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Pigments of the Crinoid *Florometra Serretissima*

Guy Allen Howard
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PIGMENTS OF THE CRINOID FLOROMETRA SERRETISSIMA



A Thesis
Presented to
the Graduate Faculty
Central Washington State College



In Partial Fulfillment
of the Requirements for the Degree
Master of Science



by
Guy Allen Howard
July, 1967

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At this time I would like to thank Dr. Richard A. Neve', Dr. Robert D. Gaines and Dr. Richard W. Hasbrouck for their help in completing this investigation.

I would also like to sincerely thank Dr. Mary Griffiths, Department of Zoology, University of Washington, for her gift of known pigments and for the many hours she spent helping me.

Finally, I would like to express my deepest regards to my wife and family who withstood my absence and encouraged me throughout my graduate program.

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INTRODUCTION

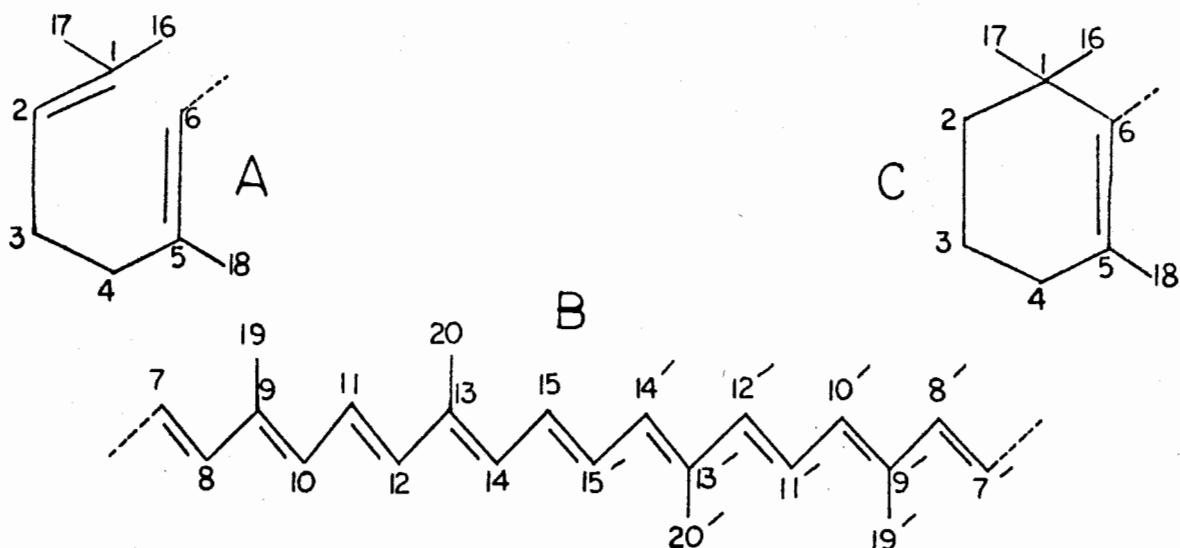
The class Crinoidea of the phylum Echinodermata contains animals which are commonly called Sea Lilies and Feather Stars. The crinoidea are stalked or stalkless pentamerous Pelmatozoa with pinnulated branched or unbranched arms containing extensions of the food grooves, coelom, and nervous, water-vascular, and reproductive systems (1). An extensive coverage of the ecological, physical, and physiological characteristics of the Crinoidea can be found in Hyman's series, The Invertebrates (1). In many zoology textbooks crinoids are discussed as rare, curious remnants of an interesting animal type now on the verge of extinction. The discoveries of the past few years have shown, however, that the crinoids are quite abundant as individuals and as species (2).

Yellow is the most common color of the crinoids, although they may be almost any color; some being multi-colored in bands or spots (3). Other than the recorded colors of crinoids, knowledge of the pigments of these animals is incomplete (4). Little work has been done toward identification of the specific pigments responsible for the colors of crinoids. Kruckenbergl (5) in 1882 found a red pigment in Comatula mediterranea and Antedon rosacea which he called comatulins. Further studies of Antedon rosacea by MacMunn (6) in 1889 revealed a specific red

pigment which he did not characterize. Abeloos and Teissier in 1929 (7) isolated what appeared to be the same red pigment. A yellow pigment present in the same animal was shown to be a derivative of the red pigment. The structure of the pigment called comatulins has not been elucidated. Studies by Karrer and Solmssen (8) in 1935 indicate that carotenoids are not responsible for the color of Antedon rosacea.

Lonnberg (9), in studying another type of crinoid, Antedon petasus, found that the coloring matter was mostly carotenoids. Antedon bifida was shown by Dimelow (10) in 1958 to contain β -carotene, astaxanthin, esterified astaxanthin, xanthophyll, and some hydroxynaphthaquinones. No evidence was found of any echinenone or vitamin A in the particular animals studied. Sutherland and Wells in 1959 (11) reported that the main pigments of Comatulina pectinata are hydroxyanthraquinones.

In the work cited above the presence of carotenoids was noted in at least two of the species investigated. It appeared that the animals investigated in the study reported herein could contain carotenoids. Carotenoids are thought to be combinations of three basic structures, A, B, and C (see below), or derivatives of these three structures formed by cyclization, aromatization, dehydration, oxidative degradation or introduction of functional groups (12).



Examples of natural carotenoids as combinations of these three basic structures (A, B, C) are lycopene (A+B+A), γ -carotene (A+B+C), and β -carotene (C+B+C).

Almost a hundred different carotenoids are now known (12). For a recent, comprehensive account of the chemistry of carotenoids refer to The Carotenoids by Karrer and Jucker (13). Carotenoids may be grouped as hydrocarbons, e.g., the carotenes, and their oxygen-containing derivatives, e.g., zeaxanthin (3,3'-dihydroxy- β -carotene) and xanthophyll (3,3'-dihydroxy- α -carotene).

The carotenoids are biologically important because many of them are provitamins A, e.g., β -carotene, cryptoxanthin, and echinenone, since they can be converted in the animal body into vitamin A (12, 14, 15). The wide occurrence of carotenoids in nature suggests that there may be some factor or property through which all of the various

functions ascribed to them may be related (16). No attempt was made in this study to determine any physiological functions of the pigments present.

The purpose of the present study is to separate and identify the pigments responsible for the bright orange color of the crinoid Florometra serretissima. This is the first reported investigation of the pigmentation of this particular crinoid.

METHODS AND MATERIALS

Collection of Animals

Florometra serretissima were collected during the month of December in Satellite Channel northeast of Sydney on the east side of Vancouver Island. They were obtained by dredging in about 120 feet of water.

Preparation of the Pigment Extract

The wet animals were placed in wide-mouthed jars containing 95% ethanol. The ethanol extracted most of the pigment. The crinoids were then ground in a Waring Blendor and extracted again with ethanol. This procedure yielded very little additional pigment. The extracted pigments were transferred to petroleum ether (bp 30-60°), containing a small amount of diethyl ether, by water saturation of the ethanol solution. The residual ethanol was removed by washing the petroleum ether with a saturated NaCl solution, to minimize emulsions, followed by a water wash. The solution containing the pigments was concentrated under vacuum and stored at -4° in the dark under nitrogen.

