A STUDY OF SOME PLANT CAROTENOIDS AND OF CERTAIN COLORLESS FLUORESCING COMPOUNDS OF MARINE ORIGIN

Thesis by
Francis James Petracek

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

California Institute of Technology
Pasadena, California
1951

ACKNOWLEDGMENTS

The author wishes to express his deep appreciation to Professor Zechmeister for his guidance in the problems connected with this research and for the sincere interest he showed throughout the course of this work.

- Abstract-

The sudden color change observed during the ripening of the Pyracantha gibbsi yunnanensis berries was investigated. It was caused by the rapid formation of an anthocyanin, not by the cis→trans isomerization of a polycis carotenoid.

30 gm. of all-trans lycopene were stereoisomerized by refluxing in benzene, and the solutions were chromatographed to separate the very small amounts of polycis lycopenes that should be present according to theoretical considerations. Four chromatographically separable pigments were isolated from the filtrate which would have contained any polycis lycopenes, but none of the four pigments belonged to the stereoisomeric lycopene set.

The carotenoid pigments and fluorescing substances extracted from several marine sources were isolated and investigated by chromatographic and spectroscopic methods. A provitamin D sterol was identified and isolated in an impure form from one of these marine sources, the Thoracophelia mucronata.

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INTRODUCTION

In all of the experimental work reported in this thesis, certain standard techniques, equipment, and reagents were used. In order to avoid a useless repetition of detail, a description is now given which applies to all the work herein, unless specific mention is necessary for a particular case.

Adsorbents - The lime used was Sierra Hydrated Lime, Superfine, United States Lime Products Corp., Los Angeles, California. It was necessary to mix this lime with Celite, # 545, Johns-Mansville Co., in order to obtain a better rate of filtration.

The alumina used was Activated Alumina, Grade F, 80-200 mesh, Aluminum Ore Co., East St. Louis, Ill. The alumina was reground to -200 mesh, and mixed with celite and/or lime.

The calcium carbonate was Merck's reagent calcium carbonate.

Solvents - Petroleum ether was used in most chromatographic experiments. The brand was Skellysolve B, boiling range 60° - 70°. Benzene used for chromatographic work, spectra and crystallization was reagent grade. Acetone and ethanol refer to c.p. acetone and to 95% ethanol. A commercial grade of methanol was used. Hexane used for spectral work was prepared by treating Phillip's commercial grade hexane, or Eastman practical grade hexane repeatedly with fuming sulfuric acid until its optical density was near that of distilled water. Untreated commercial hexane replaced petroleum ether for chromatographic purposes in the latter sections of this work.

Spectral Measurements - Visual Spectra were taken on an Evaluating Grating Spectroscope, Zeiss, light filter BG-7. Quantitative extinction curves and qualitative curves were taken using a Beckman photoelectric spectrophotometer.

General Notes - Evaporations and concentrations were performed *in vacuo* (water aspirator) while a stream of nitrogen or carbon dioxide bubbled through the solution. Sintered glass funnels were used for elutions, and in general, where possible, all-glass apparatus was used for evaporations, washing, etc.

Chromatographic Columns -

Column Size	Inner Diameter (mm.)	Length (mm.)
NO. 2	19	200
NO. 3	38	230
NO. 4	48	270
NO. 5	58	300
NO. 6	80	330

I. AN INVESTIGATION OF THE PIGMENTS OF RIPE AND UNRIPE PYRACANTHA BERRIES

A. INTRODUCTION

The phenomenon of *cis-trans* isomerization of the C_{40}-carotenoids has been studied in detail during the last ten years[1]. Isomerization of the poly*cis* lycopenes by the standard methods of iodine, refluxing, or melting of crystals produces an equilibrium mixture of *trans* and several *cis* forms with an attendant shift in the absorption maxima to a longer wave length[2]. At the same time a deepening of the color is observed.

During the ripening of the *Pyracantha gibbsi yunnanensis* berries a sudden color change (occurring within two days) from light orange to a deep red was noticed. Possible explanations for this change might be (a) the independent formation of a new pigment other than those present in the unripe berries; (b) an *in vivo* isomerization of a *cis* carotenoid; (c) the sudden formation of large amounts of the original pigment. Schroeder[3] has found that when the petals of the *Mimulus longiflorus* flower were allowed to open under varying intensities of light they yielded carotenoids differing both qualitatively and quantitatively from each other. The petals which were allowed to open in diffuse light contained substantially more of the *cis*-forms, prolycopene and pro-γ-carotene.

A qualitative and quantitative determination of the

pigments in the ripe and unripe berries by chromatographic methods as described below has given evidence for the cause of the color change mentioned above.

B. EXPERIMENTAL

Samples of the ripe and unripe berries, each weighing two hundred grams were obtained from the same plant early in January. The ripe berries were bright red, whereas the unripe fruit showed a pale orange color. Both samples were placed under methanol immediately after removal from the plant.

The extraction of each sample was carried out in the following manner: The berries were ground to a fine slurry in a Waring Blendor with 1 l. of methanol. The methanol was filtered from the pulp by means of a large Buchner funnel. The extract of the unripe berries was light yellow, while that obtained from the ripe fruits was deep red. The color remained in the aqueous phase when attempts were made to transfer this pigment to petroleum ether by the addition of water.

The pulp was then extracted three times with 300 ml. of methanol-petroleum ether mixtures (1:1, 1:2, 1:3). The combined extracts were washed methanol-free. The light orange extract obtained from each sample of the fruit was dried with sodium sulfate and concentrated to 100 ml. Each extract was chromatographed on a No. 6 column of 3:1 lime-celite and developed for 30 minutes with petroleum ether.

The chromatograms of the ripe and unripe berry extracts were almost identical in appearance (the figures on the left denote the width of the respective zones, in mm.).

60 undefined, orange, pink, yellow streaks
50 colorless
10 light orange
60 orange: all-trans β-carotene
5 light orange
5 pink } β-carotene stereoisomers
5 light yellow
40 blue fluorescing: phytofluene

The β-carotene zone was eluted with acetone, transferred into petroleum ether and purified further by chromatography. The amount was estimated by means of extinction measurements in the Beckman spectrophotometer. The three pale colored zones appearing immediately below β-carotene on the column were separated into four distinct zones by chromatographing the combined zones on a No. 2 column of 3:1 lime-celite. Extinction curves in hexane solution showed that all four pigments are stereoisomers of β-carotene.* The amount of each pigment was calculated on this basis. The amount of phytofluene was also determined by extinction measurements obtained with the Beckman spectrophotometer(4).

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* Several or all of these pigments may be formed by the spontaneous isomerization of β-carotene in solution.

Table 1.

Comparison of carotenoid pigments in ripe and unripe Pyracantha gibbsi yunnanensis berries.

(figures denote mg. pigment per 100 g. fresh berries)

	Ripe	Unripe
β-carotene	7.3	7.6
β-carotene steroisomers	2.4	1.9
phytofluene	0.2	0.15

As shown in Table 1. the amounts and kinds of carotenoids appearing in the ripe and unripe berries were very nearly identical. Therefore the sudden color change observed during the ripening process was certainly not due to a cis→trans isomerization of a polyene pigment.

Detection of the anthocyanin pigment - A second 200 g. sample of the ripe berries was extracted with methanol. The solution, as in the previous extraction, was deep red. Treatment of this red methanol extract (not obtainable from the unripe berries) with small amounts of potassium hydroxide solution produced an immediate color change from red to a dark green. Upon acidification, this green color reverted to a brilliant red. This acid-base behavior is a typical characteristic of anthocyanin pigments.

C. SUMMARY

The sudden color change observed during the ripening of the Pyracantha gibbsi yunnanensis berries was caused by the rapid formation of an anthocyanin from colorless or weakly colored materials and not by the cis→trans isomerization of a polycis carotenoid.

II. THE FLUORESCING SUBSTANCES AND PIGMENTS OF AN INTERTIDAL OCEAN MUD.*

A. INTRODUCTION

Description of phytofluene - The discovery of phytofluene, a colorless, fluorescing polyene, occurred while some investigations were being carried out with the colored extracts of carotenoid-containing plants(6). During the development of these pigment extracts and illumination of the Tswett column with ultraviolet light, a strong, greenish-gray fluorescing zone of phytofluene appeared on $Ca(OH)_2$ immediately below the zone of α-carotene and of some isomers of α-carotene. The separation of phytofluene from carotenoids is achieved by chromatographing an extract, such as that obtained from tomatoes, repeatedly on columns of $Ca(OH)_2$ and developing with petroleum ether mixed with small amounts of acetone(4).

The analysis and molecular weight determinations of the oily substance(4) gave C and H values corresponding to the formula $C_{40}H_{56}$ (±2H). Phytofluene is light-sensitive and subject to rapid autoxidation. Its spectral curve in hexane shows three sharp maxima at 331, 348, and 367 mu. The specific extinction value in hexane $E^{1\%}_{1\ cm.}$ is about 1200, although this value will possibly have to be corrected in the future since the oily nature of the compound makes it

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* This work was carried out on the Contract NR-059-207 of the Office of Naval Research. A condensed report of this investigation has been published in the Archives of Biochemistry (5).

difficult to obtain absolutely pure samples. Like the carotenoids, phytofluene readily undergoes the phenomenon of cis→trans isomerization.

