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Isolation and Identification of the Toxic Principle in the Tissues of the Ectoproct Lophopodella carteri (Hyatt)

Donald Coleman Smith
B.S., Washington and Lee University

Isolation and Identification of the Toxic Principle in the Tissues of the Ectoproct *Lophopodella carteri* (Hyatt)

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Donald Coleman Smith

B.S., Washington and Lee University

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APPROVED:

Thesis Committee

F. B. Lefkovich

H. S. Woodcock

W. R. Penney

J. C. Strickland

Milan E. Rice

R. L. Clarke

David W. Towle

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ABSTRACT

The tissue of Lophopodella carteri (Hyatt) was isolated, and the agent toxic to fish present in the tissues was extracted. A chloroform-soluble component and a water-soluble component were separated from the filtrate of the homogenized tissue. Each component was tested for toxicity. The chloroform-soluble component was found to be extremely toxic. The water-soluble component was originally non-toxic, but after several months of freezing and defrosting the tissue, this component became toxic.

The chloroform-soluble component was purified by thin-layer chromatography. Several lipid classes were obtained, of which one was toxic. This toxic class was further purified by thin-layer chromatography resulting in five fractions, one of which was toxic. Compound-specific stains were sprayed on the thin-layer plates in order to determine the chemical nature of the toxic and non-toxic classes and fractions.

Absorption spectra were obtained for the pigments associated with the toxic principle after pigment separation by paper chromatography. Similarities between the spectra of the unknown pigments and known pigments were observed.

Evidence indicates that the toxic agent found in the tissues of carteri is a lipoprotein closely associated with two pigments, which have absorption spectra similar to chlorophyll a and neo-β-carotene B.

INTRODUCTION

Toxicity of the freshwater ectoproct Lophopodella carteri (Hyatt) was first reported by Rogick (1957), and later by Tenney and Woolcott (1964) after they discovered the presence of the organism in Virginia (Tenney and Woolcott, 1962). In 1951, Oda observed that the tissue of another freshwater ectoproct Pectinatella gelatinosa (Fleming) was toxic when crushed in the vicinity of the common Japanese fish Oryzias latipes (Rogick, 1957). In 1951, Oda proposed that the toxic agent was located in the tissue. Oda later (1958) proposed that the toxic agent in carteri and gelatinosa is located in the coelomic fluid, and is excreted through the vestibular pore upon invagination of the individual polypides. However, Meacham and Woolcott (1968) determined that the toxic agent of carteri is located in its tissues and not in the coelomic fluid, thus supporting Oda's earlier observation. Collins et al. (1964) observed that fishes that swim in the vicinity of colonies of carteri do not use the ectoproct as food. However, other freshwater ectoprocts are frequently used as food in a number of habitats (Osburn, 1921; Applegate, 1966; Bushnell, 1966). Thus, the toxicity of the tissues of carteri may enhance the survival of the organism (Collins et al., 1966).

Thomson (1969) reported that fish subjected to ostracitoxin, a mucous secretion of the boxfish Ostracion meleagris, exhibited symptoms similar to those reported by Tenney and Woolcott (1964). Similar effects observed in this study were dynamic and seem to

indicate some interference with the respiration of the test organisms.

Thomson (1969) reported that ostracitoxin is a single lipid having hemagglutination and hemolytic properties. Dolan (1971) reported that the toxic agent of carteri appeared to be a multicomponent lipid, with effects similar to those reported earlier (Tenney and Woolcott, 1964). The purpose of this study was to determine the chemical nature of the toxic agent in the tissues of carteri.

MATERIALS AND METHODS

Colonies of Lophopodella carteri were collected from the Virginia State Fish Cultural Station (VSFCS) in Stevensville, Virginia, in September, 1971. Upon returning to the laboratory, debris and other foreign material was removed from the VSFCS water leaving only the colonies of carteri, which were placed with the water medium into plastic bags and frozen at -15°C until used.

Tenney and Woolcott (1964) observed that disturbed colonies of carteri had toxic effects on gilled vertebrates. To test toxicity of the ectoproct tissues, bluegills (Lepomis macrochirus) collected from Westhampton Lake on the campus of the University of Richmond served as test organisms. The weights of the fish ranged from 210-550 mg, with a mean weight of 350 mg. The fish remained in the capture vessel overnight to allow them to acclimate before use.

A bag of ectoprocts was defrosted and approximately 5.0 g of tissue (wet weight) were removed for assay. This tissue was suspended in 10 ml of chloroform/methanol (2:1, v/v) prepared daily and homogenized with a glass pestle in a Potter-Elvehjem glass tissue grinder for 3-5 min. Five milliliters of distilled water were added before homogenizing the tissue to insure separation of the chloroform-soluble and water-soluble components of the tissues. The tissue suspension was then filtered through Whatman #1 filter paper using a Buchner funnel-suction flask apparatus with a faucet aspirator. The suspension was washed several times with chloroform/methanol. The filtrate was poured into a separatory funnel, and the solid material was discarded. The chloroform-soluble and water-soluble components were separated and retained.

The water-soluble component was heated at 70°C in a water bath to evaporate the methanol. The water-soluble fraction was brought to volume (25 ml) with distilled water. A fish weighing 350 mg was placed in the beaker (50 ml) to test toxicity of the water-soluble fraction.

The chloroform-soluble fraction, i.e. lipid fraction, was filtered through Whatman #1 phase separating filter paper using the suction apparatus to insure complete removal of water, methanol, and solid material from the lipid fraction. The filtrate was then placed in a ground glass, round-bottomed flask which was placed on a rotor-evaporator connected to the aspirator and a trap. The chloroform evaporated by having the flask rotating in a water bath at 60°C. The evaporation continued until all of the chloroform

had been removed. The crude lipids were resuspended in 10 ml of chloroform/methanol. This lipid fraction was used to spot silica gel plates for thin-layer chromatography.

