliquots were added to 0.02 ml of sodium chloride (0.9%) and 0.08 ml of water . These were then injected intraperitoneally into mice which weighed approximately 8 grams. The amount injected was adjusted to the weight of the mouse, and the units of activity were assayed as previously described (4).

RESULTS

Distribution of Toxic Activity Along the Venom Duct

Previous observations by Endean and Rudkin (9, 10) and So (11) have indicated the occurence of higher activity in the posterior as compared to the anterior segments of the venom duct. Figure 2A substantiates these observations. Total activity per segment is shown to increase toward the posterior end, except for the segment nearest the bulb which showed a decrease in total activity. The same pattern is observed using either time of onset of partial paralysis or death as a measure of toxicity. The specific activity of the whole venom was similarly found to increase from the anterior to the posterior duct segments, again with a noticeable decrease at the region nearest the bulb (Figure 2B).

Protein and Toxicity Distribution in Venom Fractions

Centrifugations at 10,000 rpm effected separation of venom extracts into soluble and granular fractions. Figure 3 shows that except in the anterior portion, the protein content of the soluble fractions is greater than that of the granular fractions and the protein level decreased with distance from the pharynx. On the other hand, the protein content of the granule extracts

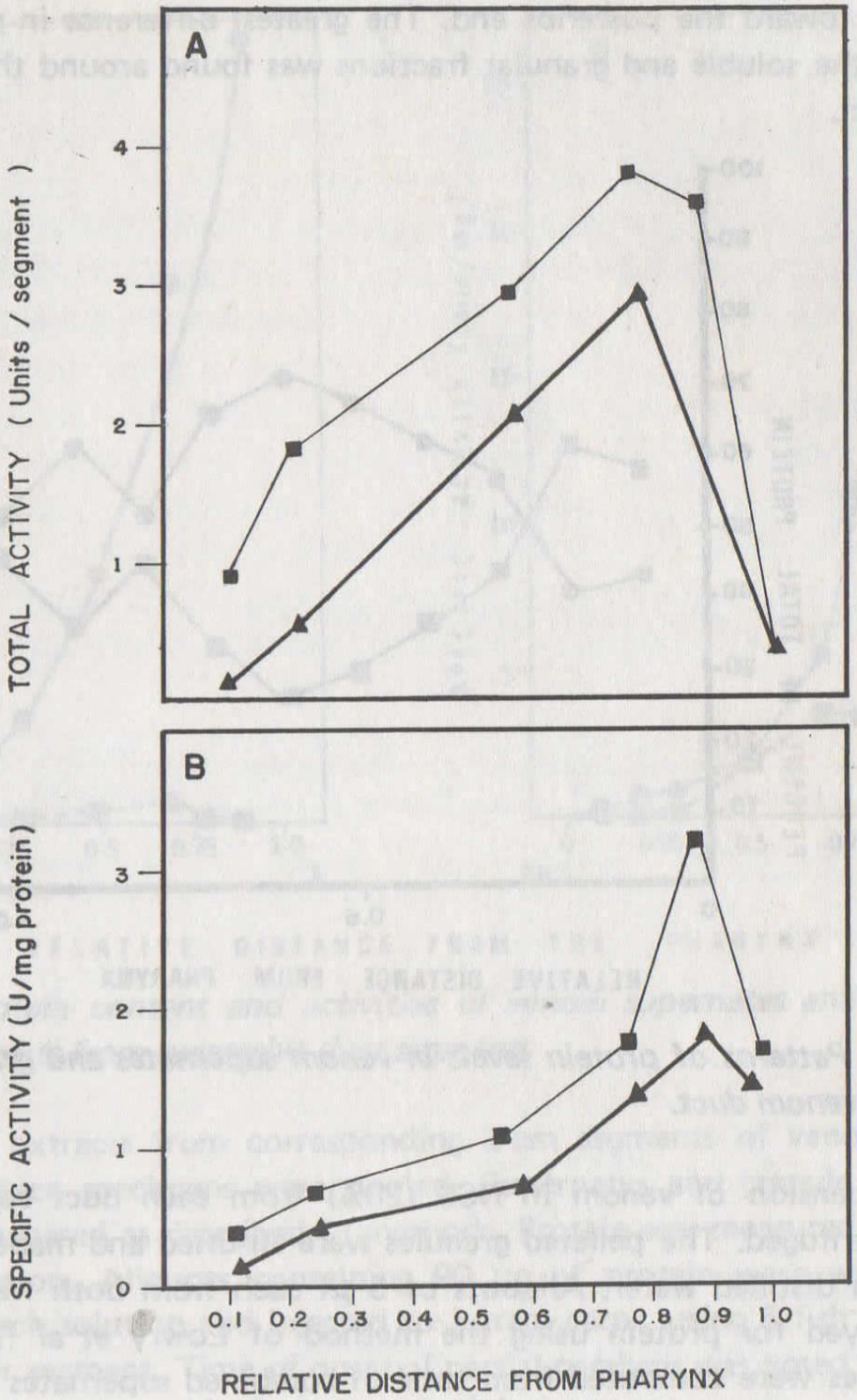

Figure 2. Distribution of toxic activity along the venom duct

Venom extracted from 2-cm duct segments was taken up in NSS to make a 20% suspension. An aliquot of the suspension containing 90 μg of protein was injected into 5 two-month old gurami. Time of onset of partial paralysis and death were noted.

(▲_____▲) Activity computed from the time of onset of partial paralysis

(■_____■) Activity computed from death time

increased toward the posterior end. The greatest difference in protein level between the soluble and granular fractions was found around the mid-point of the duct.