Evidently the formation of phytofluene is in some way connected with the biosynthesis of the carotenoids, perhaps as a colorless intermediate. Its probable role in plant metabolism is further substantiated by its widespread occurrence in the vegetable kingdom, especially in those plants which are rich in carotenoid pigments(7).

Another colorless, fluorescing polyene, termed phytofluenol, has been found in small amounts in the extracts of tomato paste(8).

Fluorescence of mud extracts - The occurrence of phytofluene in intertidal ocean muds seemed to be probable considering the presence of carotenoids in the mud(9) and the observation that such mud extracts show an intense bluish fluorescence similar to that of phytofluene.

Earlier Investigations of Ocean Mud - The first investigation of sea mud was conducted with samples of material classed as deep-sea mud, estimated to be about 1000 years old(10). The conclusions of this investigation confirmed the presence of carotenoid pigments as reported earlier(9), but the bluish-gray fluorescence was found to be caused by four chromatographically homogeneous compounds showing extinction maxima around 230 mμ in hexane solution. Phytofluene or similar fluorescing polyenes were absent.

Although phytofluene is not present in sea mud 1000 years old, it was of interest to know whether it would be found in a mud of recent origin.

Present Investigation - In the work described below, a chromatographic analysis was carried out with an intertidal mud sample collected from the exposed flats in Mission Bay, San Diego, California. The result was, that like the much older deep-sea material, this fresh mud of recent history failed to yield any detectable amounts of phytofluene.

Conclusions - The bluish-gray fluorescence of the extract was found to be caused by four homogeneous compounds, whose spectral curves are shown in Figure 1. These fluorescing compounds are, however, distinctly different from those occurring in the deep-sea mud(10). Their extinction maxima are located at markedly longer wave lengths than those characteristic for the deep-sea mud.

Figure 2. shows the spectral curves of the carotenoid pigments present in this mud sample. Their aggregate weight was about 0.2 mg./kg. of dry material. The preponderant fraction showed epiphasic behavior, thus agreeing with some data published earlier by Fox et al(5).

B. EXPERIMENTAL

Starting Material - The starting material was a 20 kg. wet sample obtained at low tide from the top three inches of surface mud. This sample was transported under methanol

from its point of collection at Mission Bay to Pasadena. The original water content of the sample was 55%; the organic matter content of the dry material as determined by the chromic acid method averaged 1.85%.*

The sample was dried by allowing it to stand overnight thoroughly mixed with an equal volume of methanol. The liquid was separated from the mud in a basket centrifuge (diameter of basket, 20 cm.), and the green solution was extracted with petroleum ether. This extract showed a dark red fluorescence, indicative of chlorophyll or related compounds.

Extraction Procedure - The dehydrated mud sample was extracted four times with 4 l. of 3:1 petroleum ether-methanol mixtures by shaking for twenty minutes in two 15 l. wide-mouthed jars on a mechanical shaker. The last extract showed hardly any color or fluorescence when illuminated by ultra-violet light. The extracts were combined and washed methanol-free with water in 20 l. separatory funnels. The petroleum ether extract obtained from the initial treatment with methanol and the main extract were now combined and washed thoroughly with water to remove any remaining methanol. The wet petroleum ether extract was then dried over sodium sulfate. Finally the light green extract was concentrated in vacuo at 50° from 20 l. to 1 l.

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* These analyses were carried out by Dr. Denis L. Fox, Scripps Institute of Oceanography of the University of California, La Jolla, California.

Saponification of Extract - The dark green extract was then saponified overnight by allowing it to stand over a layer of 20% methanolic KOH. After saponification the petroleum ether layer was much lighter in color and exhibited an intense greenish fluorescence in ultra-violet light (the red fluorescence now appeared in the lower alkali layer). After decantation the petroleum ether extract was saponified a second time in the same manner as before. After the second treatment the petroleum ether layer was washed thoroughly to remove the methanol and KOH, dried, and concentrated to 150 ml.

Chromatographic Operations - The dark brown solution was chromatographed on four No. 6 columns of 3:1 lime-celite. The chromatograms were developed with a mixture of 3% acetone-petroleum ether for one hour.

In the chromatograms listed below, the figures on the left denote the height of the zones in millimeters, while those on the right (for the first chromatogram only) designate mg. pigment/kg. dry mud. The final resolutions were achieved by repeated rechromatographing on various adsorbents as outlined in the following flow chart scheme:

150 ml. of the purified and concentrated extract on four No. 6 columns of 3:1 lime-celite; developer, 3% acetone-petroleum ether.

4 reddish-brown	(0.0025)	Pigment IA	Section I
2 orange	(0.0025)	Pigment IB	
2 red	(0.01)	Pigments 5 and 6	
3 yellow	(0.005)	Pigment 4	Section II
10 colorless, weakly fluorescing			
7 pale pink	(0.006)	Pigment 3	
15 colorless, strongly fluorescing;		Compound I	
30 colorless, weakly fluorescing			Section III
5 yellow	(0.04)	Pigment 1	
5 fluorescing; Compound II			Section IV
15 orange; β-carotene (0.13)		Pigment 2	
20 pale yellow (overlapping), strongly fluorescing; Compound III			
2 yellow			Section V
15 colorless, strongly fluorescing; Compound IV			

Section I; one No. 3 column of $CaCO_3$ (Merck); developer, petroleum ether.*

5 yellow 10 purple	Section IA
8 strongly fluorescing 2 yellow, fluorescing	Section IB
4 colorless 2 pink 5 colorless 2 yellow 10 weakly fluorescing	(added to Section II)

Section IA; one No. 3 column of $CaCO_3$; developer, 0.5% acetone-petroleum ether.

8 yellow	Pigment IA
6 colorless	
12 purple	Pigment IB

Section IB; one No. 3 column of $CaCO_3$; developer, 20% benzene-petroleum ether.

30 colorless 5 yellow, fluorescing 3 blue fluorescing 4 colorless	(discarded)
12 blue fluorescing	Compound V (no extinction maxima)
15 colorless 9 pale yellow	(discarded)

* All zones were eluted from the adsorbents with acetone, transferred to petroleum ether, washed, and dried with sodium sulfate before rechromatographing. When necessary the solutions were concentrated in order to obtain the most effective development on the columns.

Section II; one No. 5 column of 3:1 lime-celite; developer, 12% acetone-petroleum ether.

15 fluorescing	(discarded)
10 red 7 orange	Section IIA
20 fluorescing 3 pale pink 10 fluorescing 3 yellow 12 fluorescing	(discarded)

Section IIA; one No. 4 column of $CaCO_3$; developer, petroleum ether.

15 light brown 4 colorless	(discarded)
7 purple 3 colorless 10 purple	Section IIA'
15 pale pink 20 colorless 30 fluorescing	(discarded)
5 orange	Pigment 4

Section IIA'; one No. 3 column of $CaCO_3$; developer, petroleum ether.

36 colorless	
12 purple	Pigment 6
10 colorless	
16 purple	Pigment 5
6 pink, weakly fluorescing	(discarded)

Section III; one No. 5 column of 3:1 lime-celite; developer, 2% acetone-petroleum ether.

30 pale pink	Pigment 3
20 pale yellow 10 colorless	(discarded)
30 strongly fluorescing	Section III'

Section III'; one No. 4 column of 3:1:1 alumina-lime-celite; developer, 10% benzene-petroleum ether.

20 fluorescing 18 colorless	(discarded)
55 strongly fluorescing	Section III''

Section III'' was rechromatographed on a similar column to obtain homogeneous Compound II.

Section IV; One No. 6 column of 3:1 lime-celite; developer, 4% acetone-petroleum ether.

8 fluorescing	Section IVF
10 pale yellow	Section IVA
10 fluorescing	Section IVF
15 orange	Section IVB
7 fluorescing	Section IVF

(three separate zones of Section IVF were combined)

Section IVB; one No. 6 column of 3:1 lime-celite; developer, 5% acetone-petroleum ether.

10 colorless	
20 fluorescing	added to Section IVF
10 pale yellow	(discarded)
5 fluorescing	added to Section IVF
30 orange	Pigment 2 (β-carotene)
60 fluorescing	added to Section IVF

Section IVA; one column of 3:1 lime-celite; developer, 5% acetone-petroleum ether.

15 fluorescing	added to Section IVF
18 yellow	Pigment 1
10 fluorescing	added to Section IVF
4 pale yellow	(discarded)
8 fluorescing	added to Section IVF

Section IVF; one No. 3 column of 3:1 lime-celite; developer, 5% acetone-petroleum ether.

30 colorless	
15 fluorescing	Compound I
12 colorless	
35 fluorescing	Compound III

Section V; one No. 4 column of 3:1 lime-celite, developer, petroleum ether.

18 pale yellow (discarded)

15 fluorescing (rechromatographed) Compound IV

8 colorless

15 fluorescing (rechromatographed) (no distinct maxima) Compound VI

Description of Pigments - Only the two pigments isolated from the uppermost part of the initial column (Pigments IA and IB, spectral curves not shown) were hypophasic when partitioned between petroleum ether and 90% methanol-water. The six remaining pigments which were more weakly adsorbed (Figure 2) showed very definite epiphasic behavior. The ratio of epiphasic to hypophasic pigments was approximately 40 to 1.

Weight calculations of the pigments, except for β-carotene, were based on molecular extinction values of known carotenoids whose spectral data were nearest to those of the mud pigments.

Table II

Weights of epiphasic and hypophasic pigments present in 1 kg. of intertidal mud sample (dry).