Silica gel G plates were prepared by mixing 90 g of silica gel G with 180 ml of distilled water, shaking vigorously for 90 sec, and applying a uniform 1 mm thick layer with an adjustable spreader. The coated plates were allowed to air dry at room temperature for 8 hrs, and were activated by incubating them for 1 hr at 110°C. The plates were allowed to cool in a dessicator. Thin-layer plates were spotted with the lipid fraction and placed in chromatography tanks which had been lined with filter paper and equilibrated for 1 hr with 150 ml of petroleum ether/ diethyl ether/acetic acid (84:15:1, v/v/v).

In all separations 150 ml of solvent were used. The plates were developed in the solvent for 25 min, removed, and air dried at room temperature. One edge of each plate was stained with 1 % iodine in methanol to determine the location of the lipid bands (Randerath, 1966). Only the unstained portion was removed for the toxicity tests. The unstained silica was removed in 7 strips, which corresponded to the bands on the stained portion of the plates. The bands were numbered according to their migration rates, from the origin up, and labelled as Classes I-VII, respectively. Chloroform/methanol was added to the gel in order to elute the lipids. The lipid-gel suspensions were filtered with a suction apparatus, and the filtrates were poured into tared 50 ml beakers. The solvent was evaporated by passing a stream of nitrogen over the beaker until only a dry powder was present. The beakers were weighed again and the weights were recorded to

determine exactly to how much lipid the test organism was subjected. A fish was added to each test beaker and to control beakers containing only distilled water. The elapsed time of death was noted and recorded. The experiment was replicated 5 times.

The toxic lipid class was separated further into fractions on plates coated with silica gel H. The silica gel H plates were prepared by mixing 80 g of silica gel H with 160 ml of 0.001 N Na_2CO_3, shaking vigorously for 90 sec, and applying a uniform 1 mm layer. The plates were air dried at room temperature for 8 hrs, and activated by incubating them for 1 hr at 110°C. The plates were allowed to cool in a dessicator. The thin-layer plates were spotted with the toxic lipid extract and placed in chromatography tanks lined with filter paper and equilibrated for 1 hr with 150 ml of chloroform/methanol/acetic acid/distilled water (25:15:4:2, v/v/v/v).

The plates were developed for 25 min and air dried at room temperature. One edge of each plate was stained with 1 % iodine in methanol to determine the location of the bands. Again, only the unstained portion was removed for the toxicity tests. The unstained silica was removed in 5 strips corresponding to the bands on the iodine-stained portion of the plates. The removed silica was treated as before. The amount of each lipid fraction was recorded as was the elapsed time of death.

Various stains were sprayed on both the silica gel G plates and silica gel H plates in an attempt to identify the lipid compounds present (Skipski and Barclay, 1971). To test for the presence of cholesterol and cholesteryl esters, the plates were

sprayed with a mixture of conc H_2SO_4 and HAc (1:1, v/v) and heated for 15 min at 90°C. If the test was positive, cholesterol and cholesteryl esters appeared as red spots on a white background. Unsaturated lipids appeared as pink brown spots. Cholesteryl stearate served as the standard. To test for esterified fatty acids, two reagents were used. Reagent I(R_I) consisted of 10 g of hydroxylamine hydrochloride ($HONH_2 \cdot HCl$) dissolved in 25 ml of distilled water, diluted to 100 ml with ethanol and mixed with 26 ml of a saturated aqueous solution of NaOH diluted to 200 ml with ethanol. NaCl precipitates were removed by filtration. Reagent II (R_{II}) consisted of 10 g of $FeCl_3 \cdot 6H_2O$ and 20 ml of HCl (37 %, w/v) ground together in a mortar and shaken with 300 ml of ether to form ethereal acid ferric chloride. The plates were sprayed with R_I, allowed to dry briefly and sprayed with R_{II}. If positive, purple spots appeared on a yellowish background.

To test for the presence of phospholipids, the plates were sprayed lightly with molybdenum blue reagent until the adsorbent was moistened uniformly. Neutral lipids and glycolipids do not give positive tests. Phospholipids show up immediately as blue spots on a white or light blue background. To test for glycolipids, the plates were sprayed with orcinol-H_2SO_4 spray. Two-hundred milligrams of orcinol were dissolved in 100 ml of 75 % H_2SO_4 (v/v) and stored in the dark under refrigeration. The entire plate was sprayed until the surface became moist and was placed in an oven at 100°C for 15 min. Most glycolipids yield blue violet spots on a white background. Phosphatidylserine served as the standard in the phospholipid test, and bovine cerebrosides served as the

standard for the glycolipid test.

In order to distinguish between phospholipids, two group-specific stains were used. Ninhydrin, which detects free amino groups, was sprayed on the plates, which were heated for 5 min at 100-105°C in an atmosphere saturated with water. Lipids with free amino groups appear as red-violet spots on a white background. To detect choline-containing phospholipids, a spray composed of two solutions was used. For Reagent I (R_I), 1.7 g of bismuth nitrate were dissolved in 100 ml of 20 % HAc. For Reagent II (R_{II}), 40 g of KI were dissolved in 100 ml of distilled water. Twenty milliliters of R_I were mixed with 5 ml of R_{II} and 70 ml of distilled water just before use (Dragendorff reagent). The plates were sprayed with the Dragendorff reagent. Choline-containing phospholipids appaar as orange to orange-red spots immediately or after gentle warming.

Silica gel H plates were sprayed with Coomassie blue to test for the presence of proteins in the toxic band. One-half gram of Coomassie blue was dissolved in 100 ml of methanol. The presence of proteins is indicated by blue spots. Oil red stain was sprayed on the silica gel H plates to determine whether lipoproteins were associated with the toxic lipids. Oil red stain was prepared by dissolving 0.5 g of oil red in 100 ml of methanol. The presence of lipoproteins is indicated by red spots.

The eluent of the toxic, pigmented band on silica gel H was spotted on Whatman #1 chromatography paper and developed in 90 % petroleum ether in acetone. The two resultant pigments were eluted from the filter paper, and absorption spectra of the eluents

were obtained. The spectra of the unknown pigments were compared with similar spectra of known pigments reported by Goodwin (1954), Marks (1969), Lehninger (1970), and Britton and Goodwin (1971).

