A Preliminary Study of *Conus* Venom Protein

BY

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(2 Plates)

INTRODUCTION

ALTHOUGH THE TOXICITY of venoms of the genus *Conus* has been known since a report by RUMPHIUS in 1705 (VAN BENTHEM-JUTTING, 1959), these venoms have been studied only intermittently since. Biochemical studies have not been carried out to any great extent; the last major study on the biochemistry of the venom of *Conus* was carried out by KOHN, SAUNDERS & WIENER in 1960.

In this communication, we summarize some preliminary studies on the protein of these venoms. It will be demonstrated that the venoms have a high protein concentration, and evidence will be presented that the proteins are responsible for toxicity. In addition, gel electrophoresis analyses indicate that there are only a few major protein species in each venom, with widely varying molecular weights.

METHODS

(1) Materials

Specimens of mollusks belonging to the genus *Conus* were obtained from several sources in the Philippines. Specimens were collected either near the islands of Cebu, Marinduque or in Batangas Province, Luzon Island. In early studies, the mollusks were frozen in dry ice, and stored at −5° C until the specimens were dissected. Extracted venoms were also stored at −5°, usually diluted with distilled water. In most studies to be described, mollusks were kept alive in salt water aquaria for several months, and the venom was extracted only when it was to be used immediately. Most studies were carried out with *Conus textile* Linnaeus, 1758 and *C. geographus* Linnaeus, 1758. Typical specimens actually used are shown in Figures *1* and *2*.

Reagents used for the preparation of polyacrylamide gels for electrophoresis came from Canal Industrial Corporation, Rockville, Maryland. All organic and inorganic chemicals used were reagent grade. Pronase (B grade) was obtained from Calbiochem, San Diego, California.

(2) Venom Extraction

The venoms from the different species of *Conus* were obtained from venom ducts by placing each duct on an ice-cold metal spatula, then cutting out the duct in 2 cm segments, and squeezing out the contents with a pair of forceps. The venom from each specimen was weighed, then suspended either in distilled water or normal saline solution.

(3) Protein Determination

An aliquot of the venom (usually 5 μl of a 20% suspension) was diluted with 0.4 ml of distilled water. Proteins were precipitated from the diluted sample by adding 0.1 ml of 50% trichloracetic acid to give a final trichloracetic acid concentration of 10%. The mixtures were allowed to stand over crushed ice for 10 min and centrifuged at 10 000 RPM in an SS-34 Rotor in the refrigerated RC2B Sorvall Centrifuge. The precipitate obtained was dissolved in 0.1 M NaOH prior to protein determination according to the method of LOWRY (1951).

(4) Disc Gel Electrophoresis

Disc gel electrophoresis was carried out in a Canalco Disc Electrophoresis chamber, model 1200, using formu-

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Table 1

Venom samples were prepared by squeezing fresh venom out of the venom ducts, weighing the venom in a tared vial and adding enough 0.15 M sodium chloride to yield a 20% venom solution. The venom (or control solutions, 0.15 M NaCl or a solution containing 50mg/ml of bovine plasma albumin in 0.15 M NaCl) were injected into mice either intraperitoneally or intracisternally. Protein determinations were carried out as described under Methods; the designation "no data" indicates that these were venom samples on which protein determinations were not carried out.

