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Pigments and Neurogenic Secretions of Dermasterias Imbricata

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PIGMENTS AND NEUROGENIC SECRETIONS OF *DERMASTERIAS IMBRICATA*

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Master of Science

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I. INTRODUCTION

The behavior of the sea anemone Stomphia coccinea is unlike that of most other anemones. When disturbed, all sea anemones will contract their body, expell water, and pull their tentacles into the interseptal chambers to contract the oral disc, forming a ball. But when Stomphia is brought into contact with the asteroid Dermasterias imbricata, it can detach and rapidly move away by a series of whip-like motions.

Sund (1) showed that direct contact by the starfish is necessary to evoke the escape response. After stimulation, the anemone's reaction is pictured as: first, withdrawal of its tentacles to form a ball; second, a rising or elongation of the body; third, a rocking motion from side to side while still attached; fourth, release of the basal disc, opening up; and lastly, a series of whip-like gyrations to propel itself through the water. After escape, the basal disc becomes cone shaped. When ready to reattach itself the cone gradually disappears and the disc becomes flattened.

Wilson (2) and Hoyle (3) have investigated the neurophysiology of Stomphia in reference to its swimming behavior. Robson (4) has studied its anatomy, neuroanatomy and neuromotor coordination. Ward (5) has studied sixteen different species of asteroids commonly found in Puget Sound, and found that only Dermasterias imbricata

and Hippasteria spinosa would elicit the response. He made tissue extracts of integument, digestive system, gonads, tube feet and aboral surface. He found that only the homogenate from the aboral surface would evoke reaction. He also found that the source of the activity was dialyzable and that heat treatment up to 100°C did not alter activity. He reported that the active substance was possibly a carbohydrate of three glucose units.

Ward (6) also studied Dermasterias histologically. He found that Dermasterias is atypical of other asteroids studied. The dermis of the aboral surface is unique. The tissue has many granulated spherule cells scattered throughout it. The contents of these dermal spherules were found to give a positive toluidine blue test for polysaccharides. He proposed that this is the site of storage for the active substance.

The asteroid Dermasterias imbricata is covered with a thick soft membrane concealing the spines. The membrane contains hidden spicules of lime in the shape of rods and may be covered with mucus (7). Coloration is a mottled lead blue and dull red.

My early work with the red pigment has led me to believe that it is a carotenoprotein. Cheesman (8) has defined carotenoproteins as those proteins in which carotenoids are present as prosthetic groups. This may be demonstrated if the colored aqueous extract, upon

treatment with organic solvents, results in liberation of carotenoid. Other than the studies of Cheesman, very little work has been done on the carotenoproteins. Ovoverdin, the green pigment of lobster eggs, has been the most studied and is typical of this class of substances.

Cheesman lists over one hundred different carotenoproteins in invertebrates but only three have ever been isolated in a state in which they satisfy even electrophoretic homogeneity.

The carotenoproteins occur primarily in two general body areas, the epidermis and the ovaries. Blue or red is the most common color. Astaxanthin is by far the most commonly found carotenoid, but canathaxanthin and xanthophyll are also found.

The purpose of the present study is to isolate and identify the active compound for swimming response, the carotenoprotein, and the carotenoids from Dermasterias.

II. EXPERIMENTAL

Collection of Animals

Dermasterias imbricata were collected in about 20 feet of water at various locations near San Juan Island and near the Narrows Bridge in Tacoma, Washington.

Specimens of *Stomphia coccinea* were obtained by dredging in the San Juan Channel. The animals were most frequently found at depths of 80-100 feet attached to shells of *Modiolus modiolus*. Both starfish and anemones were maintained in aquaria at the Friday Harbor Laboratories, Friday Harbor, Washington.

Preparation of the Pigment Extract

The aboral skin was first removed from fresh starfish and any adhering digestive or gonadal tissue was removed. The skin was then cut into small pieces and homogenized at high speed in a Waring Blender for fifteen minutes. The crude extract was then centrifuged for 15 minutes at 8000 RPM to pelletize the cell debris. The dull red supernatent was heated to 65° C for fifteen minutes and centrifuged at 12,000 RPM for 30 minutes. The resulting supernatent was a clear ruby red.

Separation and Purification of Pigments

When the clear red supernatent is passed through a column of Sephadex G25, two distinct bands of about equal size can be seen. The band that comes through

first is ruby red, and the second band is bright yellow. When acetone is added to the red fraction a large amount of white precipitate is formed and the color changes from red to orange. When acetone is added to the yellow fraction no precipitate is formed and no color change occurs, indicating no protein binding in the latter pigment.

The red pigment was checked for purity by cellulose acetate electrophoresis. The yellow and orange pigments were checked for purity by thin layer and aluminum oxide column chromatography.

Column Chromatography on Sephadex G25

Sephadex G25 was obtained from Pharmacia Company of Uppsala, Sweden. The column was 16 cm X 2 cm and was packed by gravity using 0.025M phosphate buffer pH 7.0 as a solvent. Five ml samples of extract were run on the column and the same phosphate buffer was used to elute it.

The column was monitored visually and the samples were collected in 10 ml fractions.

Cellulose Acetate Electrophoresis

A 25 microliter sample was run for 20 minutes on a Beckman model R-101 electrophoresis at 250 volts in a sodium barbitol buffer, 0.075, ionic strength pH 8.6. Detection was by development with ponceau-S stain. The strips were then read on a Beckman model R-110 densitometer.