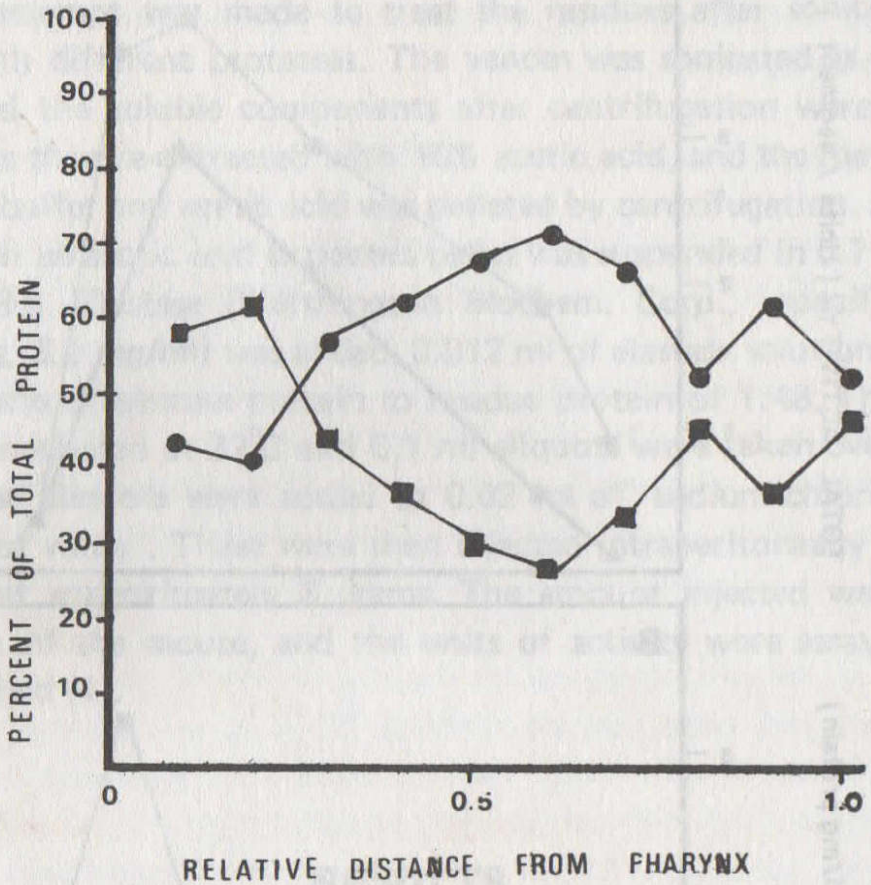

Figure 3. Patterns of protein levels in venom supernates and granules along the venom duct.

Suspension of venom in NSS (20%) from each duct segment were centrifuged. The pelleted granules were air-dried and made up to 20% with distilled water. Aliquots of 5 μl each from both fractions were assayed for protein using the method of Lowry *et al* (22). Plotted values were computed from total of combined supernates and granule proteins.

(●_____●) soluble fraction (■_____■) granular fraction

The plot of total activities of soluble and granular fractions (Figure 4A) clearly shows that paralytic activity is associated with the soluble fraction. Although some activity could be detected in the granule extracts, these were negligible compared to the supernates. Specific activities of the two fractions (Figure 4B) in the anterior portion were approximately the same. Although the specific activities of both fractions increased toward the posterior end, the increase for the soluble fraction was much more dramatic (Figure 4B).

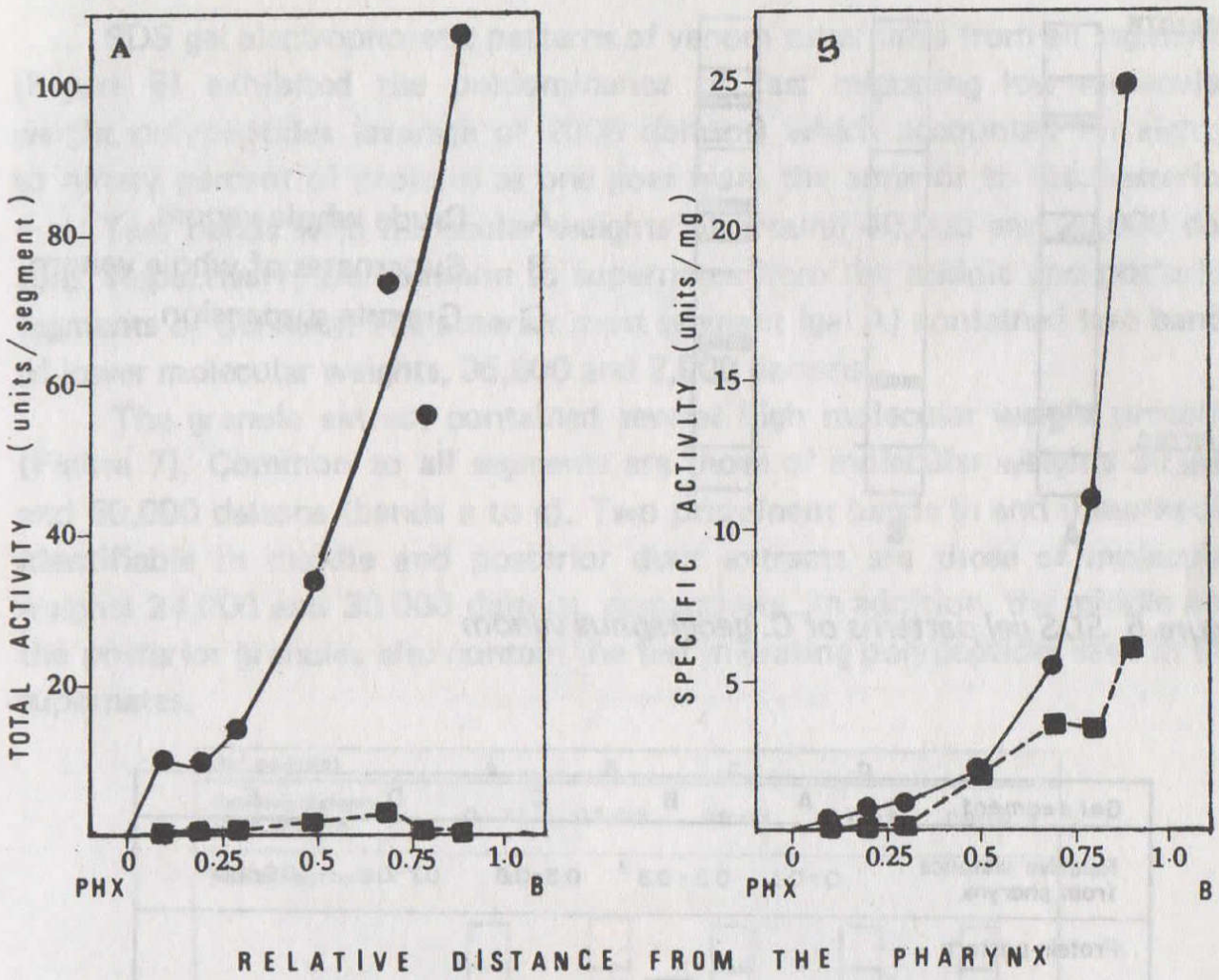

Figure 4. Protein content and activities of venom supernates and granule extracts from successive duct segments

NSS extracts from corresponding 2-cm segments of venom ducts from three specimens were pooled. Supernates and granule extracts were prepared as described in methods. Protein was measured for each preparation. Aliquots containing 90 μg of protein were withdrawn from each solution and injected to 1-gram carps, using 5 fish per fraction per segment. Time of onset of partial paralysis was noted. Activity units were obtained from the standard dose-response curve.

