Spring 1971

A determination by thin-layer chromatography of the chemical nature of the poison produced by the bryozoan lophopodella carteri (Hyatt)

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A Determination by Thin-layer Chromatography of the Chemical Nature of the Poison Produced by the Bryozoan *Lophopodella carteri* (Hyatt)

A Thesis

Presented to the faculty of the Graduate School

of the University of Richmond

in Partial Fulfillment of the Requirements for the

Degree of Master of Science

by

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June, 1971

A Determination by Thin-layer Chromatography of the Chemical Nature of the Poison Produced by the Bryozoan *Lophopodella carteri* (Hyatt)

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ACKNOWLEDGEMENTS

I present my sincere thanks to the following members of the faculty of the Biology Department of the University of Richmond: Dr. William S. Woolcott, for suggesting the problem and for his guidance in writing the manuscript; Dr. David W. Towle, for his suggestions and for his guidance in outlining the problem; Dr. Wilton R. Tenney, for his critical reading of the manuscript; and Dr. Francis B. Leftwich, for his many helpful suggestions with technical problems.

I also sincerely appreciate the assistance of my fellow students, Mr. Harold S. Millsaps, Jr., and Mr. Alfred P. Chestnut, for their help in collecting the fish used as test organisms.

Finally, I greatly appreciate the patience, understanding, and tolerance of my wife and children, whose cooperation made this study possible.

ABSTRACT

A study was undertaken to isolate and characterize by thin-layer chromatography the poisonous principle produced by the freshwater bryozoan, Lophopodella carteri (Hyatt).

The bryozoan homogenate was divided into a lipid and non-lipid portion and each was assayed for toxicity. Only the lipid extract was lethal to fishes. The lipid part was divided into six classes by thin-layer chromatography. When tested, four of the six classes were toxic. Thin-layer chromatography was used to subdivide each class into fractions. Assays showed toxicity in nine of fourteen fractions.

Evidence indicates that the poisonous principle is a lipid and the large number of toxic lipid fractions suggests that it is a multi-component toxin.

INTRODUCTION

Toxicity of the freshwater bryozoan, Lophopodella carteri (Hyatt), to fish was first reported by Rogick (1957) and later by Tenney and Woolcott (1964). In 1958, Oda proposed that the toxic agent and that of another freshwater bryozoan (Pectinatella gelatinosa Fleming) is located in the coelomic fluid and is excreted through a vestibular pore upon invagination of the individual polypides. However, Meacham and Woolcott (1968) found that the toxin of L. carteri was located in the body tissues rather than the coelomic fluid. They also postulated that the toxin might produce its effects by interfering with the function of the carbonic anhydrase system or the respiratory chain. Collins et. al. (1964) observed that fish swim in the vicinity of L. carteri but apparently do not use it as food. As other freshwater bryozoans are used as food by fishes (Bushnell 1966; and Applegate 1966), Collins et. al. proposed that the toxin of L. carteri might enhance its survival.

Before the question of the toxin's action on fish tissues could be resolved more data were needed. Tenney and Woolcott (unpublished data) showed that the toxin's efficacy was not affected by proteolytic enzymes or boiling and from this deduced that it was probably non-proteinaceous. The purpose of this study was to determine the chemical nature of the poison produced in L. carteri.

METHODS AND MATERIALS

Colonies of L. carteri were collected from the Virginia Fish Cultural Station, Stevensville, Virginia during the summer of 1970. In the laboratory, colonies were washed with spring water and debris was removed. The bryozoans were placed in plastic bags and frozen at minus 15 C until used.

Tenney and Woolcott (1964) showed that aquatic gilled vertebrates (e.g., fish and salamander larvae) were susceptible to the toxin. The fish used to detect toxicity in this study were bluegills, Lepomis macrochirus, collected from Westhampton Lake on the University of Richmond campus. These were acclimated for one week in aquaria that contained tap water that had been aged for two days.

