THE ISOLATION AND STUDY OF SOME NATURALLY OCCURRING FLUORESCENT ORGANIC COMPOUNDS AND STEREOISOMERIZATION OF PROLYCOPENE, PRO-γ-CAROTENE, AND LYCOPENE IN CHICKENS AND HENS

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The author wishes to express his deep appreciation to Professor Zechmeister for his guidance and interest in the problems connected with this research.

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ABSTRACT

A report on the detection, analysis, spectrum, and identification of several polycyclic aromatic hydrocarbons as lipoid-soluble, fluorescent organic compounds occurring in certain marine invertebrates (barnacles) is presented. Crystalline preparations of these compounds have been obtained from the intensely fluorescent zones resulting from chromatographic resolution of the crude barnacle extracts. These hydrocarbons include anthracene, phenanthrene, fluoranthene, chrysene, 1,12-benzperylene, coronene, and the strongly active carcinogen, 3,4-benzpyrene. Similar experiments carried out with the beach worm have revealed only traces of polycyclic aromatic compounds. They have not been found in deep-sea mud samples or in some seaweeds tested. Some suggestions are made as to the origin of these compounds in barnacles (and beach worms).

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When poly*cis* carotenoids are fed to chickens and hens over a period of several days, and the crude extracts of the different tissues, gut washings, and feces of these animals are submitted to chromatographic analysis, the pigments recovered are found to consist of the unchanged starting compound and stereoisomerized forms. This bio-stereoisomerization results in the formation of not only the all-*trans* and neo-forms of the carotenoid administered but also poly*cis* isomers, some of which possess more *cis* double bonds than the starting material.

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I. THE ISOLATION AND STUDY OF SOME NATURALLY OCCURRING FLUORESCENT ORGANIC COMPOUNDS.

A. DETECTION AND IDENTIFICATION OF CERTAIN FLUORESCENT COMPOUNDS EXTRACTED FROM MARINE MATERIALS.

1. THEORETICAL PART.

a. Introduction.

In recent years the investigations by Zechmeister and collaborators of lipoid-soluble, fluorescent organic compounds occurring in plant tissues have resulted in the discovery and characterization of a colorless, green-fluorescent C_{40}-polyene, phytofluene (64), and a heterocyclic, aromatic, sulfur-containing, blue-fluorescent compound, α-terthienyl $C_{12}H_8S_3$ (65).

Phytofluene is the first representative of the class of colorless carotenoid hydrocarbons and exhibits the characteristic spectrum for these compounds, except that its three extinction maxima (located at 331, 348, and 367 mμ. in hexane) have been shifted towards much shorter wave lengths (64). The probable structure of phytofluene is that of a partially hydrogenated lycopene, with two of its seven double bonds located in the terminal isopropylidene groups and the remaining five conjugated in the central section of the molecule. Because of its widespread occurrence in carotenoid-containing plant tissues (63), it has been suggested that phytofluene could be an intermediate in the biosynthesis of carotenoid pigments (5, 41).

Recently, Porter and Zscheile isolated another colorless polyene

of the C_{40}-class from tomatoes (40). This new compound, termed "phytoene", differs from phytofluene in having two double bonds less in the conjugated system (maxima, in isooctane: 275, 285, 297 mμ.).

In this Thesis similar investigations of naturally occurring, lipoid-soluble fluorescent compounds were extended to seaweeds, invertebrates, and mud samples collected from the sea shore of Southern California in order to test the occurrence of phytofluene in various materials of marine origin. In the course of these studies several intensely fluorescent, crystalline compounds belonging to a class of compounds entirely different from the aliphatic polyenes described above, namely, the polycyclic aromatic hydrocarbons, were isolated from barnacles. A description of these organisms as well as the other marine materials investigated and the fluorescent substances obtained upon chromatographic resolution of their respective extracts are given on the following pages.

b. Fluorescent Compounds Obtained from the Thatched Barnacle (Tetraclita squamosa rubescens).

Barnacles belong to the Crustacea, class Cirripedia, and can be described as shrimp-like animals enclosed by a calcareous shell (35). The larvae are free-swimming, but the adult barnacles assume a sessile existence, attaching themselves firmly by means of a secretion from special cement glands to foreign objects, such as rocks, wharf pilings, drifting wood, seaweeds, ship bottoms, whales and even to other barnacles or mussels, etc. (50).

The barnacles studied in the present Thesis belong to the order Thoracica ("true barnacles"). The shell enclosing the body contains movable opercular plates which are tightly closed when the tide is out. The animal has six pair of legs (cirri) which are divided at their ends into two long, many-jointed, hairy branches curled towards the mouth and giving the appearance of feathery plumes. When the animal is covered by the sea, the valves of the shell open to allow the protrusion of the feathered appendages. This casting net of legs, by making continuous grasping movements and withdrawing into the shell between each sweep, combs the water for finely particulate matter (detritus) which constitutes the food of the barnacle (for an excellent photograph, see reference 50).

The true barnacles are divided into two suborders, the stalked barnacles (Lepadomorpha) or goose barnacles (discussed in the following section) and the acorn or rock barnacles (Balanomorpha). In the latter type the shell of the animal is attached directly onto some solid object and consists of limy plates rigidly fitted together in a tepee-like form around the sides of the animal. At the top of the shell there are two pairs of hinged plates (operating like double doors) through which the cirri protrude during feeding.

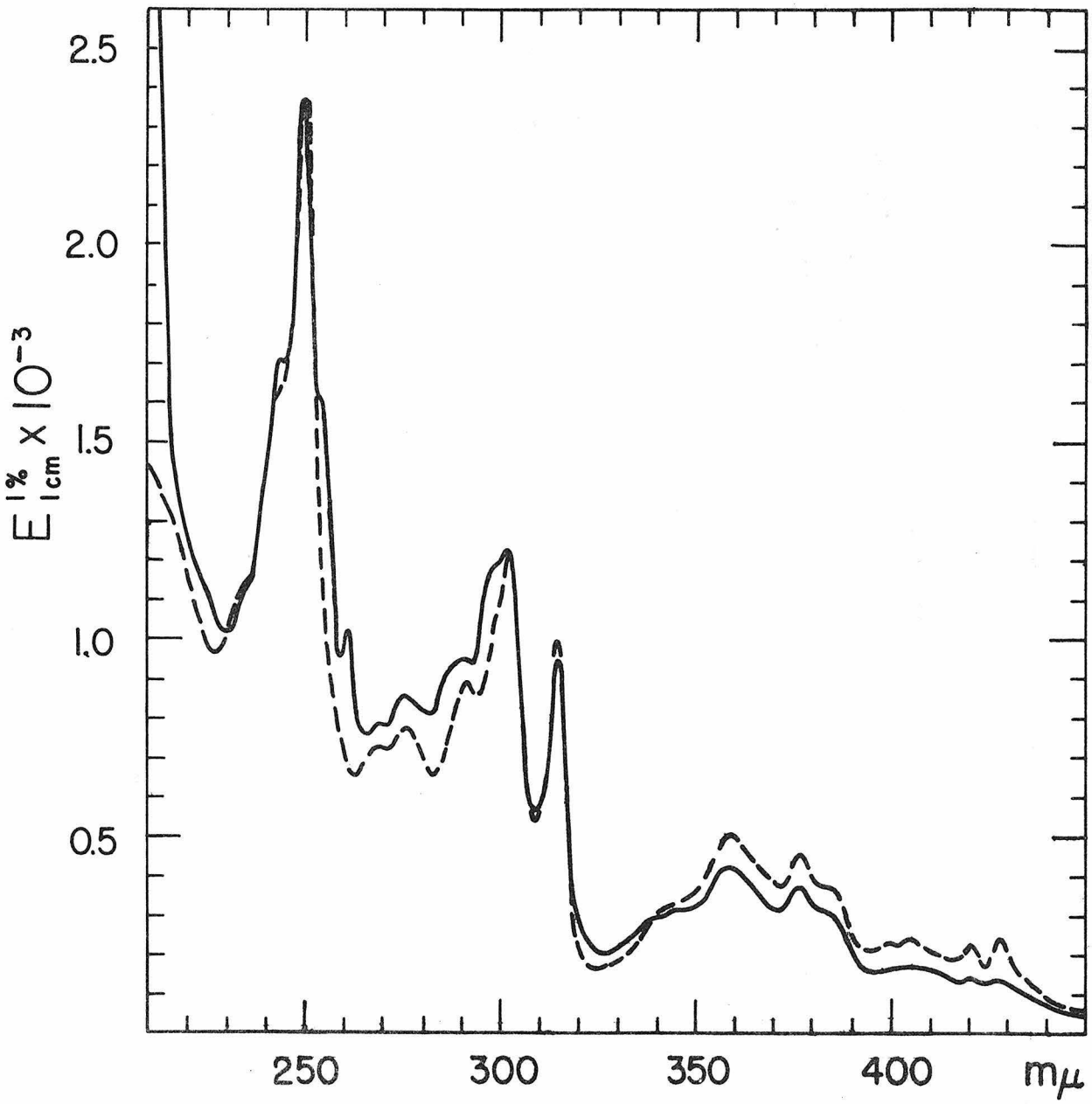


Figure 1. Specific extinction curves, in hexane: _____, crystalline compound A ex Tetraclita (maxima at 244, 249.5, 261, 269, 275-6, 291, 302, 315, 345, 358, 377, 404-5, 421, and 428 mμ.); - - - -, crystalline compound E ex Mitella (maxima at 250, 269, 276, 291, 302, 314.5, 359, 377, 400, 404-5, 421, and 428-9 mμ.). Unidentified.

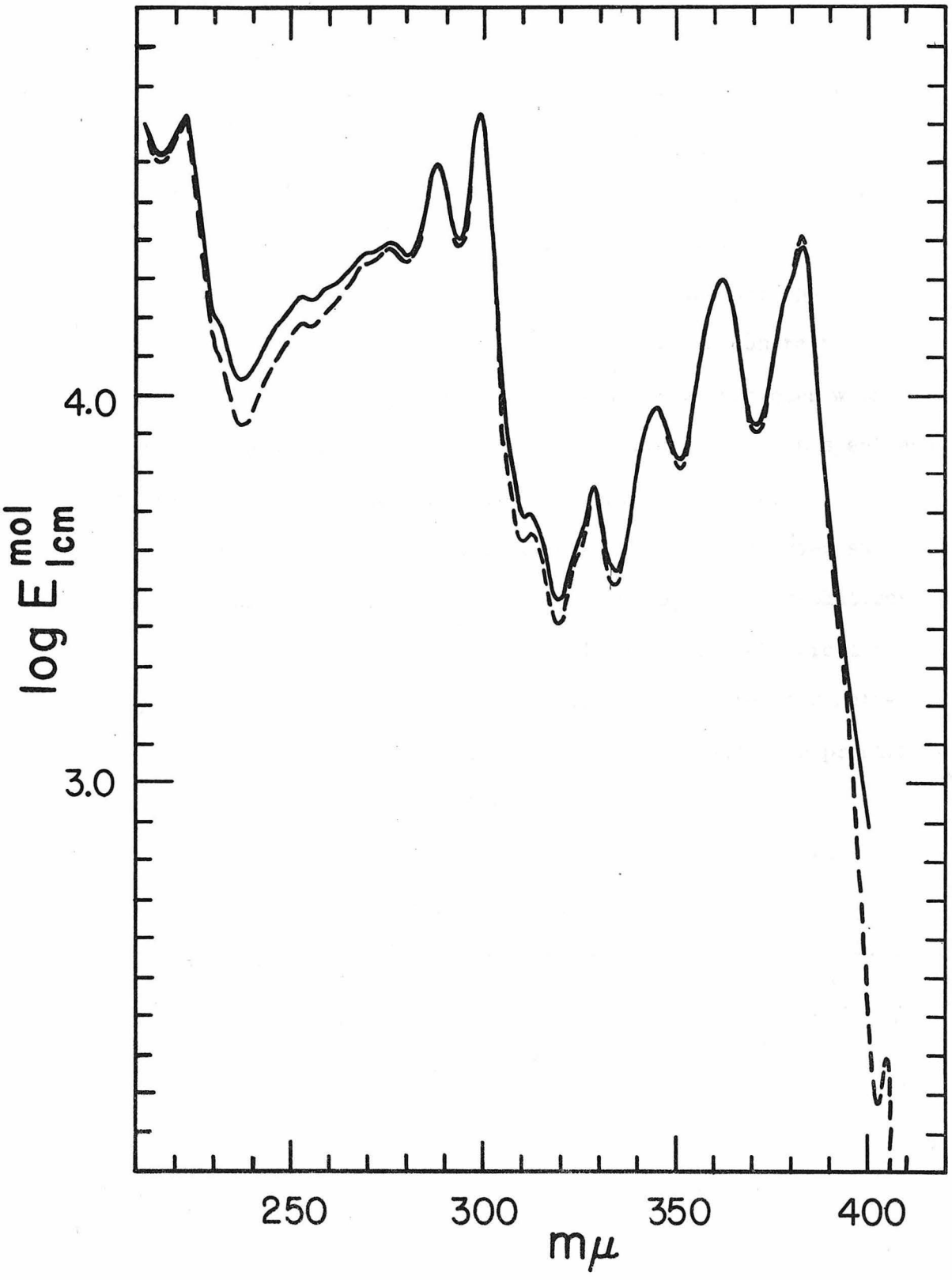


Figure 2. Molecular extinction curves of 1, 12-benzperylene, in hexane: _____, crystalline fraction ex Tetraclita; - - - -, synthetic 1,12-benzperylene (ex perylene).

(1). Thatched Barnacles from Corona del Mar, Calif. (55)

The first sample of thatched barnacles (Tetraclita squamosa rubescens) studied was obtained at Corona del Mar, Calif., from pier pilings near the Kerckhoff Marine Laboratory. Identification of the barnacles was made by Dr. W. R. Coe of the Scripps Institution of Oceanography, La Jolla, Calif. In contrast to the usual members of this species (relatively large barnacles with thick, brick-red shells) found on the Pacific Coast, the specimens collected were yellowish in color and smaller in size.

The crude extract of the thatched barnacles displayed an intense blue fluorescence, and after chromatographic resolution the resulting main fluorescent zones yielded spectra typical for polycyclic aromatic hydrocarbons. Following repeated chromatographic purification of each fraction, eight crystalline preparations were isolated in small quantities.

