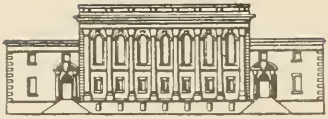

A STUDY OF THE VENOM OF *GLYCERA DIBRANCHIATA* EHLERS

by

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STATEMENT OF PURPOSE

The goal of this study is to separate the toxic principles of the venom of Glycera dibranchiata, of which little is known, and to compare these components to those partially identified in Glycera convoluta(Michel 1966,1972,1975). Gel chromatography, being a well established technique for separating large molecules according to size, is the primary separation method chosen for this study. Stability of the crude extracts as a function of temperature will be determined before the separation. This study is expected to reveal biological and chemical information concerning the poison glands of Glycera dibranchiata, which could prove to be of biomedical importance.

INTRODUCTION

Background

Glycera dibranchiata, commonly known as the bloodworm, is a marine annelid of the family Glyceridae. It was first described by Ehlers in 1868 and is an errant polychaete. The common name refers to the reddish color of the worm which is due to the hemoglobin found in the coelomic cells(Klawe & Dickie 1957). The bloodworm inhabits subtidal zones from northeast Canada to North Carolina(Klawe & Dickie 1957).

The anatomy of the bloodworm has been described in detail by Ehlers(1868) and Klawe and Dickie(1957), and in less detail by Halstead(1959) and Heacox(1974). *Glycera dibranchiata* has been known to reach a length of 37 cm, but the average length is about 25 cm(Klawe & Dickie 1957). The worm has a long cylindrical body which is tapered at both ends. At the anterior end of the body the bloodworm has an eversible pharynx. This first third of the digestive tract can be rapidly protruded, and has four sharp, black, chitinous jaws at its anteriormost point(Fig 1). Four glands, first referred to as poison glands by Ehlers(1868), are each separately associated with a jaw by a connecting duct. It is believed that the proboscis, and its associated poison-gland complex, is used for procurement of food and burrowing purposes.

Morphology of the Proboscis

Heacox(1974), in a histological study of the poison-gland complex, refers to three parts of the proboscis: an eversible buccal tube, a pharynx with jaws and associated glands, and an esophagus(Fig 2).

Fig 1. Extended proboscis of Glycera dibranchiata. A. Longitudinal section. B. Enlargement of proboscis. Notice jaws at tip of arrow. (from Halstead 1965).

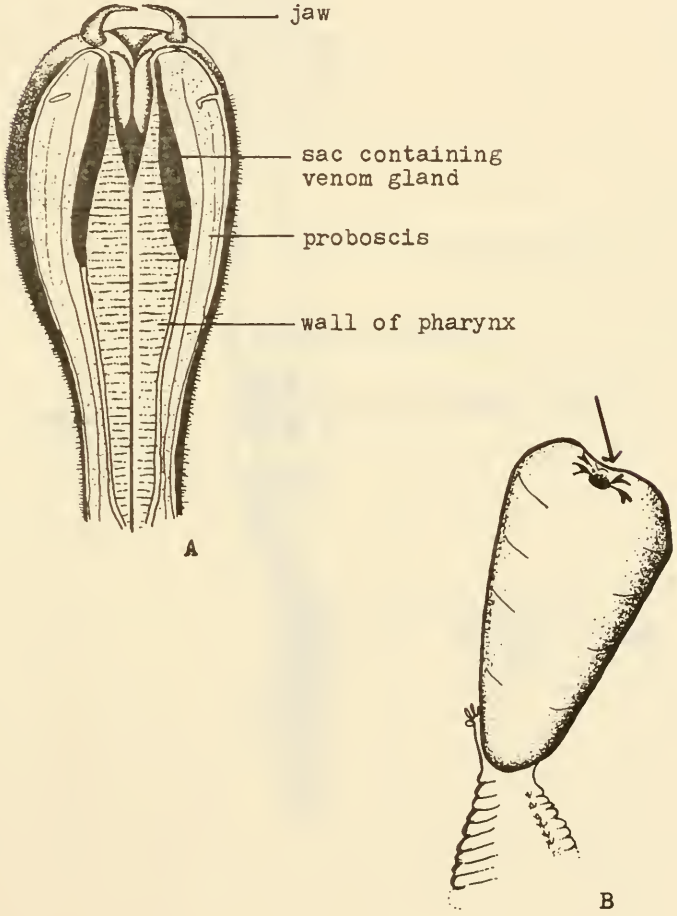
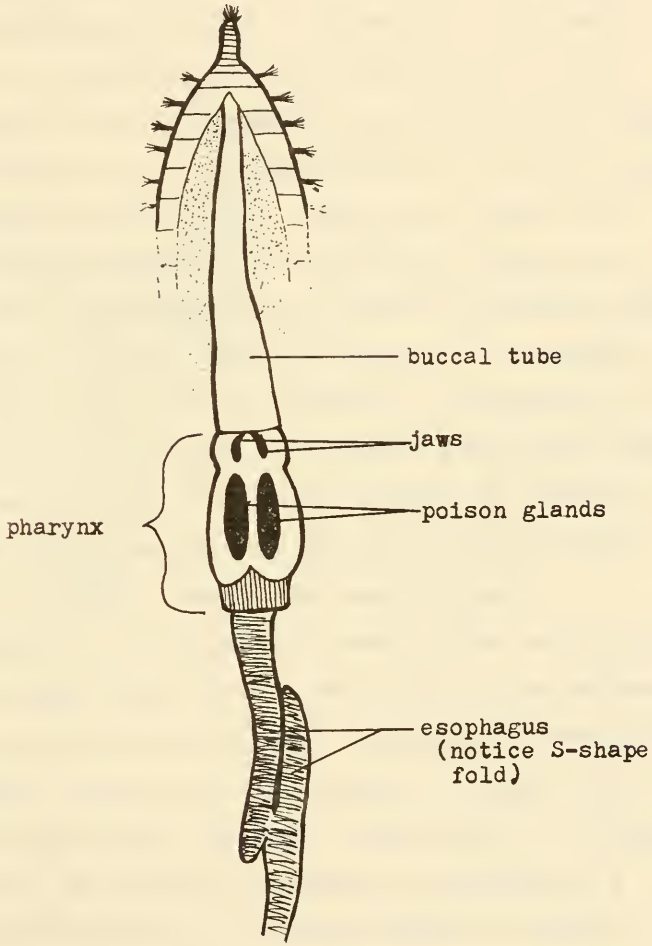


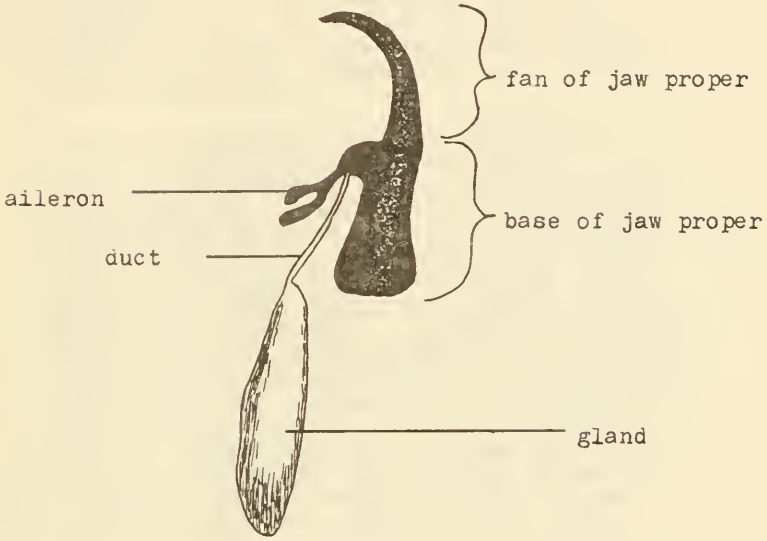
Fig 2. First third of the digestive tract(inverted) as seen after a longitudinal cut in the anterior end of the worm.