Thin Layer Chromatography

Eastman chromatogram sheets K301-R silica gel obtained from Eastman Kodak Co. were used for thin layer chromatography of the carotenoids.

When the orange and the yellow carotenoids were run in a solvent system of hexane-ethyl acetate (3:1 by volume) they were found to be chromatographically pure and definitely distinct from one another.

Column Chromatography on Aluminum Oxide

In all separations the solid phase was chromatographic grade acid aluminum oxide 80-200 mesh from Merck and Co. The column was 20 cm X 2 cm and was packed by gravity using hexane as a solvent.

When the yellow or the orange, or a mixture of the two pigments was placed on the column, the following step-wise elution pattern was followed:

1. 20 ml hexane; 5 ml diethyl ether
2. 25 ml hexane
3. 20 ml diethyl ether; 5 ml methanol
4. 20 ml hexane; 5 ml diethyl ether
5. 15 ml hexane; 10 ml diethyl ether
6. 20 ml diethyl ether; 5 ml methanol
7. 20 ml hexane; 5 ml diethyl ether
8. 25 ml methanol

If the 10 ml sample was a mixture, the yellow pigment was eluted first in the hexane-diethyl ether mixture (step 5). The orange pigment was then eluted in the diethyl ether-methanol mixture (step 6).

Spectroscopic Measurements

Visible absorption spectra were obtained by means of a Beckman DB spectrophotometer. The solvents used are noted in each case.

Infrared spectra were obtained by means of a Perkin-Elmer model 521 infrared spectrophotometer. Again the solvents used are noted in each case.

Partition Coefficients

The method of Petracek and Zechmeister (9) was used for determination of the partition coefficient of the pigments between hexane and 95% methanol. The pigment was dissolved in that phase in which it was most readily soluble and the optical density was recorded at 500 mμ for the orange pigment and at 450mμ for the yellow 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 of characteristic absorption when treated with anhydrous antimony trichloride in chloroform. The pigments were taken up in chloroform and then treated with the reagent. The absorption spectra was then obtained with a Beckman DB spectrophotometer.

Isolation of the Compound Which Causes Stomphia to Swim

To determine whether a given solution would elicit the swimming response in Stomphia, the anemones were first allowed to attach to small watch glasses. Just before a solution was to be tested, an anemone was carefully taken out of the water and exposed to the air. Twenty-five microliters of the solution were then applied to its tentacles. If the anemone escaped within a one minute interval after application, the test was interpreted as being positive. If no escape occurred, the test was negative.

Attempts to isolate the active compound were initiated by absorbing the heated starfish extract on a column of Sephadex G10 or G25 and eluting 10 ml fractions with distilled water. Each fraction was then tested for sea anemone activation. All fractions indicating activity were combined and concentrated to 10 ml in a rotary evaporator. All concentrated samples were then combined and the total volume reduced to 10 ml in the rotary evaporator. The result of all this very time consuming process was usually loss of activity.

The next method tried was to completely denature the protein remaining in the heat treated extract by adding acetone. This resulted in a yellow-orange solution. This solution was then heated to remove the acetone, but the pigment remained. To remove the pigment,

activated charcoal was added until the color disappeared. The result of this procedure was a clear solution which was sometimes active and sometimes not. This suggests that the active compound may possibly be insoluble in the acetone-water mixture or that charcoal may absorb it from solution.