Comparison of the Toxicity of Venoms from Different *Conus* Species

Venom sample injected	Net wt. of mouse, g	Volume injected (μl)	Total mg protein injected	Observations
		A. Intraperitoneal Injections		
0.15 M NaCl	26	500	–	alive, no significant effect
Conus textile	26	500[1]	no data	alive, drowsy after injection but became very
	25	50	2.5	active again; scratched the abdominal area with
	30	50	2.5	hind legs
Bovine plasma	21	50	2.5	found dead after 2 days
albumin (50mg/ml)	20	50	2.5	alive
Conus aulicus	30	50	1.73	alive, normal and active right after injection
	31	50	0.93	"
	28	50	1.98	found dead on the following day
Conus geographus	23	50	0.53	died after 7 min — normal breathing right
	29	50	0.53	died after 10 min — after injection; after 6 min
	27	50	0.53	died after 12 min — labored breathing started; jumped and jerked before death
Conus tulipa	30	50	no data	died after 36 min
	31	50	no data	died after 27 min gasped and jerked
	25	50	no data	died after 45 min
Conus marmoreus	25	50	0.68	alive; no observed effect
		B. Intracisternal injection (All mice were chloroformed before injection)		
0.15 M NaCl	24	50	–	controls looked drowsy most probably from
(control)	23	50	–	$CHCl_3$ but recovered after some time
	24	50	–	
	21	50	–	no deaths
	16	50	–	
	19	50	–	
Bovine plasma	29	50	2.5	alive; practically same behavior as controls
albumin	21	50	2.5	"
50 mg/ml	18	50	2.5	"
	30	50	2.5	"
	23	50	2.5	"
	27	50	2.5	found dead the following day
	35	50	2.5	alive
	21	50	2.5	alive
	29	50	2.5	alive
Conus textile	22	50[1]	no data	labored breathing; paralysis of right arm; tremor, then death after 30 min.
	26	50[1]	no data	same difficulty with breathing; gasped for air, eyes turned brownish red after 30 min, died after 50 min; no tremors.
	33	50	2.5	died after 5 min; gasped for air.
	28	50	2.5	died after 25 min; tremors before death.
	32	50	2.5	died after 2 min.
	26	50	2.5	died after 7 min, 30 sec.
	20	50	no data	died after 20 min.

Table 1 (continued)

Venom sample injected	Net wt. of mouse, g	Volume injected (μl)	Total mg protein injected	Observations
Conus aulicus	27	50	1.73	alive; quickened & shallow breathing; recovered after 22 min.
	25	50	0.93	alive; gasped for air; shallow breathing; recovered after 10 min.
	27	50	1.73	alive; weak; labored breathing; recovered after 6 min.
	no data	50	1.98	alive; weak; normal breathing.
	no data	50	1.0	alive; fast shallow breathing; slight trembling; jumpy.
	25	50	0.93	died after 13 min & 30 sec; weak breathing, foaming of mouth before death.
Conus geographus	28	50	0.53	died after 2½ min; no tremors.
	30	50	0.53	died after 3 min.
	29	25	0.26	died after 5 min.

[1]10% venom. All other samples were 20% venom.

lation prescribed by Canalco for RDS Gels. All electrophoreses were done at 3 milliamperes per tube for at least one hour. Protein bands were visualized by staining the gel with amido black, and destaining the background electrolytically with Canalco quick gel destainer.

RESULTS

(1) Biological Assay for Toxicity

In order to study biological effects of *Conus* venoms, a preliminary survey of toxicity of these venoms was carried out with mice. The results of these studies are shown in Table 1. It is clear that of the species examined, *C. geographus* and *C. tulipa* Linnaeus, 1758 are the only two that are toxic when the venom is injected intraperitoneally. *Conus textile, C. aulicus* Linnaeus, 1758 and *C. marmoreus* Linnaeus, 1758 did not cause death of mice under these conditions. However, it was found that if the venoms were injected intracisternally, the *C. textile* also showed a definite toxic effect. These results are in agreement with the previous biological effects demonstrated by WHYTE & ENDEAN (1962), ENDEAN & RUDKIN (1965), and by KOHN *et al.* (1960).