The buccal tube primarily consists of circular and longitudinal muscle, with many papillae projecting into the lumen of the inverted tube. The function of the papillae is not clearly understood; however, they are believed to have a secretory function related to digestion(Hartman 1950). The junction of the buccal tube and pharynx is associated with two structures: the four jaws on the luminal side and four membranous lobes, called languettes, on the coelomic side. The languettes are thin leaf-like structures of unknown significance. Between the pharynx and esophagus is a slight constriction, which is the location for the joining of four longitudinal muscles from the posterior part of each poison-gland complex. When the proboscis is inverted, the esophagus folds over on itself, forming an s-shape(Fig 2). When the worm everts its proboscis, the buccal tube turns inside out - exposing the papillae, the pharynx becomes the anteriormost point of the worm - terminating with the four sharp jaws, and the esophagus unfolds - thereby becoming straight.

Closer examination of the pharynx reveals three more divisions; the one of major importance to this study is the poison-gland complex. Heacox(1974) thoroughly describes the histology of the poison-gland complex, which is composed of jaws, ducts and poison glands surrounded by layers of muscle(Fig 3). Heacox further divides the jaw into the jaw proper, consisting of a base and fang, and the aileron. He reports that the fang is actually on the luminal side of the pharynx, while the base of the jaw is embedded in connective tissue on the coelomic side. The aileron is attached to one side of the jaw base. Near this point is a small pore through which a duct connects the jaw and

Fig 3. Poison-gland complex(diagram after Ehlers 1868).



poison gland. Both Heacox and Michel(1966) believe that this duct continues through, or almost through, the entire length of the fang, ending externally in a terminal pore.

Heacox describes the poison glands as follows:

> The wall of the gland is made up of long narrow cells which taper slightly toward the lumen [Fig 4]. They are almost entirely composed of a large apical vacuole, with the remaining cellular contents forced into the base of the cell. These cells may or may not contain nuclei.
>
> All the cells have a similar shape, but contain different types of secretions. The cells in the anterior of the gland contain relatively few, if any, secretory granules.... The majority of cells contain one of three types of secretions. The first type is composed of small granules which nearly fill the vacuole. The second type appears as more tightly packed granules. At times the secretion is not as tightly packed near the luminal surface giving an appearance similar to type one. Type three stains darkly, is solid, plate-like, and gives no granular appearance. The cells containing the type one secretion are more frequent in the anterior region of the gland, and the second and third types increase toward the posterior of the gland. Type one is the only secretion ever seen leaving the secretory cells and entering the lumen of the gland. (pp 41-43)

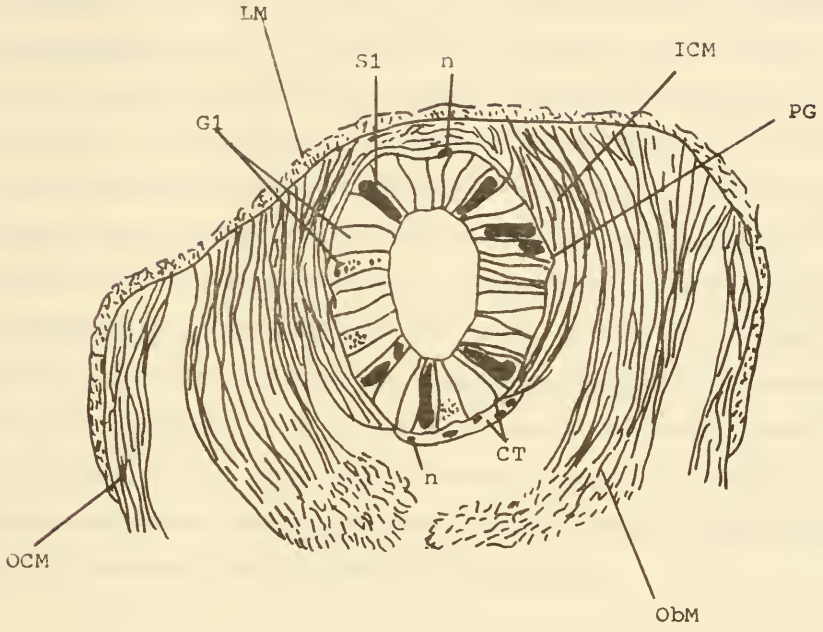
Venom Analysis

Ehlers(1868) was the first to suggest that the glands of *Glycera* could be poison glands:

> ...Concerning the nourishment of the species *Glycera*, we know from the information of Schmarda that *Glycera ovigera* waits on its prey, and also pursues it, and quickly kills smaller animals with its proboscis, by moving the jaw and producing a secretion from the jaw gland, which is probably poisonous.... (Ehlers p 643, translated by Prof. Ronald E. Horwege)

This idea is supported by the studies of Michel(1966,1972) and

Fig 4. Cross-section mid-way through a poison-gland complex (from Heacox 1974).



CT	connective tissue
Gn	granular secretion
ICM	inner circular muscle
LM	longitudinal muscle
Lu	lumen of poison gland
n	nucleus
Ob M	oblique muscle
OCM	outer circular muscle
PG	poison gland
S1	solid secretion

Heacox(1974) who report, respectively, that venom extracts of Glycera convoluta and Glycera dibranchiata are highly toxic to crustaceans. They believe the venom to be a neurotoxin. Histochemical work by Michel(1966) on the poison glands of Glycera convoluta and by Heacox(1974) on the poison glands of Glycera dibranchiata shows the presence of proteins, indole groups, PAS-positive material, disulfide groups, sulfydryl groups, and protease. The protease is thought to be the cause of local tissue histolysis observed when prey animals were bitten by Glycera alba(Ockelmann & Vahl 1970) and when shrimp(Palaemontes) and a marine fish were injected with a crude venom extract from Glycera dibranchiata(Curtis 1974,unpublished). Results from Sephadex G-75 and G-200 separations of Glycera convoluta venom indicate two toxic substances: low molecular weight compounds, possibly containing biologically active amines, and high molecular weight compounds, containing proteinases - specifically trypsin-like and collagenase-like proteases(Michel 1975).

MATERIALS AND METHODS

Glycera dibranchiata and Uca pugilator were obtained from Maine Bait Company, Newcastle, Maine, and Gulf Specimen Company, Panacea, Florida, respectively. The bloodworms were kept at 10°C in plastic containers, approximately 11 by 7 inches, 15 worms per container, with enough Instant Ocean to completely cover them. The crabs were kept in similar containers, 20 crabs per container, with a few cm of sea water. A solution of equal parts of 7.5% $MgCl_2 \cdot H_2O$ and sea water was used to anesthetize the bloodworms

before dissection. Two separate sets of equipment were used for dissection the poison glands and the muscle control to avoid any contamination. Iridectomy scissors were used to dissect the specific tissue sample.

Dissection Methods

The anterior end of the worm was determined by locating the brain and/or the jaws, both visible through the body wall as dark spots(Fig 5). The body wall was opened by a longitudinal cut just posterior to the jaws - or about a quarter of the way posteriorly form the front end, if the jaws were not readily visible(Fig 6). Then the proboscis was looped over the scissors (Fig 7). If the proboscis was everted to any extent, it could now be re-inverted by gently pulling on the anterior end of the proboscis loop. Lifting the inverted proboscis out of the coelomic cavity, the esophagus was held between the index finger and thumb(Fig 8), and the buccal tube between the index and middle finger(Fig 9). Downward pressure on the tips of the scissors around the gland(Fig 10) made it easier to obtain the glands, as well as insuring that the whole gland would be removed, instead of merely a slice off the top(Fig 11).