Pigment	Most closely related carotenoid	mg./kg. dry mud
1	β-carotene	0.04
2	known to be β-carotene	0.13
3	capsanthin	0.005
4	lycopene	0.006
5	lycopene	0.004
6	lycopene	0.006
IA,IB	cryptoxanthin	0.005

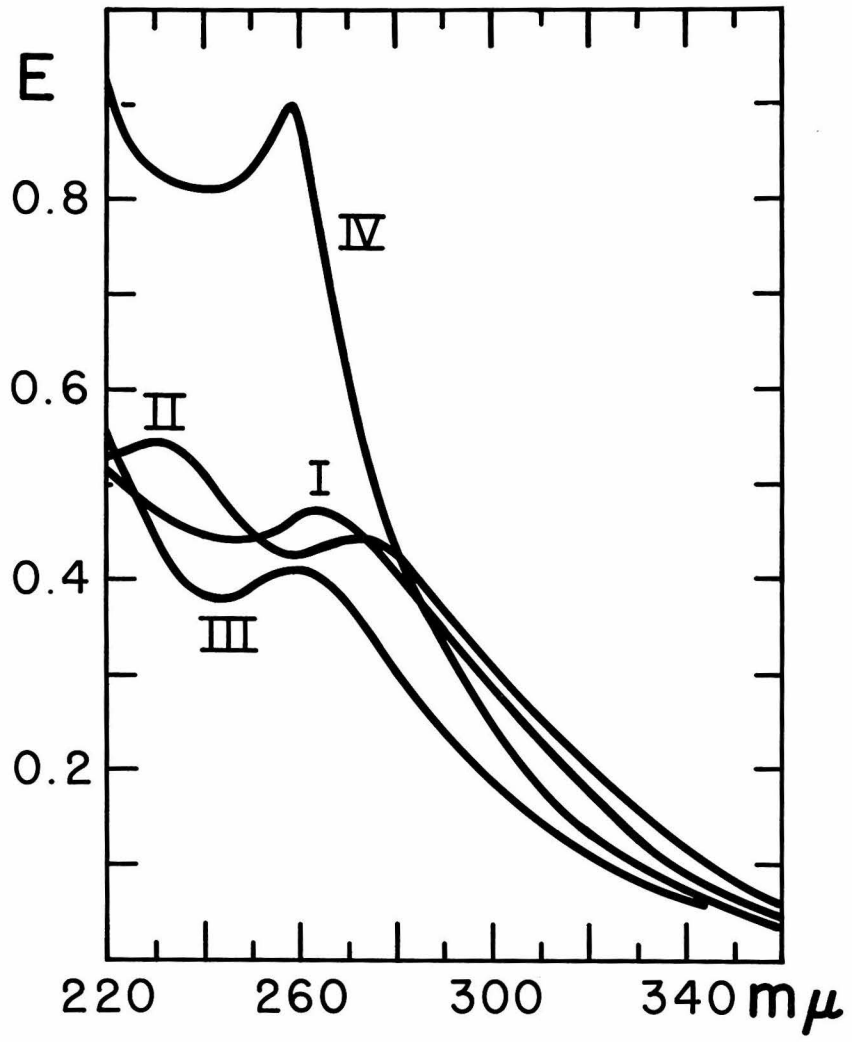

Figure 1. Spectral curves (in hexane) of some fluorescing compounds contained in a fresh, intertidal mud-sample; E designates optical density.

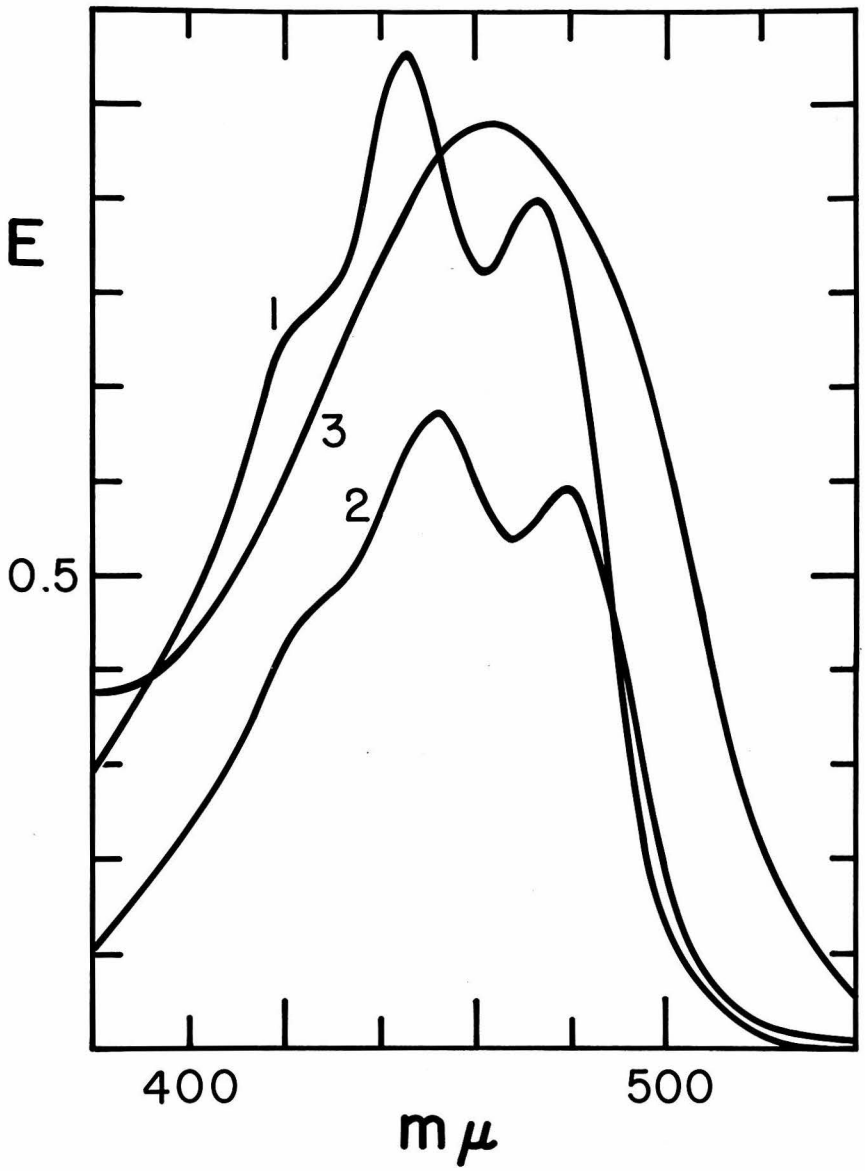

Figure 2. Spectral curves (in hexane) of epiphasic pigments in an intertidal mud sample.

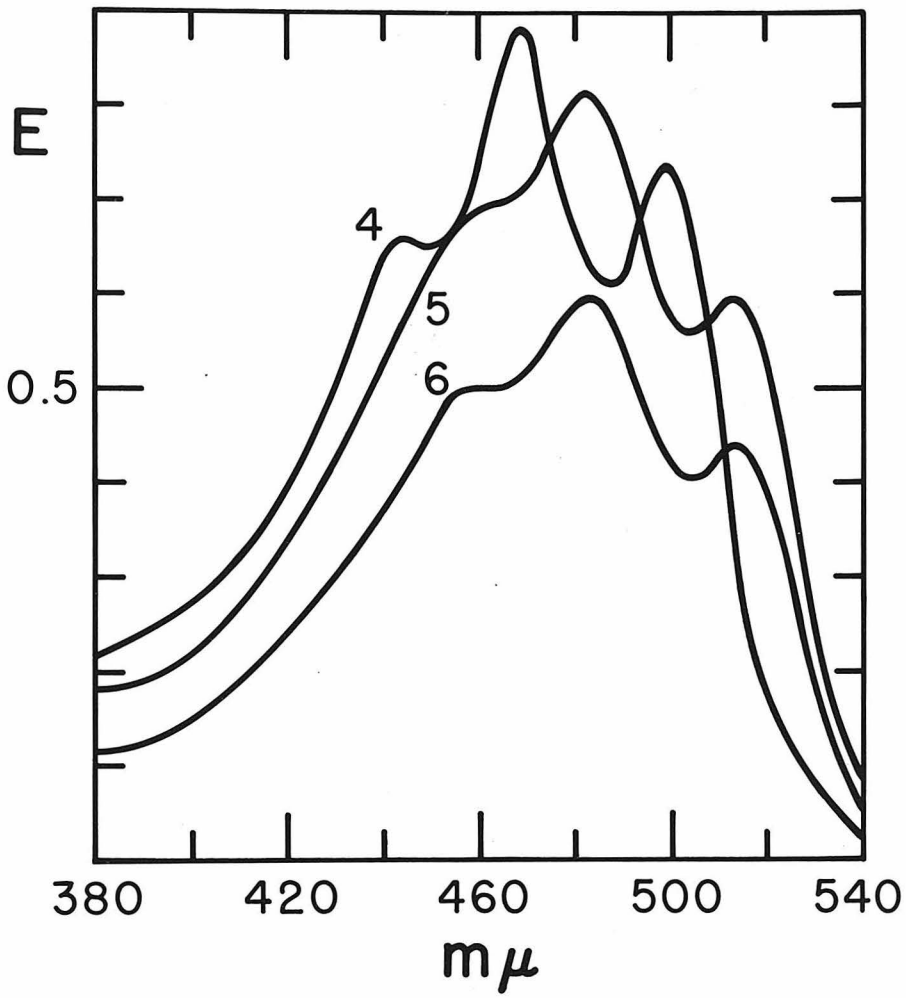

Figure 2. continued --

C. SUMMARY

The observed fluorescence of crude and purified extracts of a fresh, intertidal ocean-mud sample was caused by six colorless, chromatographically separable compounds. Four of these compounds showed extinction maxima around 260 mμ in hexane; two compounds showed no distinct maxima. Eight carotenoid pigments were observed. Six of these showed epiphasic behavior and comprised the preponderant amount of the total weight of all pigments. Phytofluene or a similar fluorescing polyene was absent.