A Total activity pattern of granules and soluble fractions along the venom duct.

B Specific activities of granular extracts and soluble fractions of duct segments. (●_____●) Soluble fraction, (■_____■) granule extracts

Qualitative Protein Patterns

SDS gel electrophoresis of equal amounts of proteins from soluble fraction and granular extracts from different segments depicted the localization of protein bands unique to each fraction. Crude whole venom of *C. geographus* show a number of high molecular weight proteins plus a fast migrating diffuse band close to the dye front and the bacitracin standard (Figure 5).

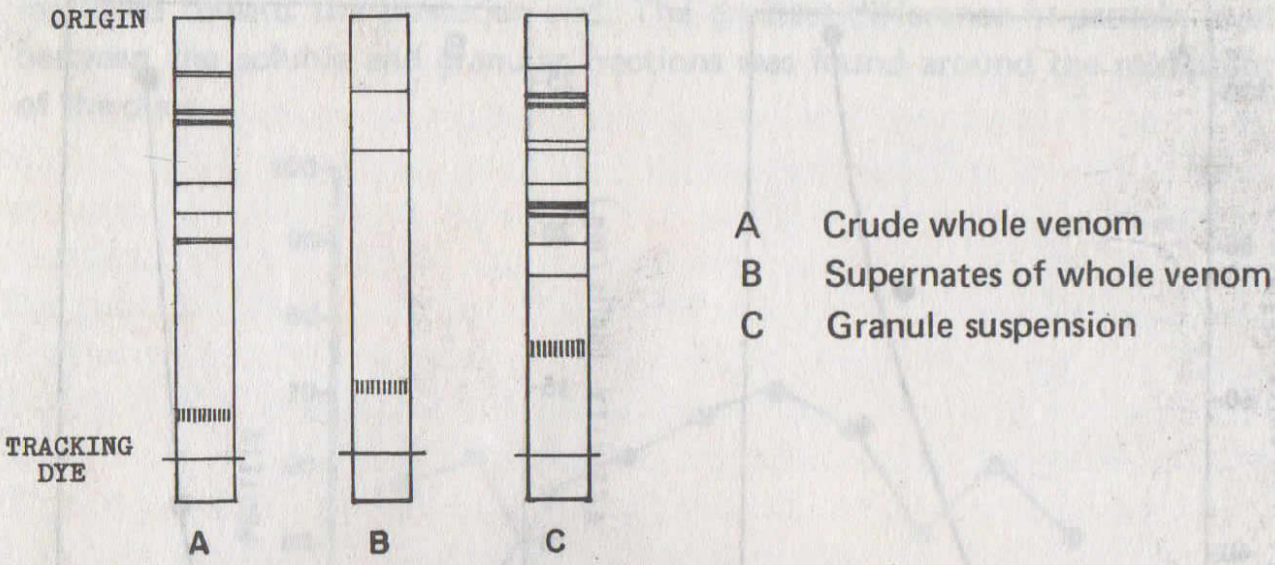

Figure 5. SDS gel patterns of C. geographus venom

Gel segment	A	B	C	D	E
Relative distance from pharynx	0-0.1	0.2-0.3	0.5-0.6	0.7-0.8	0.9-1.0
Protein pattern					
Molecular weight					
a	36,000		40,000	43,000	41,000
b		26,500	20,500	20,500	20,000
c		10,200			
d	7,900	5,600			
e	2,000	2,000	1,800	1,900	1,200

Figure 6. SDS electrophoresis patterns of venom supernates from successive duct segments

50 μg per sample were applied in 7.5% acrylamide gel. Electrophoresis was run at 4.5 mA per gel for 7 hours in 0.05 M sodium phosphate buffer, pH 7.0. Capital letters indicate duct segments and small letters designate the major protein bands.

SDS gel electrophoretic patterns of venom supernates from all segments (Figure 6) exhibited the predominance of fast migrating low molecular weight polypeptides (average of 2000 daltons) which accounted for eighty to ninety percent of proteins as one goes from the anterior to the posterior end. Two bands with molecular weights of around 40,000 and 20,000 daltons respectively, are common to supernates from the middle and posterior segments of the duct. The anterior most segment (gel A) contained two bands of lower molecular weights, 36,000 and 7,900 daltons.

The granule extract contained several high molecular weight proteins (Figure 7). Common to all segments are those of molecular weights 30,000 and 60,000 daltons (bands a to g). Two prominent bands (h and i) markedly identifiable in middle and posterior duct extracts are those of molecular weights 24,000 and 30,000 daltons, respectively. In addition, the middle and the posterior granules also contain the fast migrating polypeptides seen in the supernates.