The poison glands are embedded in several layers of muscle, as determined by Heacox(1974). Due to the size of the glands it was not possible to separate them from these muscle layers. Therefore, esophageal muscle from each worm was used as a control(Fig 12).

Fig 5. Jaws of Glycera dibranchiata can be seen through the body wall.

Fig 6. Longitudinal cut from just posterior of jaws toward anterior end of bloodworm.

Fig 7. Looping proboscis over scissors. BT-buccal tube, J-jaws, G-glands, E-esophagus.

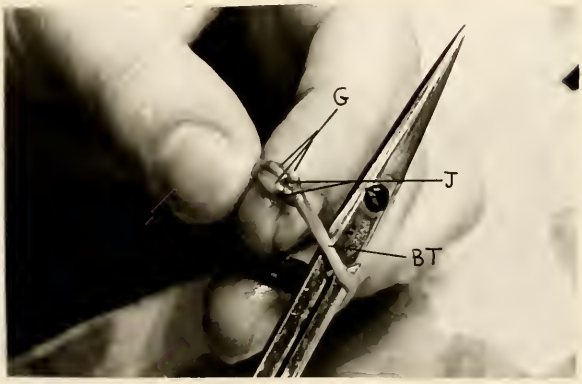


Fig 8. Holding the esophagus between the index finger and thumb. BT-buccal tube, J-jaws, G-glands.

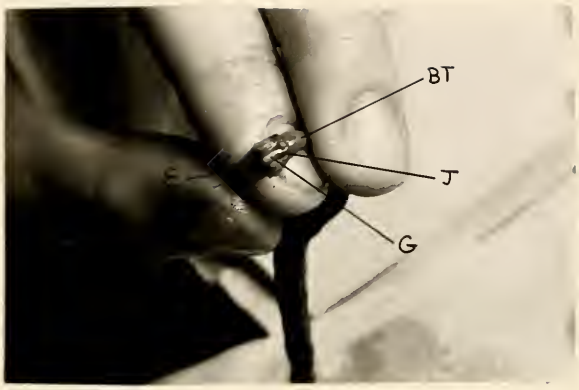


Fig 9. Holding the buccal tube between the index and middle fingers. BT-buccal tube, J-jaws, G-glands, E-esophagus.

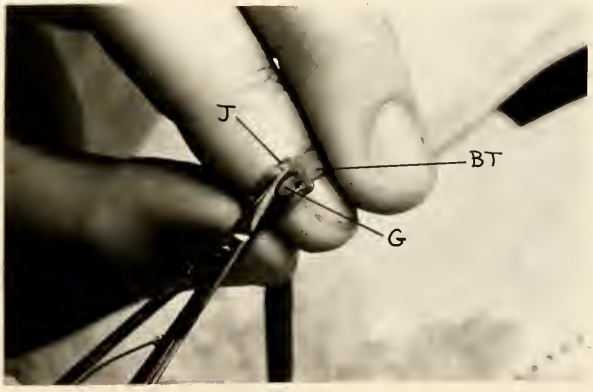


Fig 10. Dissection a gland. BT-buccal tube, J-jaw, G-gland.

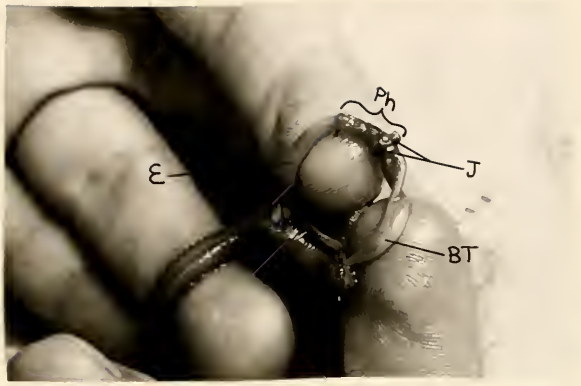


Fig 11. First third of digestive tract with glands removed. BT-buccal tube, J-jaws, Ph-pharynx, E-esophagus.

Fig 12. Dissecting esophageal muscle for control. Ph-pharynx, E-esopnagus.

Preparation of Crude Extracts

All equipment which was in contact with the tissues for any length of time was kept on ice throughout the procedure to help prevent histolysis. The dissected poison glands and muscle controls were placed in separate small, plastic weighing trays during the dissection. The trays contained a few drops of sea water to prevent the tissue from drying out. Each sample was then triturated in a homogenizer or, if more than 15 worms per sample was used, the tissue was ground in a mortar and pestle with clean sand. Depending on the sample size, up to 1.0 ml of sea water was added throughout this process. The samples were centrifuged in a Beckman TJ-6 at 3000 RPM, 6°C, for 10 min. The extract used on the Sephadex and Sephacryl columns consisted of 400 glands in 4.0 ml of 5mM NH_4HCO_3, centrifuged at 5800 RPM, 6°C, for 2.5 hrs.

Method of Extract Analysis

Toxicity Assay

The toxicity of the extracts was determined by injecting Uca with a small amount of crude extract. These animals are hardy and readily obtainable, and it had previously been established by Curtis(1974, unpublished) that the venom was highly toxic to them.

The 'standard' dose was determined in the following manner: first, the total weight of the tissue(gland or muscle) and sea water was recorded. From the measured volume and specific gravity of sea water, the weight of the tissue alone was determined. The density of the extracts was calculated in terms of grams of

tissue per ml sea water. The 'standard' was set at 6.0 mg tissue per unit(1.70 gm) of crab weight. Injection of this standard dose caused complete cessation of movement in a crab within 5-10 min.

The stability of the venom extract was determined in terms of the standard crab toxicity. Extracts were subjected to the following conditions: -20°C up to 35 days, 22°C up to 2.5 hrs, and 100°C for 10 min. The muscle control extract was tested under the same conditions. The extracts were found to be stable for at least 35 days at -20°C. This form of storage was used throughout this study.

Acetone Assay

The gland and muscle extracts were first tested for the presence of protein. This was done by adding varying amount of acetone to the extracts(Clark & Switzer 1977); the ratios of acetone to extract were approximately 0.5:1, 1:1, and 2:1. After each addition of acetone, the extracts were thoroughly mixed and then centrifuged at 2000 RPM, 6°C, for 10 min. The supernatant was poured off and re-extracted with acetone to obtain the next ratio. The precipitates were redissolved in sea water and tested for crab toxicity.

Trypsin Assay

Both crude extracts were tested for trypsin activity according to the procedure of Schwert and Takenaka(1955). Trypsin activity was measured in terms of the change in spectrophotometric absorbance at 253 nm and 20°C over time; the absorbance

was a measure of the amount of N-∝-benzoyl-L-arginine ethyl ester(BAEE) hydrolyzed by the trypsin.

The solutions needed for this assay were 0.001M BAEE in 0.05M tris-hydroxymethylaminomethane and a trypsin solution in 0.001M HCl. The absorbance was set at zero with 0.001M BAEE in 0.05 tris buffer in the reference cell. 0.2 ml of the trypsin solution was then added to 3.0 ml of this BAEE solution in the sample cuvette. The optical density initially decreased due to the dilution of the BAEE by the trypsin solution. However, the absorbance then increased linearly until about 90% of the substrate was used(Rick 1965). The crude extracts were tested at varying amounts, up to 0.50 ml.

Folin-Ciocalteu Assay

Protein concentration of the extracts were determined according to the Folin-Ciocalteu assay. The reagent yields a bluish color when mixed with free or protein-bound tyrosine and tryptophan(Clark & Switzer 1977). The protein concentration was measured spectrophotometrically. The presence of these two amino acids is a good measure of protein concentration since their total quantity in most soluble proteins is constant. This assay is very sensitive and is quantitatively accurate up to 300 μg protein.