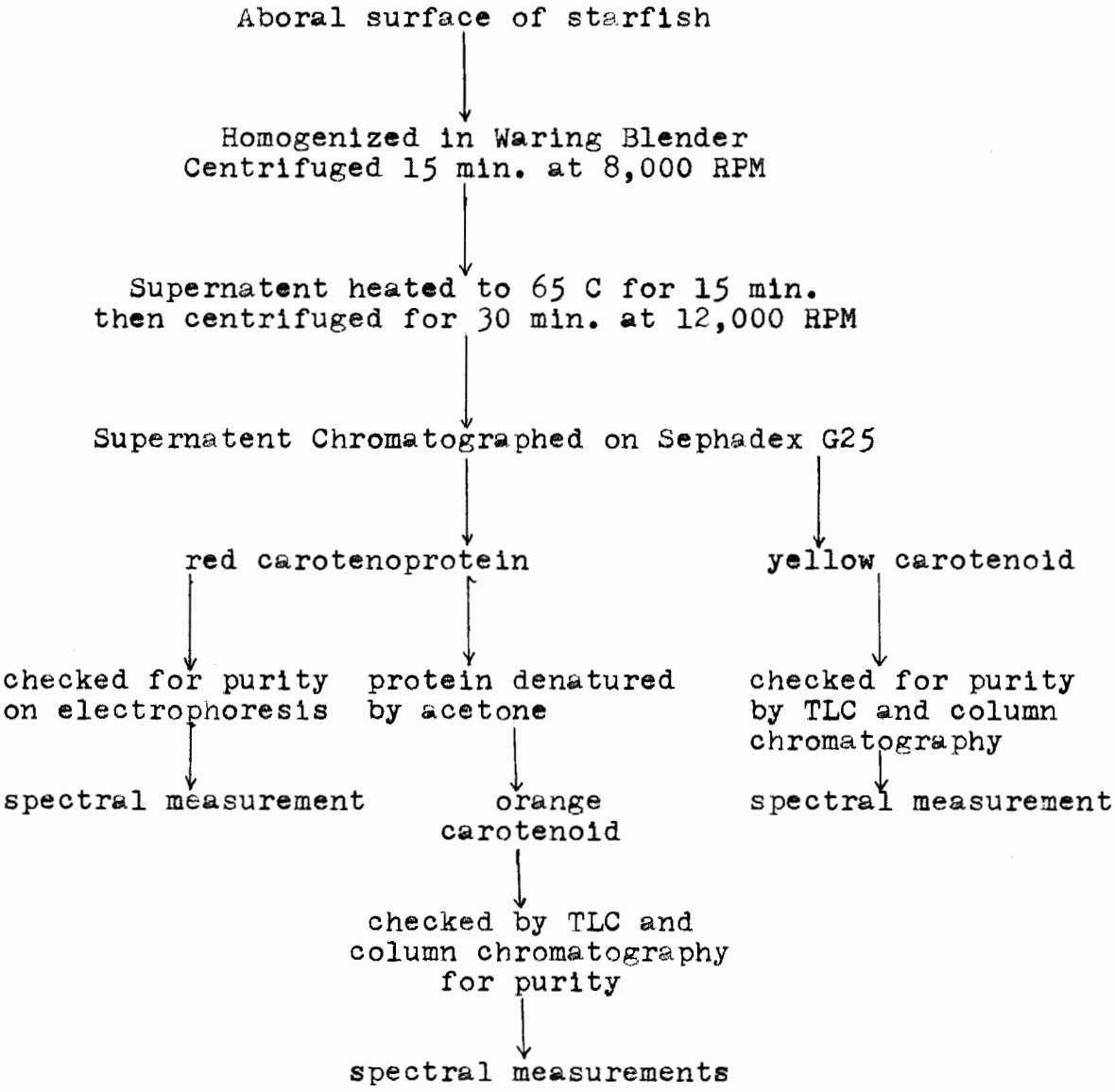
The next procedure tried was to salt out the protein in the heat treated extract with ammonium sulfate. Addition of sulfate to 70% saturation, resulted in an orange precipitate and a yellow supernatent. The precipitate was discarded and the supernatent was passed through Sephadex G10 to remove the sulfate. The 10 ml fractions that were collected were tested for activity on Stomphia. The active fractions were combined and freeze dried. After drying, a few small yellow crystals were observed. These crystals were taken up in a minimal amount of water, decolorized with charcoal, and freeze dried again. At this point, a few colorless crystals were found. When these crystals were taken up in a minimal amount of water, the solution was not active but a more dilute solution of the crystals was active. This procedure was repeated many times to give a combined yield of 0.4 gm of the crystals. This sample was sent to Schwarkopf Laboratories, Woodside, New York for carbon, hydrogen and nitrogen analysis. The results were 2.76% carbon, 5.82% hydrogen and 17.73% nitrogen. I expected

approximately 40% carbon, 6% hydrogen and 3% nitrogen. Clearly there must be a very large ammonium sulfate contamination.

To eliminate the ammonium sulfate and the time consuming column work, barium hydroxide was added to the yellow supernatent left after ammonium sulfate precipitation. The barium hydroxide was added slowly so as not to let the pH of the solution rise above 7.5. The solution was kept at 65°C and stirred continuously with a magnetic stirrer to remove the ammonia as it was produced. It required approximately 20 hours to remove the ammonium sulfate. The solution was checked for the presence of sulfate ion by the addition of lead acetate. When no white precipitate was observed, the solution was decolorized with charcoal and then freeze dried. The yield was approximately 0.2 gm of white crystals. When these crystals were tested on Stomphia no activity was observed.

TABLE 1

FLOW DIAGRAM OF PIGMENT PURIFICATION



III. RESULTS

Red Carotenoprotein

The red pigment obtained from Dermasterias met the definition of carotenoproteins in that addition of acetone resulted in the precipitation of protein and the release of carotenoid. The red carotenoprotein was concentrated by freeze drying the water solution. This concentrated solution was then subjected to electrophoresis to determine whether the pigment was protein bound and whether the protein was homogeneous. Figure 1 shows the results which were obtained. The strip was stained with ponceau-S to detect any contaminating proteins.

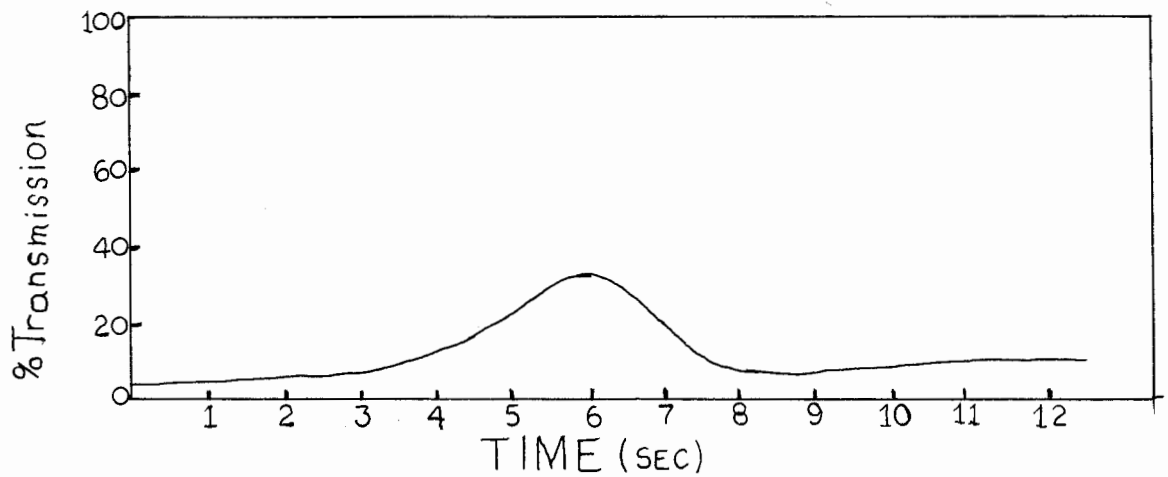


Figure 1. Cellulose acetate electrophoresis of red carotenoprotein stained with ponceau-S to detect all protein.