3,4-Benzpyrene. One of these crystalline samples whose composition and molecular weight corresponded to the formula $C_{20}H_{12}$ gave an extinction curve containing many of the maxima of the 3,4-benzpyrene spectrum (Figure 4). However, the presence of other maxima in the curve, the wide melting point range, and the shade of its fluorescence in solutions, which was distinctly more bluish than that of pure 3,4-benzpyrene, indicated that this preparation consisted of a mixture of 3,4-benzpyrene and some of its

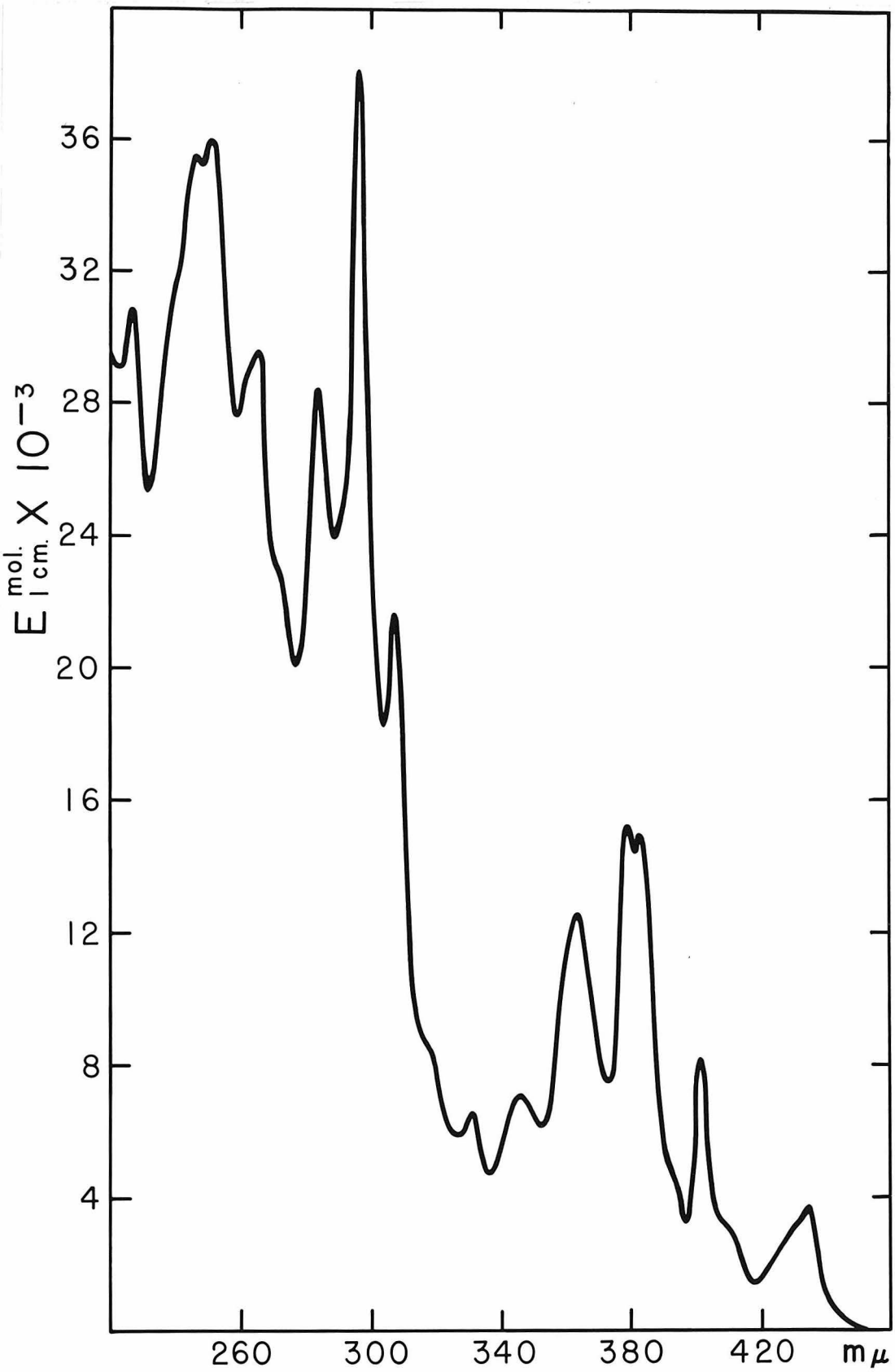


Figure 3. Molecular extinction curve, in hexane: crystalline 3,4-benzpyrene fraction ex Tetraclita.

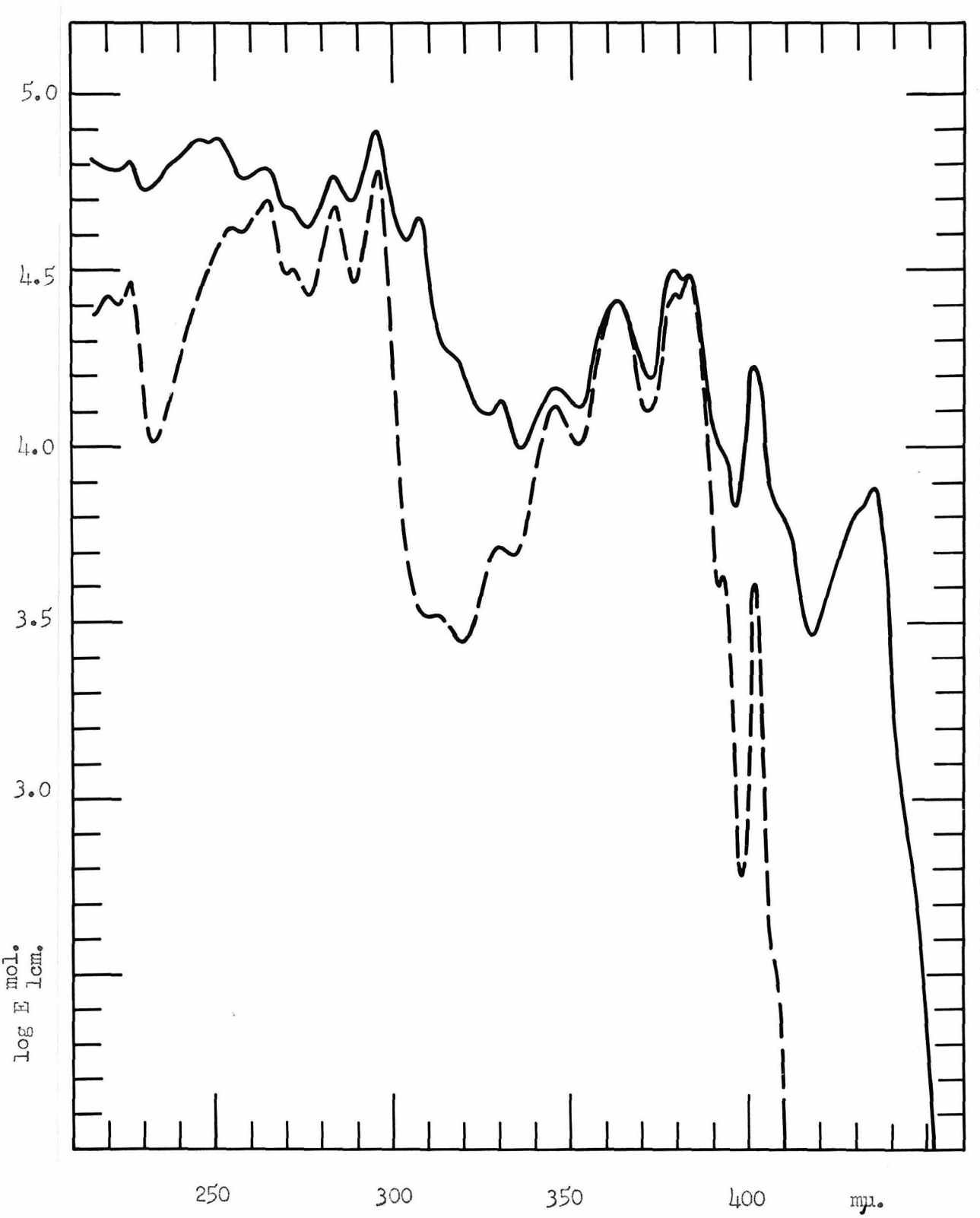
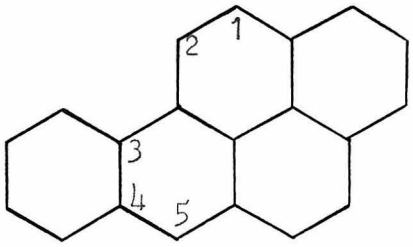


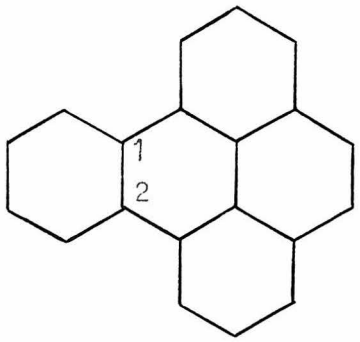
Figure 4. Molecular extinction curves of 3,4-benzpyrene, in hexane: _____, crystalline fraction ex Tetraclita; - - - -, commercial, chromatographically purified 3,4-benzpyrene.

isomers (or/and close analogs).

Since the limited amount of substance did not permit more extended resolution experiments, the presence of 3,4-benzpyrene was confirmed by means of the following observation made by Fieser and Campbell (16). These authors found that 3,4-benzpyrene is able to couple with p-nitrobenzenediazonium chloride to give an azo dye. This coupling is one of several special substitution reactions which this hydrocarbon readily undergoes at the reactive 5 position. The latter corresponds to the meso 10 position in the 1,2-benzanthracene ring system and is absent from the isomer 1,2-benzpyrene (cf. formulae below). Consequently, our crystalline preparation as well as a commercial but chromatographically purified sample of 3,4-benzpyrene was treated according



3,4-benzpyrene



1,2-benzpyrene

to the directions of Fieser and Campbell. Each of the two dye solutions obtained was then submitted to chromatographic purification, until homogeneous zones resulted. After elution it was

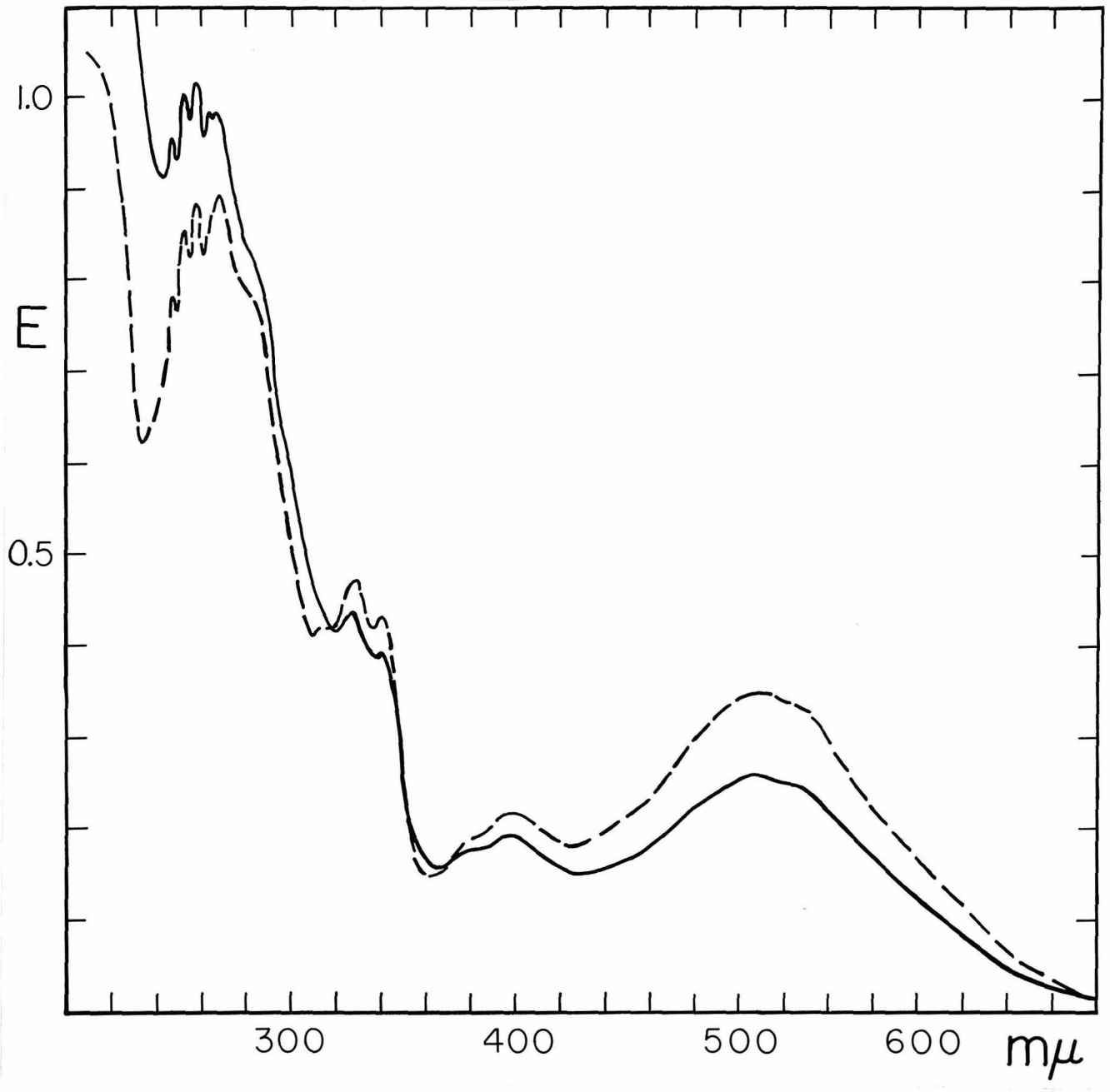


Figure 5. Extinction curves of the coupling product of 3,4-benzpyrene and p-nitrobenzenediazonium chloride, in hexane: _____, dye obtained from crystalline fraction ex Tetraclita; - - - -, dye from commercial 3,4-benzpyrene.

found that the two dyes could not be separated in the mixed chromatogram test. Furthermore, the spectral differences observed between the crystalline product ex barnacles and authentic 3,4-benzpyrene were mostly eliminated. Although the respective extinction curves as given in Figure 5 do not appear to be strictly congruent, the identity of these samples can hardly be doubted.

Since the evidence accumulated definitely indicated that our preparation contained the strongly carcinogenic 3,4-benzpyrene, the sample was tested for carcinogenic activity. This assay was kindly conducted by Dr. M. B. Shimkin of the Laboratory of Experimental Oncology, National Cancer Institute, San Francisco, Calif.

The following data taken from Dr. Shimkin's report clearly substantiated the presence of an active carcinogen in the barnacles studied (43):

"The material was dissolved in tricaprylin, 5 mg/ml. Twelve male C_3H mice, 3 months old, received a single subcutaneous injection of 0.5 mg in 0.1 ml; 12 additional mice were injected with 0.25 mg in 0.05 ml. The mice were maintained on Purina dog chow and an unlimited supply of water. They were examined weekly for the presence of progressively growing tumor at the site of injection.

Four of 12 mice receiving 0.5 mg of the material developed subcutaneous tumors in 16, 17, 19, and 20 weeks following injection. Two of 12 mice receiving 0.25 mg developed tumors in 17 and 19 weeks. The remaining 18 mice were alive and free of tumor 36 weeks after injection.

The mice with tumors were sacrificed when the tumors reached 1-2 cm in diameter. On histologic examination, all 6 were seen to be spindle-cell sarcomas with local invasion of areolar and muscular tissue. Morphologically they were indistinguishable from

tumors induced with 3,4-benzpyrene and other polycyclic carcinogenic hydrocarbons. The first tumor to be noted was transplanted into six C_3H mice and grew vigorously within 10 days, maintaining its sarcomatous appearance.

Previous data showed that 80-90% of C_3H male mice developed sarcomas within 20 weeks after the subcutaneous injection of 0.25-0.5 mg. of 3,4-benzpyrene dissolved in tricaprylin. The incidence of approximately 25%, and the longer latent period of the tumors in this investigation, suggest that the material tested contained 10-40% of the active carcinogen, assuming that 3,4-benzpyrene was the only such compound present and that other substances in the sample did not alter the carcinogenic reaction."