RESULTS

Seven classes of lipids were obtained after the crude extract was separated on silica gel G (Fig. 1). Toxicity was obtained in Classes I and III, but the frequency of positive toxic tests was much greater for Class I (Table 1). Class I, the only pigmented class, remained at the origin, as did the two standards, phosphatidylserine and bovine cerebrosides. Class VI migrated like the standard cholesteryl stearate. Class III migrated like sterols, and Class IV like fatty acids. Classes II, V, and VII were not related to any of the standards (Dolan, 1971).

Class I elicited the greatest toxic response from the fish (Table 2). The killing time ranged from 30-345 min, with a mean killing time of 113.6 min. Twelve of 18 fish died during the experimental period. The concentration of lipid ranged from 1.2-69.4 mg of lipid/25 ml of distilled water. The minimal lethal dosage was 3.0 mg of lipid/25 ml of distilled water. The mean concentration was 16.7 mg/25 ml of distilled water.

Class II lipids did not elicit any toxic response. The concentrations to which the fish were subjected ranged from 1.1-4.3 mg/25 ml of distilled water, with a mean concentration of

2.5 mg/25 ml of distilled water. Fewer fish were tested in this class than in the other lipid classes because the presence of this class was not observed until the staining tests were performed.

In Class III, 1 out of 18 fish died within the experimental period. Fish were subjected to concentrations ranging from 1.0-25.4 mg/25 ml of distilled water. The mean concentration was 6.1 mg/25 ml of distilled water, and the lethal dosage was 22.2 mg/25 ml of distilled water. None of the fish tested with the lipids from Classes IV-VII died during the experimental observation period. Concentrations ranged from 1.1-68.5 mg/25 ml of distilled water for Class IV, with a mean concentration of 8.6 mg/25 ml of distilled water; 0.6-26.2 mg/25 ml of distilled water for Class V, with a mean concentration of 4.8 mg/25 ml of distilled water; 1.0-19.5 mg/25 ml of distilled water for Class VI, with a mean concentration of 4.6 mg/25 ml of distilled water; and 1.3-53.5 mg/25 ml of distilled water for Class VII, with a mean concentration of 5.0 mg/25 ml of distilled water.

The presence of the Class II lipids was not known in the earlier tests, and therefore fewer tests for toxicity were run on this lipid class. Its presence was observed after staining the silica gel G plates with the sulfuric acid/acetic acid spray and heating in an incubator. The first detection spray tests with 1 % iodine in methanol only indicated the presence of six lipid classes, as reported by Dolan (1971). After spraying the silica gel G plates with the sulfuric acid/acetic acid spray, all seven lipid classes herein reported were observed. Subsequent toxicity

tests included all seven lipid classes.

During all toxicity tests control fish were tested in 25 ml of distilled water only. Of eighteen fish tested, no fish died within the experimental period. In several cases, a few of these control fish lived for periods of time equal to three times the experimental period. Fish were placed into the water in which the ectoproct tissue was maintained, and died within 3 min after placing the fish into the water. The fish showed stress movements, and at times, these movements were greater than those of the fish subjected to the individual lipid classes. Fish were also subjected to the crude extract of the ectoproct tissue after the solvent had been evaporated with nitrogen. The mean killing time for fish tested with this substance was 50 min.

Class I lipids were eluted from silica gel G and were chromatographed on silica gel H. Sulfuric acid/acetic acid stain was used because the 1 % iodine in methanol stain normally used did not detect the presence of the lipids. Five bands (one of which was pigmented) were observed after staining (Fig. 2). Fish were subjected to each of these fractions in two tests, each with 3 fish per lipid fraction. Only the pigmented fraction (Fraction 5) was toxic. The killing time for the 5 of 6 fish that died was recorded (Table 3). The mean killing time for Fraction 5 was 91.5 min. The concentrations of the test solutions ranged from 2.4-3.5 mg/25 ml of distilled water, with a mean concentration of 2.9 mg/25 ml of distilled water and a minimal lethal dosage of 2.4 mg/25 ml of distilled water.

Toxicity tests were also run on Fractions 1-4, but no toxicity was observed. Concentrations ranged from 2.2-3.0 mg/

25 ml of distilled water for Fraction 1, with a mean concentration of 2.6 mg/25 ml of distilled water; 2.7-3.8 mg/25 ml of distilled water for Fraction 2, with a mean concentration of 3.2 mg/25 ml of distilled water; 2.7-3.4 mg/25 ml of distilled water for Fraction 3, with a mean concentration of 3.1 mg/25 ml of distilled water; and 2.4-2.6 mg/25 ml of distilled water for Fraction 4, with a mean concentration of 2.5 mg/25 ml of distilled water. As in the tests with the lipid classes, control fish were placed in 25 ml of distilled water only. Again, none of these control fish died within the experimental period.

Stain tests were used to determine the nature of the seven lipid classes that were chromatographed on silica gel G. The test for the presence of cholesterol and cholesteryl esters was negative for the seven lipid classes (Table 4), however, it was observed that Classes III, IV, V, and VII contained unsaturated lipids. The test for esterified fatty acids was negative for all classes. The test for the presence of phospholipids was positive for Class I but negative for all the other classes. The test for glycolipids was positive for Classes II and III but negative for the other classes. Two stains were used to determine the nature of the phospholipids in Class I. The test for free amino groups of phospholipids proved negative as did the Dragendorff test for choline-containing phospholipids.

Stain tests were also used to determine the nature of the lipids of Class I that were chromatographed on silica gel H. (Table 5). The test for phospholipids was positive for Fractions 1-4 but negative for Fraction 5, the toxic fraction. The test

for glycolipids was performed again to be certain that other compounds did not conceal the presence of glycolipids in the test on silica gel G. The glycolipid test was negative for all five fractions. The Coomassie blue test for the presence of proteins was positive for Fraction 5 but negative for Fractions 1-4. The oil red test for the presence of lipoproteins was positive for Fraction 5 but negative for Fractions 1-4. These tests indicate that the toxic component of the ectoproct tissue is a lipoprotein.