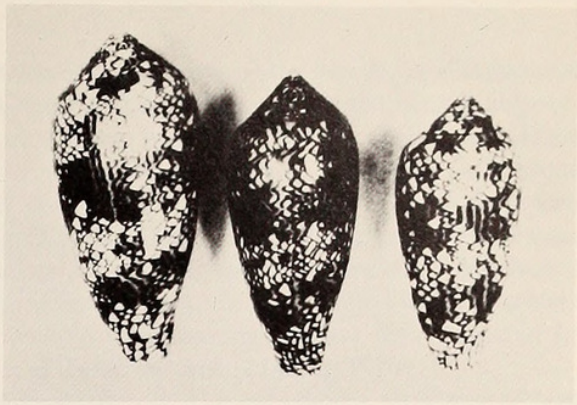
Autopsies of mice which died from intracisternal injections of *Conus textile* venom showed that lung hematomas were present. On the other hand, mice that died after intraperitoneal injections of *C. geographus* venom showed no hematomas; thus, the mechanism of toxicity may be different. There is considerable variation in the onset of death in mice after injection with 2.5 μg of *C. textile* venom (2 - 25 min); at lower venom levels, *C. geographus* venom consistently caused death in less than 5 min if injected intracisternally. This supports the general notion that *C. geographus* is probably the most dangerous of all the *Conus* species.

Explanation of Figures *1, 2*

Figure *1*: Two species of *Conus* reported to have caused injury and death in man. *Conus geographus* is a fish-eating species, while *Conus textile* preys on other mollusks. The scale is in centimeters. These are shells of mollusks actually used in this study.

Figure *2*: Venom apparatus of *Conus textile*. A, venom bulb; B, venom duct; C, radula sheath; D, pharynx; E, proboscis. The shell of the mollusk from which the venom apparatus is taken is shown for comparison.