Anthracene and phenanthrene. A second crystalline sample, $C_{14}H_{10}$, exhibited the simultaneous presence of extinction maxima typical for anthracene and phenanthrene (Figure 9). Following the method of Bradley and Marsh for separating these isomers (7), the solution of the crystals was exposed repeatedly to sunshine. The dianthracene precipitate formed by photochemical dimerization was filtered off and then depolymerized by heating; the spectrum of this product clearly demonstrated the presence of anthracene, although it was not free of contaminants (Figure 10). The filtrate from the removal of the dianthracene was rechromatographed, crystallized, and sublimed in vacuo. The crystalline substance obtained was spectroscopically identified as phenanthrene (Figure 11).

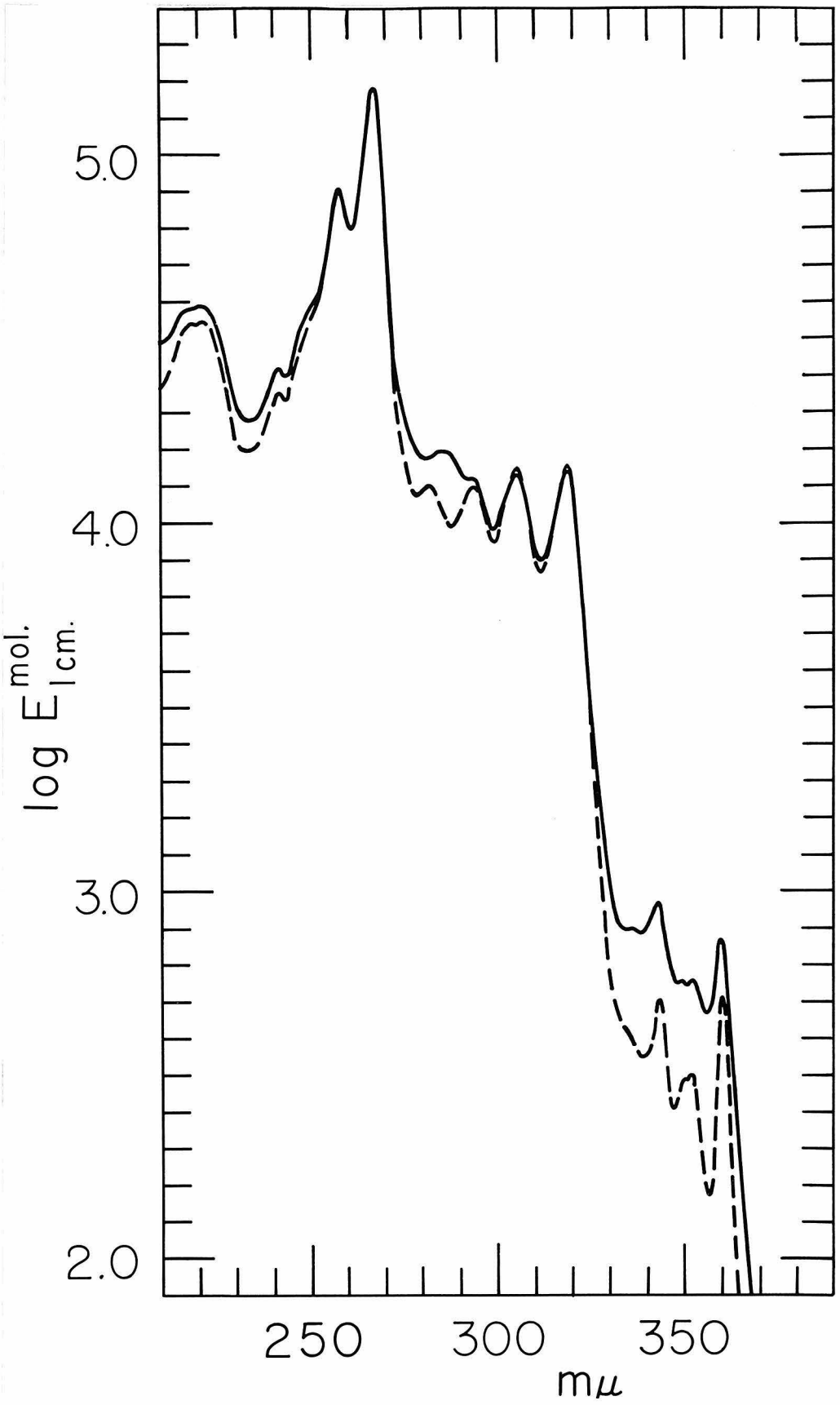


Figure 6. Molecular extinction curves of chrysene, in hexane: _____, crystalline fraction ex Tetraclita; - - - -, commercial, chromatographically purified chrysene.

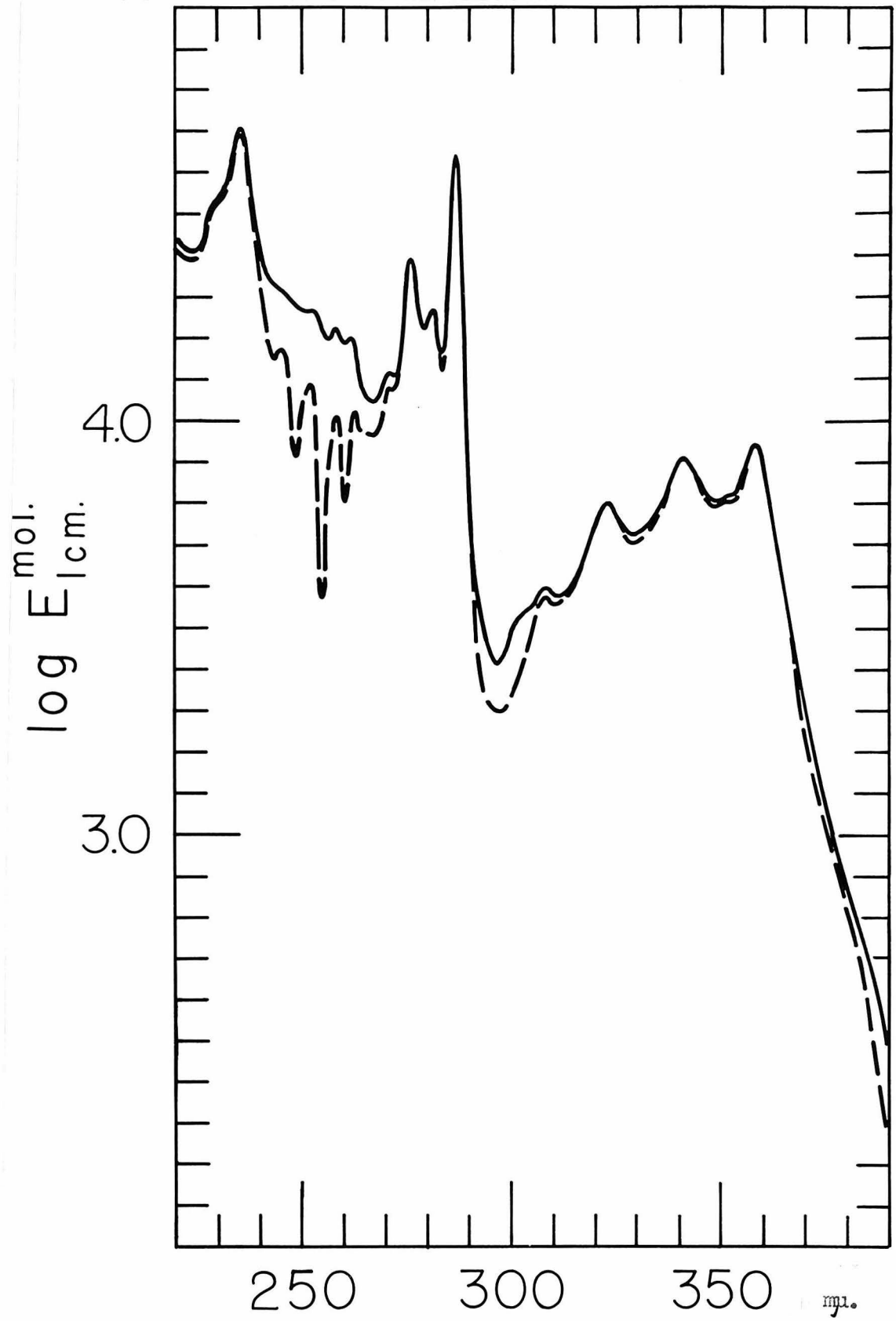


Figure 7. Molecular extinction curves of fluoranthene, in hexane: _____, crystalline fraction ex Tetraclita; commercial, chromatographically purified fluoranthene.

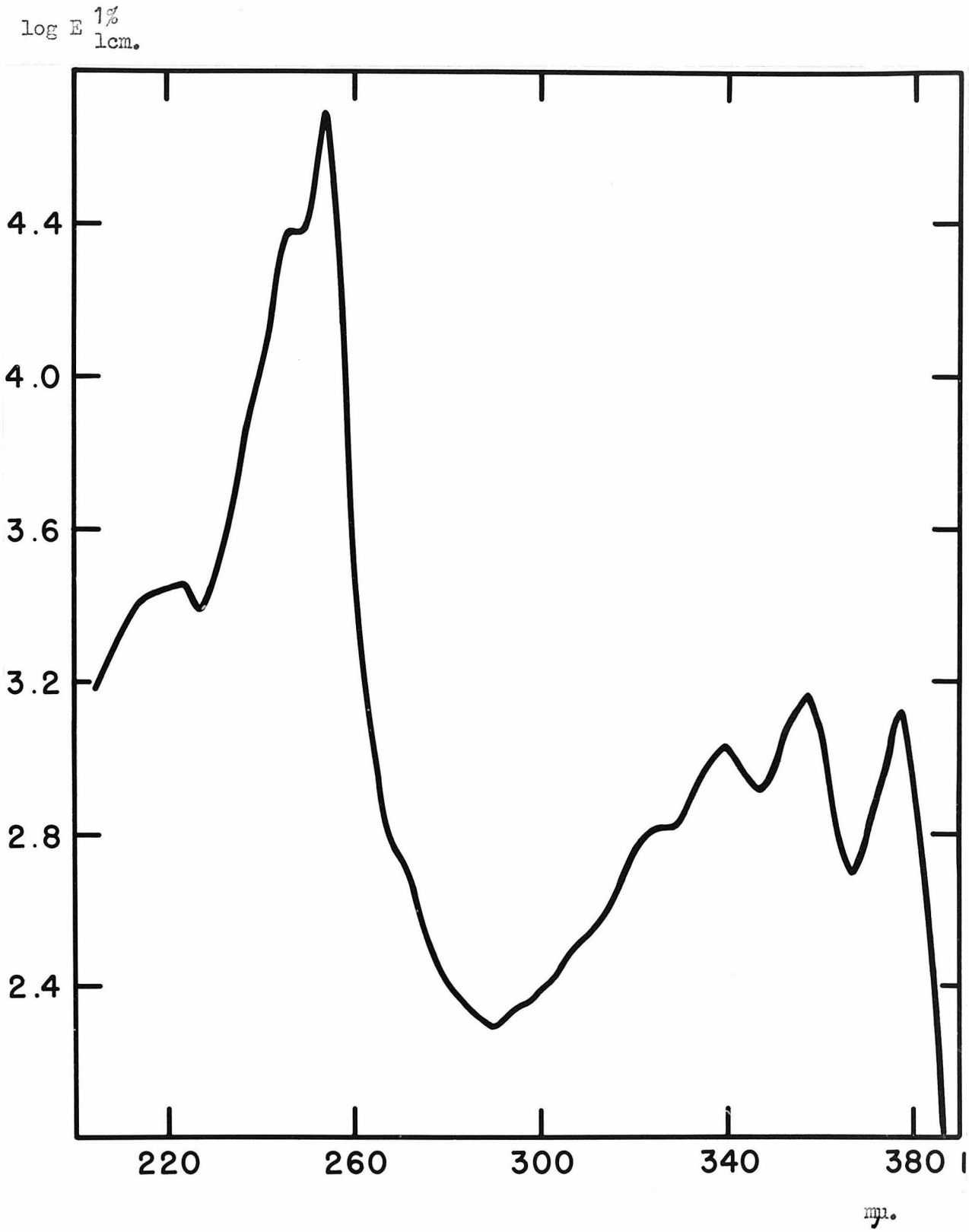
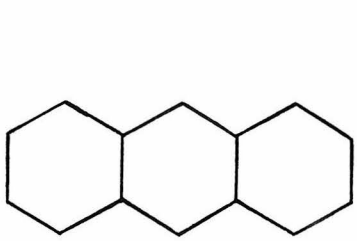
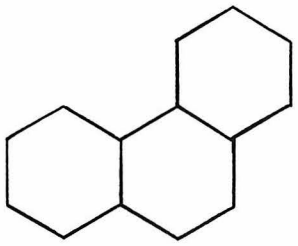


Figure 8. Specific extinction curve of unidentified crystalline compound B (ex Tetraclita), in hexane: maxima at 223, 247, 254, 326 (inflection), 340, 357, and 377 mμ.

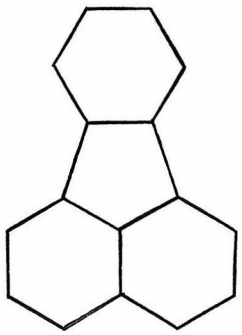


anthracene



phenanthrene

Fluoranthene. Analysis and determination of the molecular weight of a third crystalline sample yielded the formula, $C_{16}H_{10}$. The mixed melting point test, the similarity in fluorescence, and the agreement in extinction curves (Figure 7) established the identity of this preparation with fluoranthene. Moreover, the crystalline compound did not separate from an authentic fluoranthene sample on the chromatographic column.



fluoranthene

1,12-Benzperylene and chrysene. Two crystalline preparations obtained in smaller yield than those mentioned could be identified

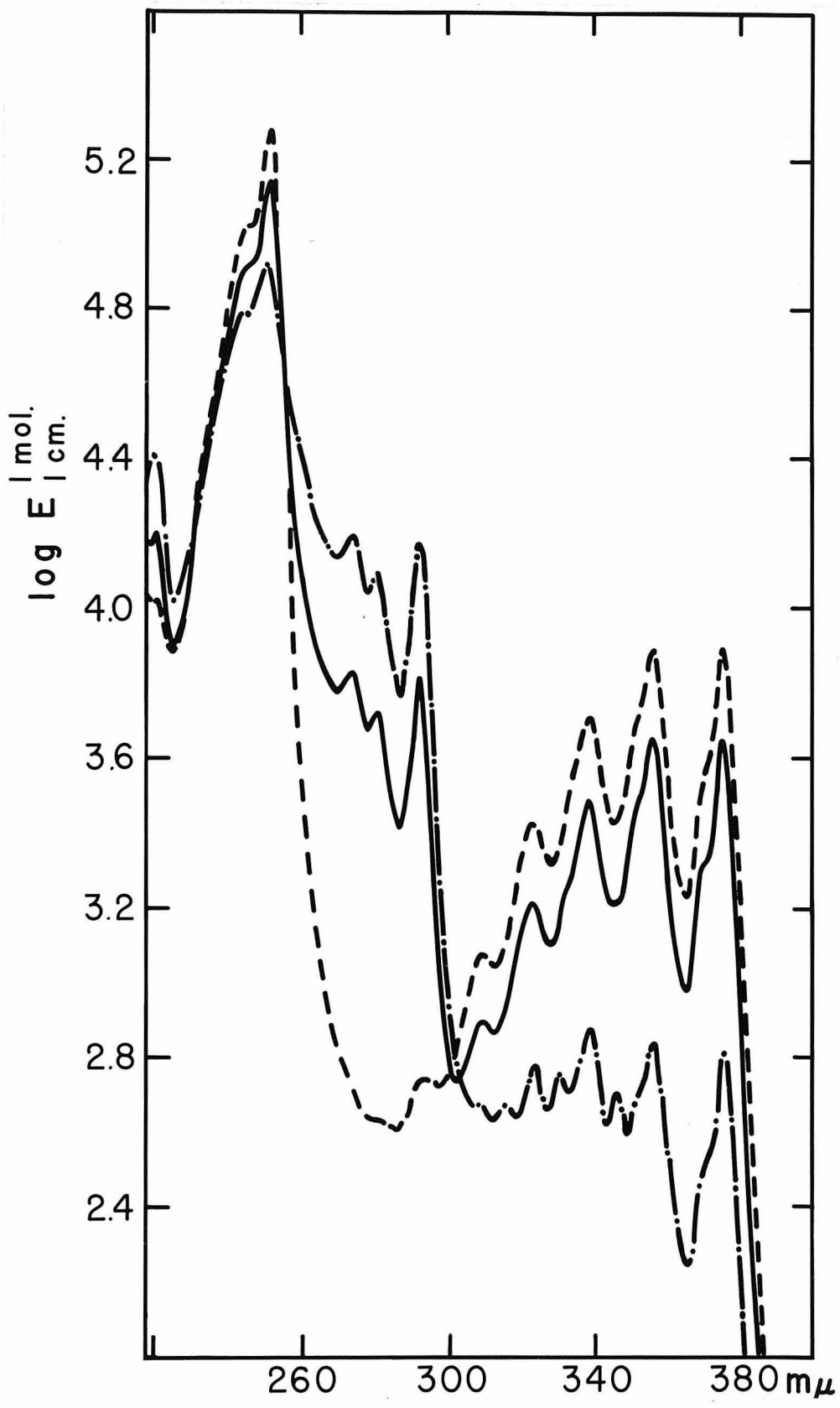


Figure 9. Molecular extinction curves, in hexane: _____, crystalline fraction ex Tetraclita; - - - -, commercial anthracene; -.-.-., commercial phenanthrene.