The protein standards consisted of lysozyme in the following concentrations: 50, 100, 200 and 300 μg. Each standard solution had a final volume of 1.2 ml. A blank, consisting of 1.2 ml of H_2O, was also used. An alkaline copper reagent was freshly prepared by mixing the following solutions in order:

1.0 ml of 1% $CuSO_4 \cdot H_2O$, 1.0 ml of 2% sodium tartrate and 98.0 ml of 2% Na_2CO_3 in 0.1N NaOH. To each standard and the blank, 6.0 ml of the alkaline copper reagent was added. After 10 min, 0.3 ml of Folin-Ciocalteu reagent was mixed in. The absorbance of each standard was read at 500 nm against the blank after 30 additional min. A range of crude extract concentrations was tested, up to 0.20 ml.

Gel Permeation Chromatography

Sephadex G-100

Gel permeation chromatography was used to separate the protein components of the venom extract. The column was a Pharmacia K16/70, 1.6 X 70 cm, including a reservoir and a 3-way valve (Fig 13). The crude venom extract used for the column, prepared similarly to the Glycera convoluta venom extract used by Michel (1975), consisted of 400 glands in 4.0 ml of 5mM NH_4HCO_3. About 1.0 ml of this extract was put on the Sephadex column and run at 6°C. Fractions of 3.0 ml were collected at a flow rate of 0.12 ml/min, which slowed down considerably as the sample passed through the column. After collecting about 67 ml the column was stopped. The fractions were monitored for protein content by spectrophotometric absorption at 280 nm. Various fractions were tested using the previously described toxicity assay, Folin-Ciocalteu assay and trypsin assay.

Sephacryl S-200

The same column, K16/70, was used as for the Sephadex G-100 (Fig 13). The column was packed as recommended by Pharmacia.

Fig 13. Pharmacia K16/70 column used for Sephadex G-100 and Sephacryl S-200 separations.

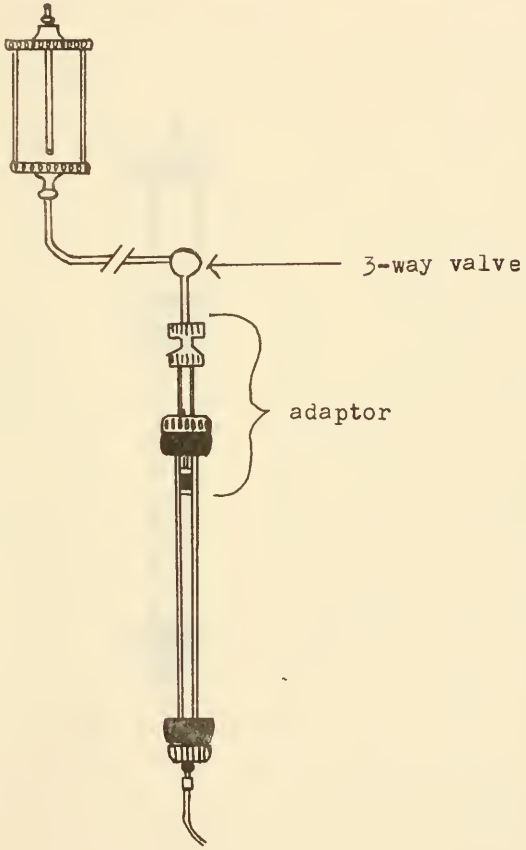


Fig 14. Pharmacia K16/70 column being packed with reservoir directly attached to column.

Approximately 200 ml of Sephacryl S-200 Superfine was used, which is about 125% of the packed bed volume. The gel was suspended in eluant buffer, 5mM NH_4HCO_3. The amount of buffer used for the suspension was about three times the volume of the settled gel. After the gel resettled, the excess buffer was decanted off. Then enough buffer was added to make a slurry of 70% settled gel and 30% excess liquid. The slurry was poured into the column and allowed to settle. The reservoir, connected directly to the top of the column(Fig 14), was filled with eluant buffer. A second reservoir was positioned above the column to obtain a flow rate of about 40 ml/cm^2-hr. The column was eluted with buffer for two hours. The reservoir attached directly to the column was removed, and a flow adaptor was added, so that it was just touching the gel surface(Fig 13). The direction of the flow was reversed and the column was eluted with the buffer at the same rate, for two hours. Upon completion of the back flushing, the column was packed and ready for chromatography at a flow rate of 30 ml/cm^2-hr or less. The packed bed volume was 124.6 cm^3. A sample of the same batch of venom extract that was used on the Sephadex G-100 column was used here. The sample consisted of 1.5 ml extract, which was eluted with 5mM NH_4HCO_3 buffer. Fractions of 3.0 ml were collected at a flow rate of about 1.0 ml/min. The protein content was again monitored by recording the spectrophotometric absorbance at 280 nm. The toxicity assay, trypsin assay, and the Folin-Ciocalteu assay were performed on selected fractions.

The Sephacryl column was calibrated by using Pharmacia high and low protein molecular weight calibration kits. Table 1

Table 1. Proteins used in Calibrating Sephacryl S-200 Column.

	Protein	Volume on column(ml)	mg protein per ml buffer
I	Blue Dextran 2000	1.0	1.0
II	Thyroglobulin	1.0	2.0
III	Thyroglobulin	1.0	5.0
	Catalase		5.0
IV	Aldolase	1.5	5.0
	Ribonuclease A		5.0
V	Thyroglobulin	1.0	2.0
	Aldolase		2.0
	Ovalbumin		7.0
VI	Thyroglobulin	1.0	2.0
	Albumin		7.0
	Ribonuclease A		7.0

shows the proteins that were used. Except for the Blue Dextran 2000, the proteins were eluted with the buffer at pH=7.8. When the Blue Dextran was run at this pH, it was absorbed by the gel. Therefore, the pH of the buffer was increased to about 10 by addition of NaOH, and the Blue Dextran was successfully rerun.

RESULTS AND DISCUSSION

Preliminary Studies on Crude Extracts

All Uca pugilator injected with a standard dose of venom extract showed the reactions of envenomation as listed in Table 2. Except for the initial scurrying, which was shown by both experimentals and controls, these symptoms are the signs of venom toxicity. The control crabs scurried around immediately after the injection, and then appeared normal.

The results of the toxicity assay are summarized in Table 3. In order to test for stability, extracts were stored at -20°C for up to 35 days. When the crabs were injected with the venom extracts(Table 3, sample #4), they exhibited the same toxic symptoms as recorded in Table 2. The control crabs did not show any unusual signs. Therefore, the extracts may be considered stable, for the purposes of this experiment, at -20°C for 35 days.

The results of the study at room temperature suggest two active principles in the venom, a quivering factor and a lethal factor. The latter showed no change when the extract was stored at 21-22°C for up to 2.5 hrs; however, the quivering factor decreased in activity under the same conditions. The injected crabs exhibited less quivering the longer the venom remained at room temperature, and after 2.5 hrs no signs of quivering were observed. Therefore, this quivering factor appears to be a temperature-sensitive protein which has an activity inversely proportional to the length of storage time at room temperature.

Heat stability of the toxin was tested by heating the extract at 100°C for 10 min. A large amount of coagulum appeared, indicating the presence of proteins. Then the extract was

Table 2. Signs of envenomation.

Time	Observation
	immediate scurrying
10-15 sec	legs on injected side of body start quivering
30 sec	legs on injected side straighten and become limp
50-90 sec	eyestalk on injected side falls
within 20 sec	other eyestalk falls
2-4 min	crabs generally tilt backwards - there are alternate periods of legs quivering, legs appearing limp, and then legs straightening out and quivering - no reaction made when threatened
5-10 min	no movement

Table 3. Results of toxicity assays.