III. ATTEMPTED ISOLATION OF POSSIBLE POLYCIS LYCOPENES FORMED BY THE LARGE SCALE STEREOISOMERIZATION OF ALL-TRANSLYCOPENE.

A. THEORETICAL CONSIDERATIONS

Cis-Trans Stereoisomers of All-trans Lycopene - As is well known, lycopene is a $C_{40}H_{56}$ polyene pigment containing 13 aliphatic double bonds, seven of which are sterically unhindered and available for trans→cis rearrangements. According to Pauling[11], 72 stereoisomeric lycopenes could be formulated by using all possible sterically non-hindered cis - trans configurations. Approximately 12 of these stereoisomers have been observed in the vegetable kingdom, although the all-trans compound is preponderant. When lycopene is subjected in vitro to catalytic isomerization, an equilibrium mixture consisting of only a few detectable members results.

Prolycopene, a Polycis Stereoisomer - If the stereoisomerization described above is a reversible one, as it is theoretically foreseen, then when any stereoisomer is submitted to the treatment by which it was formed, members of the same stereo chemical set should appear. The natural product, prolycopene, which has probably 6 cis bonds, has not been detected so far, after catalytic isomerization, in the final equilibrium mixture. According to the present theory, prolycopene should be present, if only in minute amounts.

Iodine Catalyzed Isomerization - Although stereoisomerization of carotenoids in solution is a spontaneous process, its rate can be increased very substantially by the presence of some iodine, in light.* In this manner optimum amounts of the stereoisomeric members are recovered, and not much of the starting material is lost due to competitive irreversible reactions, such as cleavage and oxidation of the polyenic structure.

Isomerization of Carotenoids by Refluxing - When refluxing in hexane solution at 67° is used for the isomerization of a carotenoid, the composition of the resulting equilibrium mixture is usually different from that obtained by the photosensitized iodine procedure. Not only are the ratios of the steric forms different, but the presence or absence of detectable amounts of certain isomers is not identical in the two methods(12). Iodine catalyzed isomerization is the most desirable method to use, since the results are more reproducible, and the reversibility of the reaction is more perfectly maintained.

Scale of Previous Experiments - Using the iodine catalysis method and three other methods -- acid, refluxing, melting of crystals -- the isomerization of all-*trans*-lycopene has been studied mainly on a 1-25 milligram scale(12). In all cases only a few neo-forms, termed neo-A and B, were

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* The method used for illumination of solutions is a 40 watt Mazda fluorescent lamp (length of tubes, 120 cm.) placed 60 cm. from a dilute solution (in pyrex glass) for a period of 20 minutes.

detected in the resulting mixture besides the unchanged all-trans lycopene.

Limitations of Previous Experiments - It seemed possible that the lycopene stereoisomers containing several cis-bonds are formed in amounts not detectable in the small scale experiments carried out previously[11]. The ratio of mono-cis/all-trans is calculated to be of the order of 0.1. For a cis-lycopene with six cis-bonds (prolycopene) the ratio would be 0.000,001, assuming that the free energy change accompanying the trans-cis isomerization of each double bond is the same.

Thus, the isomerization of 30 gm. of all-trans-lycopene should, from theoretical considerations, produce only 30 ug. of prolycopene. Even this small amount, however, brings the experiment within the range of detection when chromatographic and spectroscopic techniques are employed, and therefore, such an experiment was undertaken.

Outline of Experimental Procedure - The use of iodine for isomerizing lycopene in the necessarily concentrated solutions had to be abandoned. The light effect in promoting the reaction was practically eliminated by such concentrations and the consequent opaqueness of the solution. In the preliminary experiments it was found that long periods of time (over one hour) were necessary to attain the desirable equilibrium ratio (All-trans: neo-forms = 60:40). In such experiments the side reactions of cleavage and oxidation reduced the percentage recovery of stereoisomeric members to an impractical point (Table III). Strain[13] employed

dimethylaniline, pyridine, and other organic bases to eliminate, or at least reduce, these side reactions. However, this method was not found to be effective in the present work.

Refluxing the benzene solutions of lycopene in an atmosphere of nitrogen produced a desirable equilibrium mixture and a minimum of destruction (Table IV). Because of the large amounts of lycopene to be used, petroleum ether was not a satisfactory solvent. Procedures were worked out for benzene since this solvent dissolves almost 15 times as much lycopene (at room temperature) as does petroleum ether.

B. LARGE SCALE PREPARATION OF LYCOPENE

1. Introduction

30 gm. of chromatographically pure lycopene was desired as starting material for the stereoisomerization experiments. Isolation of lycopene approaching this magnitude had previously been carried out only by Willstätter and Escher[14], who obtained 11 gm. of once crystallized lycopene from 75 kg. of Italian tomato paste. They employed carbon disulfide as the extracting solvent. Since carbon disulfide was undesirable for our purposes, it was necessary to devise extraction and crystallization procedures for large scale operations by following a standard method using CCl_4 and benzene and introducing expedient variations.

Although lycopene is present in a wide variety of plant and animal organisms, ripe tomatoes are the most readily

available source. Fresh tomatoes, because of their high water content, are not as satisfactory as the commercial concentrated paste or puree. The canning process eliminates most of the water content without destroying the lycopene.

In our procedures the tomato paste was dehydrated with methanol and extracted with methanol-carbon tetrachloride. The crude product was then crystallized twice from benzene by the addition of methanol, which yielded a lycopene preparation of 98-99% purity. Finally, chromatographing on calcium hydroxide gave analytically pure lycopene.

Noteworthy in the following scheme is the considerable increase in yield (about 50%) obtained by isolating a second crop of crystals from the mother liquor of the initial crystallization. This mother liquor had previously been discarded.

2. Experimental

Extraction of Tomato Paste - The starting material was 180 kg. of Campania brand canned tomato paste, which is a blend of "Pearson" and "San Marzano" tomatoes packed by the West Coast Packing Corporation. 18 kg. of paste were extracted daily in the following manner: Six 3 kg. portions of paste were transferred into six 15 l. packer jars and each portion was then mixed thoroughly with an electric stirrer with 4 l. of methanol. The suspension was allowed to stand overnight at room temperature. The methanol was removed from the pulp the following morning by filtration in a

basket centrifuge (diameter of basket, 12 inches). Invariably the filter became clogged because of the gummy character of the material; however, by carefully adding small amounts of the methanol-paste suspension to the filter-clogged basket, the methanol was continually forced over the upper lip of the basket while the paste formed a solid pack on the inside. A recycling of the methanol filtrate was usually necessary to recover small amounts of pulp that had been carried over with the liquid in the first operation. The methanol was then discarded.

The residue of each original 3 kg. portion was a bright red pulp, still moist with methanol, which was returned to the packer jars and extracted by shaking with a mixture of 2 l. of methanol and 2 l. of CCl_4. The suspension was shaken mechanically for 30 minutes and centrifuged again in the basket; this was a much easier operation than before, since by the first treatment with methanol the pulp had lost most of its gummy character. The practically colorless pulp was discarded.

Treatment of the Extract - The combined methanol-carbon tetrachloride extracts (two phases) obtained from six 3 kg. portions (20 l.) were transferred into 2-15 l. separatory funnels. The dark red CCl_4 solution was drawn off and washed in 2 l.-portions by inserting a glass tube to the bottom of a 4 l. flask through which a stream of water was introduced. The washing was continued in this manner for one hour.

(The flasks were hung over a sink to facilitate disposal of the water.) The methanol phase was discarded.

After drying with sodium sulfate 10 l. of the above extract were concentrated and finally evaporated to dryness in a three-necked 5 l.-flask at 55°, in vacuo, under an atmosphere of nitrogen. The pasty dark red residue of crude lycopene was dissolved in the smallest possible amount of benzene, and again the solution was evaporated completely to dryness. The removal of the carbon tetrachloride was then considered to be complete. The material was then dissolved in benzene; a total of 3750 ml. of this solvent was required to dissolve the crude lycopene obtained from 180 kg. paste.

2. Crystallization of the Lycopene

The benzene solution of lycopene just mentioned was divided into five 750-ml. portions. Each portion, in a 1500 ml. Erlenmeyer flask, was warmed to 45°, whereupon 500 ml. of boiling absolute methanol was slowly added with constant stirring within 10-15 minutes. Crystallization of the pigment took place rapidly.

After standing at room temperature for two hours the suspension of bright red crystals was kept at 4° for two days and then filtered with suction through coarse sintered glass funnels (diameter, 10 cm.). This pasty mass of lycopene was removed from the funnels, redissolved in 4 l. of benzene, and recrystallized as before.

The dark red mother liquor, after standing for two days at 4°, yielded two grams of light orange crystals. Examination of the crystals under the microscope showed them to be rhombic plates (possibly α- and β-carotene) rather than clusters of long red needles, which is the crystal form of lycopene.