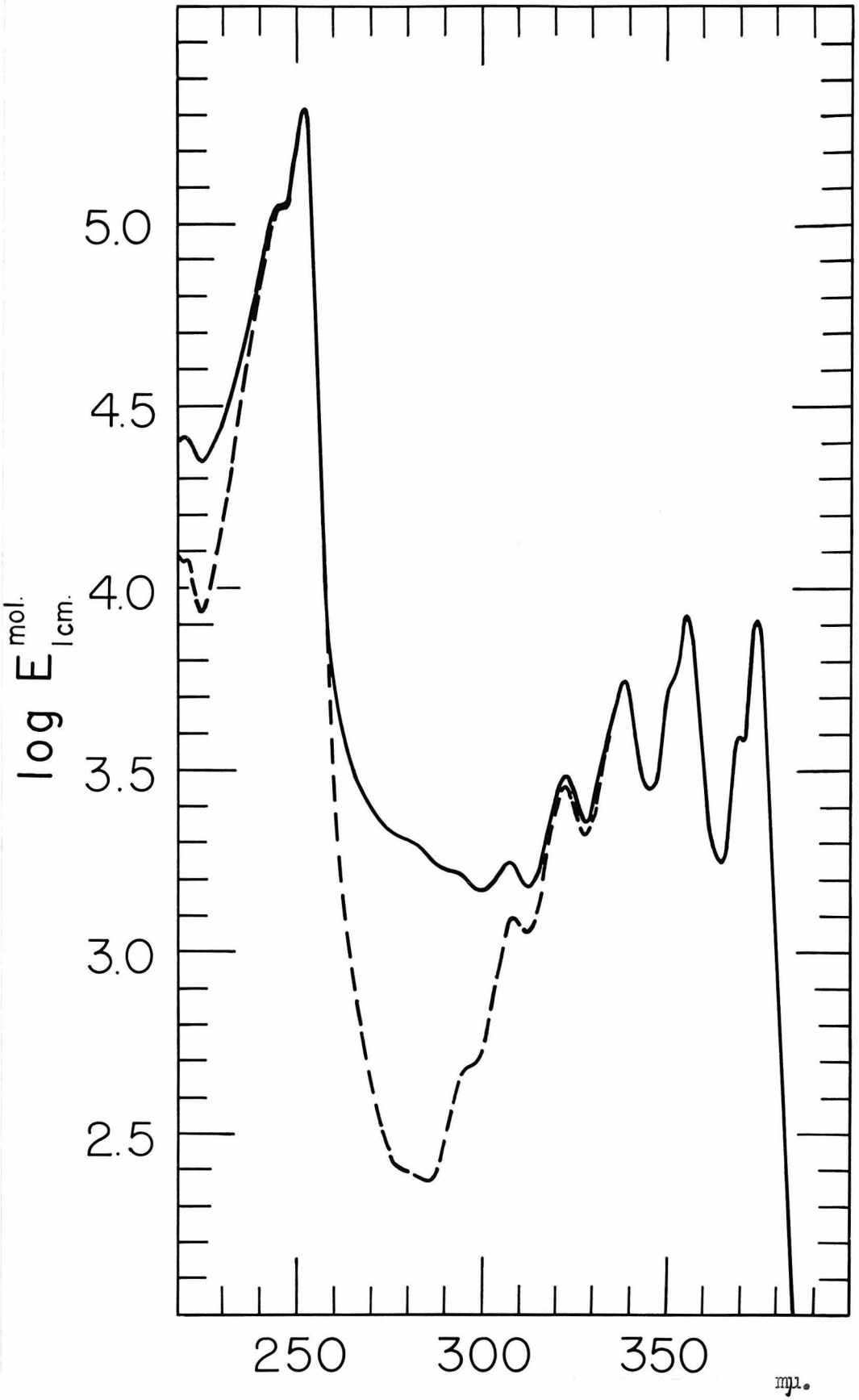


Figure 10. Molecular extinction curves of anthracene, in hexane: _____, crystalline fraction ex Tetraclita; - - - -, commercial anthracene. Both preparations were purified via dianthracene.

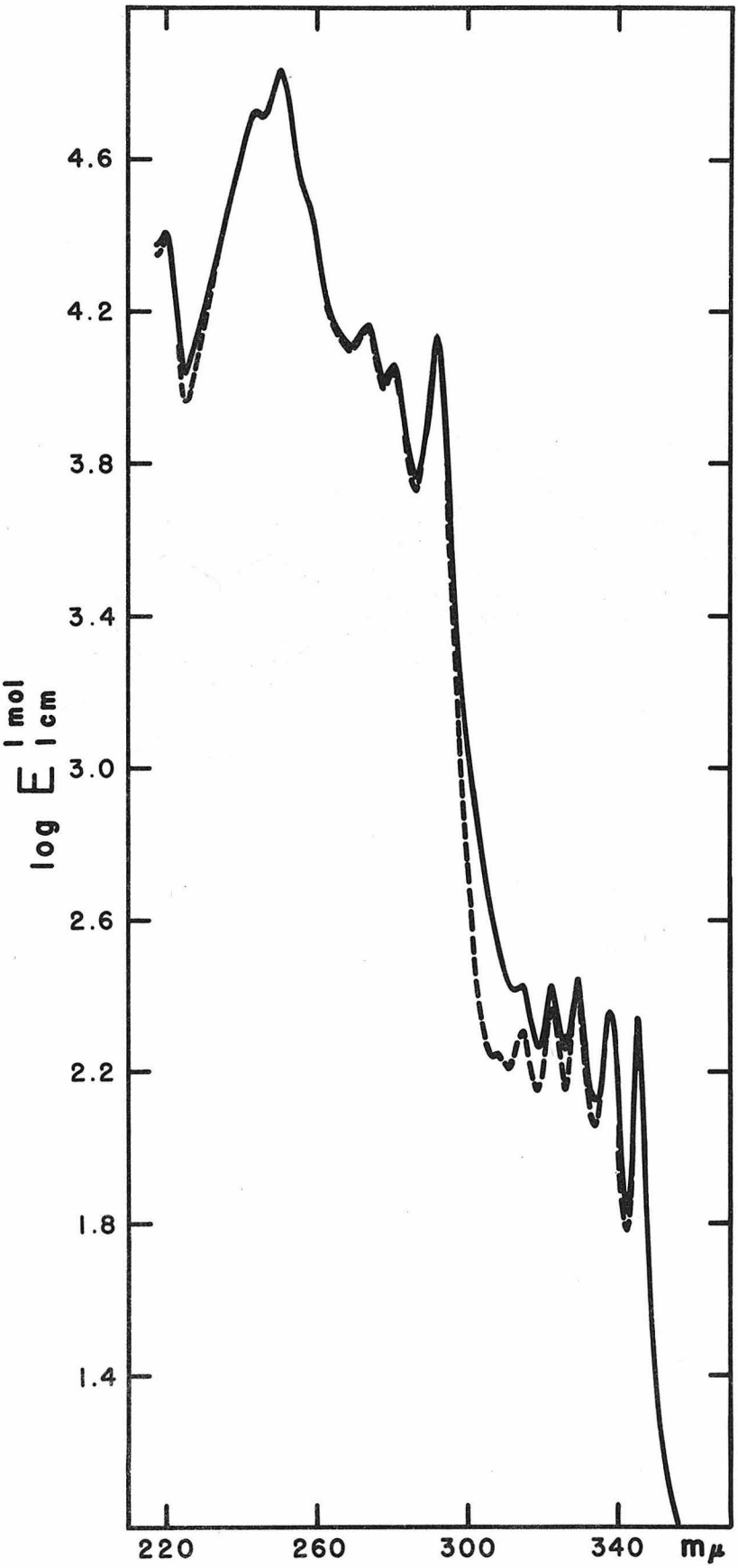
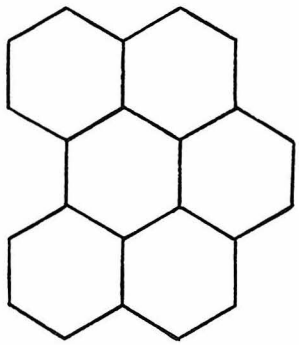
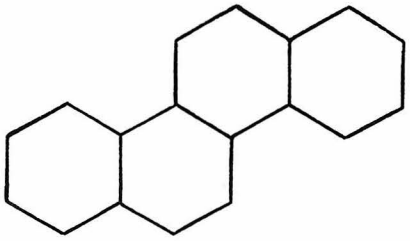


Figure 11. Molecular extinction curves of phenanthrene, in hexane: _____, crystalline fraction ex Tetraclita; - - - -, commercial phenanthrene. The anthracene content of both samples was removed as dianthracene, by illumination.

on the basis of their characteristic spectra as 1,12-benzperylene and chrysene, respectively (Figures 2 and 6). Mixed chromatogram and mixed melting point tests confirmed this identification.



1,12-benzperylene



chrysene

Unidentified crystalline polycyclic aromatic compounds, "A" and "B". Two further small crystalline samples yielded extinction curves characteristic for polycyclic aromatic compounds but could not be identified so far (Figures 1 and 8). The resemblance of the spectrum of compound B (Figure 8) to that of anthracene indicated that it was probably a derivative of the latter. On the alumina column compound A is adsorbed immediately above the 1,12-benzperylene zone. Compound B is located above anthracene from which it is separated by a clear interzone.

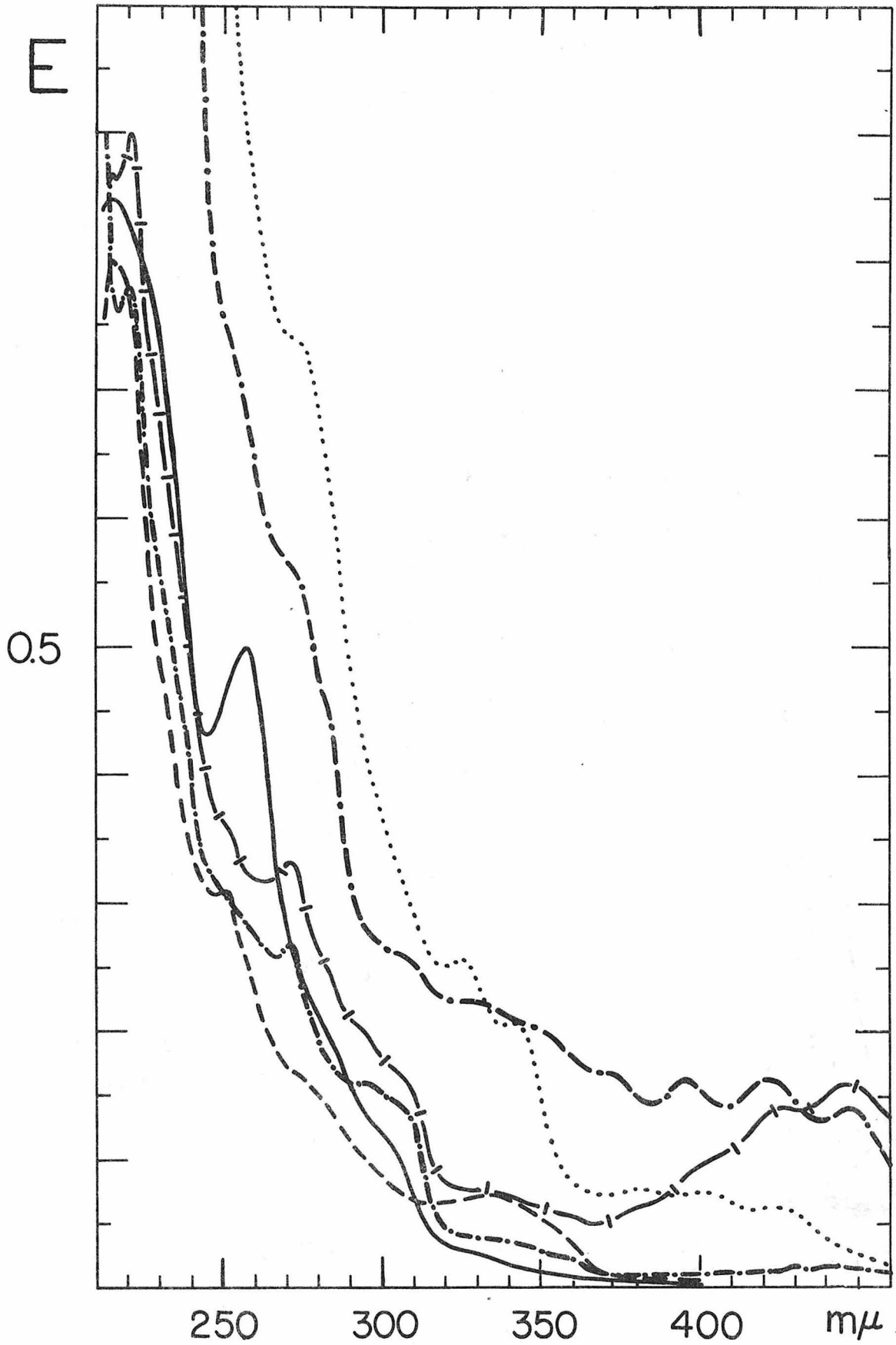


Figure 12. Extinction curves in hexane of unidentified fluorescent fractions ex Tetraclita (from La Jolla): ....., fraction IIIA; -'-'-', IIIB; --.--., VA; _____, VC; -.-.-., VIA; - - -, VIB.