Conus geographus



Conus textile

Figure 1

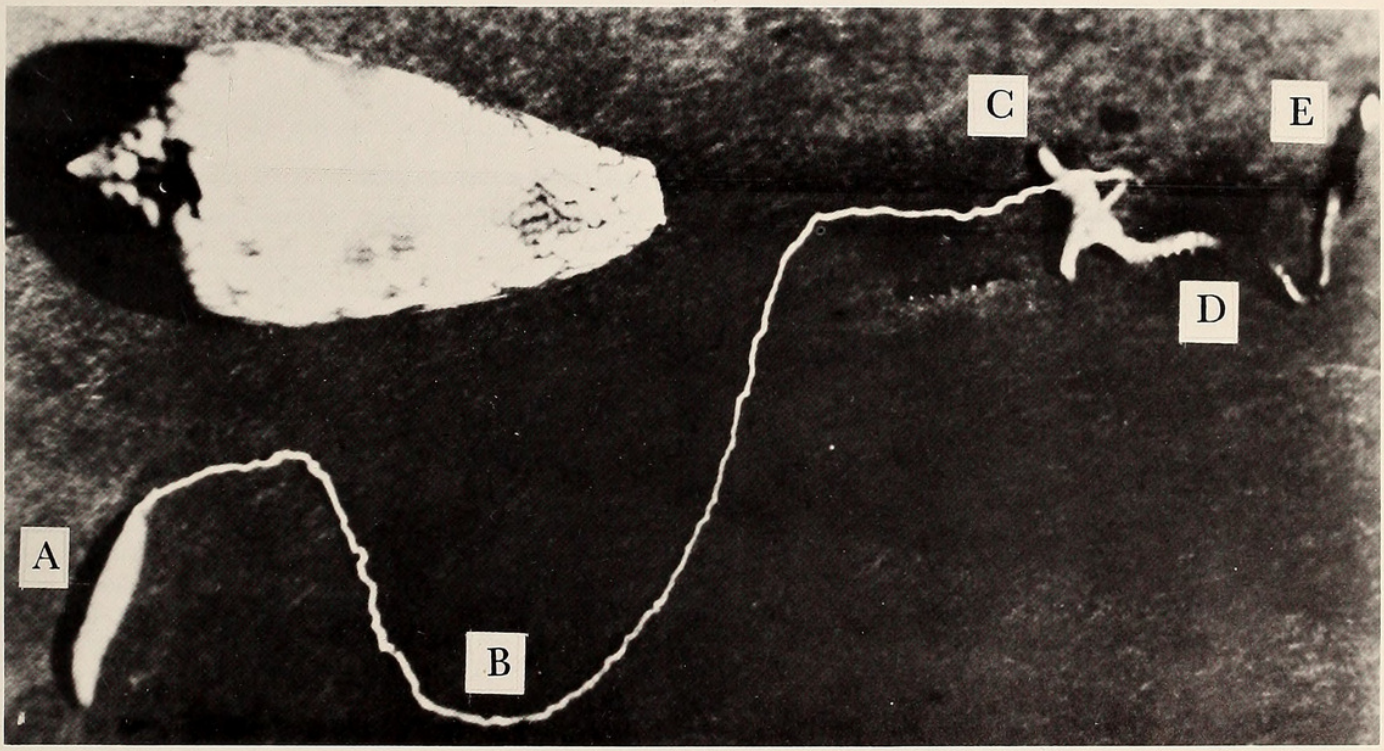


Figure 2

The results in Table 1 indicate that the toxins in *Conus geographus* and *C. tulipa* venoms can be assayed by intraperitoneal injection, but that the toxin in *C. textile* venom should be assayed by intracisternal injection.

(2) Proteins in *Conus* Venoms

Protein determinations were done on several different venoms, and it was found that typical venoms were 5 to 25% protein (Table 2). These high levels suggested that the toxic factor in these venoms might be a protein. In order to test this possibility, the venoms were treated with pronase and then assayed for biological activity. The results of this experiment are shown in Table 3. Although incubation of *Conus geographus* venom, or dialysis of this venom cause a definite decrease in the overall activity of the venom, these treatments do not abolish the ultimate effect of the venom. However, treatment with pronase renders the venom completely inactive in an intraperitoneal injection. The results may be rationalized if the venom itself contained a small amount of protease and therefore prolonged incubation or dialysis caused some inactivation of the venom. Protease activity has been reported in a number of *Conus* venoms (MARSH, 1970).

A complication in the experiment with *Conus textile* venom is that the protease itself ultimately causes death of mice if injected intracisternally. However, this is an effect that takes many hours. On the other hand, the

Table 2

Protein Nature of Toxin from *Conus geographus* venom

A. (Incubation Protocol)

Sample	Volume of Venom (μl)	Volume Pronase (μl)	Volume 0.01 M NH_4HCO_3 (μl)	Volume 0.01 M NH_4HCO_3 (μl)	Treatment
1	100	10	–	20	
2	100	10	–	20	[2]
3	100	–	10	20	
4	100	–	–	–	dialyzed
5	100	–	–	–	set aside on ice

[2]Samples in tubes 1 to 3 were incubated at 37°C for 2 hrs. Then 5λ pronase (30 mg/ml in 0.01 M NH_4HCO_3) were added to tubes 1 and 2 and all tubes (1 to 3) were incubated for another 2 hrs.

Samples 1 to 4 were dialyzed against 500 ml of 0.9% NaCl (3 hrs, overnight, then 2 hrs.) prior to injection.

B. (Results of Injection)

Sample injected	Net wt. of mouse, g	Volume injected (μl)	Observations
1. Pronase	23	25	
digested	20	25	alive
venom	17	50	
2. Pronase	20	25	
control	17	25	alive
	18	25	
3. Incubated	25	25	died after 29 min.
venom (undigested)	28	50	died after 23 min.
4. Dialyzed	20	25	died after 56 min.
venom	30	70 μl of 4X dilution	died after 2 hrs & 10 min.
5. Untreated	26	25	died after 10 min.
	26	50	died after 16 min.
	30	50	died after 6½ min.

venom when injected intracisternally normally causes death within 5 minutes. Thus, the biological effects of the protease alone and of the venom can be differentiated. In almost all cases, the untreated venom, the dialyzed venom and the venom incubated in the absence of enzyme caused the death of the animal within 5 minutes. However, pronase-digested venom or pronase alone did not cause death until many hours afterwards. These results suggest that the intracisternal injection of *C. textile* venom causes rapid death of mice by a protein factor.