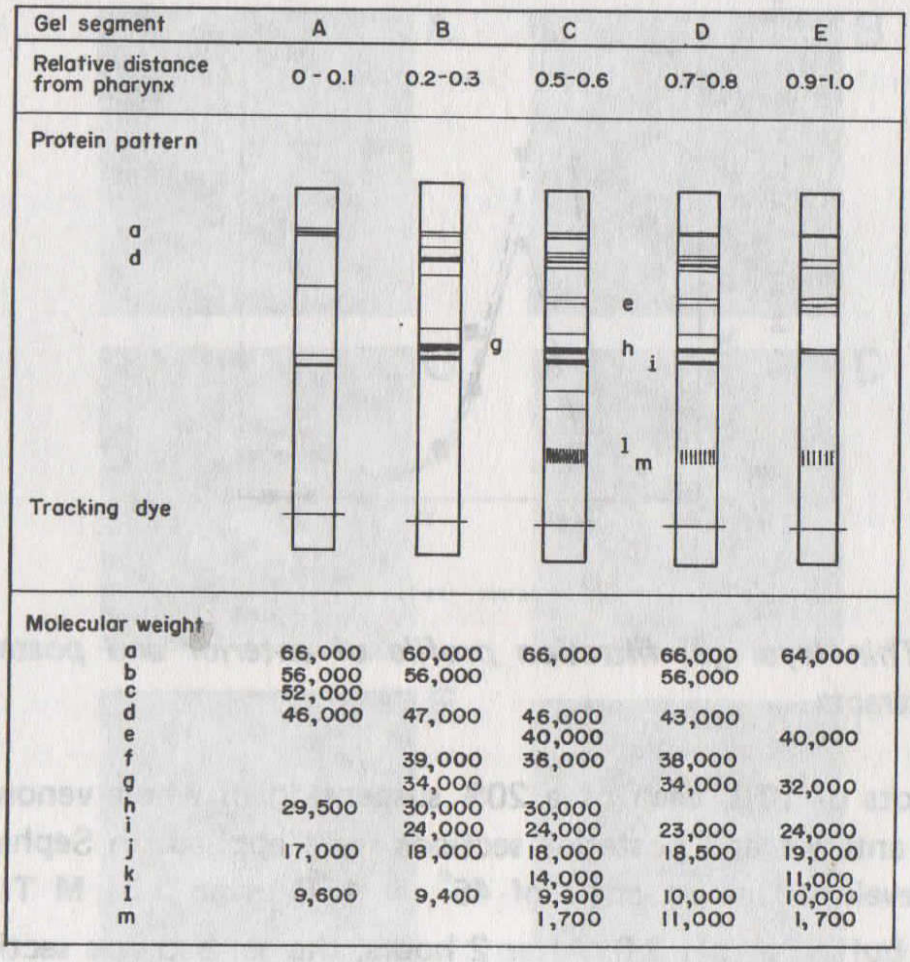

Figure 7. SDS gel electrophoresis patterns of granule proteins from successive duct segments

50 μg protein per sample were applied on 7.5% acrylamide gels. Electrophoresis was run at 4.5 mA per gel for 7 hours in 0.05 M phosphate buffer at pH 7.0. Capital letters indicate duct segments and small letters designate the major protein bands.

Thin Layer Gel Filtration

TLG chromatography of Sephadex G-50 was done using equal volumes of 20% solutions of anterior and posterior venom extracts. Upon bioassay of the eluates from gel plate sections, two activities were detected (Figure 8). One was a low molecular weight component travelling close to bacitracin

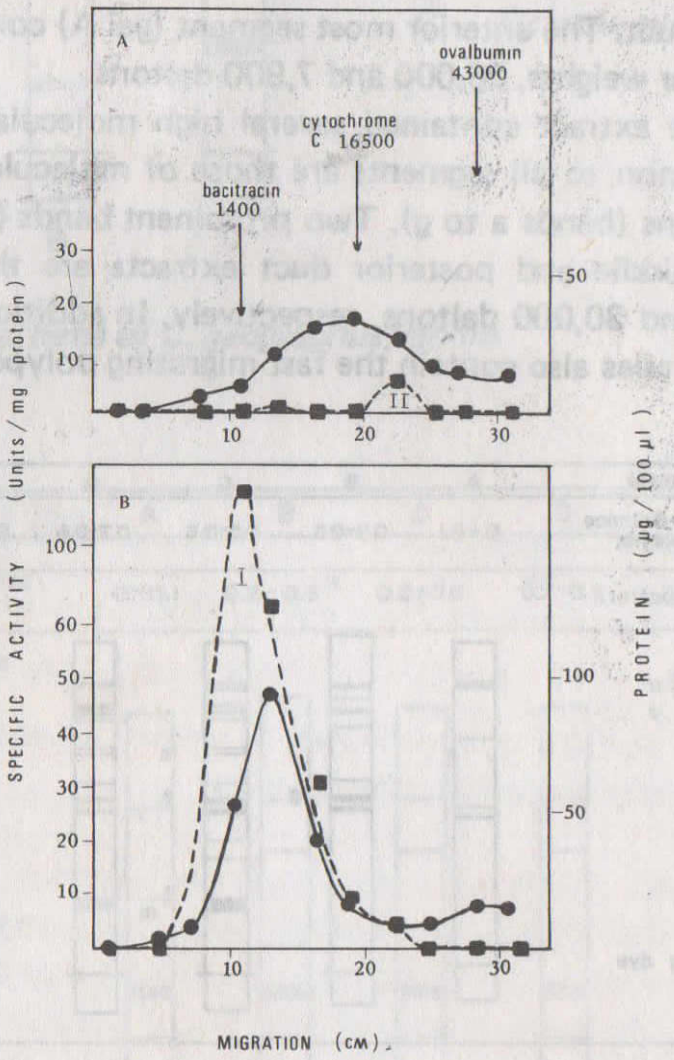

Figure 8. Thin layer gel filtration profile of anterior and posterior duct extracts

Aliquots of 10 μl each of a 20% suspension of whole venom extracts from anterior and posterior sections were applied on Sephadex G-50 gel developed at an angle of 45°, at 15°C using 0.01 M Tris-0.25 M NaCl buffer at pH 8.0. After 2 hours, the gel bed was sectioned into 2 cm x 1.5 cm areas which were scraped and transferred to tubes containing 200 μl of buffer. Aliquots were taken and subjected to protein analysis and bioassay.

A Anterior venom (●____●) Protein pattern
B Posterior venom (■____■) Lethality pattern

which elicited paralysis and death (Activity I); the other was associated with a higher molecular weight component migrating after cytochrome c, which elicited hyperactivity, followed by paralysis and death (Activity II). Of the lethal activity in the anterior extract, 93.3% was attributable to II and 6.7% to I. Lethal activity of the posterior extracts was 99.7% I and only 0.3% II. Since the total lethal activity of the posterior extract is 78 times greater than that of the anterior extract, it is evident that Activity I is mainly responsible for the lethality of venom extracts.

Histologic Studies

The venom duct had an outer diameter of 1,800 - 2,000 microns and an inner diameter of 1,400 - 1,800 (Figure 9). An outer layer of connective and muscle tissues line the periphery of the duct. The lining facing the lumen consists of a thick layer of epithelial tissues with columnar cells possessing