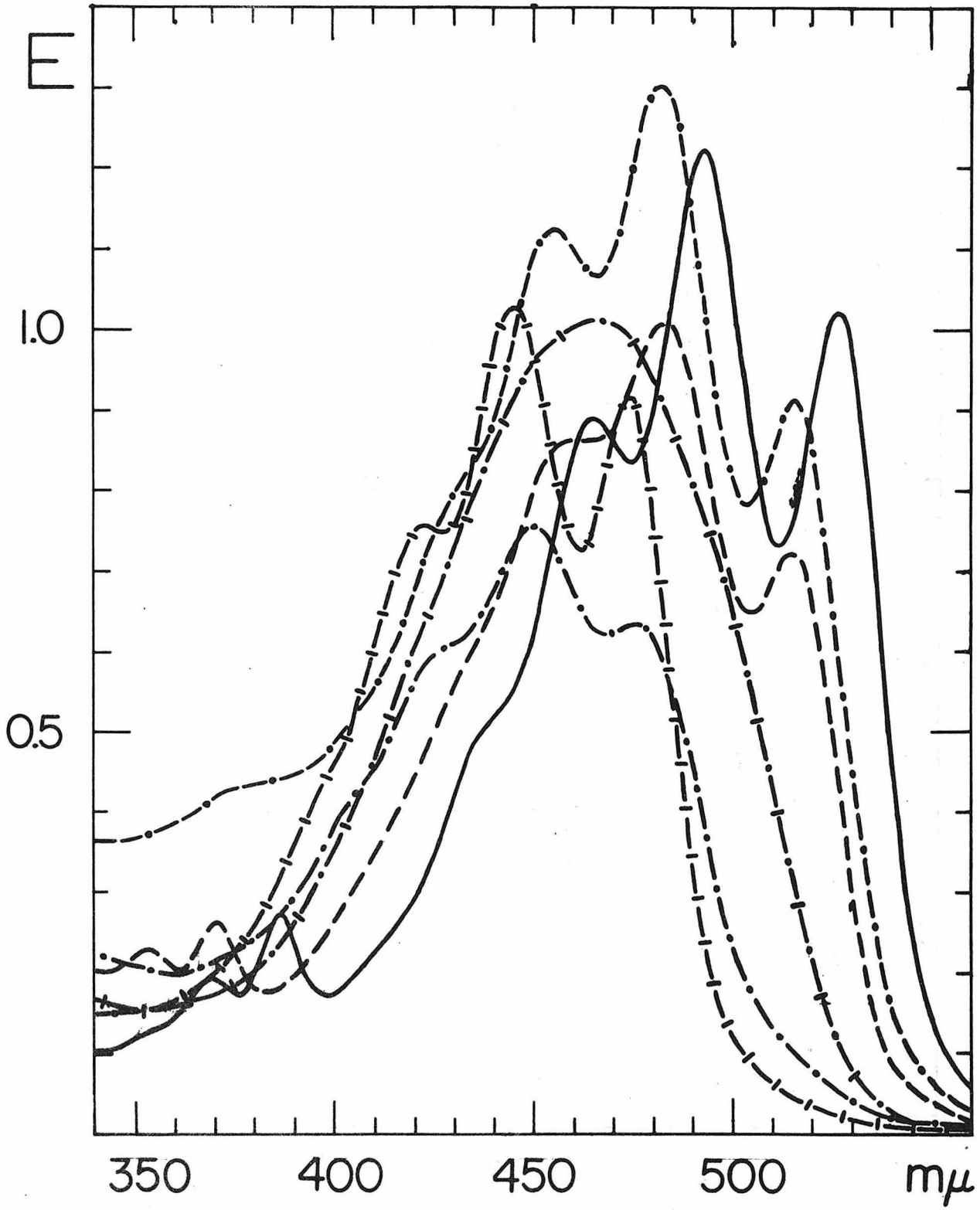


Figure 13. Extinction curves in hexane of pigment fractions ex Tetraclita (from La Jolla): _____, spirilloxanthin; --.--., unidentified; -.-.-., zeaxanthin; - - - -, unidentified; -'-'-', lutein; -.-'-., unidentified.

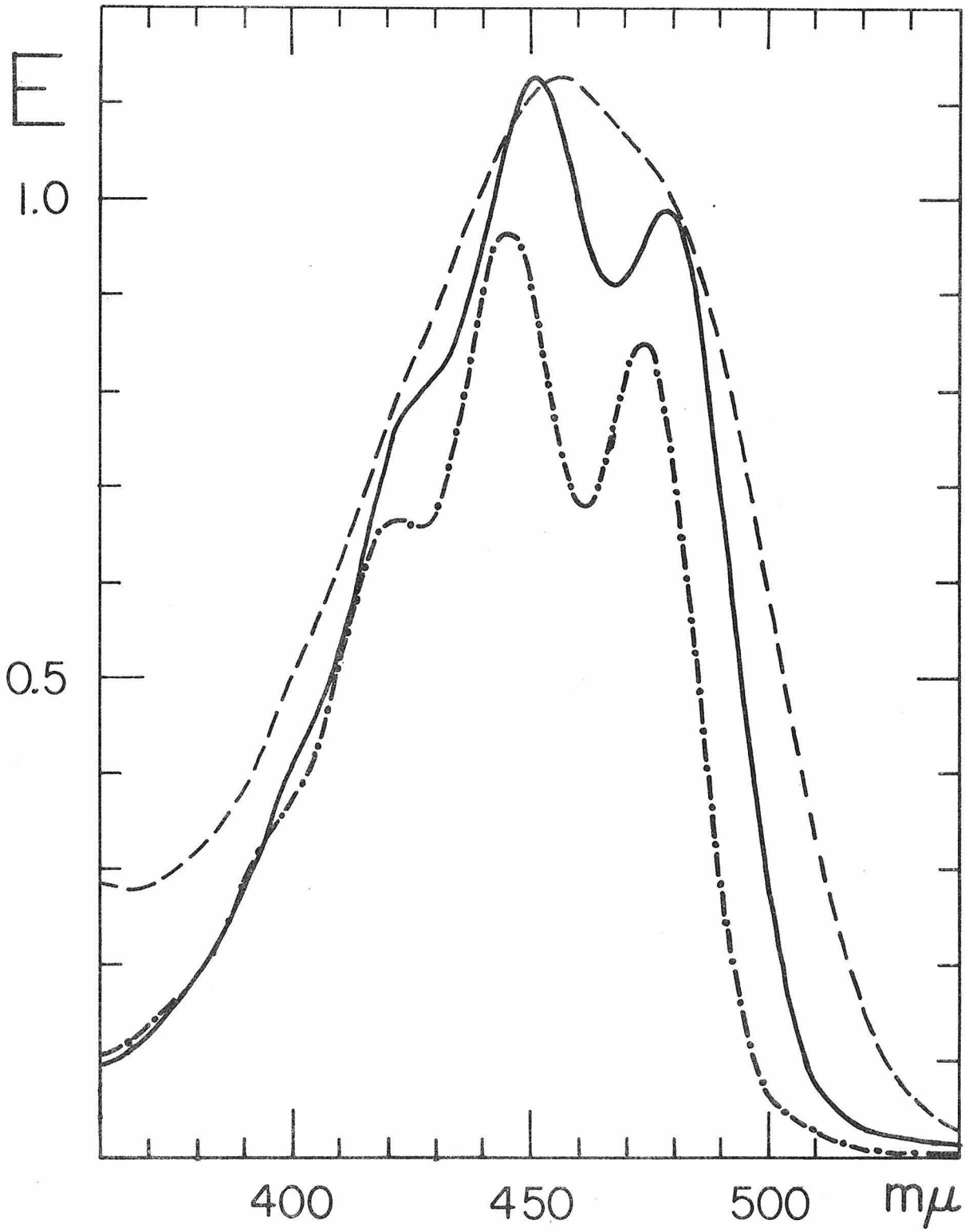


Figure 14. Extinction curves in hexane of pigment fractions ex Tetraclita (from La Jolla): _____, β -carotene; -.-.-., α -carotene; - - - -, unidentified.

(2). Thatched Barnacles from La Jolla, Calif.

In sharp contrast to the Corona del Mar sample of Tetraclita which yielded the polycyclic aromatic compounds just described, the fluorescent fractions obtained from thatched barnacles originating from rocks near La Jolla, Calif., were found to be of a very different nature. The extinction curves of these unidentified substances increased sharply with decreasing wave length below 300 mμ.; they were characterized by only one or two small maxima (Figure 12).

c. Fluorescent Compounds Obtained from the Goose Barnacle (Mitella polymerus).

The "goose barnacle" belongs to the suborder of the stalked barnacles (6). These animals are attached by a fleshy stalk or "peduncle" to rocks, pilings, other marine animals, etc. and only vaguely resemble the squat and heavy-shelled thatched barnacles in appearance. The peduncle is edible and is considered by the Spanish and Italians as a choice food (42). The body (capitulum) of the animal, including the cirri or "feather-feet", is enclosed within a calcareous shell consisting of two valves at the unattached end of the peduncle. Figure 15 shows a specimen of the goose barnacles investigated which were collected from pier pilings at the Scripps Institution of Oceanography, La Jolla, where they had been growing on and among mussels. Although the stalks appeared to be rather brownish on the outside, the fleshy interior was reddish orange. Only the separated peduncles were extracted in the present study.

Figure 15. Goose Barnacle (Mitella polymerus). Note the smaller goose barnacle, which had been growing on the peduncle of the larger specimen, and the presence of a small acorn barnacle on the capitulum of the latter.

The fluorescent fractions obtained upon chromatographic resolution of the crude extract of the peduncles yielded small quantities (0.1 to 1 mg.) of eleven crystalline substances, all belonging to the class of the polycyclic aromatic compounds. These crystalline preparations included coronene, 1,12-benzperylene, 3,4-benzpyrene, chrysene, fluoranthene, and six other unidentified

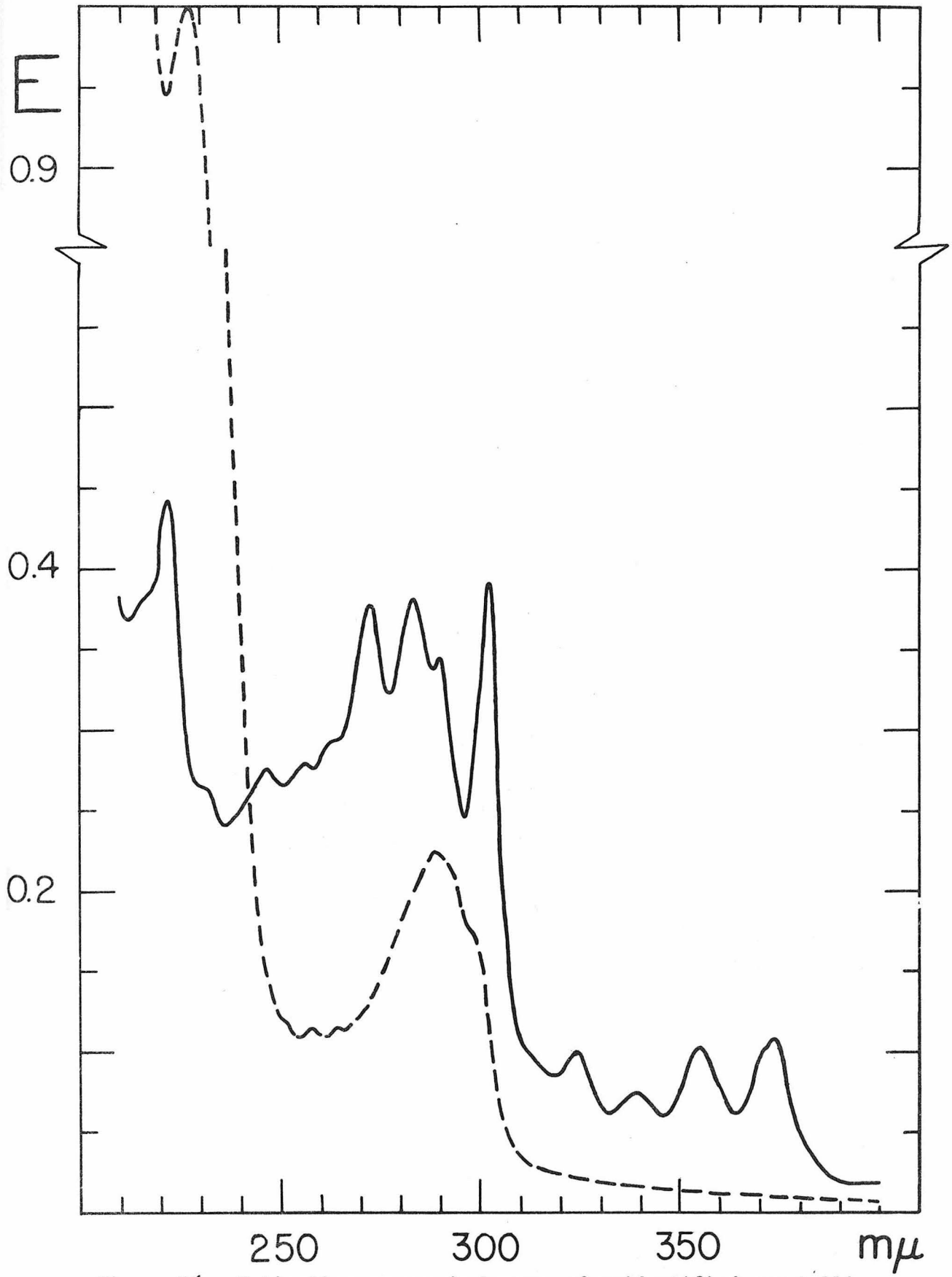


Figure 16. Extinction curves in hexane of unidentified crystalline compounds ex Mitella: _____, compound D.