The pigmented fraction eluted from silica gel H was spotted on Whatman #1 chromatography paper and developed in 90 % petroleum ether in acetone. Two pigmented regions were observed - a green pigment with an R_F value of $0.06\bar{6}$ and a yellow pigment with an R_F value of $0.53\bar{3}$. Absorption spectra were run on each of the pigments (Fig. 3 and 4). The pigment near the origin (Fig. 3) had relatively high peaks at 250-270 nm, 410 nm, and 660 nm. The yellow pigment had a shallow peak at 460 nm, a higher peak at 430 nm, and a strong peak at 260 nm (Fig. 4).

DISCUSSION

Dolan (1971), after separation of the chloroform-soluble component on silica gel G, obtained toxicity in Classes I, II, III, and VI. The present research has shown toxicity only in lipid Class I. This discrepancy can possibly be explained by differences in technique. Dolan heated the tissue homogenate in chloroform/

methanol at 60°C for 30 min in order to fascilitate lipid separation from the ectoproct tissue. In preliminary work, Dolan's procedure was followed, and no toxicity was obtained. In the present study the heating procedure was eliminated, and toxicity was obtained only in Class I. It is suggested therefore that the heating procedure used by Dolan caused a disruption of the toxic materials in Class I which then migrated to other classes.

Tenney and Woolcott (1964) noted the responses of fish subjected to an extract of the tissues of carteri. The fish exhibited frantic swimming, as if to jump out of the beaker, and erratic opercular movements. These actions were observed in the present study when the fish were subjected to the Class I lipids from silica gel G and to Fraction 5 from silica gel H. After several minutes, the fish calmed, settled to the bottom with a reduction in opercular movements, and began to lose stability in the water. Death occurred soon after the loss of stability, and was characterized by the mouth and opercles gaping open. Some of the experimental fish in this study exhibited gasping movements at the surface before settling to the bottom. Other fish exhibited spasmodic regurgitating movements of the jaws in an attempt to expel water through the mouth. Thomson (1969) observed similar reactions of test fishes when subjected to the toxic stress secretion of the boxfish Ostracion meleagris. In all observed deaths in the present study, mucous secretions appeared in the water, apparently exuded through the opercles from the gill tissue of the fish.

The experimental period in which the lipid classes were

tested for toxicity was limited to 6 hrs, slightly longer than the maximum elapsed time for the death of a fish subjected to Class I. Other fish died after 12-15 hrs exposure to the test solutions. It was postulated that if the experimental period was extended to include these fish, other factors, such as O_2 tension of the medium and bacterial contamination, might affect the fish and thereby make it difficult to ascertain proper conclusions from the data obtained.

The concentrations of the various lipids in the experimental media are important in comparing the data obtained. Toxicity was observed in Class I and Class III, however the toxicity observed in Class I was much greater, i.e. 12 out of 18 fish died in Class I, and only 1 out of 18 fish died in Class III (Table 2). The minimum lethal dosage for Class I was 3.0 mg/ 25 ml of distilled water, whereas the lethal dosage for Class III was seven times greater. From the data obtained from the toxicity tests, it is quite apparent that the toxic component is restricted to Class I. The one fish that died in Class III possibly was unfit and died from another cause, such as injury in handling.

Only Fraction 5, a lipoprotein, from silica gel H was toxic. The maximum killing time for this fraction was 3.6 times less than the maximum killing time for the intact Class I lipids from silica gel G. The separation of the Class I lipids on silica gel H purified the toxic fraction. Non-toxic fractions 1-4 from silica gel H were phospholipids. The increased toxicity of Fraction 5 on silica gel H might have resulted from the elimination of the inhibiting effects of the phospholipids

associated with the toxic lipoprotein.

The absorption spectra of the two pigmented fractions, the pigments of which possibly originated from the food of the ectoproct, separated from toxic Fraction 5 of silica gel H were similar to the absorption spectra for known pigments. The absorption spectrum for the green fraction had peaks at 250-270 nm, 410 nm, and 660 nm. Lehninger (1970) states that pure chlorophyll a has a maximum absorption at 663 nm, which is relatively close to the absorption at 660 nm for the unknown pigment. The absorption spectrum for chlorophyll a (Marks, 1969) when superimposed over the spectrum for the unknown pigment indicates that the spectra are similar at 410 nm and 660 nm. Because of the similarities between the two spectra, it seems likely that the unknown green pigment is chlorophyll a.

The strong peak at wavelengths ranging from 250-270 nm suggests an association of the pigment with the lipoprotein. Proteins have a strong, characteristic peak at 280 nm. A probable reason for the shift in the peaks of the unknown pigment is the association of the pigment with the protein.

The absorption spectrum for the yellow fraction had peaks at 260 nm, 430 nm, and 460 nm (Fig. 4). The absorption peaks for α-carotene are at 445 and 470 nm, while those for β-carotene are at 450 and 475 nm (Britton and Goodwin, 1971). The absorption spectra for these two carotenes are different (Fig. 5). The spectrum for β-carotene is similar to that of the unknown yellow pigment in that the shapes of the peaks are similar. Thus, it seems likely that the unknown yellow pigment is a β-carotene. The peaks for neo-β-carotene B are at 440 and 475 nm. The peaks

for neo-β-carotene U are at 450 and 480 nm. The spectra for the unknown pigment and neo-β-carotene B are similar in that the peaks of the unknown pigment approximate the same magnitude as those peaks of neo-β-carotene B. The two peaks for neo-β-carotene U are higher than those for neo-β-carotene B, and are quite different from those for the unknown yellow pigment. Therefore, it seems likely that the unknown yellow pigment is neo-β-carotene B. The strong peak at 260 nm is close to the representative 280-peak of proteins. Thus, the toxic lipoprotein is also associated with the carotene, and the shift of the peak from a standard 280 nm to 260 nm may be due to this association (Fig. 4).