The blended material was extracted with a solution of diethyl ether containing a trace amount of 2 N HCl to remove any potentially present echinochromes after carotenoid extraction had been completed. The echinochromes are extractable only after acidification (17, 18). The diethyl

ether extract was dried over anhydrous sodium sulfate, concentrated, and stored at -4° in the dark under nitrogen.

Saponification

It is usual to divide the carotenoids into two groups by partitioning the extract between two immiscible solvents such as methanol and petroleum ether. Those carotenoids which contain two or more hydroxyls are found in the hypophase and those with no hydroxyls in the epiphase. Since most of the hydroxylated carotenoids occur in nature as esters called "pigment-waxes", it was necessary to saponify before partition (13). The petroleum ether extract of the pigments was mixed with 12% methanolic KOH and allowed to stand at room temperature in the dark under nitrogen for 24 hours. After saponification, petroleum ether was added followed by sufficient water to cause partition. Each of the phases was repeatedly washed with the solvent of the other, i.e., petroleum ether with methanol and methanol with petroleum ether, and the appropriate washes and fractions combined. The petroleum ether phase was dried over anhydrous sodium sulfate, concentrated, and stored in the dark under nitrogen at -4° .

The methanol phase was diluted with water and extracted with diethyl ether to remove all the hypophasic pigments. The ethereal solution was washed with a saturated NaCl solu-

tion, dried over anhydrous sodium sulfate, concentrated, and stored at -4° in the dark under nitrogen.

Separation and Purification of the Pigments

Solid-liquid (S-L) column chromatography. All of the carotenoid separations using (S-L) column chromatography were done with chromatographic grade neutral aluminum oxide, 80-200 mesh, from Merck and Co., Inc., Rahway, N. J. The glass columns used were 24 cm x 2 cm, and were packed by gravity using constant flowing petroleum ether as a solvent. The columns contained approximately 75 cm^3 of adsorbent. The crude carotenoid extract was placed on the column and eluted step-wise with petroleum ether (bp $30-60^{\circ}$) containing increasing 10% increments of diethyl ether, followed by ethanol, and finally, ethanol containing 5% acetic acid.

Separation of what were thought to be echinochromes was carried out using columns packed with Whatman Cellulose Powder CF 11, obtained from Van Waters and Rogers, Inc., San Francisco, Calif. The same size, method of packing, and type of columns were used as described above. In this case the crude extract was placed on the (S-L) column and eluted with a 1:1 mixture of diethyl ether and petroleum ether (bp $30-60^{\circ}$), followed by diethyl ether alone, and finally diethyl ether containing 5% acetic acid. Higher proportions of petroleum ether were avoided because echinochromes

are reported to be highly unstable in this solvent (17). Also, Millot reports that echinochromes are unstable on (S-L) columns of CaCO_3 , MgSiO_3 , or silica gel, but that decomposition can be minimized by using cellulose as an adsorbent (19).

In each of the above cases, the eluted pigments were collected in 2-5 ml fractions. Visible absorption spectra of these fractions were determined by scanning with a Beckman DB Spectrophotometer, and the appropriate fractions combined to give six to eight main fractions for the alumina column and three for the cellulose column. The purity of each fraction was determined by thin-layer chromatography (see below), and where more than one pigment was present, separation was effected by further (S-L) column chromatography using the same types of columns. In the case of echinochromes, evaporation to dryness was avoided whenever possible as this treatment had previously been reported to lead to large losses of pigment (17). An outline of the methods of separation and measurement of the pigments is shown in Fig. 1, p. 9.

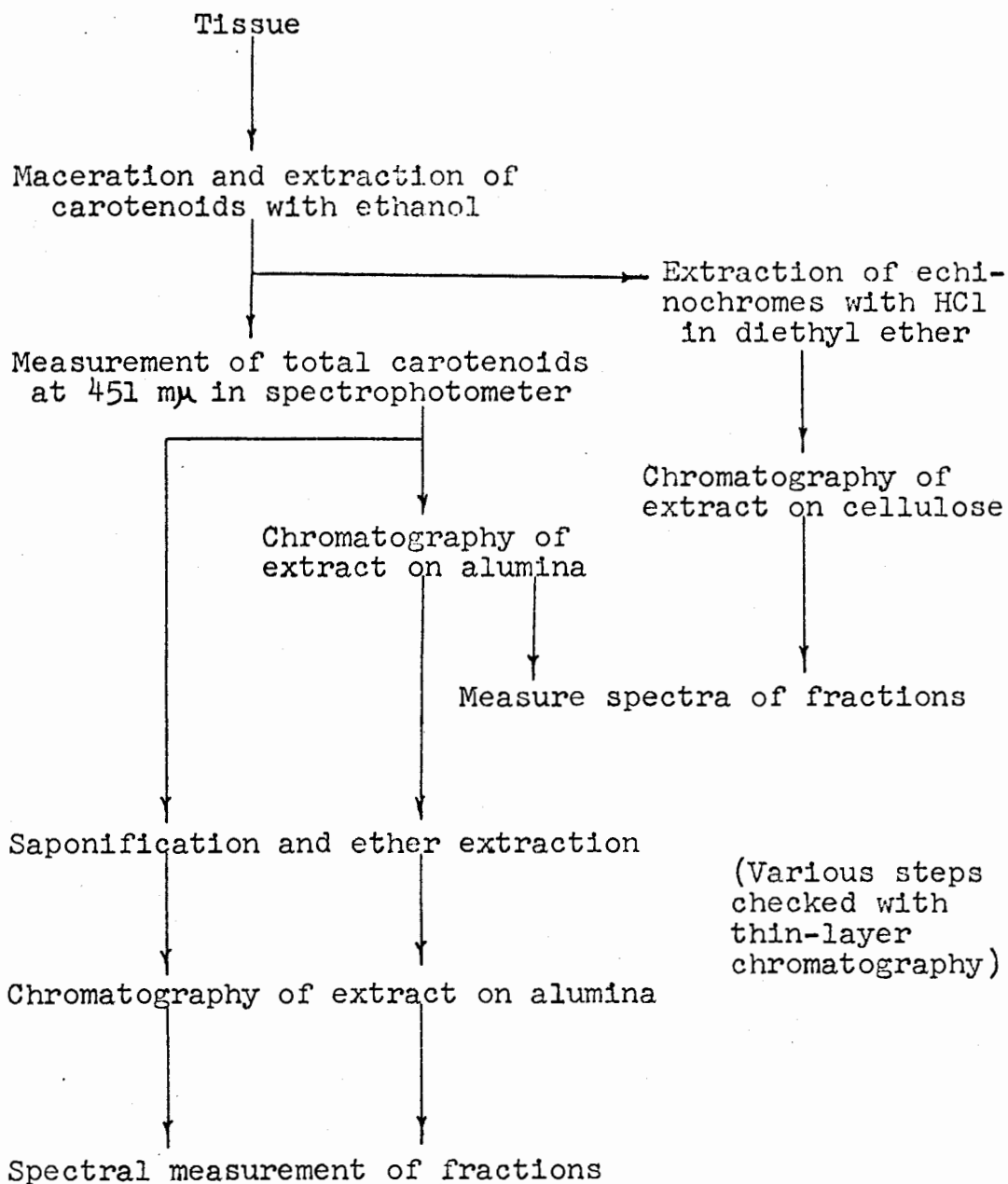


Fig. 1. Outline of methods of separation and measurement of pigments in Florometra serretissima.