Since Tenney and Woolcott (unpublished data) considered the toxin to be non-proteinaceous, attention in this study was given to the lipid part of the tissue homogenate. Separation of the bryozoans into lipid and non-lipid portions was accomplished by adding chloroform/methanol (2:1, v/v) to a known weight of frozen bryozoans in a 3:1 ratio (v/w) and homogenizing in a Potter-Elvejhem glass tissue grinder for 2 - 3 min until all but the bryozoan statoblasts were homogenized. The homogenate was placed in a covered flask and incubated in a water bath at 60 C

for 30 min to accelerate separation of the lipids from the remainder of the slurry. Subsequently, the homogenate was filtered through Whatman 31 filter paper to remove any solids. The solids on the filter paper were washed twice with 100 ml of the chloroform/methanol solvent to complete the lipid removal. The filtrate was centrifuged in an International clinical centrifuge (model CL) at maximum speed for 10 min to separate the water and methanol from the chloroform layer that contained the lipids. The top layer containing the water and methanol was carefully removed with a pipet and added to the solids from the filtering operation. The solids were refrigerated for further testing. The solvent was removed from the lipid portion by evaporation in a vacuum. A solution was prepared by dissolving 30 mg of the dried lipid extract in 100 μl of chloroform/methanol.

Silica gel G Plates were prepared by mixing 45 g of silica gel G with 90 ml of distilled water, shaking vigorously for 90 sec, and applying a uniform 1 mm thick layer with a Colab adjustable spreader. The coated plates were allowed to air dry for 15 min before activating them in a drying oven at 110 C for 30 min. The plates were cooled to room temperature in a desiccator. Thin-layer plates were spotted with the lipid extract and placed in a chromatography tank which had been lined with filter paper and equilibrated for 1 h with 150 ml of petroleum ether/diethyl ether/acetic acid (84:15:1).

In all chromatographic separations 150 ml of solvent were used. This was adequate for separation but small enough in volume to keep the solvent level below the origin on the plate. The plates were developed in the solvent for 30 min, removed, and air-dried at room temperature. A blank control plate was treated in the same way.

The developed plates were stained on one edge with a 1% iodine/methanol stain. Only the unstained portion of the plate was removed for toxicity assays, since iodine in concentrations greater than 0.002% (aqueous) killed the fish within 12 min. Neither a 2% concentration of absolute methanol (aq) nor a 10% silica gel suspension (aq) was toxic within a 1 h test period. The unstained silica was removed from the plate in 6 strips. These corresponded to the bands on the iodine-stained part of the plate. The strips were numbered according to their migration rate, from least to greatest, and labeled as Classes I - VI respectively. Chloroform/methanol was added to the gel to elute the lipid and the solution was filtered to remove the gel. The remaining lipid solution was placed in a 50 ml beaker and the solvent was evaporated by passing nitrogen over it. The lipid powder was suspended in 25 ml of distilled water. A fish about 3 cm in length and weighing approximately 300 mg was added to each class of lipid and the blank. The time required for the fish to die was recorded. The experiment was replicated once.

Each class found toxic was sectioned into fractions by thin-layer chromatography using specific solvents as given in the Experiments and Results. To determine if it was the quantity of lipid or its qualities that were toxic, a known lipid with a migration rate similar to that of each class was tested. These lipids were phosphatidylethanolamine, cholesterol, stearic acid, and cholesteryl stearate. Concentrations ranging from 15 - 40 mg/25 ml distilled water, in steps of 5 mg, were prepared. Only the highest of these concentration proved toxic, and on this basis, further testing of L. carteri lipid extract involved concentrations in the range between 15 - 30 mg/25 ml.

To determine if the non-lipid portion retained any toxic components, a weight of the non-lipid extract (112 mg) corresponding to a bryozoan weight of 2.5 g was transferred to a 50 ml beaker and the solvent was evaporated under a nitrogen gas stream. Twenty-five ml of water were added and the sides were scraped with a clean glass rod to remove the non-lipid sediment. A fish was added to the beaker. For a control, 25 ml of distilled water were placed in a 50 ml beaker and a fish was added.

EXPERIMENTS AND RESULTS

Four of the six lipid classes (I, II, III, and VI) were shown to be toxic. The migration rates of the four classes were compared to the rates of the standards

(phosphatidylethanolamine, cholesterol, stearic acid, tristearin, and cholesteryl stearate from lowest to highest respectively). Class I migrated like phospholipids, Class II like sterols, Class III like fatty acids, and Class VI like the cholesteryl esters. Classes IV and V were not related to any of the standards (Fig 1).