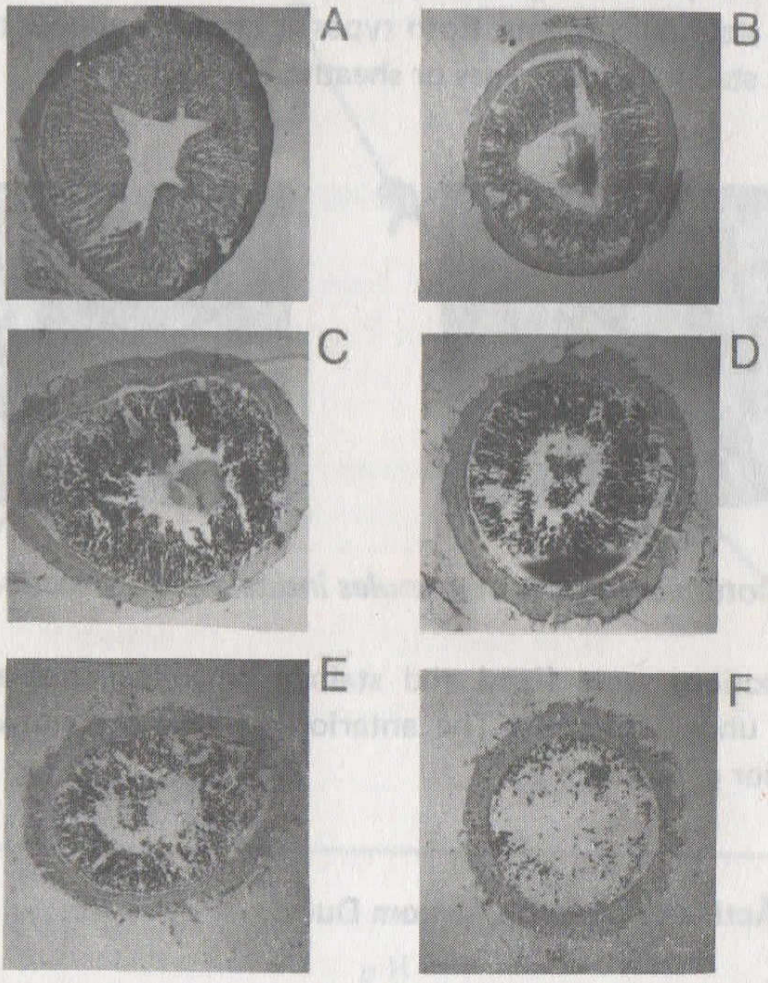

Figure 9. Transverse sections of the venom duct

The duct was fixed and stained with eosin-hematoxylin as described under methods. Sections A, B, C, D, E and F were taken at relative distances of 0.1, 0.2, 0.5, 0.6, 0.8 and 0.9 from the pharynx.

basal nuclei. Granular bodies abound in this inner lining and two forms can be identified. Granules found in the anterior regions are ellipsoidal bodies which took the pink eosin stain, showing interaction of the acidic stain with basic components of granules. Those found in the other sections are of similar shape but approximately 1.3 times longer and reacted more with the deep purple hematoxylin stain, suggesting interaction with acidic components of the granules. This latter type of granules were found to be most densely packed in the middle duct sections becoming sparsely distributed near the venom bulb. Histologic studies done by Endean and Duchemin (12) have shown a similar abundance pattern of granules and also a difference in size between the anterior and posterior granules from the venom duct of *C. magus*. However, for this species, anterior granules were found to be spherical rather than elongated.

On higher magnifications, the anterior bodies in the venom duct of *C. geographus* seemed transparent since underlying bodies can be clearly seen (Figure 10). The posterior granules, however, seemed to be filled with more of the dense staining contents. Both types of granules appear to be enveloped by thin eosin staining membranes or sheaths.

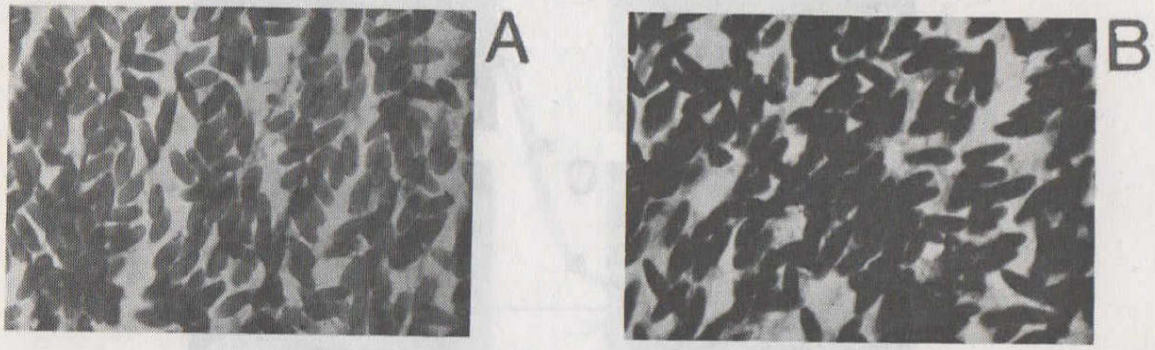

Figure 10. Photomicrographs of granules inside the duct sections

The sections were fixed and stained with eosin-hematoxylin as described under Methods. The anterior granules are shown in A and the posterior granules in B.

Proteolytic Activity Along the Venom Duct

Preliminary screening on gelatin plates was done to see if there is a variation of proteolytic activity from the anterior to the posterior segments of the duct. The data indicated that the anterior venom had the highest proteolytic activity. To quantitate the results, the azocasein method (8) was adopted. Using azocasein in solutions of different pH ranges (7.5 - 10.0) the optimum pH for the *Conus geographus* proteases was found to be at pH 8.5,

(Figure 11). The activities of duct segments pooled from four cones were determined using the same procedures carried out at pH 8.5 and physiologic temperature. Results in Figure 12 show highest proteolytic activity in the region at a relative distance of 0 - 0.4 from the pharynx.

Using the gelatin plate method, preliminary studies were also done to find out whether the proteases are found in the soluble or granular fraction of the venom. Results shown in Table 1 indicate that highest activities are found in both the granular and soluble fractions of the anterior region. In the middle and posterior duct regions, activity was localized in the soluble fraction and none was detectable in the granular extracts. The results also show that the esophagus contains some proteolytic activity.