Thin-layer chromatography (TLC). Eastman Chromagram Sheets, K301-R-silica gel, obtained from Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y., were used for TLC of the carotenoids. Prepared cellulose sheets (MN Polygram cel 300, Brinkmann Instruments, Inc., Westbury, N. Y.) were used for TLC of the suspected echinochrome pigments. The sheets were developed with hexane-ethyl acetate, 3:1, unless otherwise noted (20). The solvent front was allowed to travel about 20 cm in each case. Some identification of pigments was done by TLC of mixtures of the unknowns with known pigments (β -carotene, echinenone, zeaxanthin and astacene, obtained from Dr. Mary Griffiths, Zoology Department, University of Washington).

As stated above, TLC was used routinely after every step of the separation procedure to rapidly and efficiently check the purity of the various fractions obtained. Detection in all cases was by visual observation. The location and color of the spots were marked as soon as development was complete as they tended to fade fairly rapidly. An example of a silica gel thin-layer chromatogram of the carotenoid pigments along with some known carotenoids is shown in Fig. 2, p. 11.

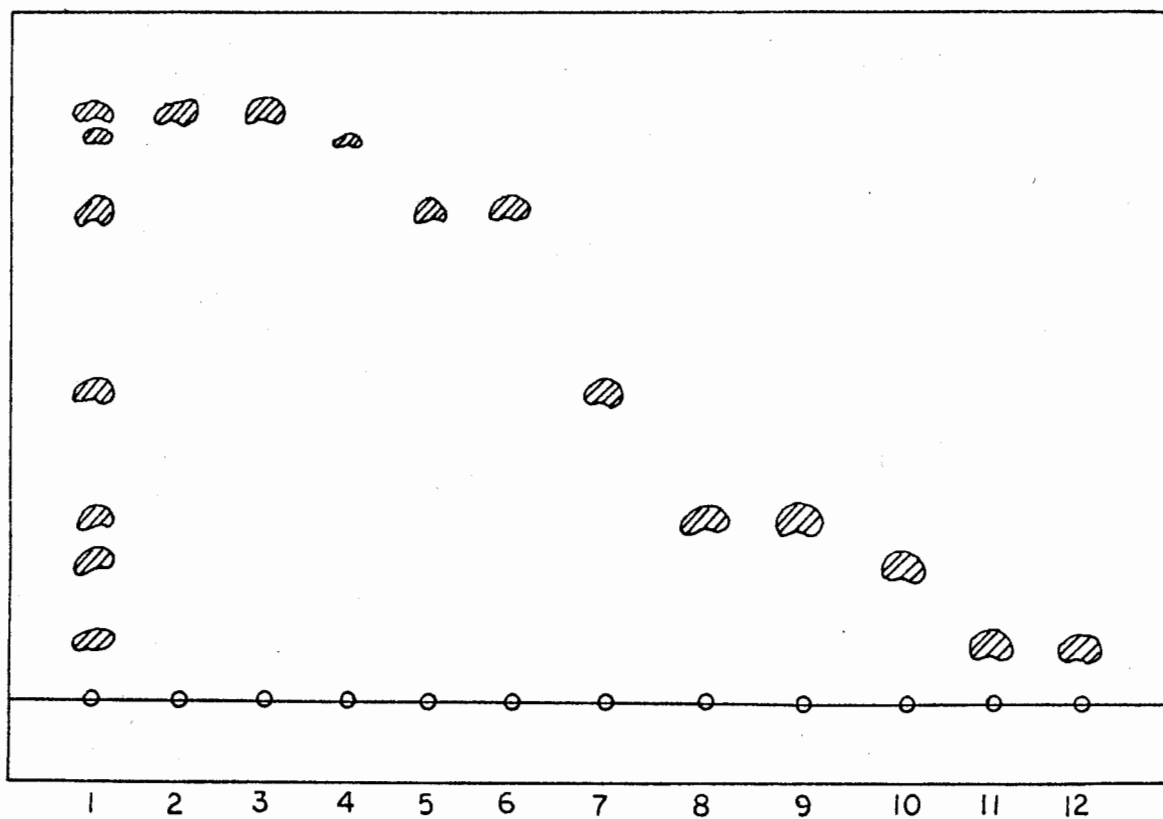


Fig. 2. Thin-layer chromatogram of carotenoids from *Florometra serretissima* along with some known carotenoids. Adsorbent: silica gel; solvent: hexane-ethyl acetate, 3:1. 1-Total pigment, 2-Fraction I, 3- β -carotene, 4-Fraction II, 5-Fraction III, 6-echinenone, 7-Fraction IV, 8-Fraction V, 9-zeaxanthin, 10-Fraction VI, 11-Fraction VII, 12-astacene.

Spectroscopic Measurements

Absorption spectra. Visible absorption spectra of the pigments were determined with a Beckman DB Spectrophotometer. The various solvents used are noted in each case.

Infrared spectra. Infrared spectra were determined with a Perkin-Elmer model 137 B Infrared Spectrophotometer with the pigments neat.

Partition Coefficients

The method of Petracek and Zechmeister (21) was used for determination of the partition coefficient of a pigment between hexane and 95% methanol. Just prior to use, each solvent phase was saturated with the other. The pigment was dissolved in that phase in which it was most readily soluble, and the optical density was recorded at λ_{\max} of the pigment. The solution was then shaken with an equal volume of the second solvent phase, and the optical density of the first phase was recorded again.

Carr-Price Test

Carotenoids give dark blue colorations with the Carr-Price reagent which often have characteristic absorption maxima (22). Two milliliters of a 30% solution of anhydrous antimony trichloride in chloroform was added to 0.5 ml of an approximately 20% chloroform solution of the carotenoid. The absorption spectra was then determined by scanning with a Beckman DB Spectrophotometer.

RESULTS

Carotenoids of Florometra serretissima

(S-L) column chromatography of the non-saponified carotenoid extract with alumina yielded the seven fractions listed in Table I in the order in which they were eluted. Chromatography of the saponified extract gave the same seven fractions with the exception of fraction VII. In the saponified extract fraction VII was astacene, not astaxanthin.

The relative amount of each pigment is given as a percentage of the total pigment present. This figure is based on the extinction at λ_{\max} ; however, the values are only approximate, as no allowance was made for differences in molar extinction values.