Each class was separated into fractions, which were then designated by Arabic numbers according to migration rates from least to greatest (Table I). All experiments except for Class I were replicated once. Solvents used in the following experiments were from Randerath (1966).

Class I lipids were dissolved in chloroform/methanol and were spotted on a silica gel H plate that was prepared by spreading a slurry (40 g of silica gel H in 80 ml of 0.001 N sodium carbonate) to a thickness of 1 mm. This class was subjected to chromatography as described above, except for the solvent system which consisted of 150 ml of chloroform/methanol/acetic acid/water (25:15:4:2) (Fig 2). When stained with iodine stain, three fractions were visible. The unstained portion of the fractions was removed for toxicity assays and all three were toxic; in addition the controls died within seven minutes. A measurement indicated a low pH of 4.0 and control solutions were comparable to a pH of 6.3 in the aquaria. Further, during the drying process the Class I lipids did not become completely dry as did the other lipid classes. Considering the high

boiling point of acetic acid (118 C), which might have prevented evaporation, the acid became suspect. Fish in 25 ml of distilled water, adjusted to pH 4.0 with acetic acid died within approximately 10 min. In subsequent tests pH was controlled by two different methods. Phosphate buffers (Na_2HPO_4 /KH_2PO_4) were added to maintain pH at 5.5 which corresponded to that of the distilled water being used. The control fish lived and fractions 2 and 3 were lethal within 32 and 23 min respectively. The second method followed the procedure of Rathbone et. al. (1965) which involved repeated washings with chloroform/methanol/water. The fractions were washed three times and each time the washings were discarded. The pH of the fractions after washing ranged from 5.4 to 5.7, and fraction 3 was toxic within 56 min. The control fish lived until the termination of the experiment. It is possible that enough lipid was lost from fraction 2 during the washing process in the second method to render that fraction non-toxic.

Class II was treated the same as the whole lipid extract except that the solvent system was chloroform/methanol (95:5) (Randerath, 1966) (Fig 3). Three fractions, two of which migrated from the origin, were toxic to fishes. Fractions 1 and 2 were lethal within 21 min while fraction 3 was lethal within 32 min.

Class III was also treated the same as the whole lipid extract except that the solvent system was chloroform/

acetic acid (96:4) (Fig 4). The pH was adjusted to 5.5 with phosphate buffers and the fish were tested. Three fractions were isolated. Fractions 2 and 3 were toxic. The actual time of death was not recorded, but was less than one hour.

Class VI received treatment identical to the whole lipid extract except that the solvent was 100% chloroform (Fig 5). Four fractions were isolated, three of which were toxic. Fraction 1 was lethal in 14 min, fraction 3 in 45 min, and fraction 4 in 28 min. All control fish for experiments II, III, and VI survived until the termination of each experiment.

Inasmuch as preliminary experiments showed the non-lipid fractions of the homogenate to be non-toxic, no further work was done with them.

CONCLUSIONS

The tests indicated toxicity only in the lipid extract of L. carteri. The proposal that the toxin is non-proteinaceous is supported by Tenney and Woolcott (unpublished data) who found that the toxicity was not altered by boiling or after digestion with proteolytic enzymes. Lipids have been shown to be the poisonous principle in the toxin, ostracitoxin, released by the box fish, Ostracion meleagris Shaw, by Boylan and Scheuer (1967). Halstead (1967) cites several authors who found that lipids were

the substances that caused illness when the muscles and organs of certain fishes were consumed by man.

Testing of the lipid classes revealed that toxicity existed in those that migrated like phospholipids, sterols, fatty acids, and cholesteryl esters. Nine out of fourteen fractions within the classes were toxic to fishes. Halstead (1967) refers to reports of instances where toxicity was exhibited in more than one fraction of a toxin. He suggested that this behavior could result from contamination, formation of toxic by-products, or a multi-component toxin like that found in the toxic serum of eels (Anguilliformes).