Toxicity tests were also run on the water-soluble component of the extracted ectoproct tissues. Earlier workers (Tenney and Woolcott, 1964) noted that this component was non-toxic to fish. Early tests in this present study with the ectoproct tissue obtained in September, 1971, substantiated these findings. However, after several months of maintaining the tissue at -15°C alternating with defrosting periods, the water-soluble component became toxic. Fish placed in test solutions composed of this component died within 2 min. This fact casts doubt on the non-toxicity of the component, but it also intimates that bacterial degradation of the tissue had altered the water-soluble component thereby making it toxic. Also, bacterial endotoxin may cause the toxicity, but only further work with fresh ectoproct tissue will answer this hypothesis.

In conclusion, the tissues of carteri contain a toxin that has been identified as a lipoprotein through thin-layer

chromatography and compound-specific stains. Other invertebrate toxins have been reported in the literature, such as the toxin of the jellyfish and that of several dinoflagellates, but only the toxin from the tissues of carteri has been shown to be a lipoprotein. The lipoprotein is associated with two pigments, which have absorption spectra similar to chlorophyll a and neo-β-carotene B, respectively. The role of the pigments in the toxicity of the lipoprotein was not determined.

TABLE 1. Toxicity of the seven lipid classes extracted from the tissues of Lophopodella carteri (Hyatt) as eluted from silica gel G.

Lipid class	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
I	+ (3/3)	+ (3/3)	+ (3/3)	+ (1/3)	+ (2/3)	+ (3/3)
II	*	*	*	*	-	-
III	+ (1/3)	-	-	-	-	-
IV	-	-	-	-	-	-
V	-	-	-	-	-	-
VI	-	-	-	-	-	-
VII	-	-	-	-	-	-

* Not aware of the presence of this class until later tests following detection with stains.

() Figures in parentheses are the number of fish killed out of the number tested, e.g. 2/3 = 2 out of 3 fish were killed.

\+ Toxicity

\- Non-toxicity

TABLE 2. Killing time (min :he lipid classes of the tissue of Lophopodella carter :ica gel G.

Lipid class	No. of runs	No. fish per run	No. runs all died	No. tested	No. killed	Killing time range	Killing time mean	Conc (mg/25 ml dist H_2O) range	Conc (mg/25 ml dist H_2O) minimal lethal dose	Conc (mg/25 ml dist H_2O) ^{2} mean
I	6	3	1	18	12	30-345	113.6	1.2-69.4	3.0	16.7
II	2	3	0	6	0	-	-	1.1-4.3	-	2.6
III	6	3	0	18	1	99	99	1.0-25.4	22.2	6.1
IV	6	3	0	18	0	-	-	1.1-68.5	-	8.6
V	6	3	0	18	0	-	-	0.6-26.2	-	4.8
VI	6	3	0	18	0	-	-	1.0-19.5	-	4.6
VII	6	3	0	18	0	-	-	1.3-53.5	-	5.0
Dist H_2O control	6	3	0	18	0	-	-	-	-	-

TABLE 3. Killing time (min) of fish subjected to the lipid fractions from the tissues of Lophopodella carteri after elution from silica gel H.

Lipid fraction	No. of runs	No. fish per run	No. runs all died	No. tested	No. killed	Killing time range	Killing time mean	Conc (mg/25 ml dist H_2O) range	Conc (mg/25 ml dist H_2O) minimal lethal dose	Conc (mg/25 ml dist H_2O) mean
1	2	3	0	6	0	-	-	2.2-3.0	-	2.7
2	2	3	0	6	0	-	-	2.7-3.8	-	3.2
3	2	3	0	6	0	-	-	2.7-3.4	-	3.1
4	2	3	0	6	0	-	-	2.4-2.6	-	2.5
5	2	3	1	6	5	43-249	91.5	2.4-3.5	2.4	2.9
Dist H_2O control	2	3	0	6	0	-	-	-	-	-

TABLE 4. Record of the stain tests on the lipid classes from the tissues of Lophopodella carteri chromatographed on silica gel G.

Lipid class	Test[1] for cholesterol and cholesteryl esters	Test[2] for esterified fatty acids	Test[3] for phospho-lipids	Test[4] for glyco-lipids	Test[5] for free amino groups for phospho-lipids	Test[6] for choline-containing phospho-lipids
I	-	-	+	-	-	-
II	-	-	-	+	-	-
III	*	-	-	+	-	-
IV	*	-	-	-	-	-
V	*	-	-	-	-	-
VI	-	-	-	-	-	-
VII	*	-	-	-	-	-

1...H_2SO_4/HAc (conc, 1:1)

2...See Materials and Methods for the reagents

3...Molybdenum blue reagent

4...Orcinol test

5...Ninhydrin spray

6...Dragendorff test

*...Unsaturated lipids

+...Positive test

-...Negative test

TABLE 5. Record of the stain tests on the lipid fractions of Class I chromatographed on silica gel H.

Lipid fraction	Test[a] for phospho-lipids	Test[b] for glyco-lipids	Test[c] for proteins	Test[d] for lipoproteins
1	+	-	-	-
2	+	-	-	-
3	+	-	-	-
4	+	-	-	-
5	-	-	+	+

a Molybdenum blue reagent

b Orcinol test

c Coomassie test

d Oil red test

\+ Positive test

\- Negative test

FIGURE 1. Diagramatic representation of the separation of the seven lipid classes extracted from the tissue Lophopodella carteri as chromatographed on silica ge presence of three standards. The solvent system was ether/diethyl ether/acetic acid (84:15:1, v/v/v).