Fraction I. The first fraction was eluted with 100% petroleum ether (bp 30-60°). This fraction contained approximately 3% of the total pigment. Fraction I was shown by TLC to be one pigment. The visible absorption spectra is very similar in shape to that of β -carotene (Fig. 8), with maxima in carbon disulfide and petroleum ether (Table I) similar to those reported for β -carotene (23). When subjected to a partition test (p. 12) the pigment shows a partition ratio of 100:0 between hexane and 95% methanol. This is the figure given by Petracek and Zechmeister (21)

for β -carotene. The infrared spectra of fraction I (Fig. 3) is identical to that reported for β -carotene (24). Further, in the Carr-Price test (22) (see p. 12) fraction I gives a blue solution with absorption maximum at 590 m μ which is typical of β -carotene (25). The identity of fraction I with β -carotene was further established by TLC of fraction I mixed with known β -carotene on two different adsorbents. In no case did the pigments separate.

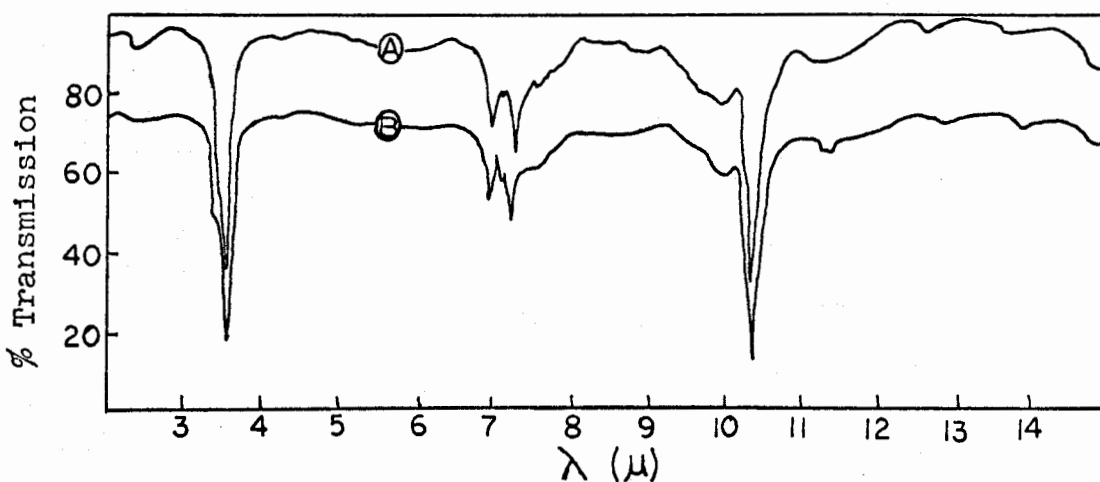


Fig. 3. Infrared spectra of carotenoid fraction I (A) and β -carotene (B) (24).

Fraction II. Only a trace amount of this fraction was present. It was eluted from a (S-L) alumina column with 1-2% diethyl ether in petroleum ether. It was shown to be slightly slower moving on TLC than either Fraction I or known β -carotene. A larger quantity of pigment is needed for further identification.

Fraction III. The third fraction contained about 46% of the total pigment. It was eluted with 25% diethyl ether in petroleum ether. TLC showed only one pigment. After further purification on a second (S-L) alumina column the pigment showed the following properties, which identify it as echinenone (4-keto- β -carotene) (14, 26). The visible absorption spectra of fraction III has only one asymmetric peak in carbon disulfide, petroleum ether, or chloroform (Table I, Fig. 9). Infrared spectra of fraction III and authentic echinenone (neat) were identical (Fig. 4). TLC of known echinenone mixed with fraction III on two different adsorbents failed to show any separation of the pigments.

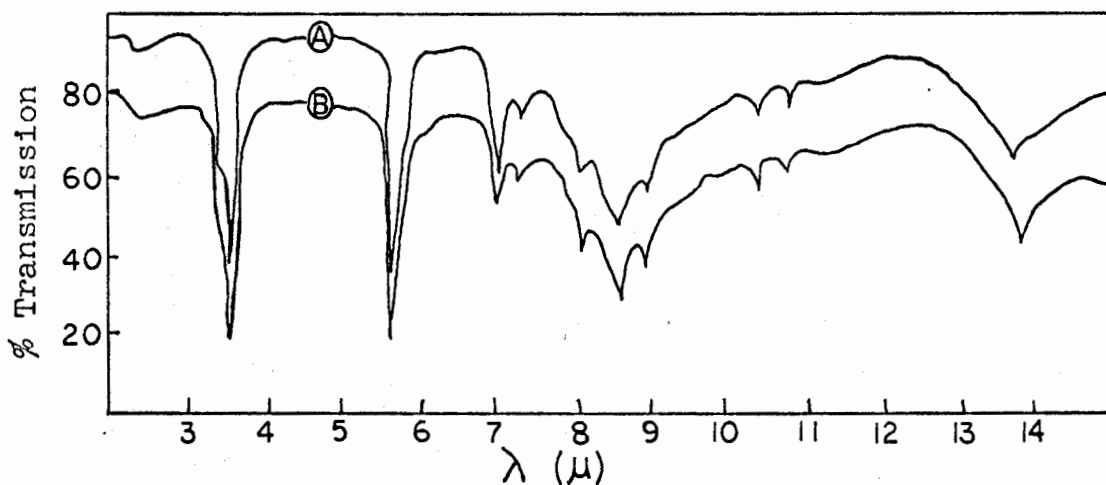


Fig. 4. Infrared spectra of carotenoid fraction III (A) and known echinenone (B).

Fraction IV. This fraction, eluted with diethyl ether containing a trace of ethanol, contained about 31% of the total pigment. It was purified on a second (S-L) alumina column. The visible absorption spectra in carbon disulfide and in hexane show single, broad peaks (Fig. 10). The maxima (Table I) are similar to those of astaxanthin (3,3'-dihydroxy-4,4'-di-keto- β -carotene) (27) and canthaxanthin (4,4'-di-keto- β -carotene) (28-30). Astaxanthin, however, can be eluted from (S-L) alumina columns only with ethanol containing 5% acetic acid (31) (see Fraction VII below). Partition of fraction IV between hexane and 95% methanol (see p. 12) gives a ratio of 50:50. This is the figure reported for canthaxanthin (21).

Fraction V. Fractions V and VI were eluted together from the first (S-L) alumina column using 100% ethanol. TLC on silica gel showed two components to be present in this fraction. Separation could not be effected on a second (S-L) alumina column. The two fractions were separated with (S-L) columns packed in the same manner as above (see p. 7), but with silica gel (140 mesh, chromatographic grade, J. T. Baker Chemical Co., Phillipsburg, N. J.) and cellulose (2:1, by volume) as the adsorbent. Passing each of the fractions (V and VI) through a second (S-L) silica gel-cellulose column purified the separated pigments.