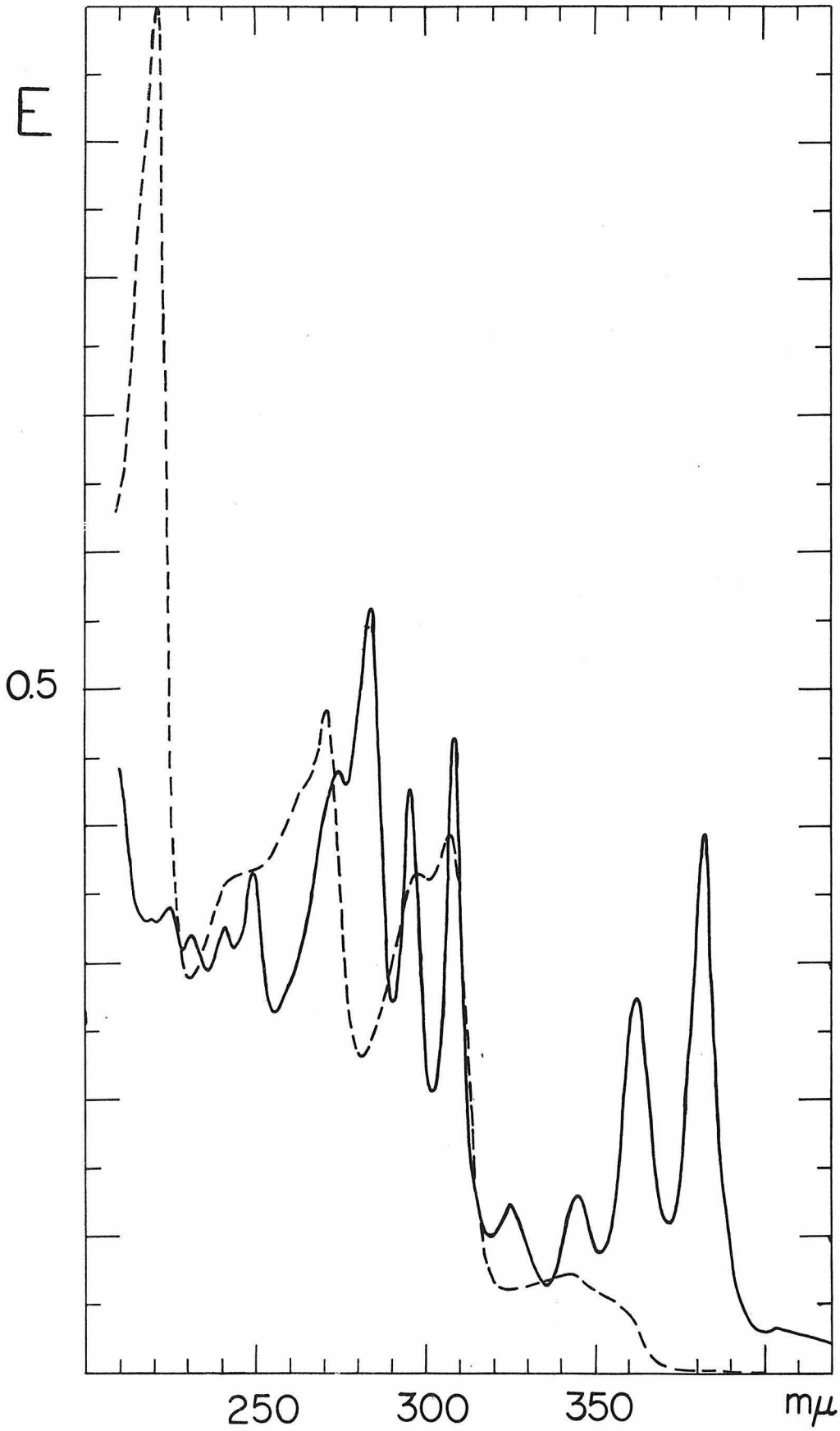


Figure 17. Extinction curves in hexane of unidentified crystalline compounds ex Mitella: _____, compound C; - - - -, compound H.

compounds. Several of these substances had also been obtained from the thatched barnacles collected near Corona del Mar. The polycyclic aromatic compounds isolated from the two species of barnacles are listed in Tables I and II. The identification of the five hydrocarbons mentioned is briefly described below.

Table I

List of Identified Polycyclic Aromatic Hydrocarbons Obtained from Barnacles

Tetraclita squamosa rubescens (ex Corona del Mar)	Extinction Curve (Figure)	Mitella polymerus (ex La Jolla)	Extinction Curve (Figure)
		Coronene	18
1,12-Benzperylene	2	1,12-Benzperylene	19
3,4-Benzpyrene	4	3,4-Benzpyrene	21
Chrysene	6	Chrysene	23
Fluoranthene	7	Fluoranthene	24
Anthracene	10		
Phenanthrene	11		

3,4-Benzpyrene. A small sample of a crystalline substance showed most of the typical maxima of 3,4-benzpyrene in its extinction curve (Figure 21). The identification of this preparation with 3,4-benzpyrene was confirmed by its non-separation on the chromatographic column from an authentic sample and furthermore, by the coupling reaction with p-nitrobenzenediazonium chloride. The dye obtained

Table II

List of Unidentified Crystalline Polycyclic Aromatic Compounds Obtained from Barnacles

Compound	Source (species)	Melting point	Color of crystals	Fluorescence in solid state	Fluorescence in hexane solution	Figure
A	Tetraclita	149-153°	yellow	yellowish	intense yellow-green	1
B*	Tetraclita	178-184°	pale yellow	intense green	blue-violet	8
C	Mitella		pale yellow	yellow-green	intense blue	17
D	Mitella		pale yellow	greenish	gray-blue	16
E**	Mitella	157-160°	yellow	yellowish	intense yellow green	1
F	Mitella	199-204°	almost colorless	greenish	intense blue	20
G	Mitella		pale yellow	yellow-green	light blue	20
H*	Mitella	107°	colorless	blue-violet	intense blue-violet	17

* Molecular weight found in exaltone: compound B, 184; compound H, 181.
** Mixed melting point of compounds A and E: 155-159°.

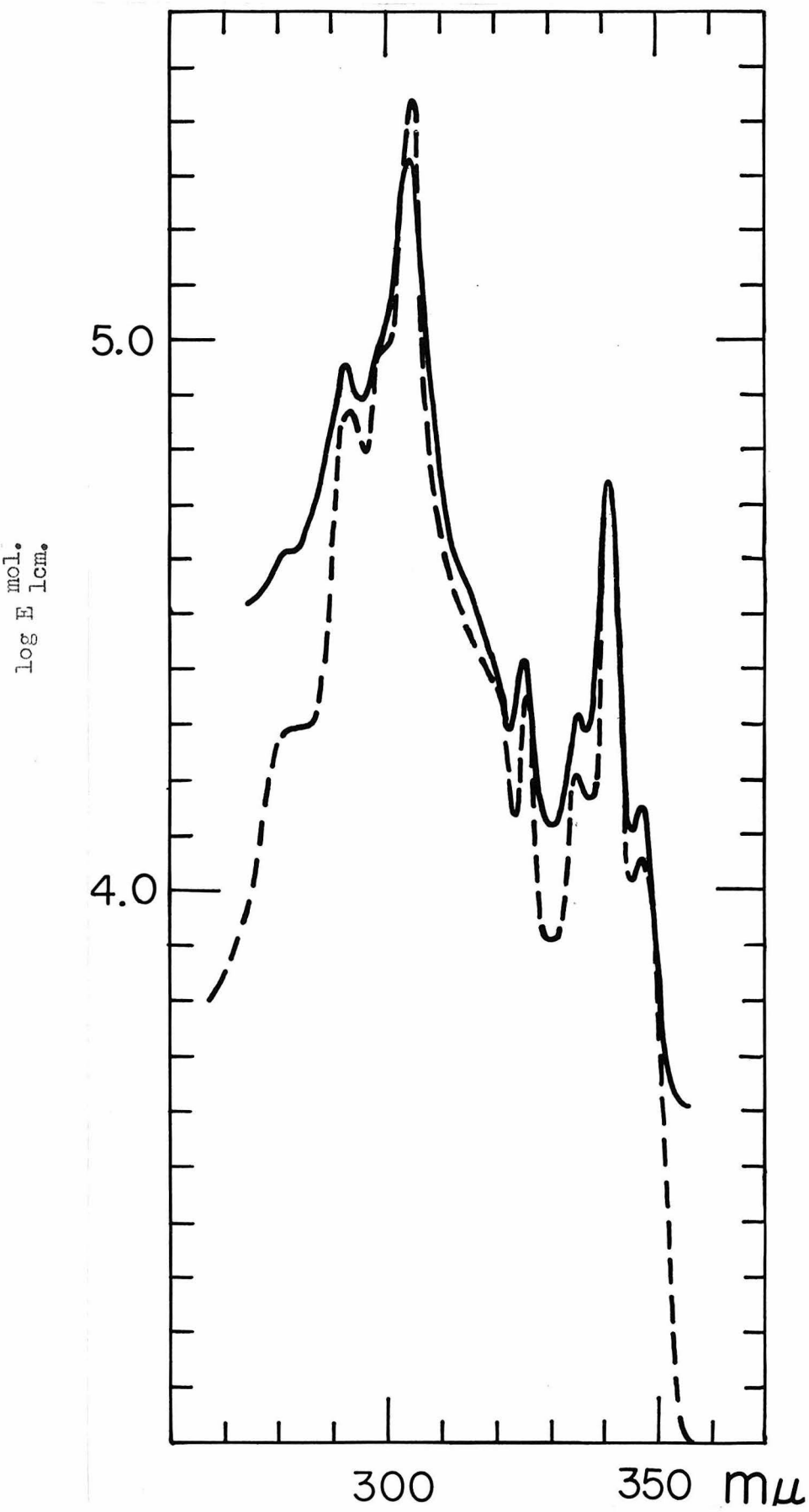


Figure 18. Molecular extinction curves of coronene, in chloroform: ——, crystalline fraction ex Mitella; - - - -, curve given by Patterson (37) for coronene.

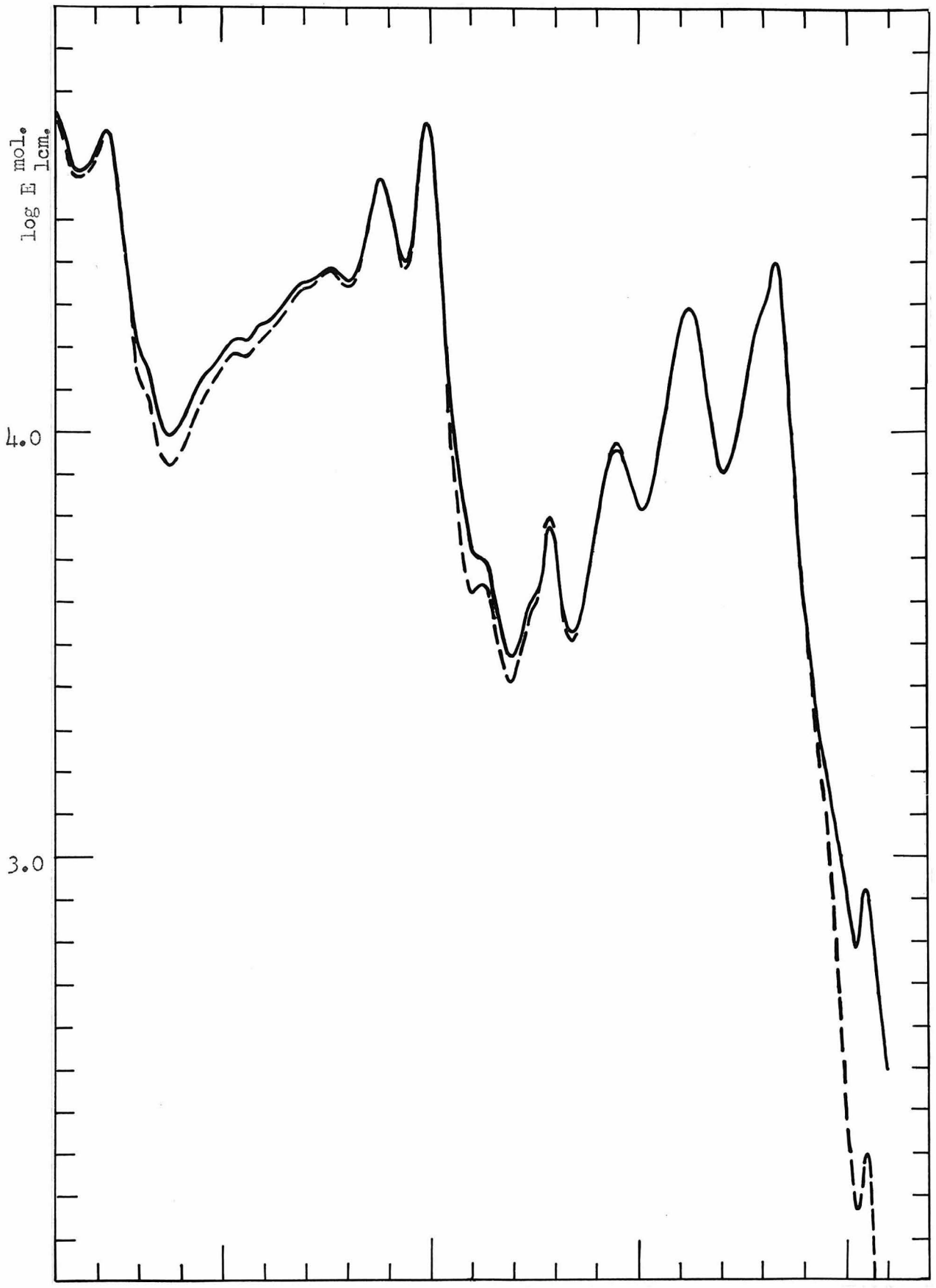


Figure 19. Molecular extinction curves of 1,12-benzperylene, in hexane: _____, crystalline fraction ex Mitella; - - - -, synthetic 1,12-benzperylene (ex perylene).

did not separate chromatographically from the one prepared from pure 3,4-benzpyrene; moreover, both dyes displayed similar extinction curves (Figure 22).

A comparison of Figures 4 and 21 shows that the spectrum of the 3,4-benzpyrene preparation ex Mitella agreed even better with that of the pure reference sample than did the curve of the corresponding preparation ex Tetraclita. After resolution of the crude peduncle extract, the "3,4-benzpyrene" fraction was observed to be chromatographically heterogeneous - consisting of three poorly separated zones. Nevertheless, the latter could be separated by careful cutting. The crystalline preparation obtained from the lower zone gave the 3,4-benzpyrene spectrum as shown in Figure 21, while the top and middle zones yielded unidentified crystalline compounds designated as F and G, respectively (Figure 20). By comparing their curves with those represented in Figures 3 and 4, it becomes evident that some of the discrepancies in the spectrum of the "3,4-benzpyrene" fraction obtained from Tetraclita may well have been caused by the presence of these two compounds (F and G) (e.g., the peak at 307 mu. which does not belong to the curve of pure 3,4-benzpyrene (Figures 3 or 4) corresponds to the main maximum of compound F). However, despite the elimination of F and G from the sample, the 3,4-benzpyrene fraction ex Mitella still contained several peaks which were not found in the curve