The lycopene from the second crystallization was washed in 5 g. fractions three times with 150 ml. portions of boiling methanol, and then dried in small vacuum desiccators. After three hours the weight of the crystals remained about constant. Yield, 35 gm. The sample was sealed under nitrogen in three 250 ml. round-bottomed flasks and stored at 4° until it was used.

The mother liquors from both crystallizations and the methanol washings were combined and concentrated to a volume of 1 l. During standing for one month at 4° 19 gm. of crystals had appeared. These were washed with cold hexane and recrystallized in the manner described for lycopene above. They consisted almost entirely of lycopene. A 60 mg. sample of the crystals was purified by chromatography and recrystallized. The spectrum, mixed chromatograms with an authentic lycopene sample, and analysis (calculated; C, 89.48; H, 10.52; found: 3, 89.36%; H, 10.62%) proved the identity of this fraction with lycopene. Less than 1% β-carotene was present as an impurity.

C. EXPERIMENTAL

1. Preliminary Small Scale Experiments

Molecular Extinction of Lycopene in Benzene - It was necessary to obtain molecular extinction data for lycopene in benzene (not reported in the literature) in order to determine the concentration of lycopene solutions during the experiments. The lycopene used for these measurements was a small sample of the large preparation which was chromatographed once on 2:1 lime-celite and crystallized twice more from benzene-methanol to insure the highest degree of purity. After the second crystallization the sample was dried for two hours in an evacuated Abderhalden apparatus at 50°. Following this, three separate samples were weighed (2-3 mg. in each sample weighed on the microbalance). Immediately after each weighing the lycopene was dissolved completely in benzene at room temperature and quantitative spectral curves were taken using the Beckman photoelectric spectrophotometer.

Isomerization with Iodine and Refluxing - Two 25-ml. portions of each of the above solutions were isomerized; one, by refluxing the benzene solution for 30 minutes at 80° in an atmosphere of nitrogen, and the other, by the iodine catalysis method. Quantitative curves were taken for each of the equilibrium mixtures, and average readings of the three separate samples were used to plot the final quantitative spectral curves of the fresh solution, after iodine catalysis, and after refluxing (Figure 3).

The three main maxima of the fresh solution were found at 458, 486, and 520 mu. They were shifted approximately 15 mu toward longer wave lengths as compared with lycopene in hexane. The average $E_{1\ cm.}^{mol.}$ at 486 mu of the three samples was 16.0×10^4, the single results being 15.9, 15.8 and 16.2×10^4.

Solubility of Lycopene in Benzene - Solubility measurements of lycopene in benzene were necessary to determine the highest possible concentration that could be used for the experiment. To determine the solubility, 250 mg. of lycopene were allowed to stand overnight with 25 ml. of benzene at 24°. A 10-ml. aliquot was filtered from the two phase mixture, and its concentration was measured with the Beckman spectrophotometer. The solubility was 1.6 g. of lycopene per liter of benzene.

During the large scale experiment a sufficient margin of safety against accidental crystallizations was assured by setting the concentration of the solutions at 1 g. per 1. benzene.

Comparison of Several Adsorbents - Three adsorbent mixtures were tested to determine their relative efficiency in separating lycopene from prolycopene using benzene as the developer. It was necessary for the large scale experiment that any prolycopene formed by isomerization should be washed through the columns leaving lycopene and its main stereoisomers well attached. In this manner any prolycopene

could be accumulated in the filtrate during chromatographic operations, and the bulk of unchanged all-*trans*-lycopene and neolycopene-A could be recovered from the adsorbent for use in further isomerizations.

The three adsorbents examined were 2:1 lime-celite, 3:1:1 alumina-lime-celite, and 4:1 alumina-celite. Benzene solutions of lycopene-prolycopene mixtures (10:1) were placed on No. 2 columns of the three adsorbents and developed with benzene. The most effective separation of the lycopene from the prolycopene was observed on the column of 2:1 lime-celite, which was used as the adsorbent in all subsequent experiments.

Comparison of Iodine Catalysis and Refluxing - To determine the better method of isomerization, a series of small scale experiments were carried out by using iodine catalysis and the refluxing of benzene solutions at 80° under nitrogen. For these experiments 1.5 gm. of analytically pure lycopene was prepared by chromatographing and recrystallizing a 2 gm. sample of the large scale preparation.

The extent of isomerization and precentage recovery were followed by chromatographing several 2-ml. aliquot fractions of the solutions on small columns of 2:1 lime-celite. After eluting from the columns and transferring to benzene, the amounts of *trans*- and neo-forms were estimated with the Beckman spectrophotometer. During the iodine catalyzed isomerizations the solutions were kept in well-filled, sealed volumetric flasks to reduce oxidation.

Results of the two separate methods of isomerization are shown below in Tables III and IV.

TABLE III

Results of Some Steroisomerization Experiments Using Lycopene and Iodine, in Light.

mg. lycopene per ml. benzene	I_2, % of pigment weight	Distance in cm. from light source	Time of illumination, in minutes	Ratio of trans-neoforms	% recovery
250/250	2	60	5	---	---
---	---	60	10	90:10	---
---	---	30	30	80:20	---
---	---	30	90	60:40	50*
125/230	2	30	60	70:30	60
100/100	1**	30	30	50:45	76

* Considerable yellow coloration in the filtrate indicated a substantial proportion of cleavage reactions which produce shorter chain chromophores. Visual spectroscopic measurements in the Zeiss grating spectroscope of the yellow filtrate in benzene showed blurred maxima at 467 and 502 mμ. No noticeable shift occurred after the addition of I_2. Thus the pigments are not members of the stereoisomeric lycopene set.

** 2 ml. of dimethylaniline was added with the iodine to the solution[13].

TABLE IV

Results of Experiments Using the Refluxing of Benzene Solutions at 80° in Nitrogen to Isomerize Lycopene.

Composition of solutions	Period of refluxing, in minutes*	Ratio of all-*trans*: neo-forms	% recovery
15 mg. in 15 ml.	35	60:40	83
1 g. in 1 l.	25	59:41	85
2.5 g. in 2.5 l.	25	61:39	83

Method Chosen for Large Scale Experiment - A comparison of the percent recovery figures obtained using the two methods of isomerization showed that the refluxing method gave a better recovery of material than the iodine catalyzed isomerizations. Also the ratio of all-*trans*: neo-forms was within the acceptable range for a good equilibrium. In order to conserve the supply of lycopene, recovery of the unchanged all-*trans* form was necessary; neolycopene-A was also recovered and recatalyzed to give another source of lycopene.

Sensitivity of Method - The sensitivity of the experimental methods depended on the efficiency in detecting even minute amounts of any poly*cis* lycopenes that might be formed during the stereoisomerization process. The following experiment showed that the overall sensitivity of the experimental methods was roughly 1:3,000,000: 10 µg. of prolycopene

* These figures do not include the seven minutes initial heating period used in the large scale experiments.

in 250 ml. of benzene were placed on a conical percolator (50 x 24 cm) containing 2:1 lime-celite, and the column was developed with benzene. The first liter of the chromatographic filtrate was collected and carefully concentrated to give 10 ml. of a yellow solution. The spectrum in benzene, observed by the Zeiss grating spectroscope, showed maxima at 485 mμ and 455 mμ, which are the two main maxima of pro-lycopene and indicated that the original material had been recovered. Using the Beckman spectrophotometer, the amount of prolycopene recovered was estimated at 6 μg. of the 10 ug. placed on the column.

2. Large Scale Experiment

Conical percolators (50 x 24 cm.) were used as the chromatographic columns. 15 percolators were required to chromatograph the isomerization mixtures of the 30 gm. of lycopene. 4 gm. of the crude lycopene preparation were purified prior to the refluxing operation by dissolving the lycopene in 4 l. of benzene and chromatographing on two percolators of 2:1 lime-celite, developing for one hour with benzene.* A brown zone, 10 mm. wide, remained on the uppermost part of the percolators. The lycopene was observed as a broad red zone (30 cm.) in the center of the percolators. After eluting the lycopene with acetone containing 5% methanol and transferring to benzene, the solutions of lycopene were

*The use of large amounts of benzene in the open percolators demanded special precautions because of its toxic effects. The laboratory was kept well ventilated by using a large electric blower.

adjusted to the refluxing concentration of 1 gm. per liter.

Refluxing of Solutions - 2 gm. of lycopene in 2 l. of benzene were refluxed in a 5 l. flask, equipped with two reflux condensers and a glass tube inlet to flush the system with nitrogen prior to the introduction of the solution. The flask was set in an electrically heated water bath maintained at 90°. After 2 l. of solution were introduced, 7 minutes were needed to bring the mixture to the refluxing temperature of 80°. The refluxing was then carried out for 25 minutes in diffuse light.

Chromatographing the Isomerization Mixture - After refluxing, the apparatus was quickly taken apart and the flask was cooled in an ice bath to room temperature. Immediately the two liters of solution were poured on one percolator and developed for one hour or until the orange-brown zone of neolycopene-A approached the bottom of the percolator. The following sequence of zones was observed (the widths of the zones in cm. are an approximate average for the 15 percolators):

10 brown, oxidation products
5 colorless
20 red, all-*trans* lycopene
10 orange-brown, neolycopene-A
5 brown, neolycopene-B

Filtrate, yellow, weakly fluorescing (possible poly*cis* lycopenes.)