Sample number	Number crabs/ test	Treatment of extracts	Cessation of crab movement Expt's(min)	Cntrl(days)
1	7	Standard	5-10	2,3,3,3,4, 21,31
1a	2	Sea-water control	-	4.32
		Frozen(-20°C)		
2	2	few days	5-10	-
3	2	5 days	5-10	5,8
4	2	35 days	5-10	14,40
		Heated(100°C, 10 min)		
5	2	cooled slowly	3,5 days	39,100
6	2	cooled slowly, centrifuged	5,8 days	5,6
7	1	cooled immed., centrifuged	4 days	-
		Room temperature(21-22°C)		
8	2	15 min	5-10	30 min 3 days
9	2	25 min	5-10	1,3
10	2	45 min: little quivering,*	5-10	1 hr, 4 days
11	2	1 hr: less quivering,	5-10	2,43
12	2	1.5 hr: less quivering,	5-10	3,43
13	2	2 hr: less quivering,	5-10	2,3
14	2	2.5 hr: no quivering,	5-10	-
		Acetone precipitation		
15	2	fraction #1	35	4,4
16	2	fraction #2	3	4,4
17	2	fraction #3	6	4,4

- not tested

* see text

allowed to cool to room temperature(samples 5,6) and injected into the crabs. The crabs were very lethargic for 10-15 min after the injection, showing only slight reactions. Initially it was thought that some of the coagulate had been injected into the crabs, and that this could somehow be causing the lethargic symptoms(sample 5). The test was repeated with another sample(6), centrifuging the heated extract(after it cooled to room temperature) at 2500 RPM, 5°C, for 20 min, so as to eliminate that possibility. However, these injected crabs showed similar behavior. A third possible explanation is that some of the protein could be renaturing(Freifelder 1976) since the extract had been allowed to cool slowly at room temperature. Therefore another venom extract was prepared(sample 7); it was similarly heated for 10 min, immediately cooled in an ice bath, and centrifuged at 2500 RPM, 4°C, for 10 min. These crabs showed no signs of venom toxicity; thereby supporting the third explanation.

The concept of renaturation seems to be a recent topic. Much of the terminology used is only vaguely defined. A 'denatured' enzyme refers to "a form of a macromolecule that has less secondary structure than that which is called native"(Freifelder 1976). The 'native' structure is defined as being the macromolecular structure as found in nature, the isolated but enzymatically active structure of a macromolecule, or that structure which retains its secondary spatial arrangement, but is biologically inactive(Freifelder 1976).

The kinetics of denaturing enzymes is very complicated (Roberts 1977). The process depends on the number of bonds altered, and is associated with a high enthalpy of activation

and a large increase in entropy(Roberts 1977). The entropy change suggests that the protein loses its ordered structure, including the active site. Most enzymes denature at 70°C, although exceptions have been found(Roberts 1977). Of the enzymes studied so far, trypsin, which has been identified in bloodworm venom, has a high degree of renaturation(Neilands 1964). Smaller proteins are able to almost completely renature if they are subjected to only mild methods of denaturation(Haurowitz 1963). The methods used in this study are considered only moderately harsh, which could account for a high degree of renaturation.

In a study of octopus toxin, Ghiretti(1960) found that it produces the following effects:

> When a drop of saliva is injected into a crab, the legs immediately contract and the animal remains in this position for about 1 min. The righting reaction is abolished, the appendages begin to tremble, the pincers open and close without external stimulation, and tetanic spasms of the body at intervals are seen. Little by little the animal becomes quiet; spontaneous movements as well as provoked reactions disappear and the aggressive animal of a few minutes before is changed to an inert limp organism.... The saliva has produced first a phase of overexcitability followed by a quiet phase and then paralysis. (p 730)

These reactions are similar to those produced in this study by injecting crabs with bloodworm venom. Several of the components identified in octopus saliva are referred to as being very heat resistant(Ghiretti 1960); they are tyramine, histamine, acetylcholine, taurine, p-hydroxyphenyl-ethanol-amine(octopamine) and 5-hydroxytryptamine(5-HT, serotonin, enteramine). When saliva was heated for 10 min at 100°C and injected, no lethal effects were observed, as was found in this study in the case of the

immediately cooled bloodworm venom. However, if there are heat resistant substances in the bloodworm venom, as in the octopus saliva, it could explain the sluggishness of the crabs ofter being injected with heated and slowly cooled venom. Ghiretti did not say how the octopus saliva was cooled, but it would be interesting to see the effects of the different treatments on crabs.

Because the appearance of a heavy coagulum in heated extracts suggested the presence of proteins, acetone precipitation was used to fractionate, or partially purify, the extracts. Precipitates were obtained by adding different amounts of acetone to extracts, with the least soluble proteins precipitating out first(Clark & Switzer 1964). After the first acetone extraction, a much larger amount of precipitate was noticed in the experimental sample than in the control. The opposite was true for the second and third extractions. When the venom precipitate was redissolved in sea water and injected into the crab, it became evident that a separation had taken place. The first fraction caused signs of envenomation, but at a very slow rate. The crabs did not completely stop moving for 35 min. However, the second and third fractions caused the same symptoms to occur at a much faster rate, 3 and 6 min respectively. Another interesting observation was that fraction 1 had a lot more precipitate than fraction 2 yet the latter fraction caused a much faster reaction to occur in the crabs. The active components of the venom must be very potent. These results seem to indicate that the toxic principles are not very soluble, and that the spreading factor(which is believed to cause tissue histolysis) is

more soluble than the toxic components themselves. If there are two such components, they appear to be complementary in action. One speedily breaks down the tissue to allow the other component to penetrate quickly. The crabs injected with control precipitate did not exhibit any venomous signs.

Acetone extraction on octopus saliva shows different results (Ghiretti 1960). When the precipitate is redissolved in sea water and injected, the crabs show only overexcitability, and no sluggishness. The crabs then appear normal after a few minutes.

Acetone precipitation may offer a second means for storing the venom; however, further studies need to be done to test its stability in this form. There is also a possibility that not all of the toxic components are proteins.

In general, none of the control crabs showed signs of envenomation. Most crabs continued moving for a week or two, although the actual extremes were from 30 min to 100 days.

The venom was tested for trypsin activity, yielding positive results(Table 4). The control was found to have very little, if any, trypsin activity. The presence of trypsin in the venom could account for the high degree of renaturation found in the heated extract.

The Folin-Ciocalteu assay measured protein concentration up to 300 μg. The control extract had 3-4 mg more tissue per ml than the venom extract, although the latter showed a higher protein concentration(Table 5). It was not possible to accurately determine the protein concentration in the venom extract because the assay is valid only up to 300 μg and the venom extract had a higher spectrophotometric reading than that of the 300 μg

Table 4. Trypsin determination of crude venom extract.

µg trypsin	$\frac{\text{abs(253nm)}}{\text{time(min)}}$	ml extract	$\frac{\text{abs(253nm)}}{\text{time(min)}}$
5.0	0.600	0.50	0.340
2.0	0.109	0.05	0.318
1.0	0.057	0.05	0.177

Table 5. Protein concentration of crude extracts.

µg protein	abs 500nm	ml venom extract	abs 500nm	ml muscle extract	abs 500nm
50	0.144	0.05	0.445	0.05	0.285
100	0.245	0.10	0.573	0.10	0.482
200	0.302	0.20	1.283	0.20	0.619
300	0.346				

standard. These results do indicate that there is a higher protein content in the venom extract than the control.