The visible absorption maxima of fraction V in carbon disulfide and in ethanol (Table I, Fig. 11) are similar to those of zeaxanthin (3,3'-dihydroxy- β -carotene) (20, 32). The infrared spectra of fraction V (Fig. 5) is identical to that reported for zeaxanthin (33). When subjected to a partition test (see p. 12) the pigment shows a partition ratio of 10:90 between hexane and 95% methanol. This agrees with the 11:89 reported for zeaxanthin (21). TLC of fraction V mixed with authentic zeaxanthin on two different adsorbents showed no separation of the pigments. In the Carr-Price test, with antimony trichloride in chloroform (see p. 12), fraction V gave a blue solution with absorption maximum at 590 $m\mu$ which is the reported value for zeaxanthin (25).

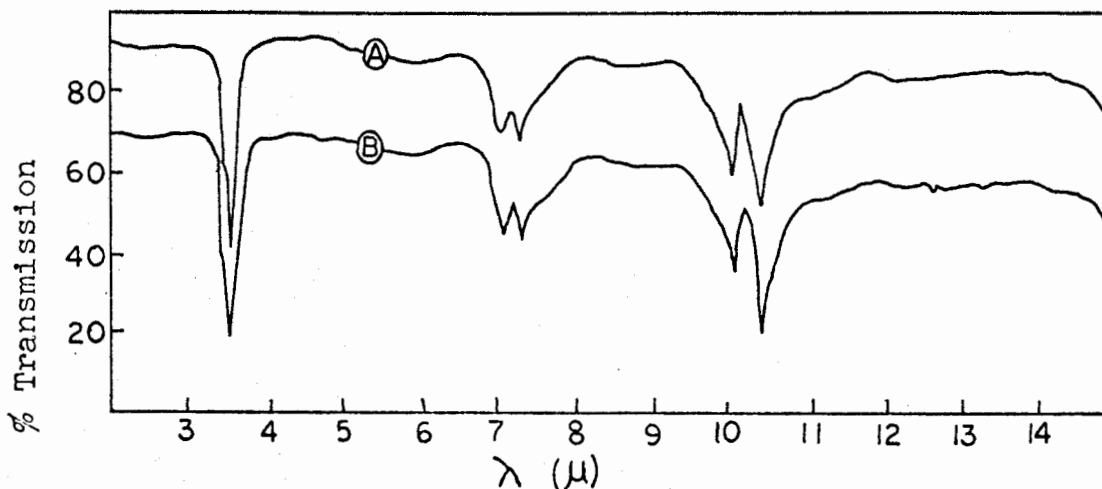


Fig. 5. Infrared spectra of carotenoid fraction V (A) and zeaxanthin (B) (33).

Fraction VI. This fraction contained about 3% of the total pigment. Its purification is described above (see Fraction V). The absorption maxima in carbon disulfide and in petroleum ether, respectively, are similar to those of xanthophyll (3,3'-dihydroxy- α -carotene) (Table I, Fig. 12) (32). Partition of fraction VI between hexane and 95% methanol (see p. 12) gave a ratio of 13:87. This agrees with the reported value of 12:88 for xanthophyll (21). The Carr-Price test (see p. 12) gave a blue solution with absorption maximum at 585 m μ which is in close agreement with the 586 m μ reported for xanthophyll (25). The infrared spectra of fraction VI (Fig. 6) is identical to that of xanthophyll (33).

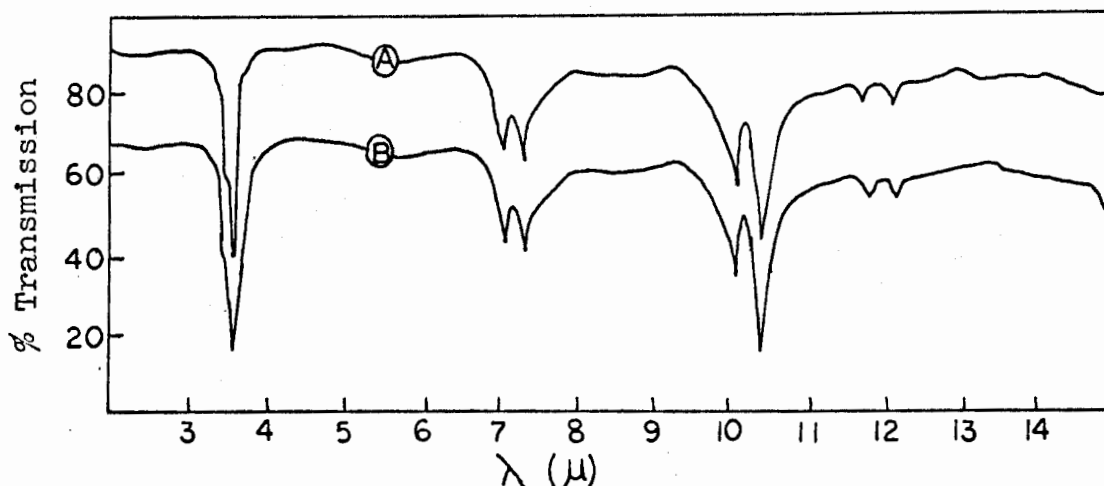


Fig. 6. Infrared spectra of carotenoid fraction VI (A) and xanthophyll (B) (33).

Fraction VII. Fraction VII was eluted from the first (S-L) alumina column with 5% acetic acid in ethanol: this is a characteristic property of astaxanthin (29, 34, 35). This fraction contained about 12% of the total pigment. The acetic acid-ethanol solution of this fraction was diluted with an equal volume of water, and the pigment extracted with diethyl ether; this extract was then concentrated and identified as described below. TLC showed this fraction to contain traces of preceding fractions (probably slight residuals). The main fraction (VII) was purified with a second (S-L) alumina column. The absorption spectra in carbon disulfide (Table I, Fig. 13) is similar in shape and maximum to astaxanthin (27). TLC of fraction VII (after saponification) mixed with authentic astacene on two different adsorbents gave no separation of the pigments. Astaxanthin is the natural pigment, for astacene does not occur in nature (35-37). Astacene is formed from astaxanthin by saponification (27). This conversion was shown to take place with fraction VII. The infrared spectra of this fraction (after saponification) is identical to that of known astacene (Fig. 7).