Recovery of All-Trans Lycopene - Complete separation of the all-*trans* lycopene from the neolycopene-A could not be achieved. This partial overlapping of zones was not important in the recovery of all-*trans* lycopene, since small

amounts of neolycopene-A should not change the equilibrium ratios of the subsequent experiments in which this partially impure all-*trans* lycopene is used. The neolycopene-A zone was eluted from all chromatograms and stored in benzene at 4° until it was reisomerized by refluxing. The all-*trans* lycopene (5 gm.) was recovered from this isomerization by chromatographing the 5-l. of solution on two percolators of 2:1 lime-celite.

Investigation of the Filtrate - The filtrate obtained from the development of each of the 15 percolators was light yellow and showed a weak blue fluorescence under ultraviolet light. During the experiment the yellow filtrate from each chromatogram was stored in air-tight, CO_2-flushed pyrex bottles. After isomerizing 30 gm. of lycopene and chromatographing the mixtures, 30 l. of the pale yellow filtrate remained. It was concentrated *in vacuo* at 45° to 125 ml. of a red solution with a strong terpene-like odor. This red solution was first chromatographed on a No. 6 column of 2:1 lime-celite and developed with benzene. After 40 minutes the following sequence of zones was observed:

20 colorless	
35, pink, all-*trans* lycopene	
30, brown, neolycopene-A	(discarded)
15 greenish-yellow	
8 light orange	(fraction possibly containing polycis lycopenes)
6 yellow	
4 fluorescing	

The four lower zones were eluted together, transferred to hexane, and evaporated to dryness. The viscous red oil which remained was redissolved in hexane and placed on a No. 5 column of alumina. The column was washed with several liters of hexane; the pigment zone which remained strongly absorbed on the upper part of the column was eluted, and transferred to 100 ml. of hexane. The oily character of the first solution was removed by this treatment.

This 100 ml. of the solution was now placed on a No. 5 column of 2:1 lime-celite, and developed with hexane containing 2% acetone. Four distinct zones were obtained which were separately cut from the column, eluted and transferred to hexane. Each of the four pigments separated sharply from an authentic sample of prolycopene when mixed chromatograms were carried out on 3:1:1 alumina-lime-celite, using hexane containing 3% acetone as the developer. Spectroscopic observations were made on each of the four pigments in the Zeiss visual spectroscope. After each reading, iodine was added to determine the shift of the bands. No shifts typical for poly<u>cis</u> lycopenes took place.

Finally, 0.01 mg. of prolycopene and then iodine were added to each solution. In all four cases the prolycopene could be identified by the spectral shift. Thus it was shown conclusively that none of the four pigments mentioned belonged to the stereoisomeric lycopene set.

TABLE V

Spectroscopic Data of Pigments Isolated From the Chromatographic Filtrate of Isomerization Mixtures.

Pigment (No. indicated position on column)	Main maxima in mμ.*	Effect after I_2 addition	Test for polycis by adding prolycopene
1	492	no change	positive
2	491.5 458.5	maxima shift to shorter wave length	positive
3	483.5 452.5	maxima shift 486.5	positive
4	no definite spectrum	no change	positive

* All bands were blurred.

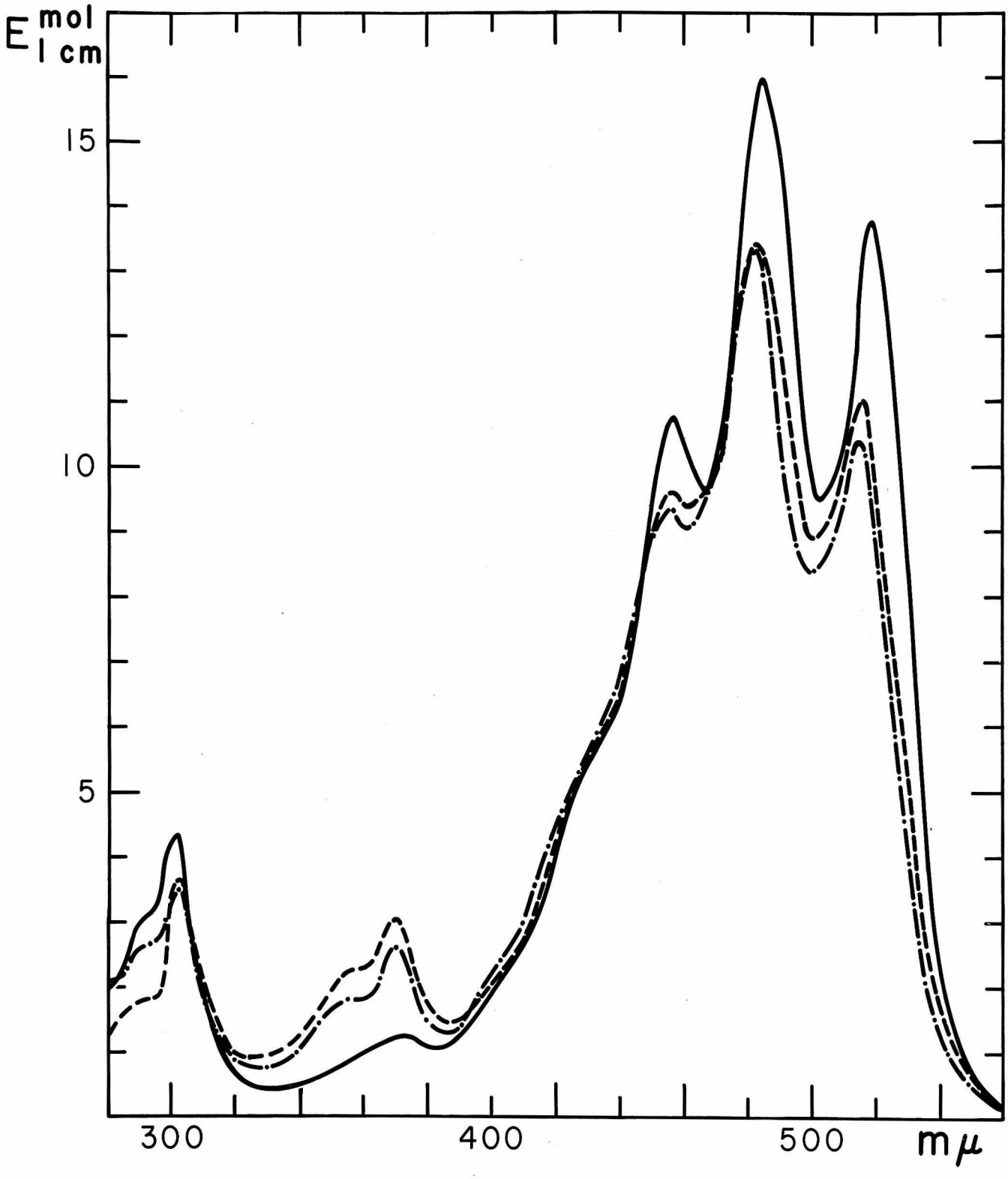

Figure 3. Molecular extinction curves of lycopene and its stereoisomerization mixtures in benzene. Lycopene in fresh solution _____. After 30 minutes refluxing_.-.-. I_2 catalyzed isomerization_____.

D. SUMMARY

30 gm. of all-*trans* lycopene was prepared by extracting 180 kg. of tomato paste, crystallizing from benzene-methanol mixtures, and purifying further by chromatography.

The 30 gm. sample was isomerized in 2 gm. fractions by refluxing in benzene, and then the solutions were chromatographed on lime-celite to separate any poly*cis* lycopenes that may have been formed.

Four chromatographically separable pigments were isolated from the filtrate which would have contained any poly*cis* lycopenes, but mixed chromatograms and spectroscopic observations showed that none of the four pigments belonged to the stereoisomeric lycopene set.

IV THE INVESTIGATION OF SOME FLUORESCING SUBSTANCES, PIGMENTS, AND A STEROL FOUND IN THE MARINE ANNELID WORM, THORACOPHELIA MUCRONATA.*

1. INTRODUCTION

Fox et alia[14] have conducted a detailed biochemical study concerning the pigments assimilated by the marine annelid, Thoracophelia mucronata. This marine animal is commonly known as the blood worm because of the large amounts of colloidal haemoglobin it contains. The animals live in colonies a fraction of an inch to a foot below the surface of the intertidal sand. Fox found that xanthophyllic pigments were almost entirely excluded in preference to the epiphasic carotenoids, especially β-carotene. By contrast, the organic matter of the sand constantly ingested by the worms contains preponderant amounts of the xanthophyllic or hypophasic pigments. Five blue fluorescing, chromatographically separable fractions were separated. These fractions showed sharp maxima from 300 mμ to 310 mμ in hexane solution. In their method[14] the worms were extracted with acetone, and the pigments and fluorescing fractions were separated by chromatographing on magnesia.

The work described below was carried out mainly to reinvestigate the fluorescing substances in the Thoracophelia mucronata, especially since the spectral readings of the previous investigation did not extend below 300 mμ. In this

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* This work was carried out on the Contract NR-059-207 of the Office of Naval Research.

laboratory, polycyclic aromatic compounds showing absorption in the region, 230 mμ to 385 mμ, have been isolated from a marine animal, Tetraclita squamosa rubescens[15]. It was therefore of interest to determine whether or not the fluorescence shown by extracts of the worm was caused by compounds of the same type.