Fish exposed to ostracitoxin exhibited symptoms (Thomson, 1969) similar to those reported by Tenney and Woolcott (1964) for fish poisoned by L. carteri. The ostracitoxin is a single lipid that has shown a variety of effects on several biological systems including hemagglutination and hemolysis (Thomson, 1969). The bryozoan toxin may be either a single component or multi-component; however in as much as nine fractions have been found toxic the latter hypothesis seems more probable.

Additional information on the bryozoan toxin is needed. A method should be developed for culturing L. carteri in the laboratory to provide a constant sourve of test organisms independent of natural environmental fluctuations. Control of the environment in the laboratory could provide evidence

to determine whether the toxin is derived from the bryozoans' food or if it is a metabolite of the bryozoan. Additional work on the toxin should involve purification and identification of its chemical structure. When the structure has been determined it would then be possible to synthesize the toxin and investigate the mechanisms by which it acts on gilled aquatic vertebrates.

In summary, evidence supports the hypothesis that the toxin produced by L. carteri is a lipid. Detailed chromatographic studies revealed toxicity in six classes of lipids and in nine of the fourteen lipid fractions. However, additional information is needed to determine if toxicity results from a multi-component toxin or a single toxin showing aberrant chromatographic behavior.

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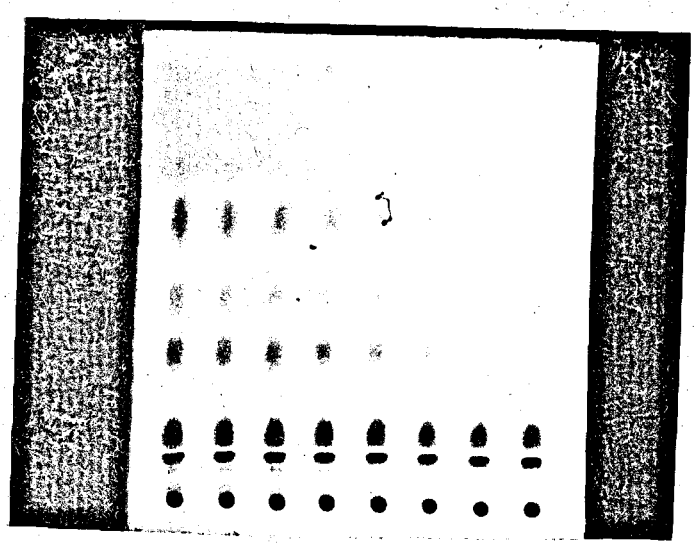
Table 1. Separation of lipids into classes and fractions and measurement of their migration rates.

Lipid	Migration Rate (R_f)
Class I	No migration
fraction 1	No migration
fraction 2	0.42
fraction 3	0.58
fraction 4	0.79
Class II	0.09
fraction 1	No migration
fraction 2	0.37
fraction 3	0.85
Class III	0.16
fraction 1	No migration
fraction 2	0.35
fraction 3	0.88
Class IV	0.34
Class V	0.47
Class VI	0.66
fraction 1	No migration
fraction 2	0.39
fraction 3	0.52
fraction 4	0.88

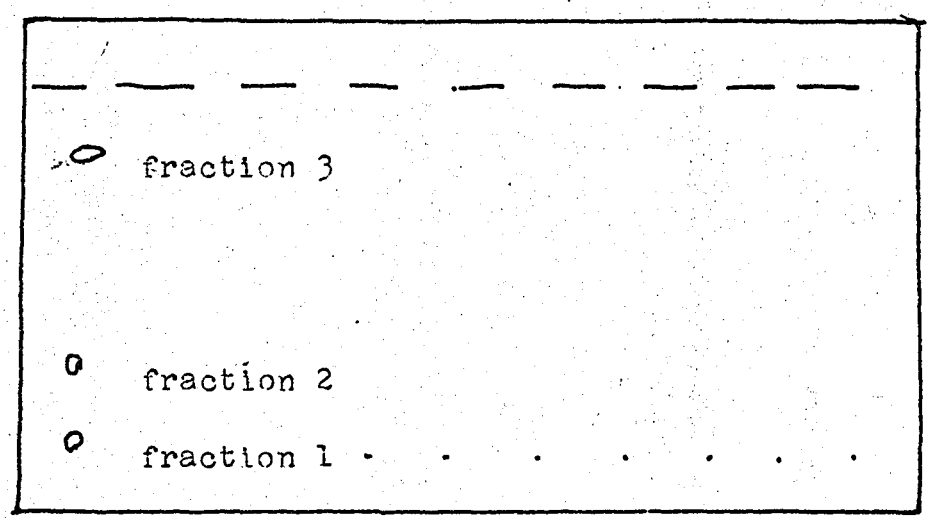
Fig 1. Separation of the whole lipid extract into classes with petroleum ether/diethyl ether/acetic acid (84:15:1) solvent.