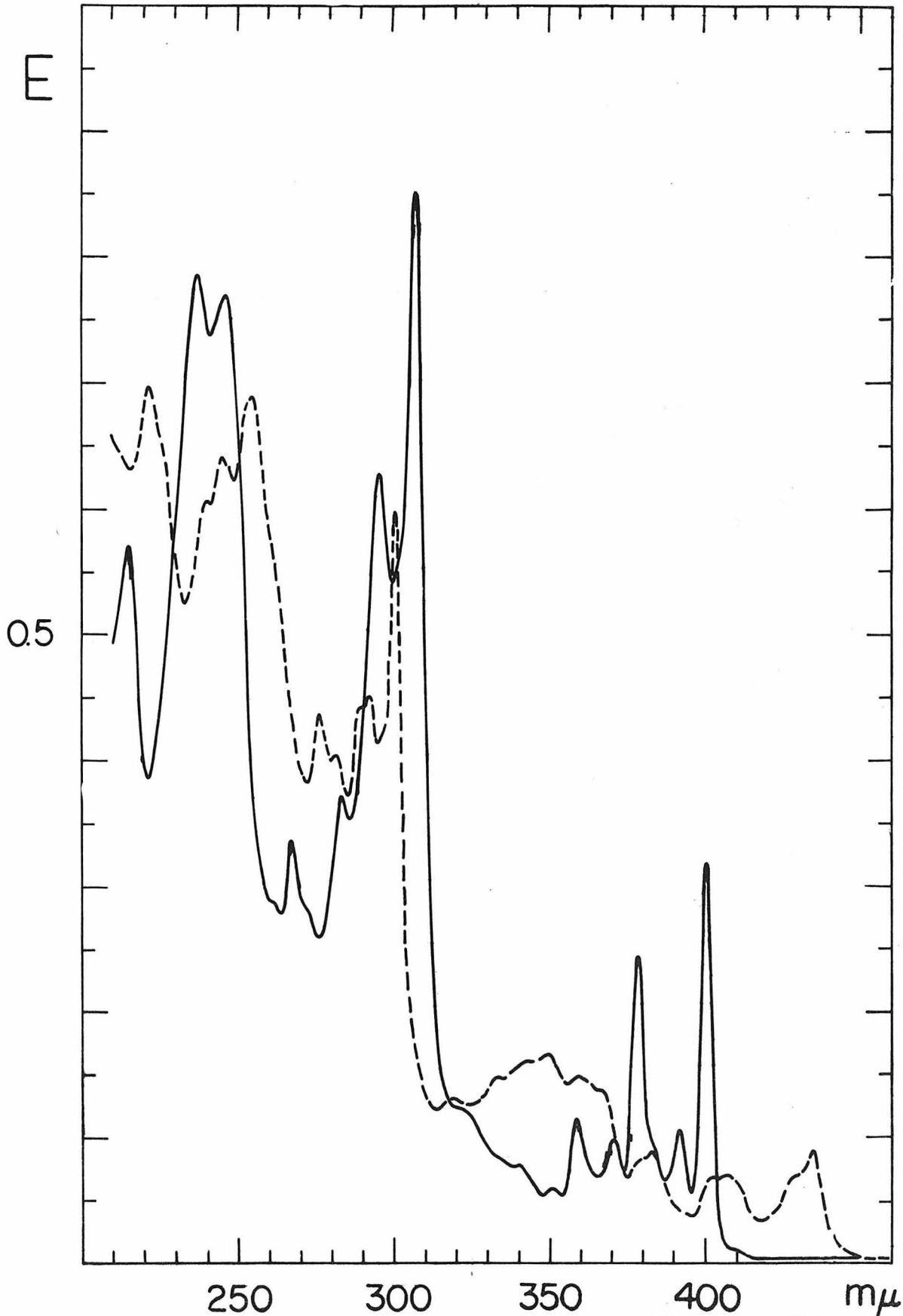


Figure 20. Extinction curves in hexane of unidentified crystalline compounds ex Mitella: _____, compound F; - - - -, compound G.

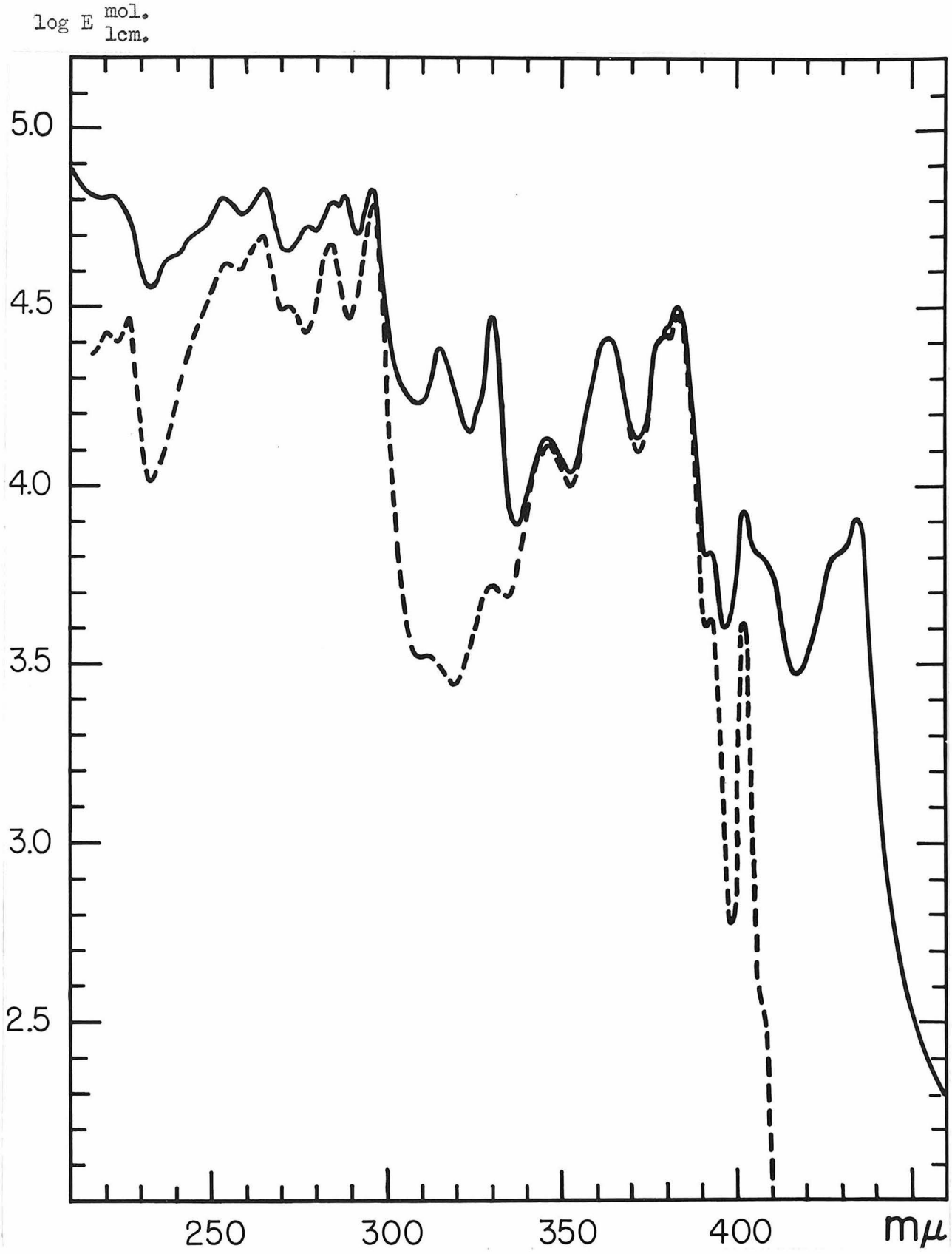


Figure 21. Molecular extinction curves of 3,4-benzpyrene, in hexane: _____, crystalline fraction ex Mitella; - - - -, commercial, chromatographically purified 3,4-benzpyrene.

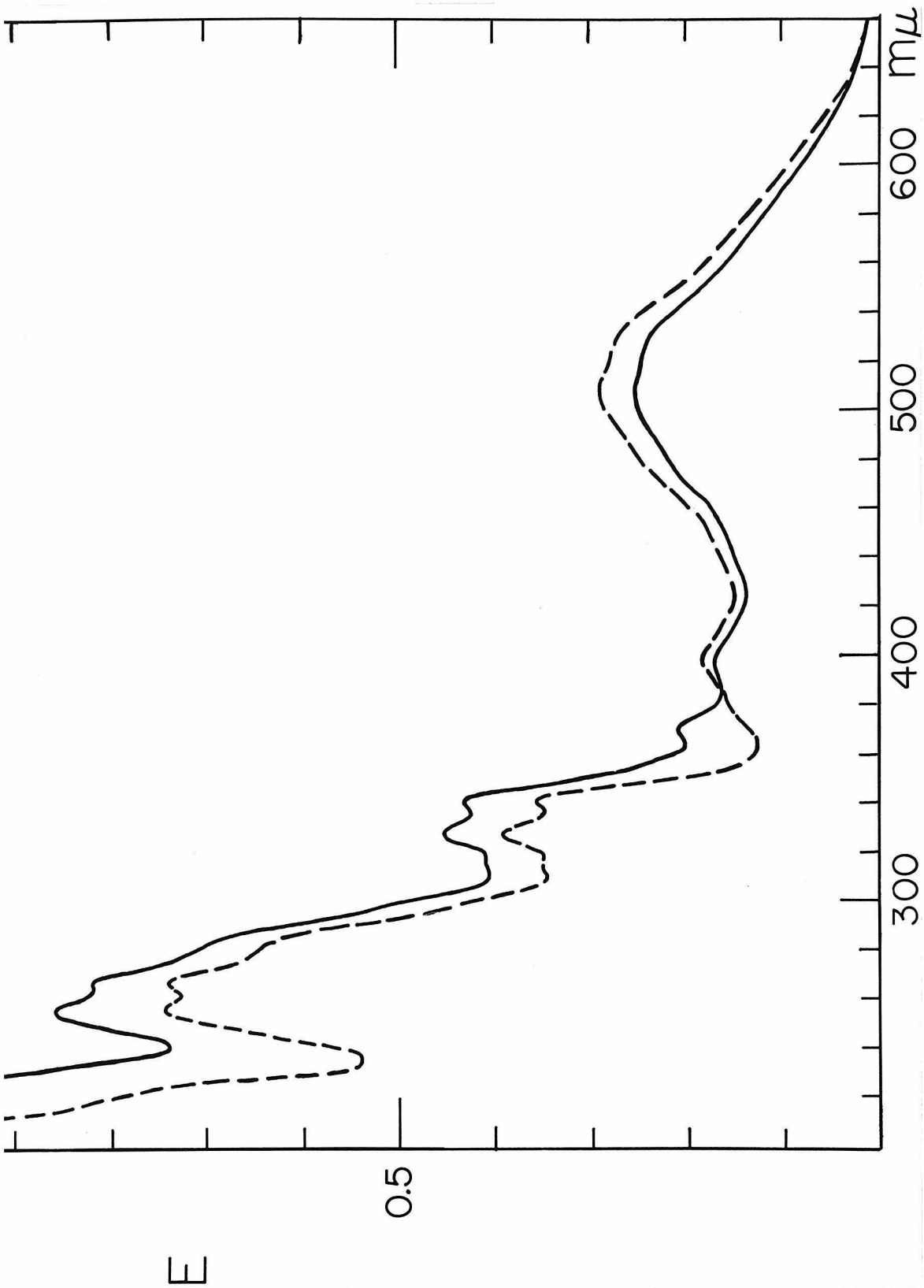
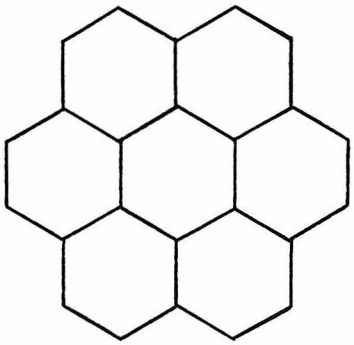


Figure 22. Extinction curves of the coupling product of 3,4-benzpyrene and p-nitrobenzenediazonium chloride, in hexane: _____, dye obtained from crystalline fraction ex Mitella; - - - -, dye from commercial 3,4-benzpyrene.

shown by the Tetraclita preparation, (277.5, 288, 316, and 330.5 mμ. in hexane). These maxima were probably caused by 1,2-benzpyrene which exhibits high absorption peaks at these very wave lengths*.

Coronene. A pale yellow crystalline preparation (a little less than 1/2 mg.) with green fluorescence was identified as coronene. Although no authentic sample of coronene could be obtained for a direct comparison, the presence of this hydrocarbon in Mitella is evident from the similarity in the extinction curve of the preparation to that reported for coronene (37) (cf. Figure 18 and Table III).



coronene

1,12-Benzperylene, chrysene and fluoranthene. The spectroscopic identification of two fluorescent crystalline samples with 1,12-benzperylene (Figure 19) and chrysene (Figure 23) was

* Wave lengths of extinction maxima of pure 1,2-benzpyrene, in alcohol: 237; 257, 267, 278, 289; 304, 316.5, 331.5; 348, 357, 366, 377, 383, 388 mμ. (11).

Table III

Wave Lengths of Extinction Maxima for Coronene in the Region from 280 mμ. to 360 mμ.*

Data for coronene ex Mitella		Data of Clar (10)		Data for coronene ex Mitella	Data of Patterson
in benzene	in hexane	in benzene	in alcohol	in chloroform	in chloroform
----	279	----	----	(282)	(282) mμ.
292.5	289.5	----	290	292	293
					(299)
304.5	301	----	302	304	305
					(316)
325.5	323	325	----	325.5	326
335.5	333	335.5	----	335.5	335
341	338	341	----	341	341
347	344.5	----	----	347	347
356	353	----	----	(355-6)	(355)

* The figures designate wave lengths, in millimicrons. Data in parentheses represent points of inflection.

confirmed by mixed melting point and mixed chromatogram tests with the authentic compounds.

The fluoranthene preparation obtained from Mitella was considerably less pure than the sample isolated from Tetraclita (Figure 24).

Unidentified crystalline polycyclic aromatic compounds. Of the six such fluorescent compounds obtained from the goose barnacles (cf. Table II), two (F and G) have already been described as being

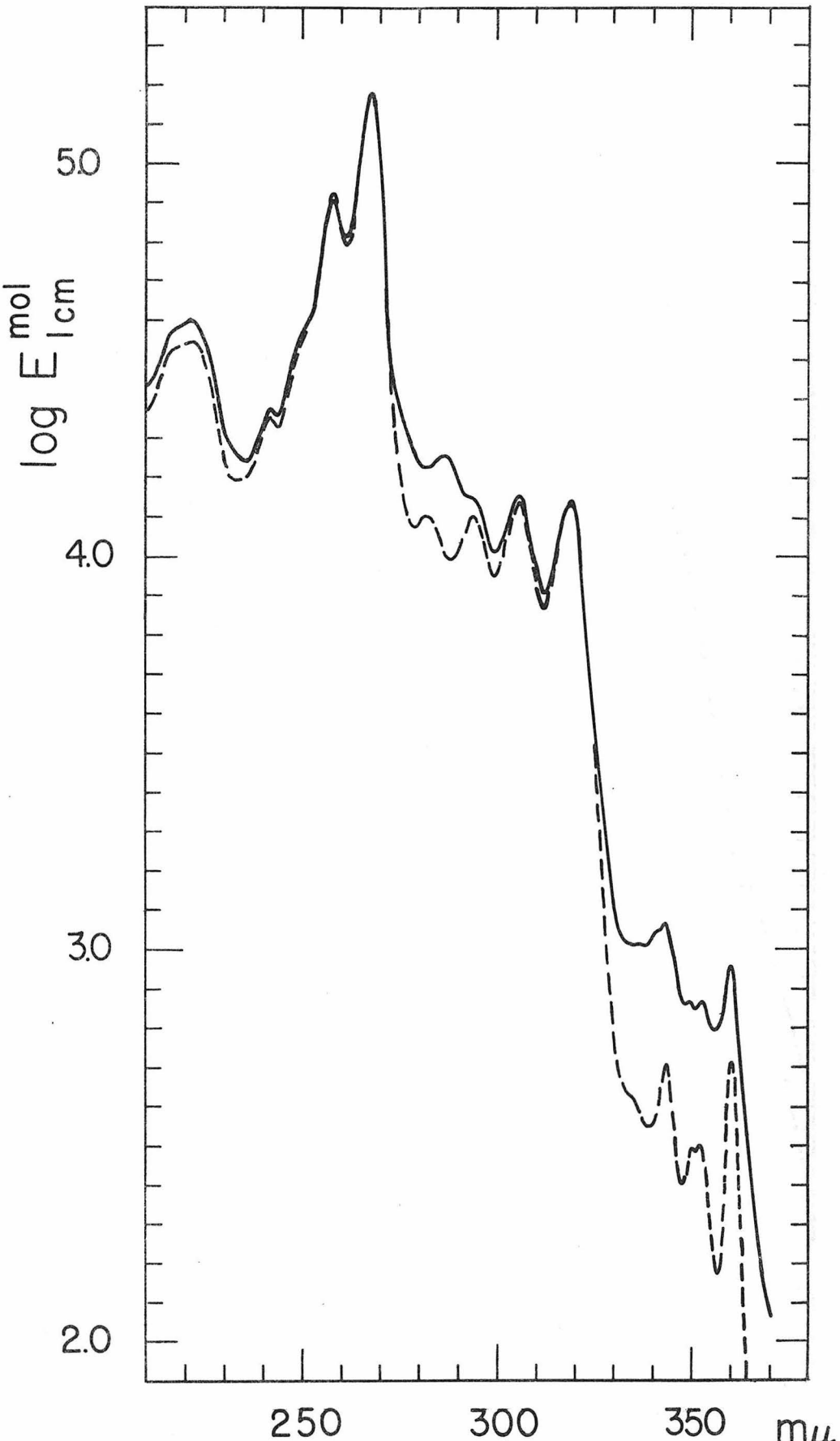


Figure 23. Molecular extinction curves of chrysene, in hexane: _____, crystalline fraction ex Mitella; - - - -, commercial, chromatographically purified chrysene.