Chromatography

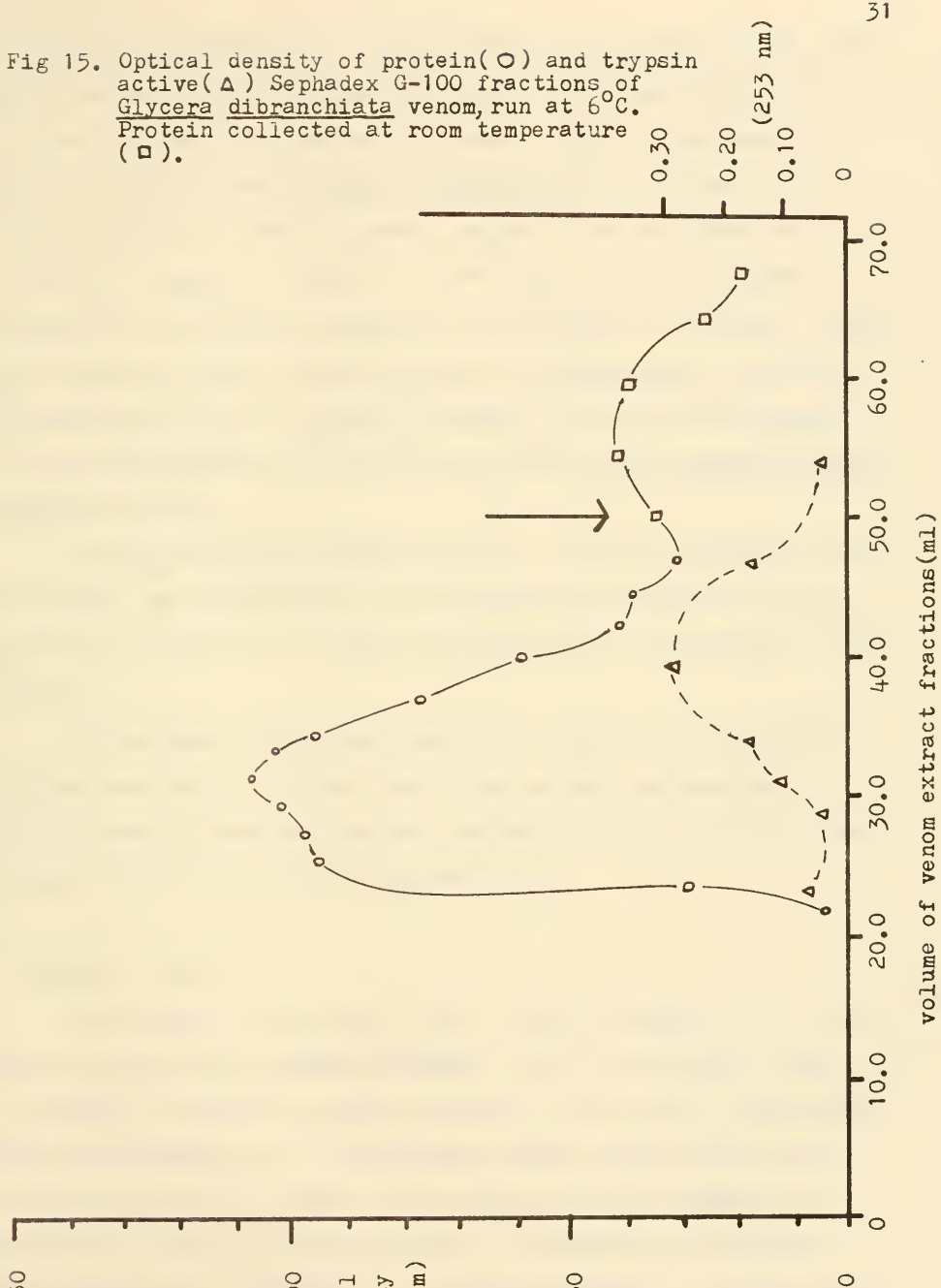
Having established the presence of proteins in the venom, the next step was to try and isolate them. Gel permeation chromatography was chosen because it is a basic separation technique. Sephadex G-100 was initially used because it was similar to what Michel(1975) used and it was readily available in this laboratory.

Sephadex G-100

During extract separation with Sephadex G-100, the flow rate of the column decreased significantly. The decrease is believed to have been caused by particles in the venom extract which clogged up the nylon netting at the bottom of the column. The netting was acting as a filter to prevent the sephadex from flowing out of the column and into the fractions being collected. After a volume of 48 ml was collected at 6°C, the column was run at room temperature. The fractions were monitored for protein content by spectrophotometric readings at 280 nm(Fig 15). The first part of this protein absorption curve is very similar in shape to that obtained by Michel(1975) on a Sephadex G-75 column, using a venom extract from Glycera convoluta. Because of the high degree of similarity, a second column was run with Glycera dibranchiata venom. Due to the flow rate problem of Sephadex G-100, Sephacryl S-200 was chosen for the column bed.

Several of the Sephadex G-100 fractions were tested for

Fig 15. Optical density of protein(O) and trypsin active(Δ) Sephadex G-100 fractions of Glycera dibranchiata venom, run at 6°C. Protein collected at room temperature (□).



trypsin activity(Table 6). The activity was plotted on the same graph as the protein absorption(Fig 15). Maximum trypsin activity occurred between the two protein peaks. This corresponds to Michel's findings with Glycera convoluta(1975). Michel did further studies on this protease enzyme to be sure that the enzyme was trypsin. However, it was found that the isolated enzyme did not act like trypsin on the β-chain of insulin. Therefore, Michel refers to this enzyme as 'trypsin-like', possibly indicating a different type of trypsin. Due to this finding a closer examination of the trypsin isolated from Glycera dibranchiata is needed.

The Folin-Ciocalteu Assay was run on several Sephadex G-100 fractions, but the results were inconclusive because the standards were not at a low enough concentration to correspond to the samples.

The Sephadex column was abandoned because of the decrease in flow rate which occurred during the extract separation. A second venom extract was run with Sephacryl S-200. This gel had a faster flow rate than the Sephadex gel.

Sephacryl S-200

The Sephacryl column also had a slight decrease in flow rate when the extract was passed through. The flow rate went from 1.0 ml/min to about 0.7 ml/min, however it was still much faster than the Sephadex gel. Four protein peaks were obtained from the extract(Fig 16). Their approximate molecular weight was calculated in the following manner, as described by Pharmacia. By experimentally obtaining the elution volumes(V_e) of the stan-

Table 6. Trypsin determination of Sephadex G-100 fractions.

µg trypsin	abs(253nm) / time(min)
2.3	0.427
4.5	0.797

0.20 ml of fraction #	abs(253nm) / time(min)
1	0.051
2	0.085
5	0.056
6	0.128
8	0.183
11	0.326
18	0.181
29	0.056

Fig 16. Optical density of protein(o) and trypsin active(□) Sephacryl S-200 fractions of Glycera dibranchiata venom, with 3.0 ml fractions collected at 4 min intervals at 6°C.

dard proteins, their K_{av} values were calculated; the formula for calculating K_{av}(partition coefficient) is $K_{av}=\frac{V_e-V_o}{V_t-V_o}$, where V_o is the column void volume(which corresponds to the V_e of the Blue Dextran 2000), and V_t is the total bed volume, 124.6 cm^3 for this column. Note that the V_e is the volume at which the highest concentration of a particular protein is collected. The K_{av} was plotted against the corresponding log molecular weight to obtain a linear relationship between elution volumes of the column and protein molecular weights. In this way the approximate molecular weight of the extract protein peaks can be determined(Fig 17). To compare these results with those of Michel (1975), the K_{av} values for Michel's Sephadex G-75 and G-200 were estimated using V_o=25.0 ml and V_t=78.5 cm^3(Table 7), and plotted (Figs 18 & 19). This study revealed four protein peaks having molecular weights of >232 000, 160 000, 121 000 and 31 000, corresponding to one high, two middle, and one low molecular weight protein peaks. Michel(1975) also obtained high, middle and low molecular weight protein peaks, as well as an additional intermediate peak in the Sephadex G-200 run. A more exact comparison cannot be made due to the lack of specific information available about Michel's columns. However, these results indicate a relationship could exist between the two venoms.