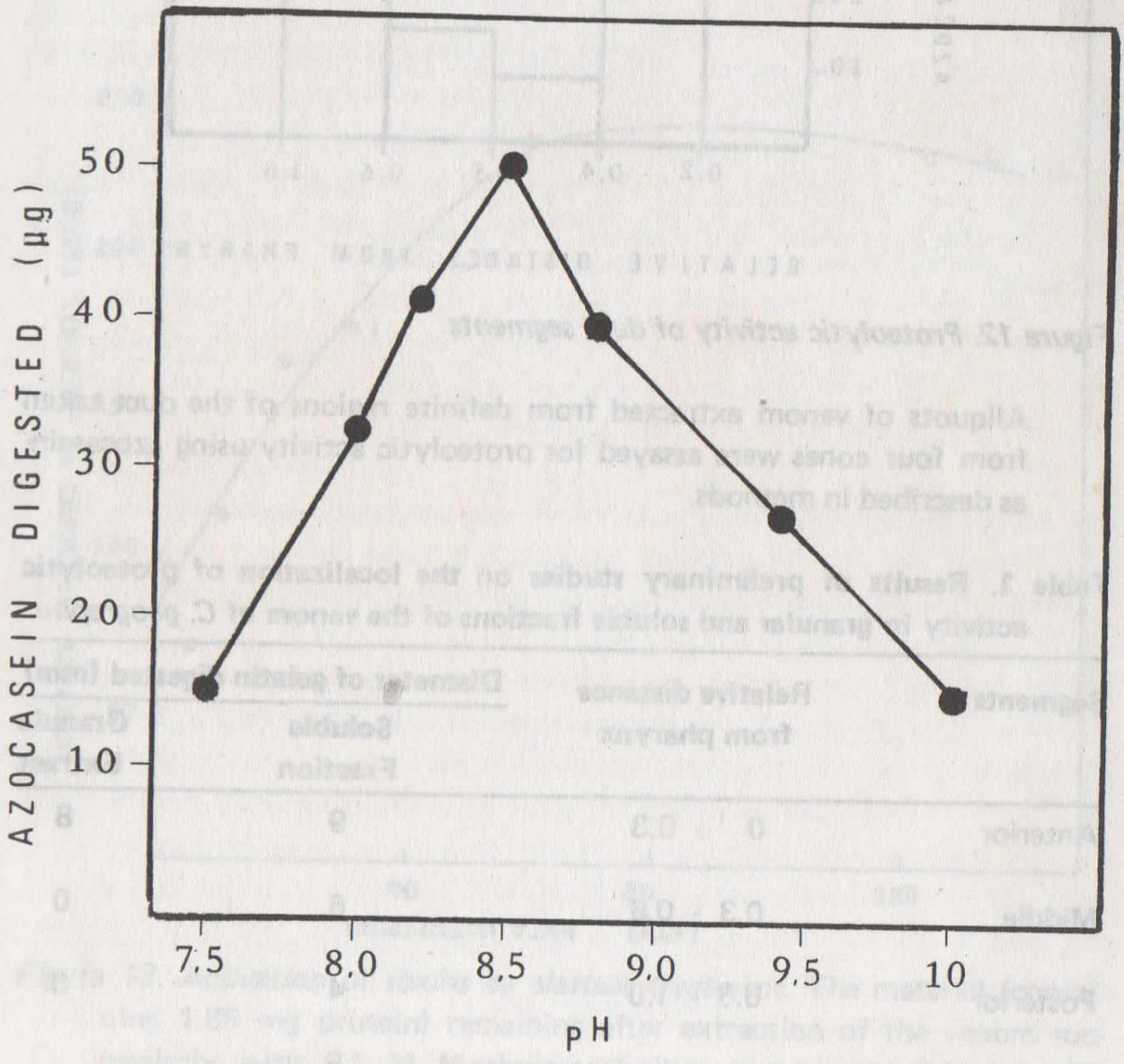

Figure 11. pH-activity profile of Conus geographus venom protease

Aliquots of a crude suspension of whole venom extracted from the entire venom duct were incubated with 50 µg azocasein in Tris buffer at pH 7.5 - 10. The assay was carried out as described in methods.

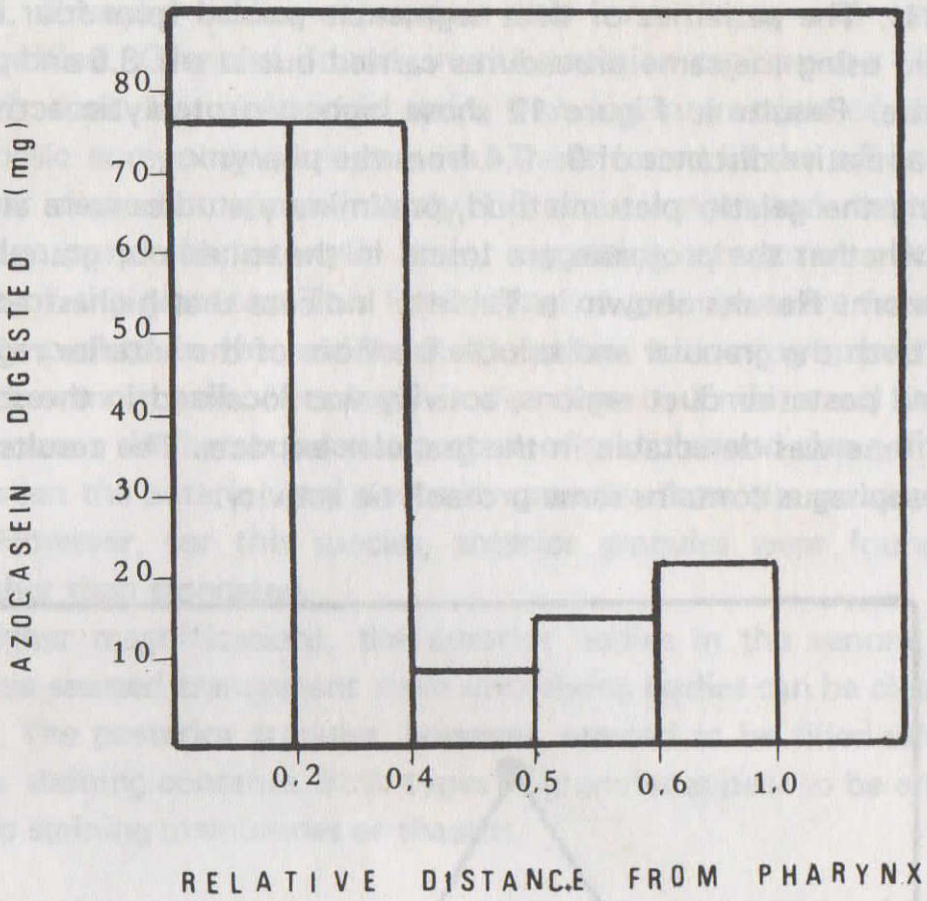

Figure 12. Proteolytic activity of duct segments

Aliquots of venom extracted from definite regions of the duct taken from four cones were assayed for proteolytic activity using azocasein, as described in methods.

Table 1. Results of preliminary studies on the localization of proteolytic activity in granular and soluble fractions of the venom of *C. geographus*

Segments	Relative distance from pharynx	Diameter of gelatin digested (mm)	
		Soluble Fraction	Granule Extract
Anterior	0 - 0.3	9	8
Middle	0.3 - 0.6	6	0
Posterior	0.6 - 1.0	4	0
Esophagus	.....	5	.....