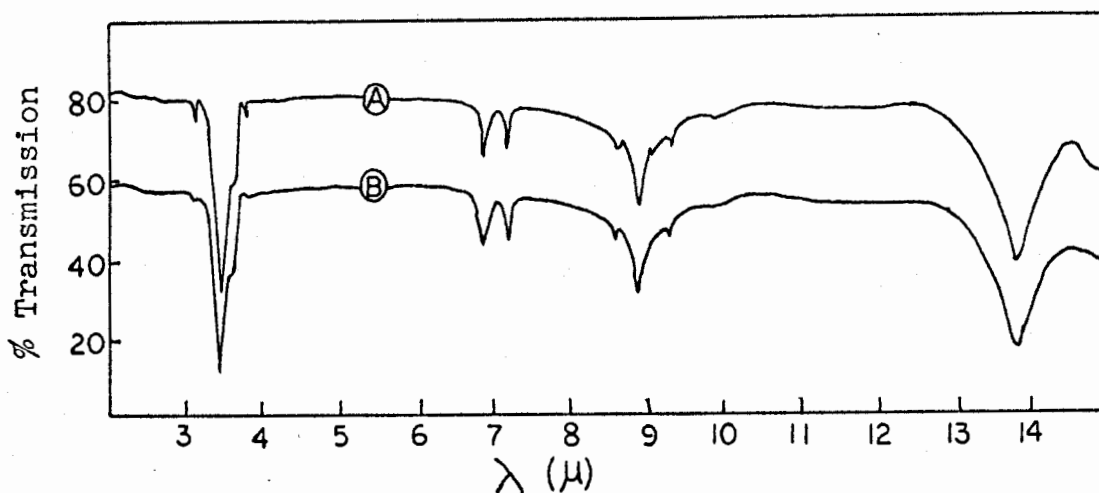


Fig. 7. Infrared spectra of carotenoid fraction VII (A) (after saponification) and known astacene (B).

Other Pigments of *Florometra serretissima*

After ethanol extraction of the macerated animals was complete, the material was slurried with diethyl ether containing a trace of 2 N HCl (see p. 5). The acidified extract was pale yellow. This solution was chromatographed on a (S-L) cellulose column (see p. 7) to yield 3 small fractions. The first fraction was eluted with a 1:1 mixture of petroleum ether (bp 30-60°) and diethyl ether. The second fraction was eluted with 100% diethyl ether, and the third fraction was eluted with 5% acetic acid in diethyl ether. These fractions were all in trace amounts and further identification studies were not run at this time.

Table I
Carotenoids of Florometra serretissima^a

Frac- tion	Relative Abundance (Approxi- mate) %	Ethyl Ether in Petroleum Ether Required for Elution, %	Absorption Maxima				Partition Coefficient (Hexane: 95% Methanol)	Probable Identity
			Carbon di- Sulfide	Petro- leum Ether	Ethanol	Chloro- form		
I	3	0	520,486 ~450 ^b	483,453 ~425	—	—	100:0	β -carotene
II	trace	1-2	—	—	—	—	—	?
III	46	25	490	460	—	475	94:6	echinenone
IV	31	100	500	463	—	—	50:50	canthaxanthin
V	5	ethanol	515,480 ~450	—	483,450 ~420	—	10:90	zeaxanthin
VI	3	ethanol	510,480 ~446	475,448 ~420	—	—	13:87	xanthophyll
VII	12	ethanol, 5% acetic acid	502	—	—	—	2:98	astaxanthin

^aThe pigments are listed in the order in which they were eluted from an (S-L) alumina column.

^b~ indicates a shoulder or inflection.

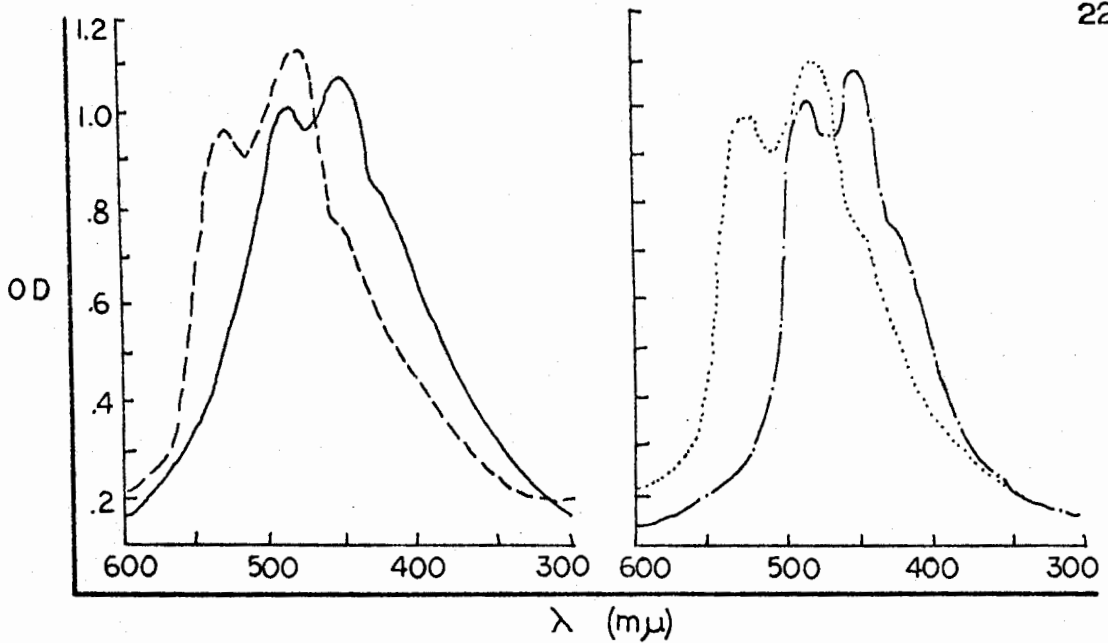


Fig. 8. Light absorption of carotenoid fraction I in CS₂ (---) and petroleum ether (bp 30-60°) (—); β -carotene in CS₂ (.....) and petroleum ether (— · —) (31).

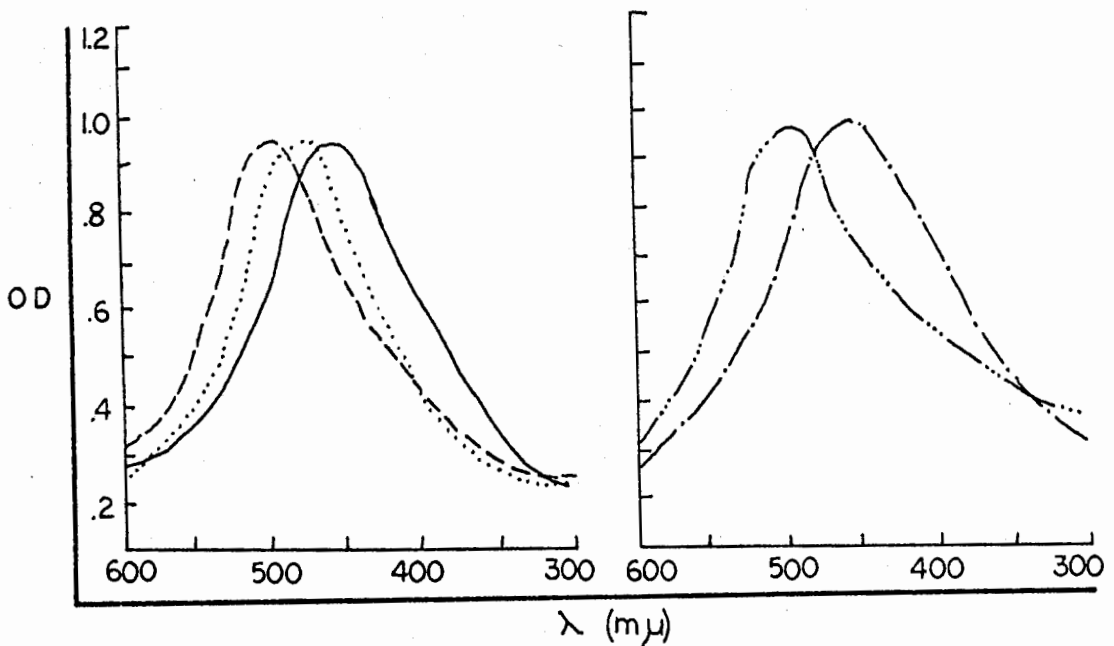


Fig. 9. Light absorption of carotenoid fraction III in CS₂ (---), CHCl₃ (.....) and petroleum ether (bp 30-60°) (—); echinenone in CS₂ (— · —) and CHCl₃ (—) (25).