Following the electrophoretic determination of a single carotenoprotein, a visible spectrum was run to determine λ_{max} (figure 2).

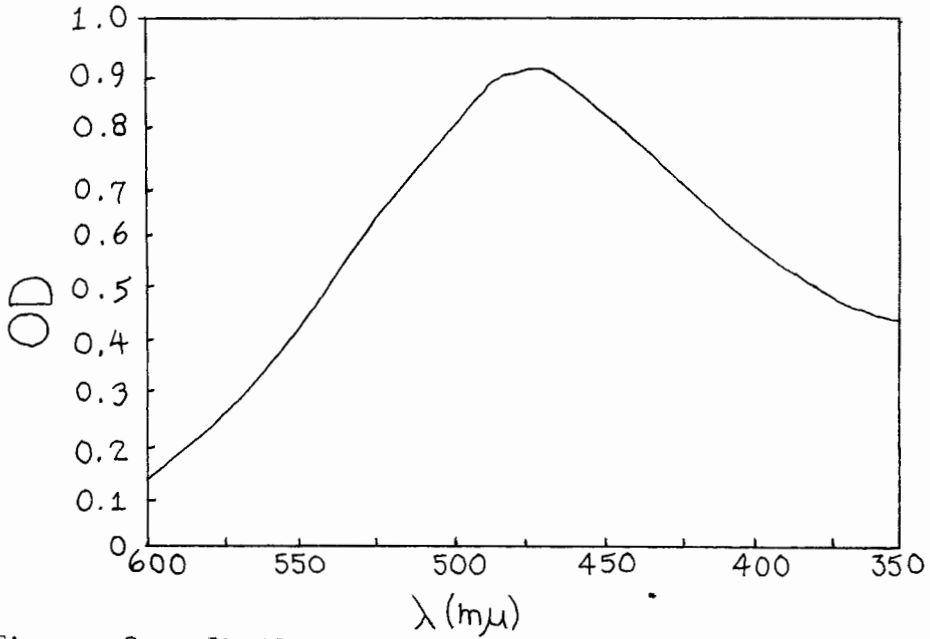


Figure 2. Visible absorption spectra of the red carotenoprotein in water after heat treatment to 65 C and passage through Sephadex G25.

To determine whether heat treatment was causing denaturation of the carotenoprotein, the visible spectra was also run on the unheated sample that had been passed through Sephadex G25 (figure 3)

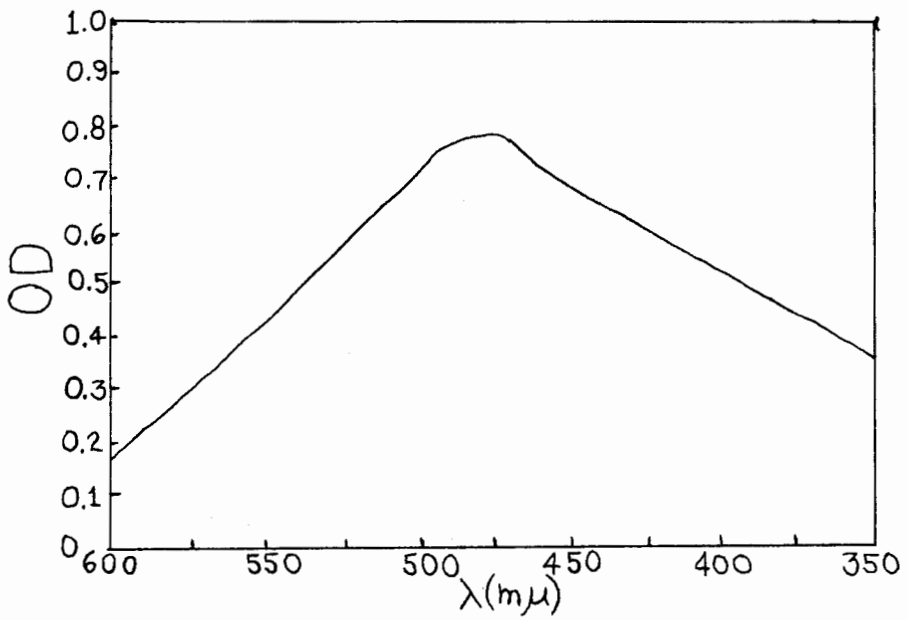


Figure 3. Visible absorption spectra of the red carotenoprotein in water after passage through Sephadex G25 and without heat treatment.

Orange Carotenoid

The orange carotenoid is the protein bound pigment. It was obtained by acetone treatment of the carotenoprotein. The resulting orange liquid was passed through aluminum oxide and checked by TLC for purity. In both instances only a single orange band was observed.