To further compare these two venoms, the approximate molecular weight of the substance responsible for the trypsin activity was determined(Table 7. Figs 17-19). The first trypsin peak had a molecular weight of 200 000, with the adjacent protein

Table 7. Molecular weight of venom protein peaks and trypsin activity for *Glycera dibranchiata* from Sephacryl S-200 and for *Glycera convoluta* from Sephadex G-75 and G-200 (Michel 1975).

Protein	Molecular Weight	
	Glycera dibranchiata	*Glycera convoluta*
Venom peaks		
I	232 000	560 000- 232 000
II	160 000	175 000- 55 000
III	121 000	44 000
IV	31 000	19 000- 10 500
Trypsin peaks		
A	200 000	88 000- 84 000
B	128 000	

Fig 17. K_{av} vs log protein molecular weight for Sephacryl S-200. Standard proteins(O), venom protein peaks (Δ), trypsin peaks(⬚).

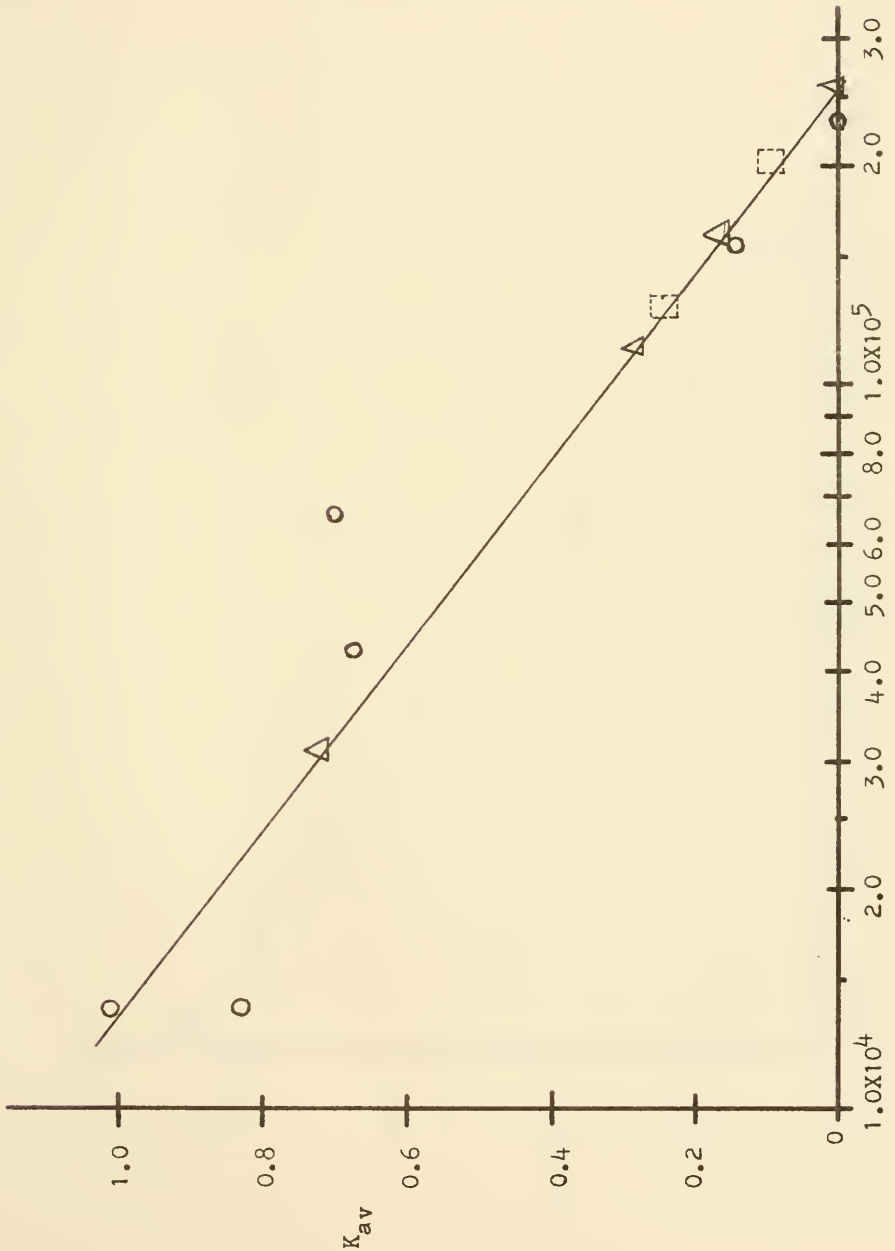


Fig 18. K_{av} vs log molecular weight for Michel's(1975) Sephadex G-75 column. Standard proteins(O), venom protein peaks(△), trypsin peak(⬚).