A ..... Bovine cerebrosides

B ..... Phosphatidylserine

C ..... Lipid classes:

I ... Class I

II ... Class II

III ... Class III

IV ... Class IV

V ... Class V

VI ... Class VI

VII ... Class VII

D ..... Cholesteryl stearate

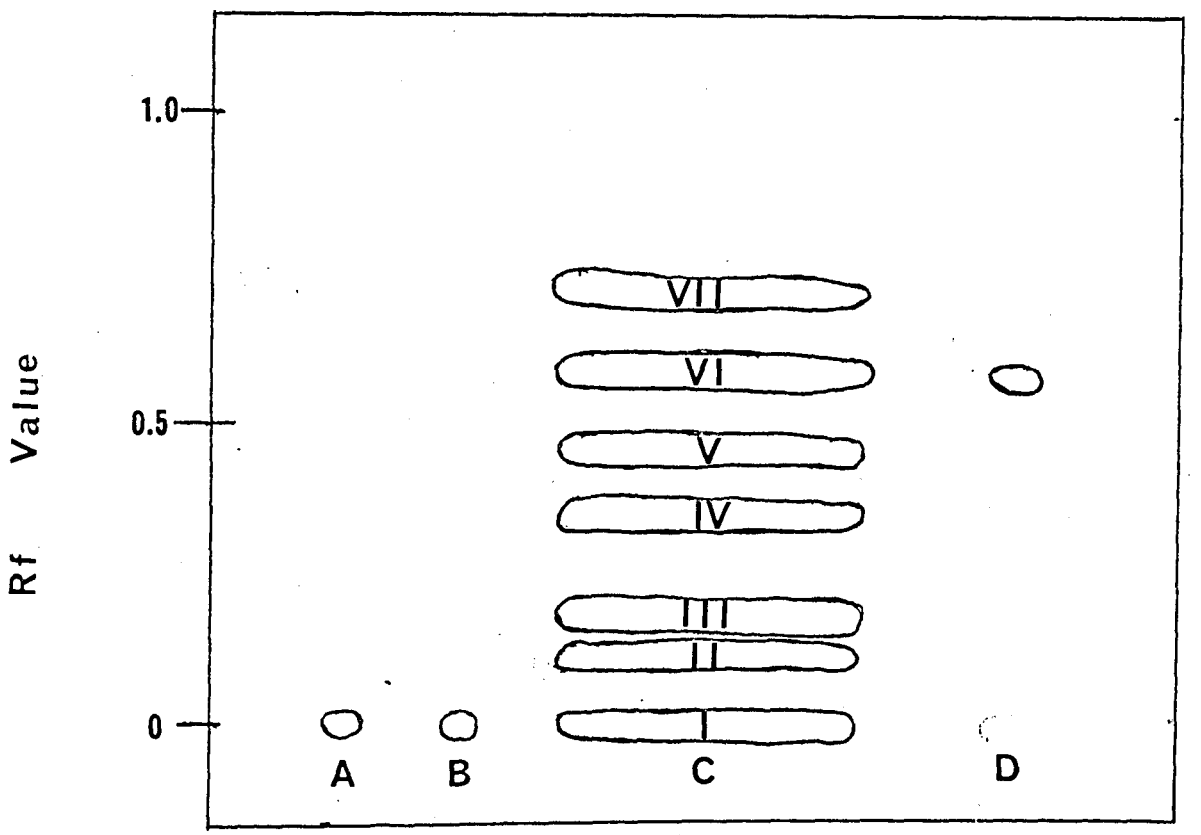

Rf Value
1.0
0.5
0
VII
VI
V
IV
III
II
A
B
C
D

FIGURE 2. Diagramatic representation of the separation of Class I from the tissues of Lophopodella carteri into its constituent fractions on silica gel H. The solvent system was chloroform/methanol/acetic acid/distilled water (25:15:4:2, v/v/v/v).

A ..... Lipid fractions

1... Fraction 1

2... Fraction 2

3... Fraction 3

4... Fraction 4

5... Fraction 5

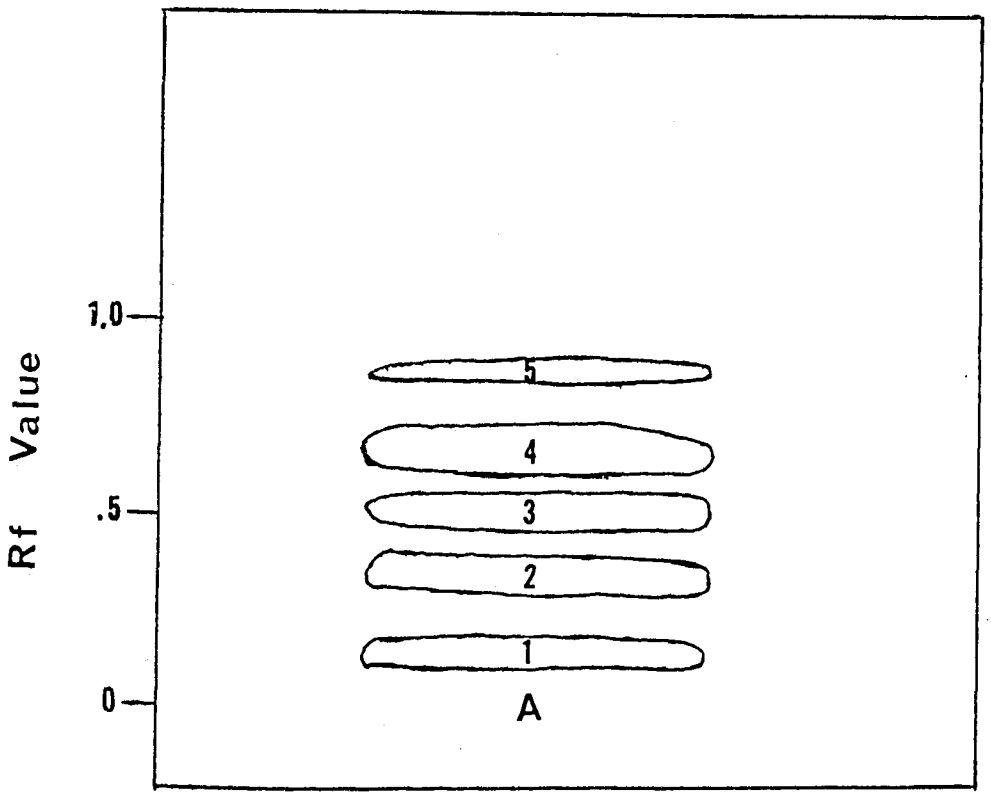

Rf Value
1.0
.5
0
5
4
3
2
1
A

FIGURE 3. Absorption spectra for the unknown green pigment, -----, extracted from the tissues of Lophopodella carteri, and chlorophyll a, _____, (taken from Marks, 1969).