The visible absorption spectrum was taken to compare its λ_{max} values with those values of known pigments. The infrared spectrum was also taken to first determine whether the pigment was a carotenoid and second to compare the pigments spectrum to that of known pigments. The following spectra were obtained:

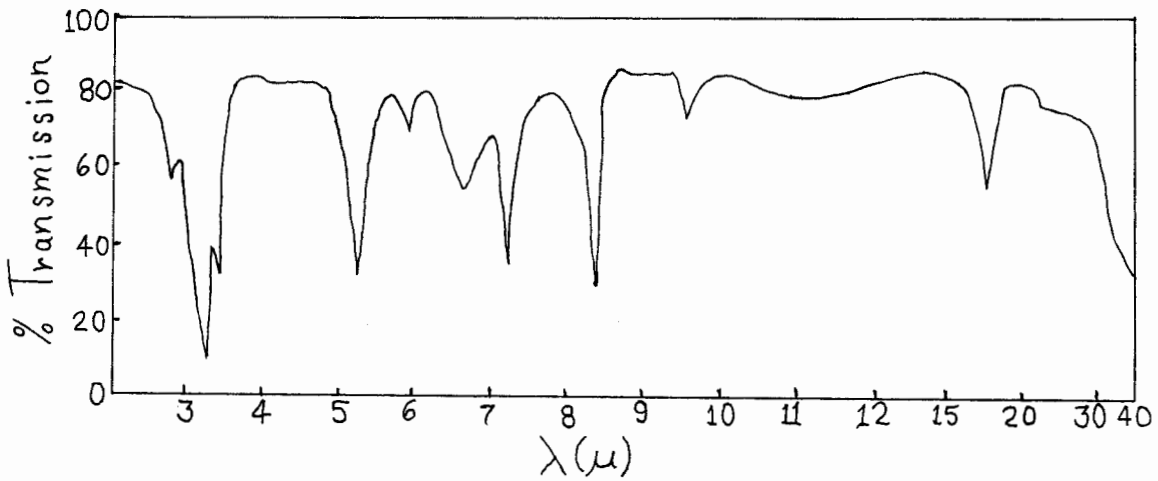


Figure 4. Infrared spectrum of the orange carotenoid in carbon disulfide.

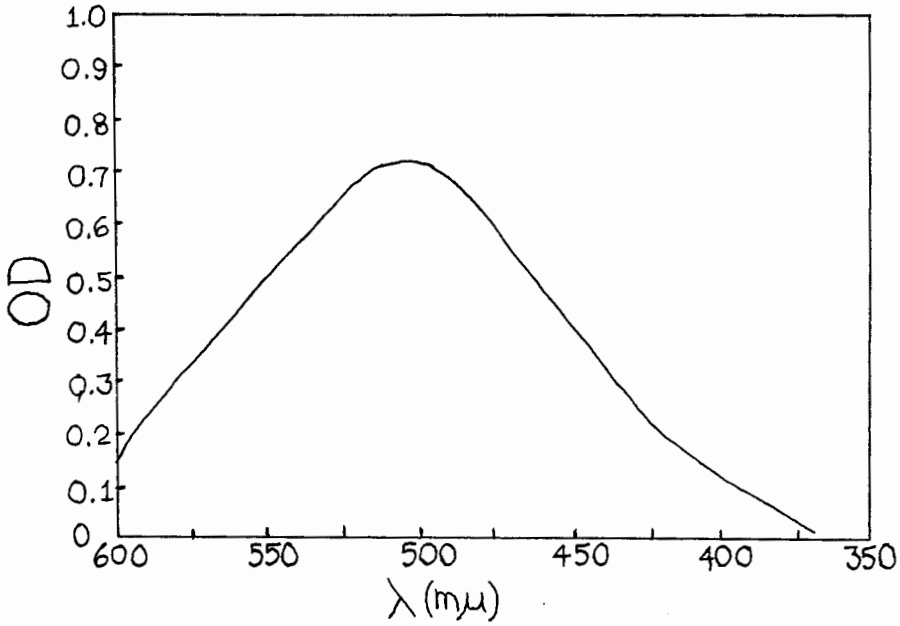


Figure 5. Visible absorption spectra of the orange carotenoid in carbon disulfide.

The Carr-Price test was also run on the orange carotenoid. The following visible spectrum was obtained:

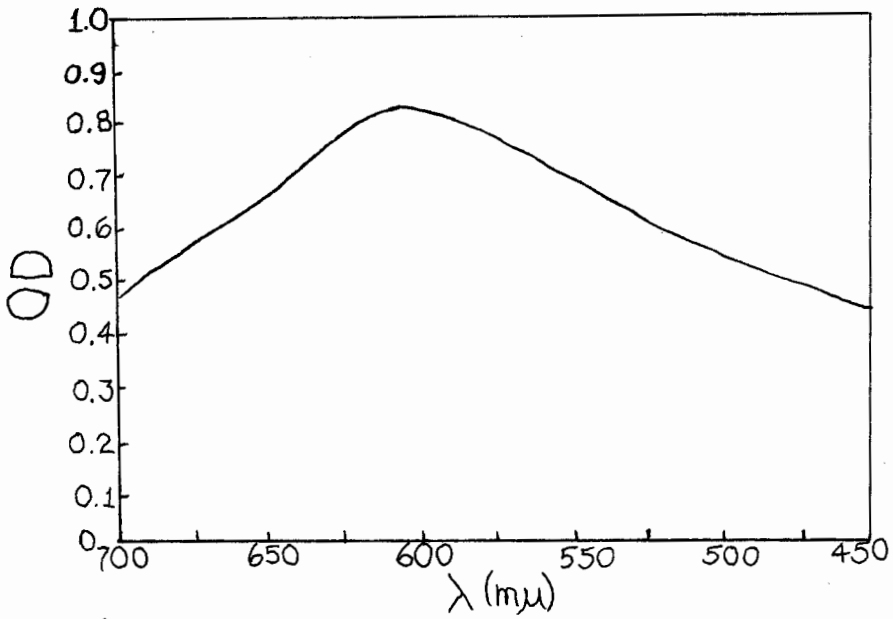


Figure 6. Visible spectrum of the orange carotenoid in chloroform after treatment with the Carr-Price reagent.