Fig 2. Separation of Class I lipids into fractions with chloroform/methanol/acetic acid/water (25:15:4:2) solvent.

Fig 3. Separation of Class II lipids into fractions using a chloroform/methanol (95:5) solvent.

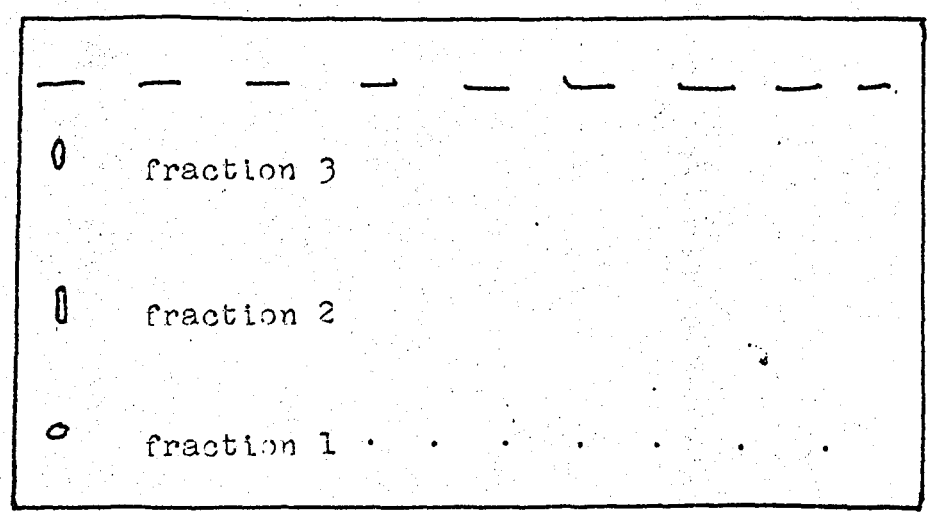


Solvent front
Class VI
Class V
Class IV
Class III
Class II
Class I(Origin)