No investigation of the sterols in the Thoracophelia mucronata has been previously reported, although the presence of cholesterol-like substances in other marine animals is well known[16].

2. EXPERIMENTAL

Starting Material - 10 kg. of worms were collected by Dr. Denis L. Fox and his group at the Scripp's Institute of Oceanography, La Jolla, California. The worms were transported to this laboratory packed in methanol. The methanol, used as a preservative en route, was colored bright red by the colloidal haemoglobin. This methanol was drained off and discarded.

Extraction - After draining the original methanol from the worms, the sample was ground to a fine pulp with fresh methanol in Waring blendors. The pulp was separated from the methanol in a basket centrifuge (diameter, 12 inches), and then extracted two times with mixtures of 2:1 hexane-methanol. The methanol and hexane-methanol extracts were combined and transferred to hexane by the addition of water. The hexane solution was then washed free of methanol and dried with sodium sulfate. The hexane solution (8 l.) showed a strong blue fluorescence. It was concentrated

in vacuo to 1 l. and then saponified by keeping it over a layer of 20%-methanolic KOH overnight. The upper layer was decanted from the alkali phase, washed free of alkali and methanol, and dried.

Removal of an Amorphous Solid - The light brown, blue fluorescing hexane solution was further concentrated to 300 ml., and the addition of 200 ml. of acetone to this solution immediately precipitated a white solid. The amorphous solid was separated from the brown extract by centrifuging and washed with small portions of acetone, the washings being added to the main extract. The total weight of the non-fluorescing substance amounted to about 2 gm. It was redissolved in 200 ml. of warm hexane and precipitated with 200 ml. of absolute ethanol. The precipitate which showed no crystal form was partially identified as a salt with weakly acidic properties.

Chromatogram of Extract - The main extract from above was then washed free of acetone, dried and developed with hexane on two No. 6 columns of 2:1 lime-celite. After one hour the following sequence of zones was observed (the figures on the left denote the width of the zones, in mm.):

20	blue fluorescing, several narrow pigment zones	Section I
40	colorless	
10	blue fluorescing	
5	orange, β-carotene	Section II
10	blue fluorescing	
40	colorless	
2	blue fluorescing, sharply defined	Section III

Isolation of Fluorescing Oil - The narrow, strongly fluorescing zone from Section III was eluted, transferred to hexane, and rechromatographed on a No. 5 column of 2:1 lime-celite. Only one sharp zone appeared which migrated rapidly when washed with hexane. It was eluted, transferred to hexane, and washed free of acetone. The hexane solution was evaporated to dryness in vacuo at 50° to 3 ml. of a yellow, strongly blue fluorescing oil, which had a strong terpene-like odor and solidified at approximately 5°. Attempts to crystallize the oil from hexane by the addition of ethyl acetate, methanol, acetone or dioxane were not successful. The oily material showed a sharp maximum at 228 mμ in hexane solution (Fig. 4). Analysis of the oil after drying for two hours at 50° in an Abderhalden apparatus gave, C = 82.42%, H = 12.94%. The molecular weight was 370 (in exaltone).

Section II - By rechromatographing Section II on 2:1 lime-celite and developing with hexane, two fluorescing zones were separated from the main orange zone. The orange pigment was shown to be β-carotene (maxima at 486 and 454 mμ). The spectral curves in hexane of both the fluorescing zones and β-carotene are shown in Fig. 4.

Section I, Isolation of Sterol - Section I was eluted from the lime with acetone, transferred to 1 l. of hexane, which was washed free of acetone and dried. The pale yellow, green fluorescing extract was concentrated to 100 ml. On cooling to 0° a white, crystalline substance appeared. The

crystals were centrifuged, washed thoroughly with hexane at 0°, and dried at room temperature. (The washings were added again to the main extract).

Chromatographic Separation of Substances in Section I - The extract, now freed of most of the sterols, was concentrated to 200 ml., and developed on a No. 6 column of 2:1 lime-celite with hexane containing 4% acetone. The following sequence of zones was observed:

10 colorless
2 orange
20 pale pink
60 weak blue fluorescing
30 several strong blue fluorescing zones
5 colorless
10 green fluorescing, with a narrow orange zone
4 colorless
10 blue fluorescing, with a narrow purple zone
40 four weakly fluorescing zones

By repeated rechromatographing, three pigment zones and five additional fluorescing zones were distinctly separated. Only those compounds which showed distinct maxima in hexane are shown in Fig. 4. The three pigment zones were completely epiphasic, and from spectral considerations they appeared to be stereoisomers of β-carotene.

Sterol From Section I - The white substance which precipitated upon concentration of the hexane eluate of Section I was recrystallized by dissolving it in warm hexane and allowing the solution to stand at 4° for three days. Microscopic examination showed long white needles. A second crystallization of the 300 mg. of material was performed by dissolving

it in the smallest possible amount of ethanol and precipitating by the slow addition of water (15% by volume). After drying for two hours at 50° in an Abderhalden 250 mg. of a white solid remained, m.p. 140°.

Preliminary Identification - The white solid had a strongly positive Liebermann reaction, changing the sulfuric acid-acetic anhydride mixture from red to deep blue. The spectral curve of the sterol containing sample in hexane showed sharp maxima at 271, 293, 281, and (263) mμ. This was clear evidence for the presence of one or both of the provitamins D, ergosterol or 7-dehydrocholesterol. Both of these provitamins D have almost identical spectra, due to the same ethylenic structure in Ring B. A commercial sample of ergosterol (Eastman) in hexane showed qualitatively the same spectral curve as that of the white solid (Fig. 5). However, the product was far from being pure since its molecular extinction amounted to only 15% of the values reported for the pure provitamins[17].

Identification of 7-dehydrocholesterol - Lamb and coworkers[18] have reported a color reaction which is able to distinguish ergosterol from 7-dehydrocholesterol even when large amounts of impurities, such as cholesterol, are present. These two closely related provitamins D show very different spectral curves in solutions of chloroform containin 18% antimony trichloride and 3% acetyl chloride. The commercial ergosterol solution showed a very sharp maximum

at 291 mμ. This peak reached a maximum height in from 5 to 10 minutes (Fig. 6). By contrast, 7-dehydrocholesterol gives a maximum at 223 mμ[18]*.

Procedure for Color Reaction - 25 mg. of the crystalline sterol were dissolved in 5 ml. of chloroform. Six drops of this solution were mixed with 5 ml. of the antimony trichloride reagent in the corex cell which was used for photoelectric measurements. The antimony trichloride-acetyl chloride reagent was also used as a blank for all readings on the Beckman spectrophotometer. A stopwatch was used in timing the reaction. In four minutes after adding the sample to the reagent a yellow color had developed. The peak at 223 mμ reached its maximum value after 12 minutes (Fig. 6). Some other maxima located at 380 and 420 mμ were very probably caused by the presence of cholesterol[18].

This test indicated that the crystalline sterol material contained the provitamin D, 7-dehydrocholesterol, rather than ergosterol, and the molecular extinction measurements showed that only about 15% of the material was the provitamin. No further attempts were made to purify the substance because of the small amounts of material remaining.

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* An authentic sample of 7-dehydrocholesterol was not available for purposes of comparison.

3. SUMMARY

The fluorescing substances of the Thoracophelia mucronata extracted with hexane-methanol solutions and separated by chromatography on 2:1 lime-celite showed maxima in the region around 220 mμ, or about 100 mμ lower than the maxima of the fluorescing compounds isolated by other authors(14).

7-dehydrocholesterol was isolated in an impure form and identified by a color reaction with antimony trichloride-acetyl chloride reagent.

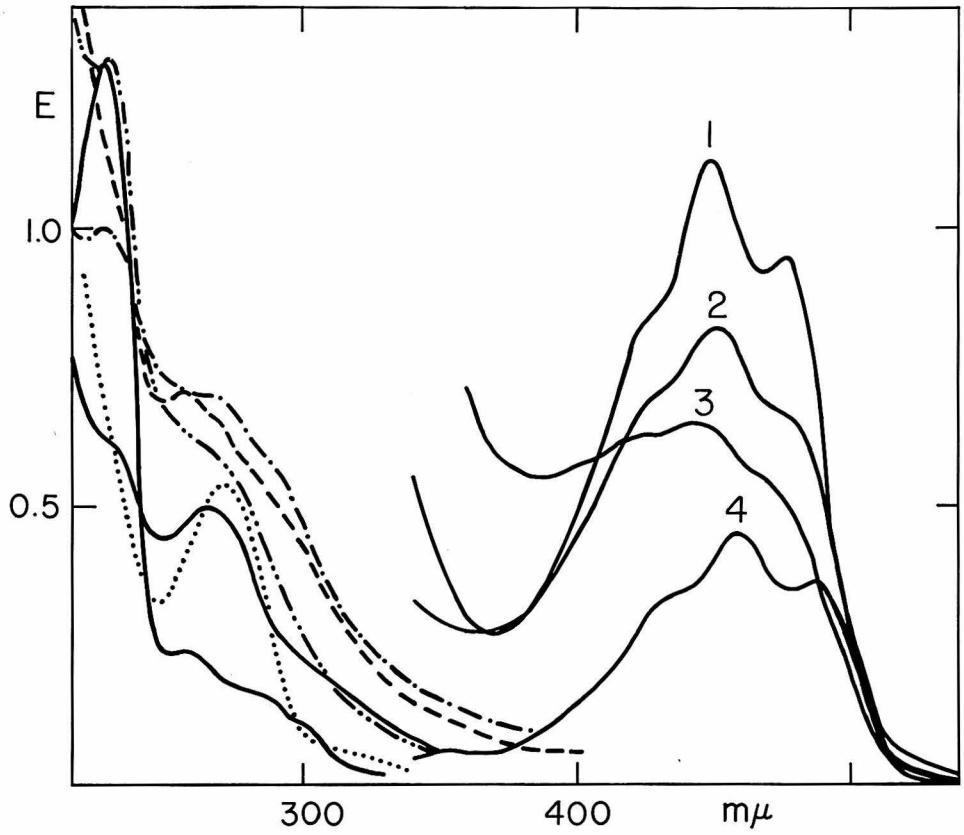

Figure 4. Spectral curves in hexane of some fluorescing compounds and pigments contained in a sample of the marine annelid, Thoracophelia mucronata. Pigments, on the right, are numbered. Fluorescing fractions are shown on the left.