When the partition coefficient of the orange carotenoid was taken, the value was found to be 50:50

Yellow Carotenoid

The yellow carotenoid is non-protein bound. It was obtained in a water solution by passing the extract through Sephadex G25. The water solution was then freeze dried and the pigment was redissolved in acetic acid or methanol. The pigment was then checked for purity by TLC and column chromatography. In both instances only a single yellow banc was observed.

The visible absorption spectrum was taken to compare its $_{max}$ values with those values of known pigments. The infrared spectrum was taken to first determine whether the pigment was a carotenoid and second to compare the pigment's spectrum to that of known pigments. The following spectra were obtained:

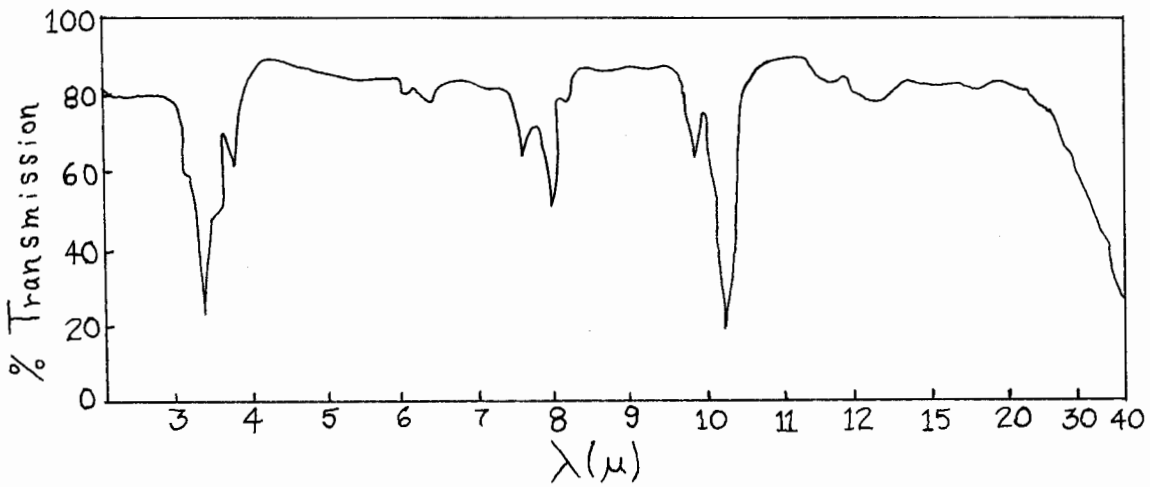


Figure 7. Infrared spectrum of the yellow carotenoid in chloroform.

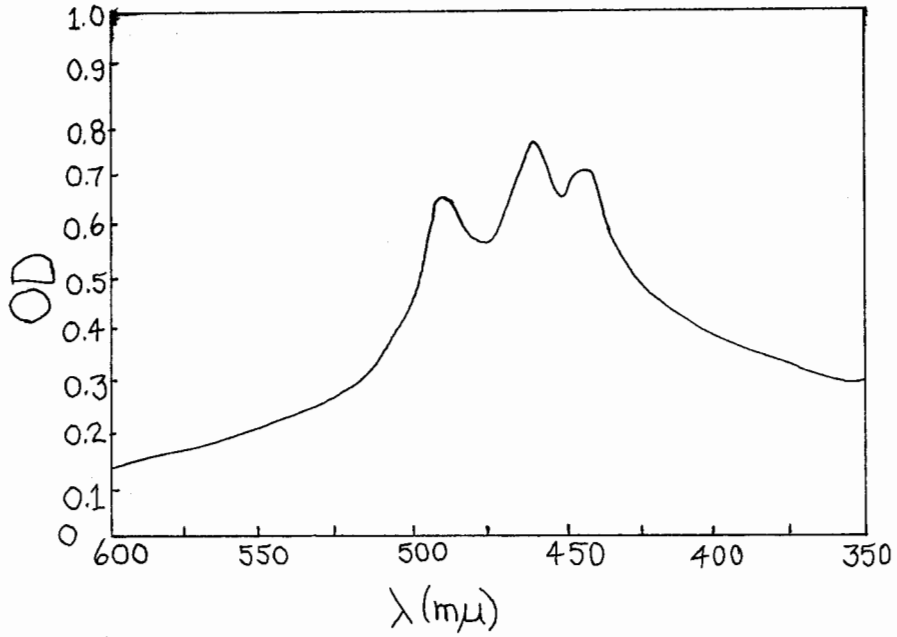


Figure 8. Visible absorption spectrum of the yellow carotenoid in chloroform.