Solvent front

Origin

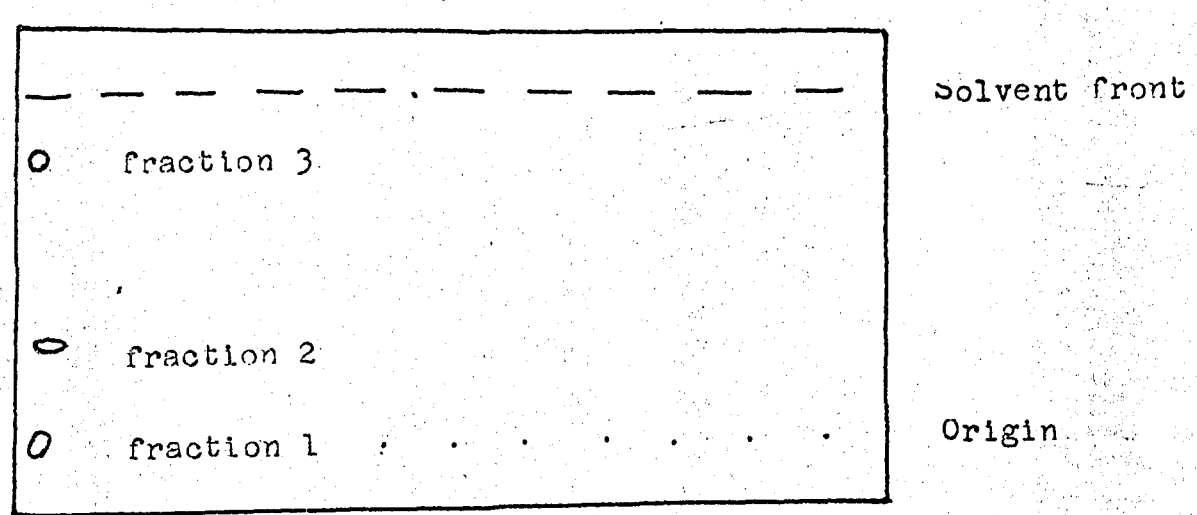


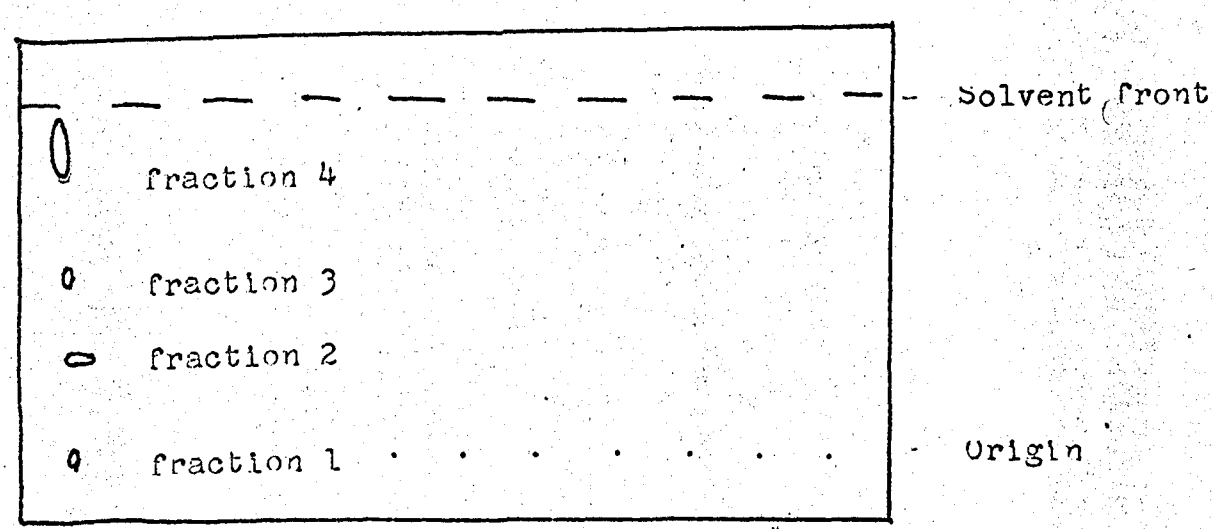
Solvent front

Origin

Fig 4. Separation of Class III lipids into fractions with a chloroform/acetic acid (96:4) solvent system.

Fig 5. Separation of Class VI lipids into fractions using a 100% chloroform solvent system.


Solvent front
fraction 3
fraction 2
fraction 1
Origin


Solvent front
fraction 4
fraction 3
fraction 2
fraction 1
Origin

VITA

John Michael Dolan, III was born in Richmond, Virginia on December 12, 1943. He attended elementary and high school there and graduated from John Marshall High School in June, 1961. In September, 1961 he enrolled at Greensboro College, Greensboro, North Carolina. After returning to Richmond in 1962, he attended Richmond Professional Institute as a part-time student until September, 1965 when he enrolled as a full-time student, majoring in biology. He was graduated in June, 1967 with a Bachelor of Science degree.

From June, 1967 to September, 1969, he was employed with the Commonwealth of Virginia in Richmond, Virginia and Union Camp Corporation of Franklin, Virginia in water pollution control activities. In September, 1969, he enrolled as a full-time graduate student at the University of Richmond and is a candidate for the Master of Science degree in August, 1971. While in Graduate School, he assisted in the general biology laboratories.

He was a member of Beta Beta Beta honorary biological society at the University of Richmond.

He was married to the former Hannah Mary Sanderson of Richmond on February 9, 1963. They have two children, Kevin Patrick and Karen Marie.

He has been accepted at Virginia Polytechnic Institute and State University where he plans to work toward a Ph. D. degree in zoology.