Pigment 1, β-carotene separated from Section II of original chromatogram. Pigments 2,3 and 4 were separated from Section I.

Fluorescing oil from Section III _____ (with maxima at 228 mu). Two fluorescing fractions from Section II ..... and _._._._._._. All other fluorescing fractions were separated from Section I.

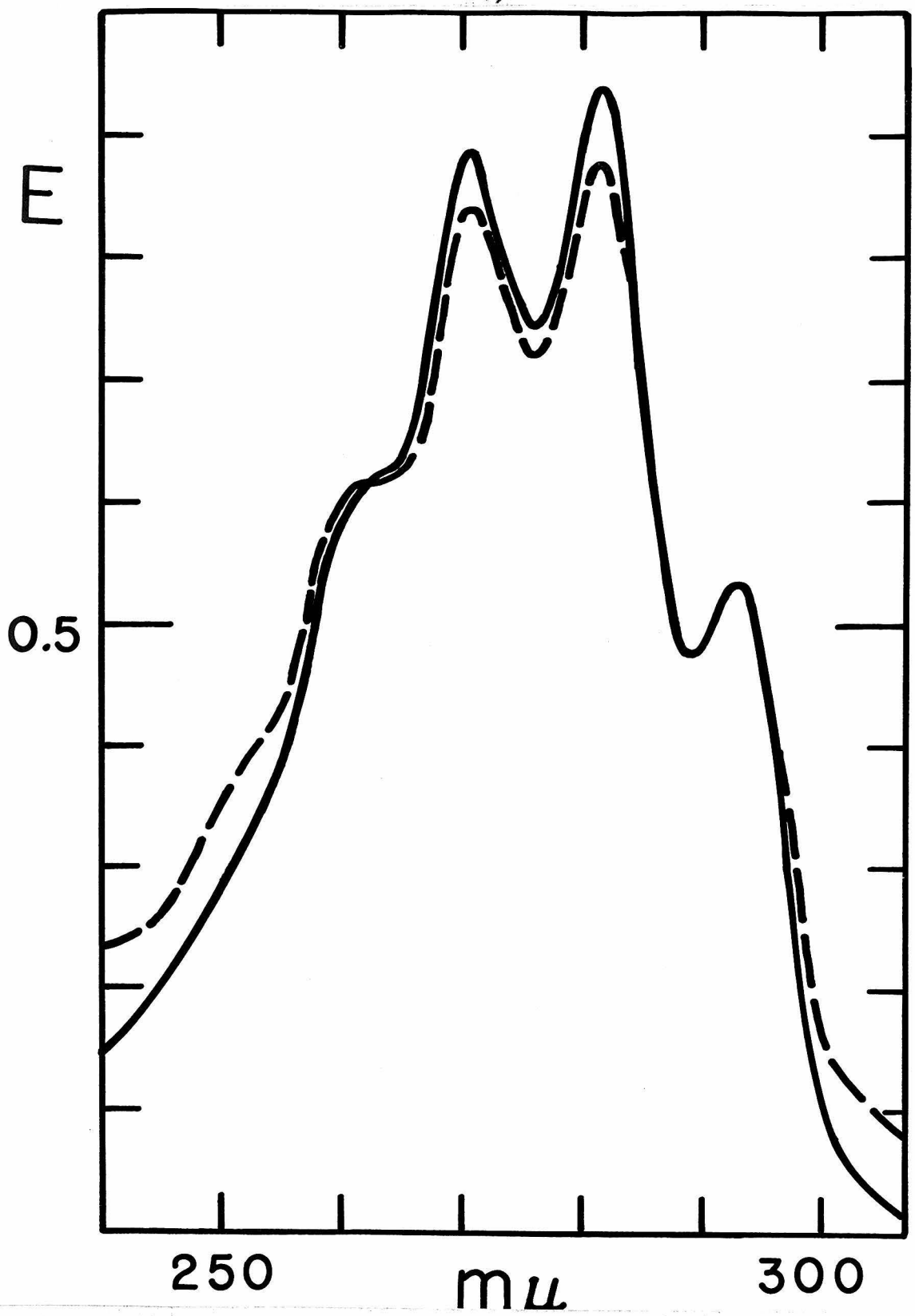

FIGURE 5. Spectral curve in hexane of commercial ergosterol - - - - - - -, and of crystalline material isolated from the Thoracophelia mucronata ————.

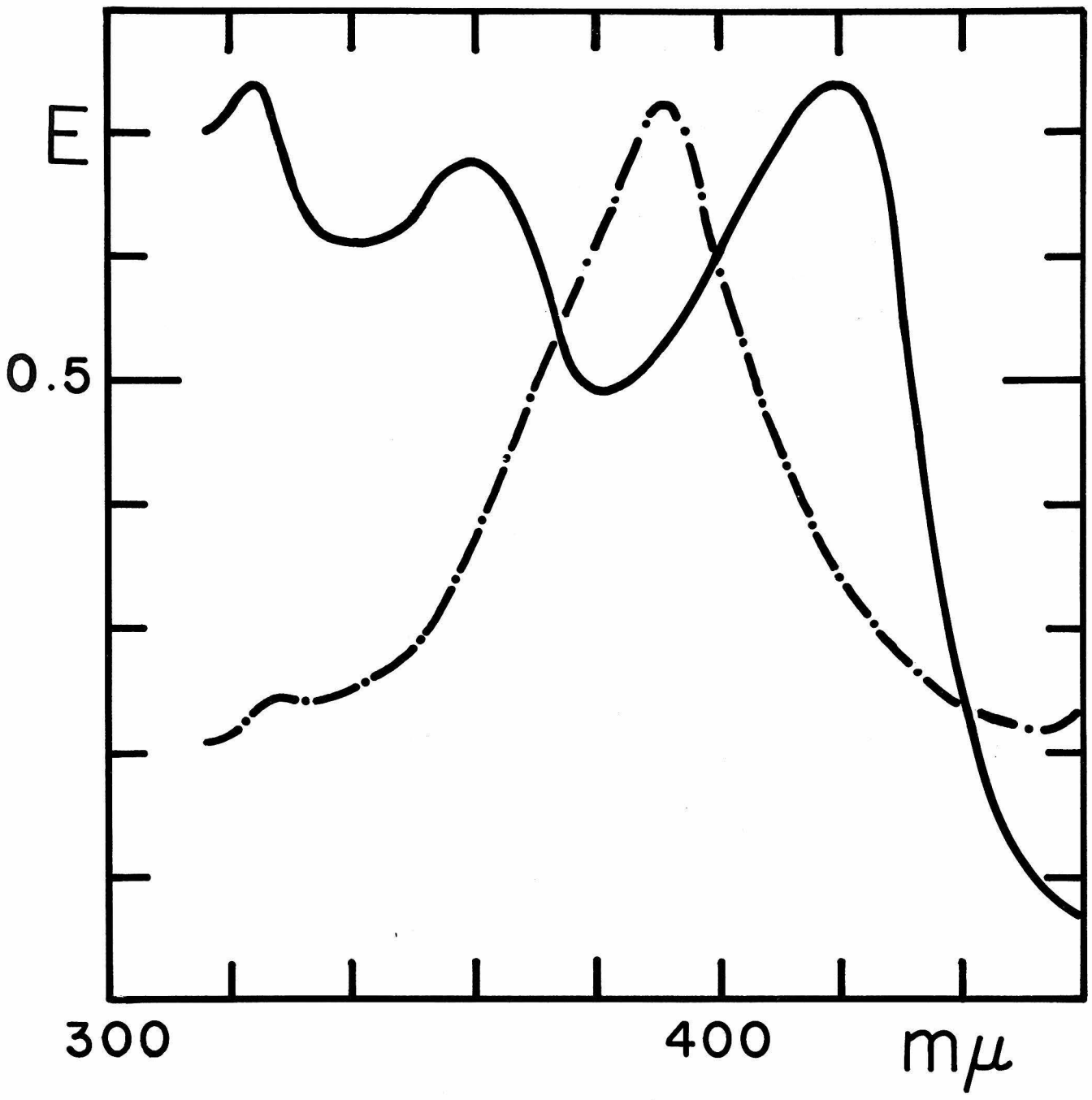

Figure 6. Spectral curves of color reactions of ergosterol and crystalline sterol from blood worms with antimony trichloride-acetyl chloride-chloroform reagent. Ergosterol -.-.-.-.-. and material from blood worms (7-dehydrocholesterol, impure) ————.

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