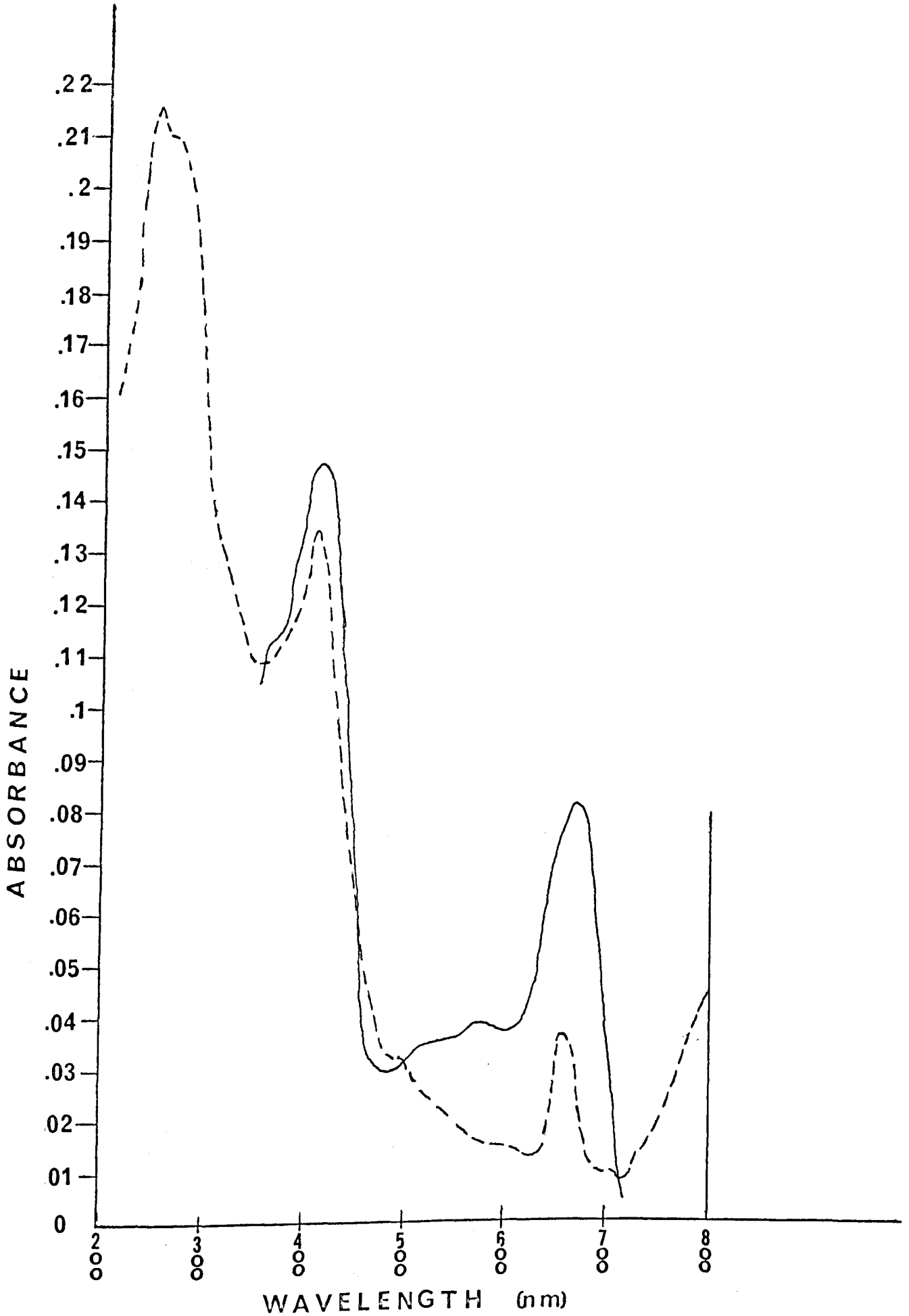

ABSORBANCE
.22
.21
.2
.19
.18
.17
.16
.15
.14
.13
.12
.11
.1
.09
.08
.07
.06
.05
.04
.03
02
01
0
200
300
400
500
600
700
800
WAVELENGTH (nm)

FIGURE 4. Absorption spectra for the unknown yellow pigment, ———, extracted from the tissues of Lophopodella carteri; for neo-β-carotene U, -----; and for neo-β-carotene B, -·-·-·-. (taken from Goodwin, 1954).