Aliquots of 5 μl each of the soluble fraction and granule extracts were applied on gelatin plates. These were incubated at room temperature for 16 hours. The gelatin plates were then washed, and the diameter of the holes made by proteolytic digestion was measured.

Reactivation and Solubilization of Residual Granular Proteins

A number of experiments were also carried out to check the toxicity of the components of the venom which remain insoluble even after extraction with 10% acetic acid; these are presumably a subset of the granular fraction. This well-washed residue retained measurable lethal activity after intraperitoneal injection into mice. These preliminary results suggested that the insoluble components might, upon protease action, be converted into soluble proteins or peptides which could then be absorbed into the blood stream and therefore eventually reach some target site.

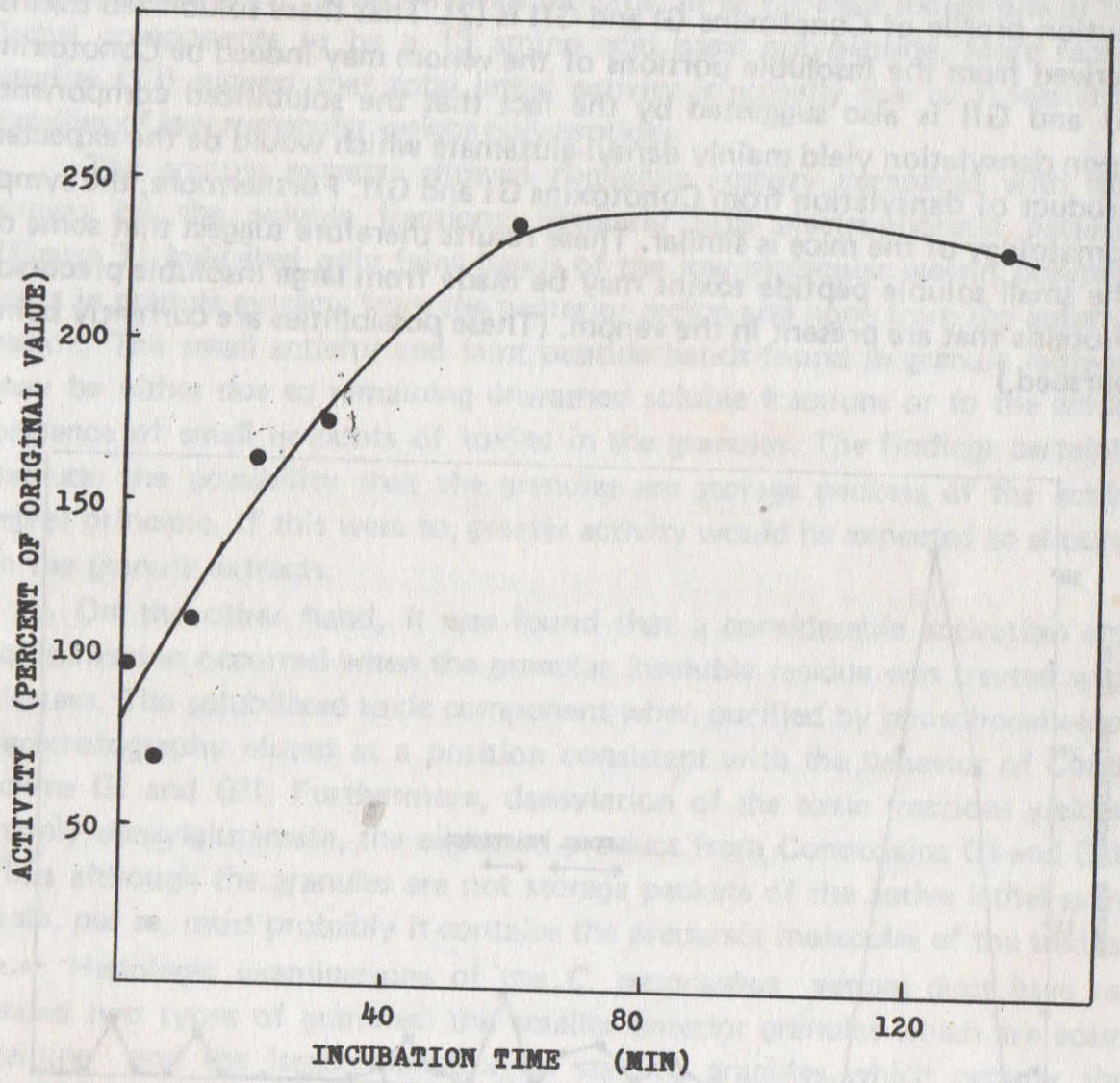

Figure 13. Activation of toxins by elastase treatment. The material (containing 1.85 mg protein) remaining after extraction of the venom successively with 0.1 M N-ethylmorpholine acetate, pH 9.0 and 10% acetic acid was washed with distilled water and suspended in 0.7 ml of 0.2 M Tris, pH 8.8. Enough elastase was added to give an elastase protein to residue protein ratio of 1:48. The mixture was incubated at 37°C and aliquots were taken for bioassay. Each point is an average of two trials.

This possibility was investigated by treating the insoluble residue with proteases. It was found that a considerable activation and solubilization occurred if the insoluble residue was treated with elastase. Such an experiment is shown in Figure 13. There is a two fold activation of the toxin upon treatment with elastase. Furthermore, much of the toxic activity has become soluber under these conditions and can therefore be analyzed by column chromatography.

An analysis of the soluble toxic material after treatment with elastase was carried out using phosphocellulose chromatography. As shown in Figure 14, the soluble toxic components eluted at a position consistent with the elution profile of Conotoxins GI and GII is (2). That these solubilized toxins, derived from the insoluble portions of the venom may indeed be Conotoxins GI and GII is also suggested by the fact that the solubilized components upon dansylation yield mainly dansyl-glutamate which would be the expected product of dansylation from Conotoxins GI and GII. Furthermore, the symptomatology of the mice is similar. These results therefore suggest that some of the small soluble peptide toxins may be made from large insoluble precursor proteins that are present in the venom. (These possibilities are currently being pursued.)