The Carr-Price test was also run on the yellow carotenoid. The following visible spectrum was obtained:

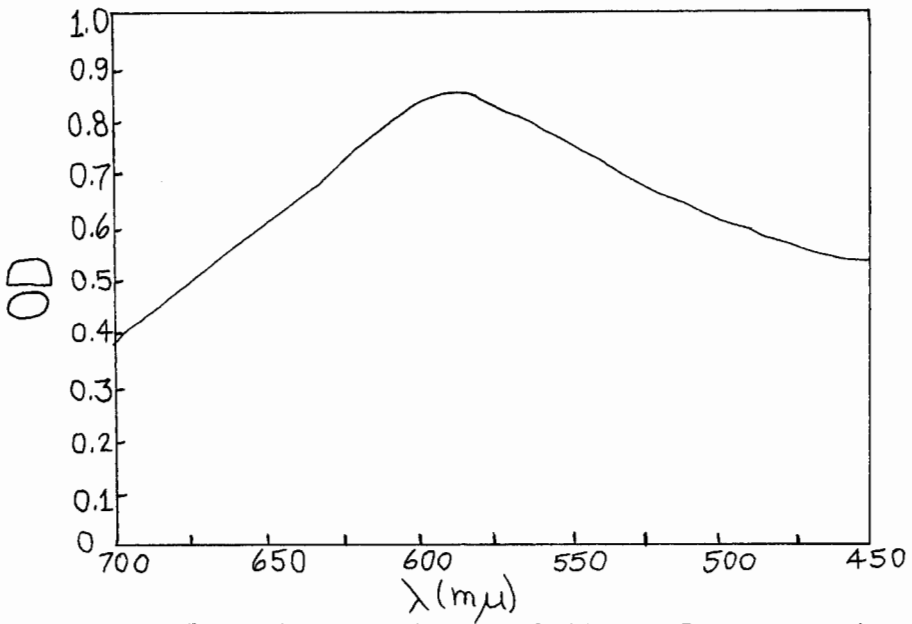


Figure 9. Visible spectrum of the yellow carotenoid in chloroform after treatment with the Carr-Price reagent.

When the partition coefficient of the yellow carotenoid was taken the value was found to be 12:88

Active Substance

It was found that when the extract was run on Sephadex G10 or G25 and 10 ml fractions were taken, fractions 11 through 24 would elicit the swimming response in Stomphia. This suggests that the compound wasn't really separating on the basis of molecular weight but rather on the basis of electrostatic interactions.

Since the compound had previously been postulated to be a carbohydrate, various carbohydrate tests such as the Anthrone, Molisch, and Nelson's were carried out on concentrated active solutions. The carbohydrate tests were positive which demonstrates the presence of carbohydrate but does not necessarily imply that the active compound is a carbohydrate.

Concentrated active solutions were also refluxed overnight in 10% HCL. When neutralized, with NaOH, the solution was still active. The solution was paper chromatographed before and after hydrolysis. Before hydrolysis, one spot was visible. After hydrolysis, two spots were visible, one much larger than the other. Neither of the spots was identifiable when run against known monosaccharides and disaccharides.

The crude extract or the active purified extracts could be greatly diluted without loss of activity. It was not uncommon for a 1:10,000 dilution of the crude or purified extract to retain activity. The dilution

technique could possibly be a method for following the purification of the active compound. But since one would be using a biological assey and there is so much variation in individual anemones, this may not prove to be a good method.

The sample that was sent for an elemental analysis was, unfortunately, too contaminated with ammonium sulfate to give any meaningful results. All attempts to remove the sulfate proved to be unsuccessful, hence a pure sample was never obtained for elemental analysis. I think that in the future acetone rather than ammonium sulfate should be used to precipitate the protein.

IV. DISCUSSION

Possible Procedure for Isolation of the Active Compound

Any future attempts to identify the active compound found in Dermasterias will have to be based on the assumption that there is very little of the compound per starfish. One must be able to work with large quantities of extract without losing activity.

The main problem of isolating the active compound is removal of the protein and the carotenoid contaminants. The bulk of the protein can be eliminated by heating the extract to 65°C for 15 minutes. But there is still a large amount of protein in the form of a carotenoprotein left in solution. This can be removed by Sephadex column chromatography, ammonium sulfate precipitation or acetone treatment. Column chromatography on a 16 cm X 2 cm column giving good results does not accomodate more than 5 ml of extract at a time. Thus, it would be too time consuming for a large volume. Ammonium sulfate precipitation is more satisfactory for large quantities, but the sulfate ifself then becomes a very hard-to-remove contaminent. In acetone treatment one assumes that the active compound is soluble in the acetone water mixture. When minimal amounts of acetone are used, the active compound is apparently not precipitated. This procedure can also be used for a large volume of extract and the

acetone can be easily removed in a rotary evaporator. The one drawback of this procedure is that it liberates into solution the carotenoid from the carotenoprotein.

The carotenoid may be partially removed by absorption on a 16 cm X 2 cm Sephadex column or completely removed by addition of charcoal. Again the column procedure would be too time consuming to handle the large amount of extract.

Addition of excess charcoal results in the loss of activity. But if the charcoal is added in many small successive portions with filtration after each addition, the solution comes out colorless and active. Thus by acetone treatment and charcoal addition it is possible to eliminate the major contaminents.