Preliminary experiments have also been done with venom from *Conus tulipa* (Table 3). It is clear that treatment with pronase abolishes the lethal effect of this venom after an intraperitoneal injection.

(3) Gel Electrophoresis Analysis

Fresh venoms were also analyzed by gel electrophoresis as described under Methods. Some of the results under non-denaturing conditions are shown in Figures *3* and *4*. It is clear that in all cases, there are a few major species of proteins which appear as bands in gel electrophoresis. The pattern of bands differs in venoms from different species of cones, although there may be some components that are common to all species.

Venoms were also analyzed by gel electrophoresis under denaturing conditions, using sodium dodecyl sulfate as the denaturing agent. Under these conditions, protein subunits dissociate, and mobility on the gel depends only on the molecular weight of each polypeptide chain. Figure *5* shows an experiment with *Conus aulicus* and *C. striatus* venoms. Again, there are only a few major protein species detected, with a wide range of mobilities.

DISCUSSION

The results presented here confirm differences in biological activity previously reported by other workers for *Conus textile* and *C. geographus* venoms. It is clear that *C. geographus* venom causes rapid death of mice if injected either intraperitoneally or intracisternally; on the other hand, *C. textile* venom has no effect if injected intraperitoneally, but kills mice in a few minutes if injected intracisternally. These observations suggest that the toxins in the two venoms are different. Indeed, previous studies by Endean & Izatt (1965) suggest that within a single venom (*i. e.*, *C. magus*), there may be two different toxic fractions.

We have demonstrated that these venoms all contain fairly high levels of protein (5 - 25%). Experiments with *Conus geographus* and *C. textile* venoms strongly indicate that the protein is necessary for the lethal effect of the venom on mice. A previous report indicated that the venom was somewhat sensitive to trypsin and that the toxic factor was non-dialyzable; however, the same report (Kohn *et al.*, 1960) indicated that *C. striatus* venom was quite insensitive to boiling for 10 min. It seems possible that at least some *Conus* toxins may be small, relatively heat stable proteins. However, other *Conus* venoms appear to be inactivated by boiling (Whysner & Saunders, 1963).

Our preliminary gel electrophoresis analyses of some of these venoms demonstrate that only a few major protein species are present in each venom. Gel electrophoresis analyses under denaturing conditions reveal a wide range of molecular weight components (from $<$ 15 000 to $>$ 100 000). These findings indicate that the venom proteins should be amenable to fractionation by standard techniques such as column chromatography. If the toxin is one of the major protein components of the venom, then the purification of the toxin to homogeneity should be straightforward. Such attempts are currently in progress.

The purification of the protein components from venoms of different species of *Conus* should permit more definitive answers to many questions remaining about these venoms. Is a single protein in each venom responsible for all toxic effects? Are the different toxins in different venoms closely related? It is quite clear that the biological

Explanation of Figures *3* to *5*

Figure *3*: Gel electrophoresis of *Conus textile* venom. Each gel represents a venom sample from a different specimen. Electrophoresis and staining were carried out as described under Methods. Note the variability in the intensity of the second fastest moving band in different gels.

Figure *4*: Gel electrophoresis of *Conus marmoreus, C. geographus, C. virgo* and *C. aulicus* venoms. In contrast to other venoms, a 20% suspension of *C. marmoreus* venom contained visible precipitates even after sonication. The suspension was centrifuged and the supernatant subjected to gel electrophoresis as described under Methods.

Figure *5*: Gel electrophoresis under denaturing conditions of *Conus aulicus* and *C. striatus* venoms. A parallel gel with DNA polymerase I from *Escherichia coli* is shown for comparison. The main band on this gel is the enzyme with a molecular weight of 109 000. Bands on the venom gels that move more slowly have a higher molecular weight; those that move more rapidly have a lower molecular weight.