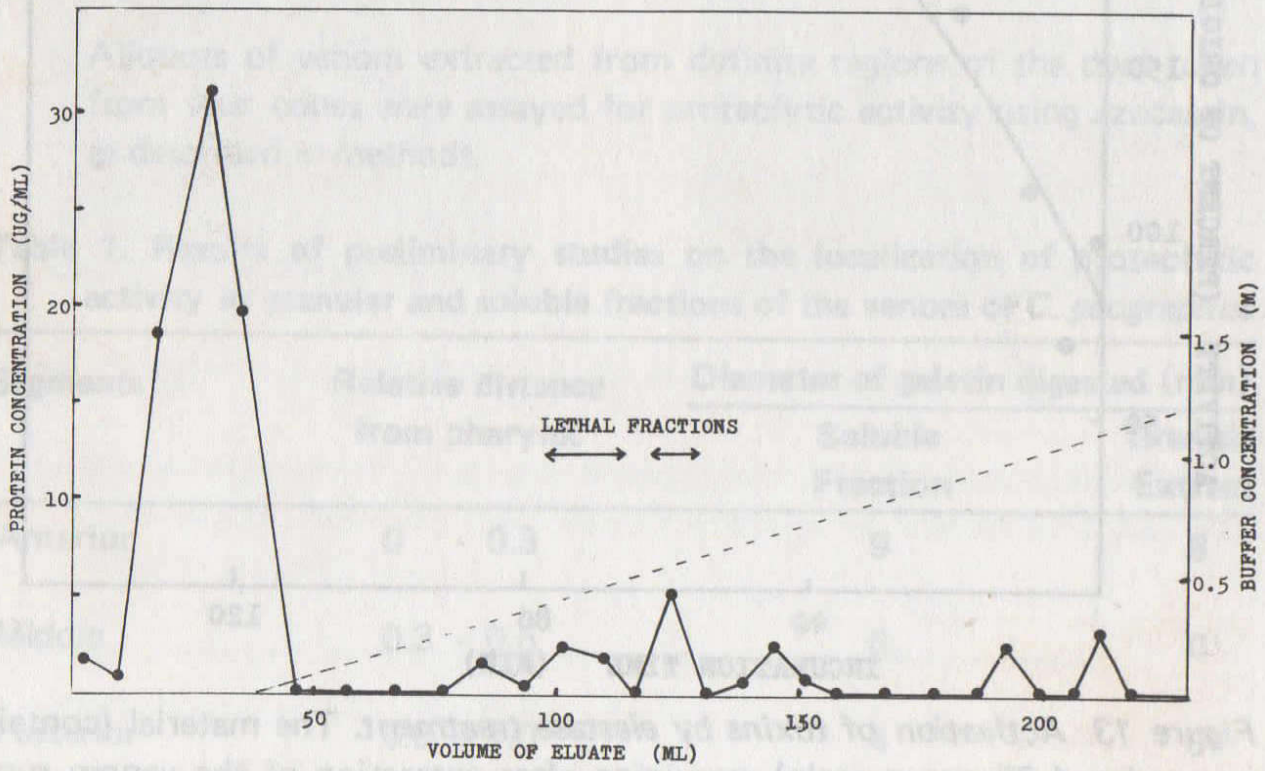

Figure 14. Chromatography of solubilized toxins through phosphocellulose column

The supernate obtained after elastase treatment was applied on to a 1.5 x 8.5 cm phosphocellulose column. The proteins were then eluted and fractions were bioassayed as previously described (2).

DISCUSSION

Previous observations (9, 10, 11) on the greater toxicity of posterior versus anterior venom is supported by the results shown in Figures 2A and 2B. Moreover, the data presented in Figure 4 clearly show that the toxic activity is mainly associated with the soluble fraction, and that this activity increases from the anterior to the posterior regions of the duct. Data from SDS gel electrophoresis (Figure 6) and thin layer gel chromatography (Figure 8) further suggest that the lethal activity of *C. geographus* venom is due mainly to the low molecular weight components found in the soluble fraction of the posterior segments. Earlier findings by Cruz, *et al* (2) have shown one of the lethal components to be a 13 amino acid basic polypeptide. More recent studies (13) suggest that total lethal activity is actually due to at least two families of low molecular weight polypeptides.

The granule extracts showed negligible activity compared with that shown by the soluble fractions. Similarly SDS electrophoretic patterns (Figure 7) indicated only faint bands of the low molecular weight polypeptides in granule extracts from the posterior region and none from the anterior region. The small activity and faint peptide bands found in granule extracts may be either due to remaining unwashed soluble fractions or to the actual presence of small amounts of toxins in the granules. The findings certainly exclude the possibility that the granules are storage packets of the active lethal principle. If this were so, greater activity would be expected to abound in the granule extracts.

On the other hand, it was found that a considerable activation and solubilization occurred when the granular insoluble residue was treated with elastase. The solubilixed toxic component when purified by phosphocellulose chromatography eluted at a position consistent with the behavior of Conotoxins GI and GII. Furthermore, dansylation of the toxic fractions yielded mainly dansylglutamate, the expected product from Conotoxins GI and GII. Thus although the granules are not storage packets of the active lethal principle, per se, most probably it contains the precursor molecules of the toxins.

Histologic examinations of the *C. geographus* venom duct have revealed two types of granules: the smaller anterior granules which are eosin staining and the larger hematoxylin staining granules which occupy the middle to the posterior sections. Both types of granules are ellipsoidal in shape and appear to be enveloped by an eosin staining sheath or membrane. Perhaps they only differ in the relative amounts of contents inside the envelope It is possible that the posterior granules are just packed with more of the acidic hematoxylin staining substances making these appear more distended, rigid and slightly bigger than the anterior granules.

The proteolytic activity of *C. geographus* venom was found to be much higher in the anterior portions (relative distance < 0.4) than in the middle

and posterior regions of the duct. Whatever proteolytic activity there was in the middle and posterior regions seemed to be associated only with the soluble fraction, the granule extracts of these regions showing no activity at all. In the anterior region where most of the proteolytic activity occurs, the supernates and granular fractions showed comparable activities. This pattern of proteolytic activity along the venom duct is consistent with the idea that the middle and posterior regions may contain zymogens which become activated as proteolytic enzymes as the granules reach the anterior duct portions. Perhaps in this region some of the activated proteases are also released to the soluble fraction thus accounting for the relatively much higher activities in the anterior supernates as compared to those of the supernatant fractions from the rest of the venom duct.

For the *Conus*, it can be postulated that granules may very well be storage bodies for precursors of toxins and digestive enzymes, perhaps analogous to the zymogen granules of the pancreas. Cleavage of precursor proteins by restricted proteolysis has been established as an extracellular process involved in the activation of several enzymes and in the release of peptides with hormonal or pharmacologic activities (14).Events in the *C. geographus* duct could be very similar to these phenomena.

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