As a speculative procedure for further purification of the active compound, the solution should then be freeze dried to remove the water. The most prominent contaminent at this point would probably be sodium chloride from the salt water. If the crystals were then taken up in a minimal amount of water and passed through a Sephadex G10 column, nearly all the salt could be removed. The active fractions could then be combined and freeze dried again. Possible impurities at this point are all low molecular weight metabolites that are water soluble and it would be very difficult, if not impossible, to separate all of them from the active compound. A reasonable expectation

would be that the quantity of the active compound exceeds the amount of the impurities. An elemental analysis and the molecular weight determination could then provide meaningful information. From this information one could ascertain whether the active compound is indeed a carbohydrate or not. If it is, procedures such as periodate oxidation, exhaustive methylation, and acid and enzyme hydrolysis could lead to the correct structure.

Possible Structure of the Active Compound

When the crude or purified extract was passed through a column of Sephadex G10 or G25 the active compound was found to be very slowly eluted from the column. This type of behavior is not characteristic of a compound that is separating on the basis of molecular weight alone. The active compound is probably being held in the column by electrostatic interactions with the Sephadex material. This suggests that the active compound is electrolytically charged or is highly polar.

Sephadex G10 excludes molecules that have a molecular weight that is greater than 700. The active compound behaves as if it was not being excluded from the beads of Sephadex G10. Thus it is a reasonable assumption that the active compound has a molecular weight that is under 700.

The stability of the compound toward acid suggests that it is not a common peptide or oligosaccharide. The compound could be a small aminoligosaccharide. This

class of compounds have been shown to be stable toward both heat and acid (10). Aminoligosaccharides are not usually charged molecules but the amino group is polar.

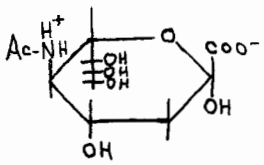


Figure 10. Pyranose form of sialic acid.

Another possible structure is that of sialic acid (Figure 10). This is a low molecular weight (M.W. 307) and charged molecule. On Sephadex G10 I would expect sialic acid to interact with the free carboxyl groups of Sephadex.

Sialic acid is a stable molecule in heat and acid, and it can form polysaccharides with other sialic acid molecules or with other common monosaccharides. It can also act as a prosthetic group in a glycoprotein. It is commonly found in the blood and nervous tissue of nearly all vertebrates and invertebrates.

Red Carotenoprotein

The red coloration is a true carotenoprotein. This was demonstrated by the fact that when acetone was added to the red aqueous extract, there resulted a release of carotenoid and a precipitation of protein.

The maximum visible absorbance was found to be 475 mμ before and after heat treatment. This implies that at least that portion of the protein to which the carotenoid was attached was not denatured during purification.

The red carotenoprotein was found to be free of any contaminating proteins or pigments. This was demonstrated by electrophoretic and chromatographic homogeneity.

More complete characterization of the carotenoprotein would possibly involve determination of the amino acid composition of the protein and a molecular weight determination with and without the carotenoid prosthetic group.

Orange Carotenoid

The orange pigment was found to be a carotenoid by a positive Carr-Price test. The carotenoid was demonstrated to be pure by both TLC and aluminum oxide column chromatography.

The maximum visible absorbance of 500 mμ and the shape of the spectrum suggests that the compound is ether astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene) or canthaxanthin (4,4′-diketo-β-carotene) both are commonly found as prosthetic groups of proteins(11).

The partition coefficient of 50:50 was identical to the value reported for canthaxanthin by Petracek and Zechmeister (9). Also it has been reported (12) that astaxanthin can only be eluted from an aluminum oxide column with an alcohol-acetic acid mixture. In elution of the orange carotenoid from the column addition of acetic acid was never necessary.

To be positive that the orange carotenoid is indeed canthaxanthin a known sample of the carotenoid must be obtained. The two could then be mixed and tested by TLC and column chromatography for homogeneity. The infrared and visible spectra of the known carotenoid could also be compared to the spectra of my carotenoid to aid in positive identification.

Yellow Carotenoid

The yellow pigment was identified as a carotenoid by a positive Carr-Price test. It was demonstrated to be homogeneous by both TLC and aluminum oxide column chromatography.

The yellow carotenoid's visible spectrum showed maxima at 485, 453, and 428 mμ. This is very similar to the values of 481, 456, and 428 mμ reported for lutein (13) (3,3′-dihydroxy-∝-carotene). The infrared spectrum of the yellow carotenoid is also similar to that reported for lutein (14).

The partition coefficient was found to be 16:84 which is more similar to lutein (12:88) than any other reported carotenoid (9).

After treatment with the Carr-Price reagent, the maximum visible absorbance was between 580 and 590 mμ which is in close agreement with the 586 mμ reported for lutein (15).

Again for positive identification a known sample of lutein would have to be obtained. Column chromatography, TLC and a comparison of spectra could then lead to a more positive identification.

SUMMARY

Identification of the compound from the asteroid Dermasterias imbricata that evokes the swimming response in the sea anemone Stomphia coccinea was attempted. Although the compound was not identified, it was postulated to be a low molecular weight highly polar molecule of possible carbohydrate origin.

The red carotenoprotein of Dermasterias was isolated by Sephadex G25 column chromatography. It was found to be electrophoretically pure. The visible absorption spectrum in water was taken.

The carotenoids of Dermasterias were isolated by aluminum oxide column chromatography. They were characterized by their visible absorption spectra before and after treatment with the Carr-Price reagent. The partition coefficient was also used for identification. Infrared spectra were obtained and compared to literature values where possible. The orange protein bound carotenoid was tentatively identified as canthaxanthin. The yellow non protein bound carotenoid was tentatively identified as lutein.

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