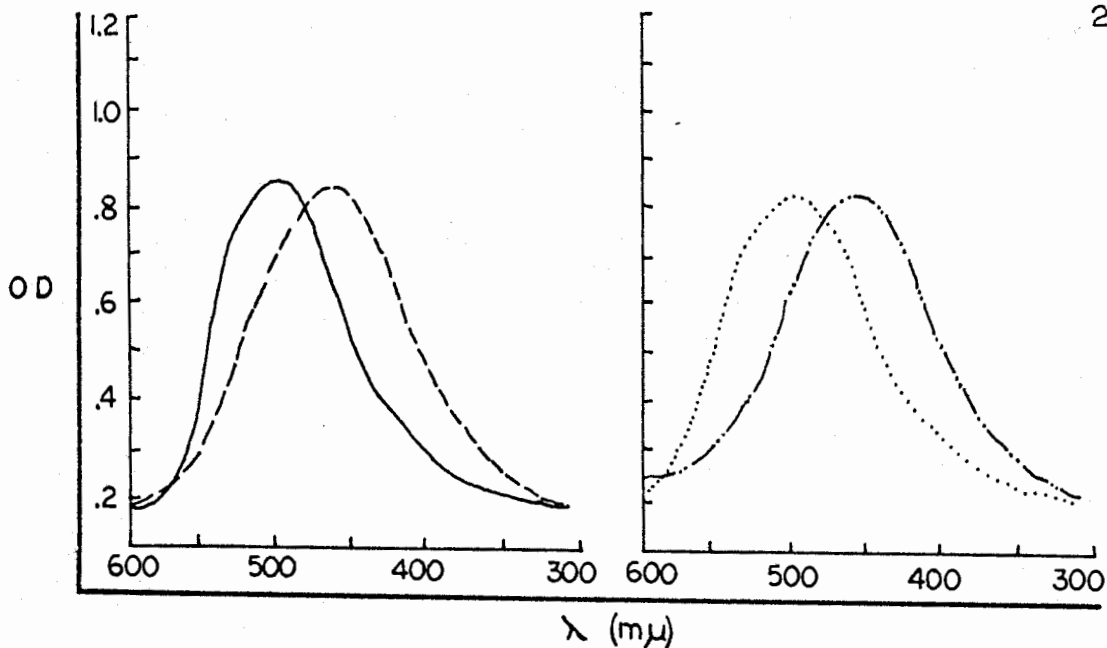


Fig. 10. Light absorption of carotenoid fraction IV in CS₂ (—) and hexane (---); canthaxanthin in CS₂ (.....) and hexane (-·-) (27).

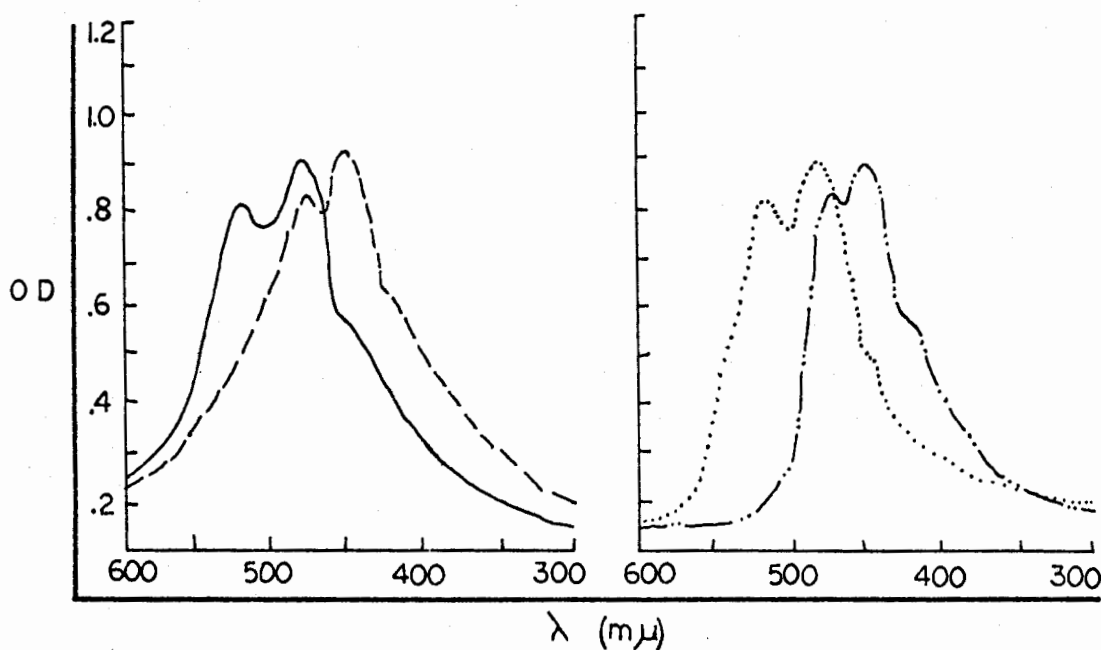


Fig. 11. Light absorption of carotenoid fraction V in CS₂ (—) and ethanol (---); zeaxanthin in CS₂ (.....) and ethanol (-·-) (31).