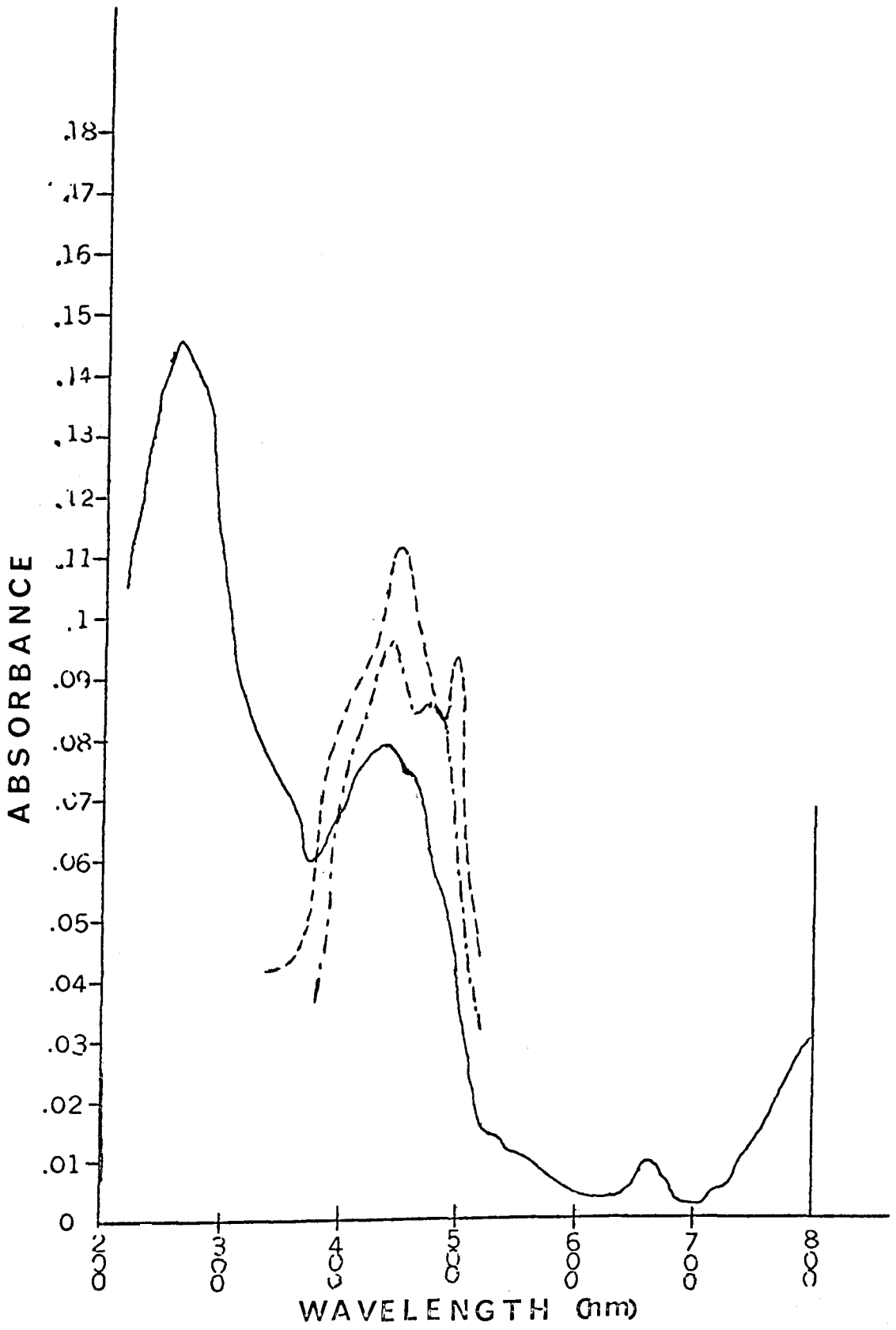
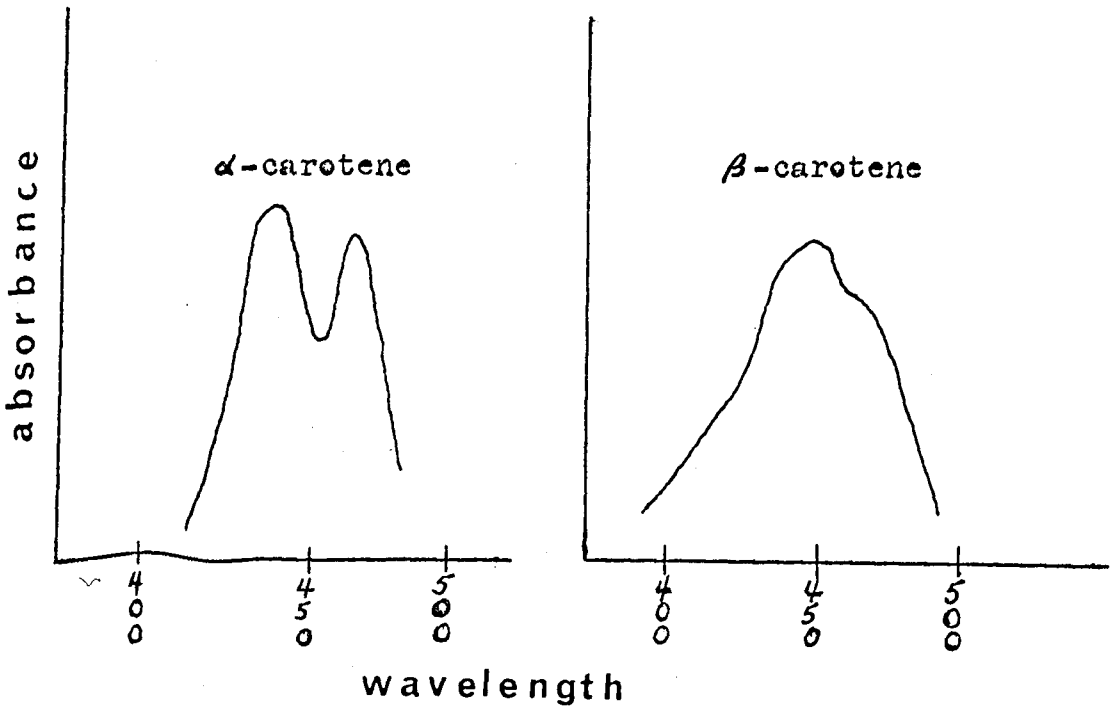

ABSORBANCE
.18
.17
.16
.15
.14
.13
.12
.11
.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
200
300
400
500
600
700
800
WAVELENGTH (nm)

FIGURE 5. Absorption spectra for α-carotene and β-carotene (taken from Britton and Goodwin, 1971).


absorbance
α-carotene
β-carotene
400
450
500
400
450
500
wavelength

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VITA

Donald Coleman Smith was born in Baltimore, Maryland on June 10, 1948. He attended elementary and secondary school in Montgomery County, Pennsylvania. He graduated from Upper Dublin High School, Fort Washington, Pa., in June, 1966. He entered Washington and Lee University in September, 1966, and majored in biology. He was graduated on June 5, 1970, with a Bachelor of Science degree. In September, 1970, he enrolled as a full-time graduate student at the University of Richmond, and completed the requirements for the Master of Science degree in biology in August, 1972. During the time as a graduate student he assisted in the Comparative Anatomy, Endocrinology, General Biology, and Plant Physiology laboratories.

In September, 1972, he will enter Emory University in pursuit of the Ph.D. degree in the Department of Pharmacology.

He was married to Randel Bliss Robbins of Fort Washington, Pa. on July 31, 1969. They have one daughter, Kelly Robin.