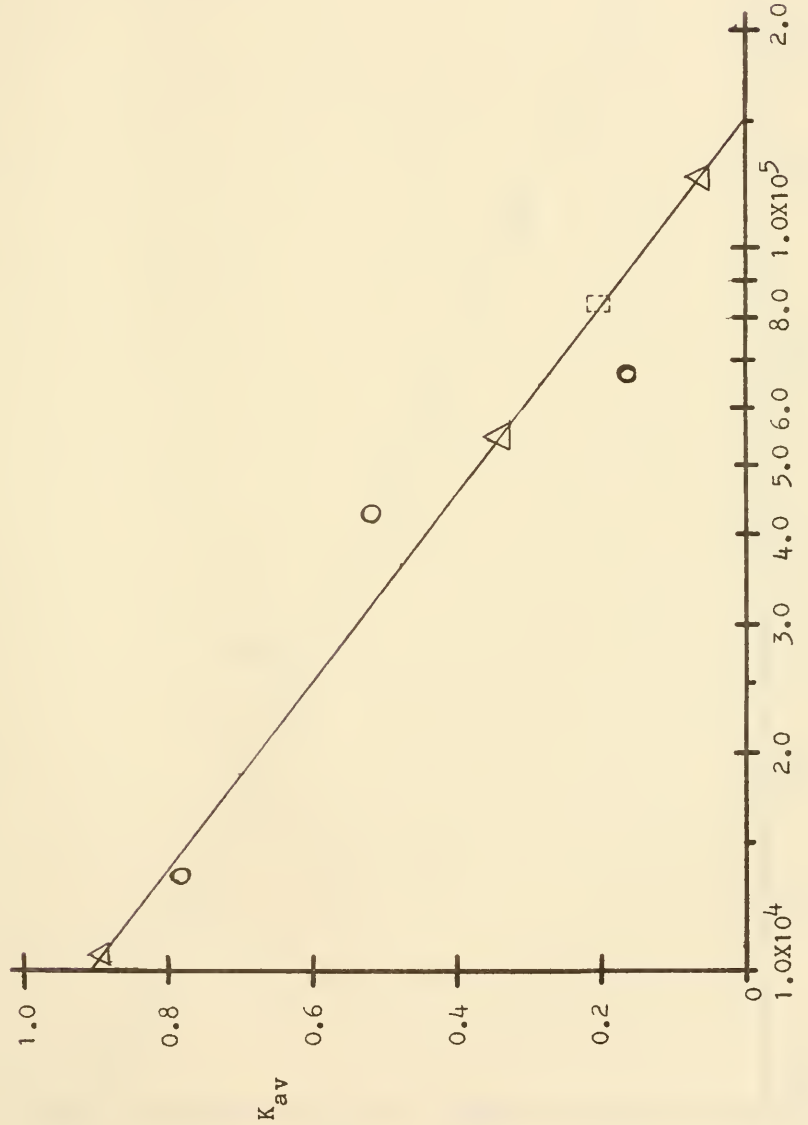
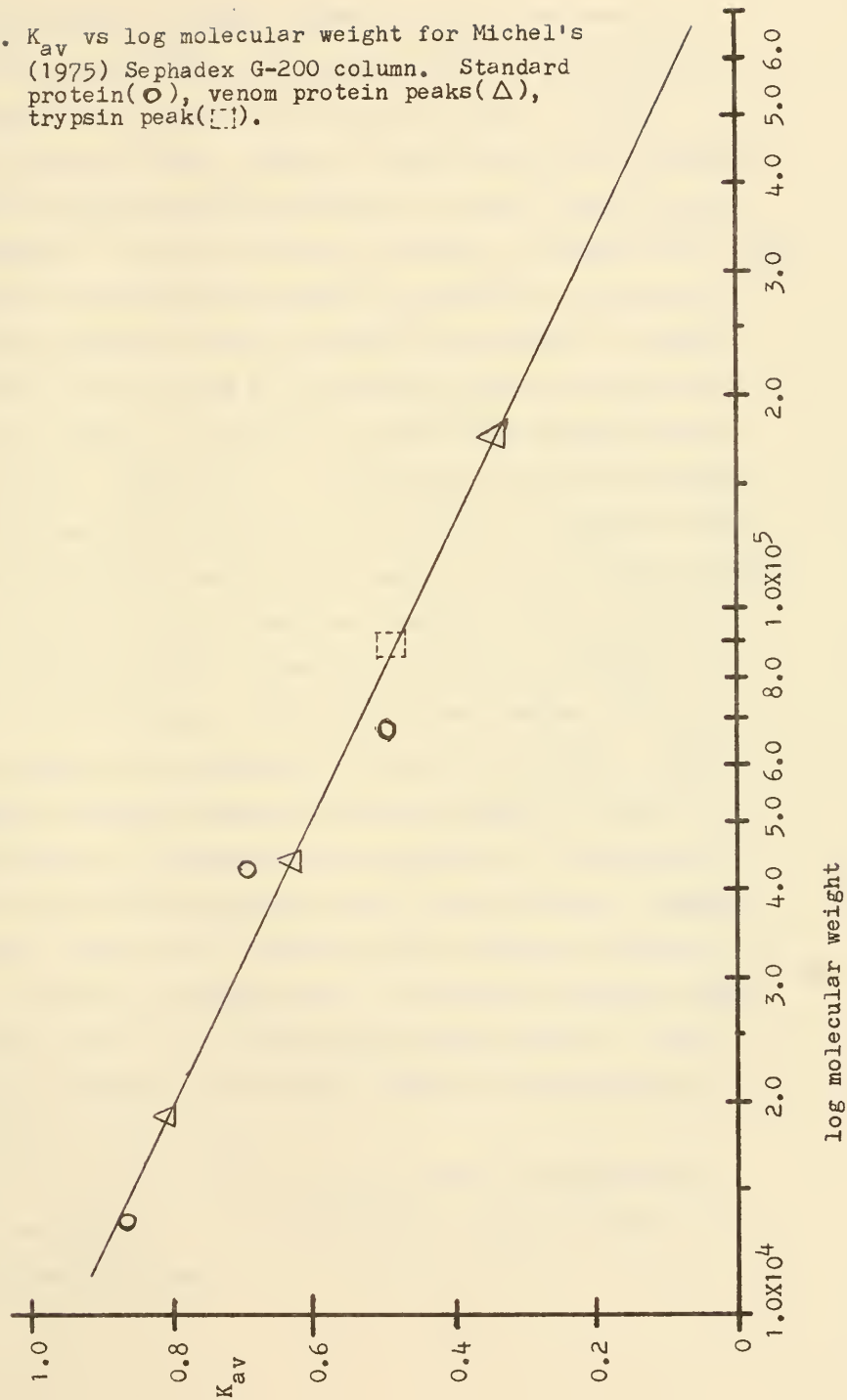


Fig 19. K_{av} vs log molecular weight for Michel's (1975) Sephadex G-200 column. Standard protein(o), venom protein peaks(Δ), trypsin peak([ ]).



peaks at >232 000 and 160 000. Michel obtained this same sequence with Sephadex G-75; a molecular weight of 84 000-88 000 for the trypsin peak, with protein peaks of about 250 000-560 000 and 55 000-175 000. However, before any final conlusions are made, the trypsin fractions should be further tested using trypsin inhibitors and the β-chain of insulin, as Michel(1975) used.

Selected fractions were assayed for toxicity. Since the extract had a solvent of buffer instead of sea water, a number of crabs were injected with an equivalent volume of pure buffer as a control measure. About 40% of these crabs stopped moving within 10-15 min. Therefore, no conclusions about the toxicity of the fractions could be made from this assay. Ammonium bicarbonate was specifically used as the buffer for the chromatographic separation in this study so that the results could be directly compared to Michel's(1975). However, in order to determine the toxicity of the fractions, either a different buffer, which is not toxic to Uca, or a different test animal is needed. Michel used Daphnia by directly applying a small amount of extract to the exposed heart. The toxicity was measured as a function of the time it took for the Daphnia heart to stop beating. Daphnia was not used in this study because of the difficult technique required to expose the Daphnia heart, and because they are fresh water animals while the bloodworms are marine animals. It is biologically important to know what effect the venom has on the bloodworm's natural prey.

Some of these fractions were also tested for protein concentration according to the Folin-Ciocalteu assay. However, no conclusions could be made.

CONCLUSIONS

The venom of Glycera dibranchiata appears to have at least two complementary components, one to increase the spreading of the venom inside the prey animal, and a lethal component, possibly a neurotoxin, which paralyzes and/or kills the prey. From the Sephacryl S-200 column four fractions were obtained with molecular weights of >232 000, 160 000, 121 000 and 31 000. These four peaks correspond to four protein peaks obtained by Michel (1975), who used venom from Glycera convoluta on a Sephadex G-200 column. A trypsin-specific assay revealed two peaks of trypsin activity, having molecular weights of 200 000 and 120 000. The position of the first peak in relation to the protein peaks directly corresponds to the trypsin peak found by Michel(1975). This preliminary study on Glycera dibranchiata venom indicates that it is very close, if not the same, as Glycera convoluta venom.

At least two more studies are needed to confirm the similarity. They are a more sensitive toxicity assay, such as the effect of the venom extract on the beating heart of a Daphnia (Michel 1975), and the effect of the trypsin substance in the venom on the β-chain of insulin. Results from these two studies would provide the needed information to fully confirm the similarity. Michel(1975) also did a collagenase assay. The assay is important because of the evidence of tissue hydrolysis upon injection of the venom in shrimp and marine fish(Curtis 1974, unpublished). The presence of collagenase would suggest that there is a component of the venom directly responsible for breaking down tissue and thereby increasing the rate at which

the venom spreads.

The presence of heat-resistant protein, revealed in this study, suggests a new area of research. It would be interesting to test the Sephacryl S-200 fractions for these specific proteins. It could reveal more about the specific components of Glycera dibranchiata venom.

SUMMARY

1. The purpose of this study was to isolate and chemically analyze the toxic principles of Glycera dibranchiata venom.

2. The crude venom extract was tested for toxicity, proteins, and trypsin content. The extract was fractionated by acetone precipitation and Sephadex G-100 and Sephacryl S-200 column chromatography.

3. Acetone precipitation results suggested the presence of at least two toxic protein components, a spreading factor and a lethal factor.

4. The crude extract was stable at -20°C for up to 35 days. At room temperature and above three protein components were found: a "quivering" factor, heat sensitive at room temperature and above, a lethal factor, heat sensitive at 100°C, for 10 min, and above, and a "lethargy" factor, heat resistant.

5. Four protein peaks and two trypsin-active peaks were obtained from Sephadex G-100 and Sephacryl S-200 chromatography. The molecular weights of the four protein peaks were >232 000, 160 000, 121 000 and 31 000 , and the molecular weights of the trypsin-active peaks were 200 000 and 128 000.

6. These results indicate that G. dibranchiata venom is probably similar to G. convoluta venom, and more work on the toxic and trypsin-active principles, according to Michel's(1975) methods, is needed.

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