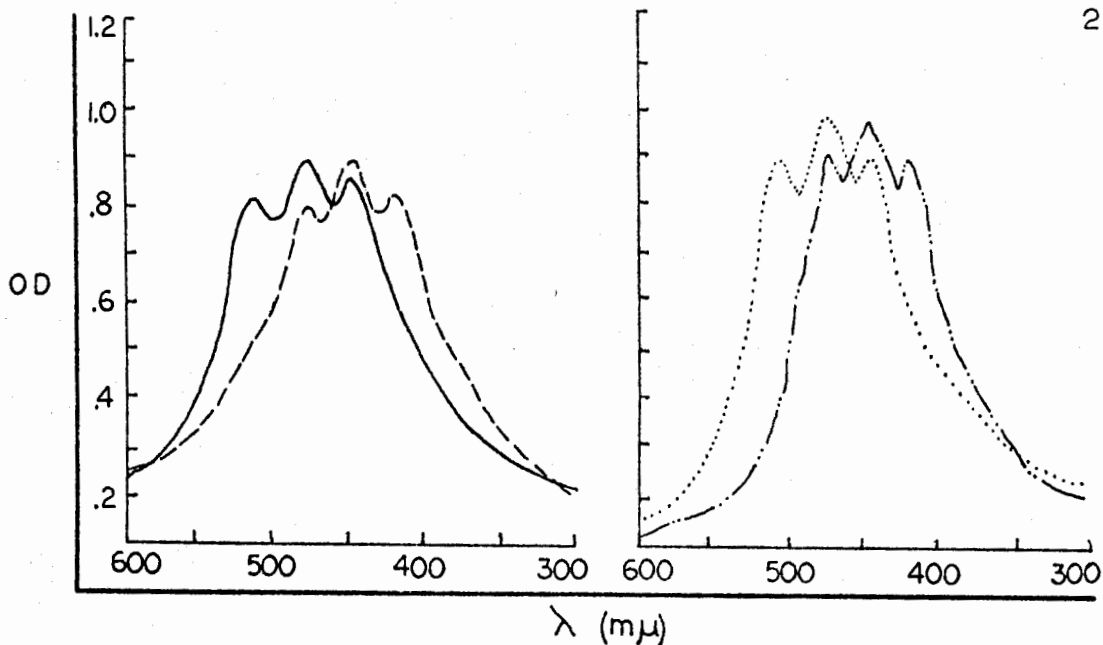


Fig. 12. Light absorption of carotenoid fraction VI in CS₂ (—) and petroleum ether (bp 30-60°) (---); xanthophyll in CS₂ (.....) and petroleum ether (— · —) (31).

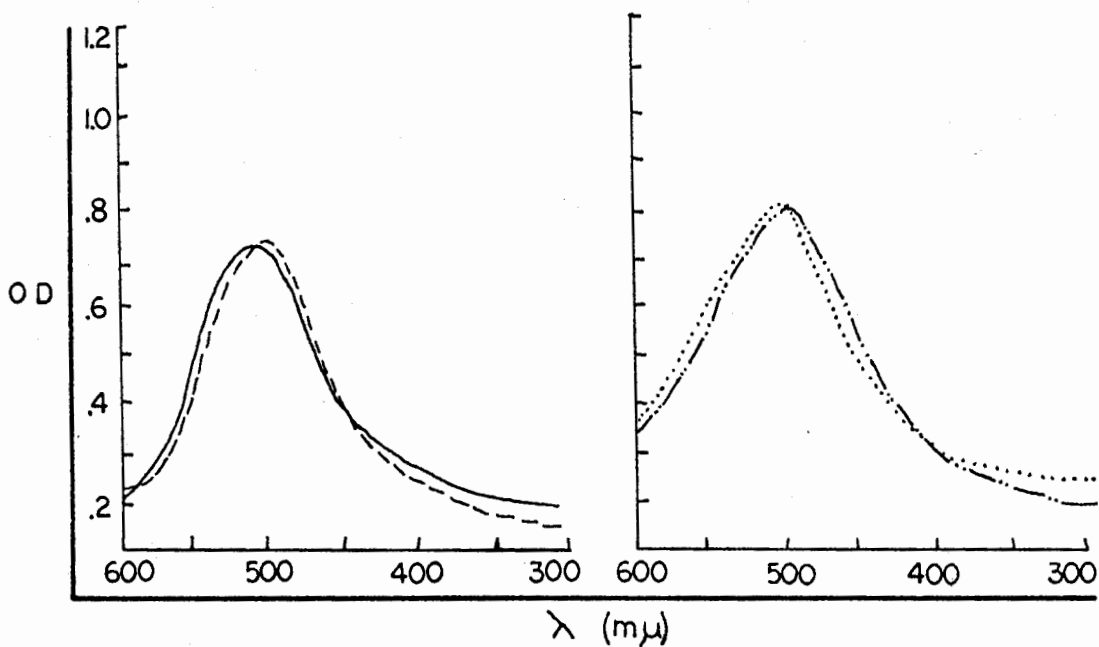


Fig. 12. Light absorption of carotenoid fraction VII in CS₂ before saponification (—) and after saponification (---); astaxanthin (.....) and astacene (— · —) in CS₂ (33).

DISCUSSION

This study shows the presence of β -carotene, echinenone, canthaxanthin, zeaxanthin, xanthophyll, and astaxanthin in the crinoid Florometra serretissima. Echinenone is present in the largest amount (46% of total pigment), followed by canthaxanthin (31%). That these various fractions have been found together is not surprising, since β -carotene is reported to be the precursor of echinenone, canthaxanthin (38), and astaxanthin (35). In fact, the sequence β -carotene \longrightarrow echinenone \longrightarrow canthaxanthin \longrightarrow astaxanthin has also been reported (39), although the exact mechanisms for these steps have not been worked out. All of the aforementioned fractions are derivatives of β -carotene, with the exception of xanthophyll, which is an α -carotene derivative (40). Other instances of xanthophyll in crinoids have been reported (41, 42). Dimelow (10) reported the presence of xanthophyll with β -carotene and astaxanthin in the same animal.

Although carotenoid fraction II was present in a trace amount and could not be identified, it is possible that this fraction is α -carotene, since xanthophyll (an α -carotene derivative) was found in a significant amount.

This study reports the first isolation of canthaxanthin from an echinoderm, although it has been found in

crustaceans (43).

The carotenoids are primarily plant pigments; as far as is known animals are unable to synthesize them (15). Clarification of this could be obtained, in part, by investigating the carotenoid content of Florometra serretissima at various growth stages. The animals studied here were not divided according to age or stage of growth.

The carotenoid content of crinoids could vary with the time of year because of seasonal differences in food supply. This study was conducted on animals collected in December. Another study on animals collected in the summer could give other results.

Traces of pigments other than carotenoids were also found (see p. 20). This is not surprising, since hydroxynaphthaquinones (10) and hydroxyanthraquinones (11) have been isolated from two different species of crinoid. Further studies with larger amounts of pigment will possibly lead to characterization of these non-carotenoid fractions found in Florometra serretissima.

SUMMARY

The carotenoids of the crinoid Florometra serretissima have been separated by adsorption chromatography and further characterized by their visible absorption spectra, behavior on partitioning between hexane and 95% methanol, and behavior on thin-layer chromatography when mixed with known pigments. The Carr-Price test, with antimony trichloride in chloroform, was performed and visible absorption spectra recorded of the resultant solutions. Infrared spectra were taken and compared to known carotenoids or to the literature. Echinenone, canthaxanthin, and astaxanthin were present in the largest amounts. Small amounts of β -carotene, zeaxanthin, xanthophyll, and an unidentified carotenoid pigment were also present.

Small amounts of three non-carotenoid pigments were found in Florometra serretissima. However, insufficient quantities were isolated for characterization.

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