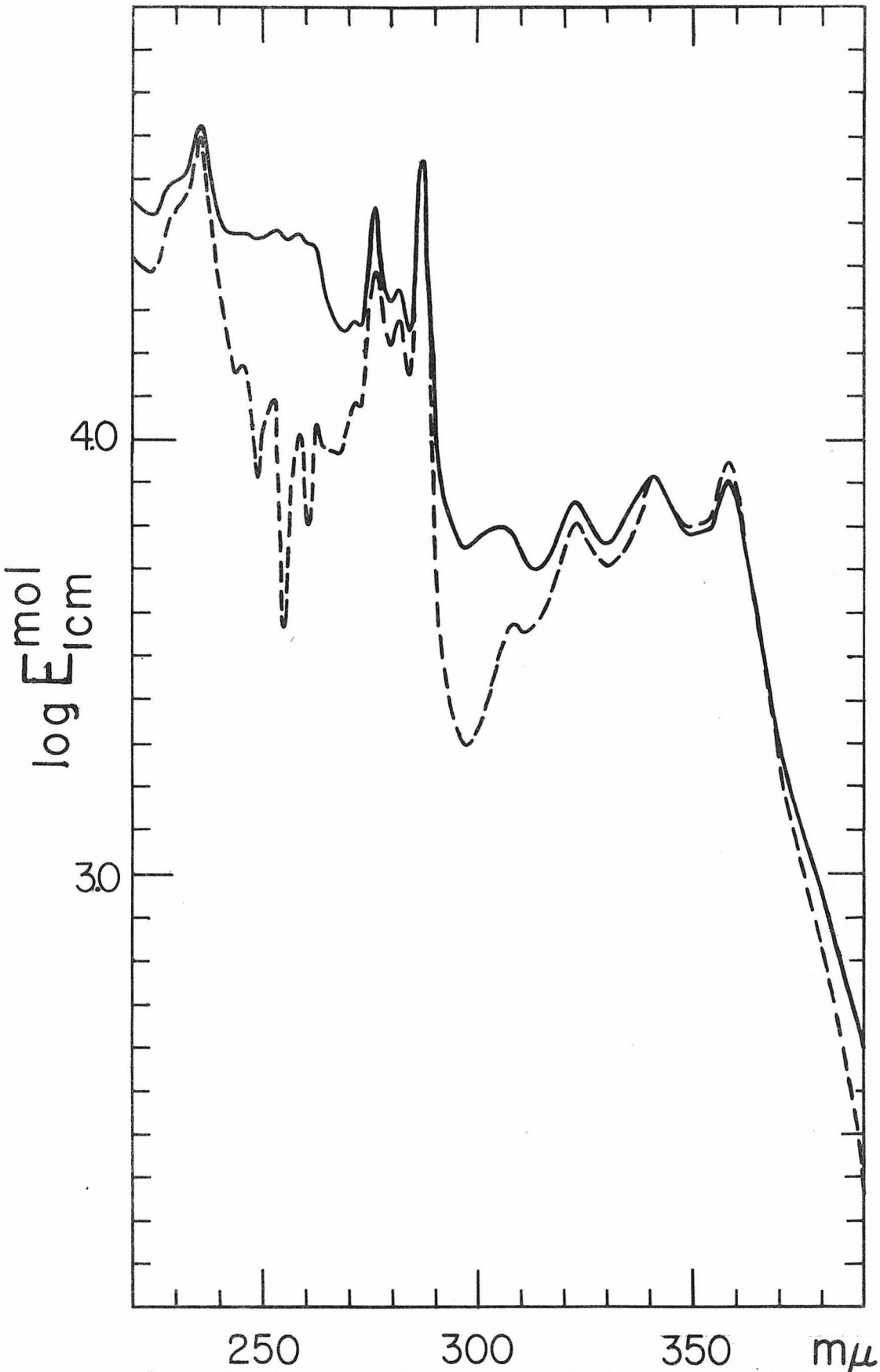


Figure 24. Molecular extinction curves of fluoranthene, in hexane: _____, crystalline fraction ex Mitella; - - - -, commercial, chromatographically purified fluoranthene.

probable contaminants of the Tetraclita sample of 3,4-benzpyrene. It should be mentioned that the general features of the extinction curve of compound F, (Figure 20) , especially the alternating high and low absorption peaks in the long wave length region, resemble those of 3,4-benzphenanthrene (34). However, the spectrum of the latter is more compressed (absorption bands located between 260-365 mμ.) than that of compound F (maxima located between 220-400 mμ.).

The spectrum of compound C (Figure 17) contained a number of maxima and features which were found in the curves of 3,4-benzpyrene (Figure 21) and 1,12-benzperylene (Figure 19) (cf. especially the region, 320-390 mμ.). Compound C, however, had a higher adsorption affinity and could be easily separated from either of these two hydrocarbons on an alumina column.

Compound E was found to be the same as compound A, an unidentified crystalline sample from the thatched barnacles (Table II). The identity of the two preparations was established by the close agreement in spectra (Figure 1), melting points, and fluorescence both in the solid state and in solutions. Moreover, they could not be separated chromatographically.

Considering the low value of the molecular weight found (181), compound H probably could at most contain three condensed benzene rings. It may be noted at this point that a fluorescent fraction obtained from the pulp of pomegranates (freed of seeds) was

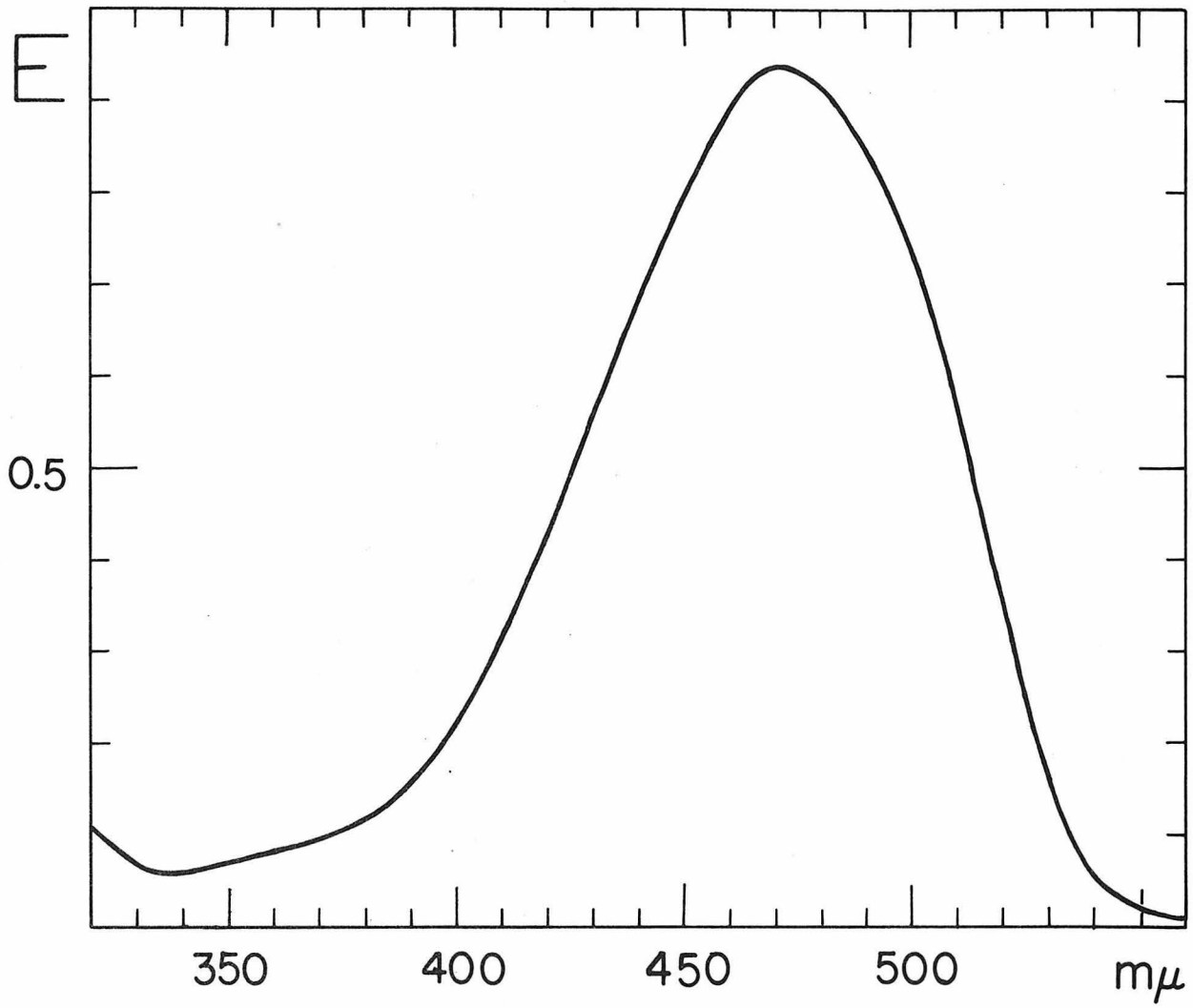


Figure 25. Extinction curve in hexane of unidentified crystalline pigment ex Mitella.

observed to be very similar to compound H (Figure 17) in adsorption affinity (alumina), in fluorescence, and in its spectrum (maxima in hexane: 220, 271, 296-7, and 306-7 mμ.).

d. Fluorescent Substances Obtained from the Beach Worm ("Blood Worm", Thoracophelia mucronata).

The beach worm Thoracophelia mucronata is a blood-red marine annelid worm which measures when mature about 30-50 mm. in length and about 0.5-2 mm. in diameter (20). The bright red and purplish colors of the worms are caused by the presence of hemoglobin, giving rise to the name "blood worms". The animals exist buried in intertidal sand mainly in the region from a fraction of an inch to a foot or more beneath the surface. They subsist on the colloidal organic matter adsorbed to the sand which they swallow and pass through their digestive system in large quantities. According to Fox, Crane, and McConnaughey (20) each worm (weighing 40 mg.) ingests 84 g. of sand per year, so that in a worm-bed 1 mile long x 10 ft. wide x 1 ft. deep (containing 158,000,000 worms) all the sand in the bed (dry weight, 2820 tons) is cycled through the alimentary tracts of the worms about once every ten weeks. The composition of a sample of fresh worms as determined by Fox and co-workers (30) is given in Table IV.

In the course of their investigations on the selective accumulation of β-carotene in Thoracophelia, Fox et al. observed the presence of several blue-fluorescent chromolipids showing single

Table IV

Composition of Fresh Thoracophelia mucronata Worms After Correction for Ingested Sand (Reference 20)

	Average Values
Sand in gut (g./100 g. of fresh tissue)	5.56 g.
Dry matter (corrected for sand)	19.76 %
Water (by difference)	80.24
Ash (corrected for sand)	2.02
Organic matter (by difference)	17.74

extinction maxima between 300 and 310 mu. in petroleum ether (20). Since the spectra of these fractions were not observed at wave lengths shorter than 300 mμ. by the authors mentioned, the beach worms had to be re-investigated in order to obtain more information as to the nature of the fluorescent substances present. In view of the polycyclic aromatic compounds isolated from two species of barnacles, it would be of interest if the presence of such substances could also be demonstrated in worms.

The first of such studies* was carried out in our laboratory by Petracek (38) who succeeded in obtaining six chromatographically separable fluorescent fractions which all displayed single extinction maxima at about 230 mμ. or 260 mμ. However, these

* These studies involved much larger samples of worms than those used by Fox, Crane, and McConnaughey. The amount of sand in these larger samples is estimated to be about 11% by Dr. Fox (17) (cf. Table IV).

spectra resembled neither those reported by Fox et al. nor those typical for polycyclic aromatic hydrocarbons.

The present study constitutes a further investigation of the blood worm. The fluorescent substances separated from the crude extract of Thoracophelia following detailed chromatographic fractionations are listed in Table V. Only fractions V and VI (the latter consisting of a colorless waxy solid) could be obtained in crystalline form. The spectra of almost all of these fractions showed one or two absorption maxima between 220 mμ. and 280 mμ. (Figures 27, 28, and 29), resembling some of those observed earlier by Petracek. None of the fluorescent substances obtained in the present investigation could be identified except for fraction IVC.

The spectrum of fraction IVC (Figure 26) is remarkable since it gives the first indication for the occurrence of polycyclic aromatic compounds in worms. Several of the peaks located in this characteristic extinction curve could be identified with those of fluoranthene. Moreover, fraction IVC was similar to the latter in fluorescence and adsorption affinity (on alumina). These observations as well as the fact that our preparation did not separate from an authentic sample of fluoranthene on the chromatographic column pointed to the presence of this hydrocarbon in the blood worms studied.

The occurrence of two peaks located at 362 mμ. and 383 mμ. (in hexane) in the spectrum of fraction IIE2 (Figure 28) and the

Table V

List of Fluorescent Fractions Obtained from Extract of Thoracophelia mucronata Following Chromatographic Resolution

(All fractions unidentified except for fluoranthene fraction, IVC)

Fraction (in chromatographic sequence)	Description of fraction in hexane solution		
	Color	Fluorescence	Absorption spectrum (Figure)
IIB2 (top)	pale yellow	weak blue-violet	27 ———
IIB3	almost colorless	bluish-violet	27 - - -
IIC2	almost colorless	grayish blue-violet	27 -.-.-.
IID2	colorless	violet-blue	27 -'-'-'
IIE1	colorless	strong violet-blue	28 - - -
IIE2	colorless	strong violet-blue	28 -'-'-'
IIF	colorless	violet-blue	28 -.-.-.
IIIA	yellow	strong gray-blue	28 ———
IIIB1	yellow	strong violet-blue	29 -'-.-'
IIIB2	yellow	moderate gray-blue	29———
IVB1	colorless	strong violet-blue	29 -.-.-.
IVB2	colorless	strong blue	29 - - -
IVC	colorless	strong greenish-blue	26 ———
V	colorless	bluish-violet	29 -'-'-'
VI	colorless	bluish-violet	29 .....

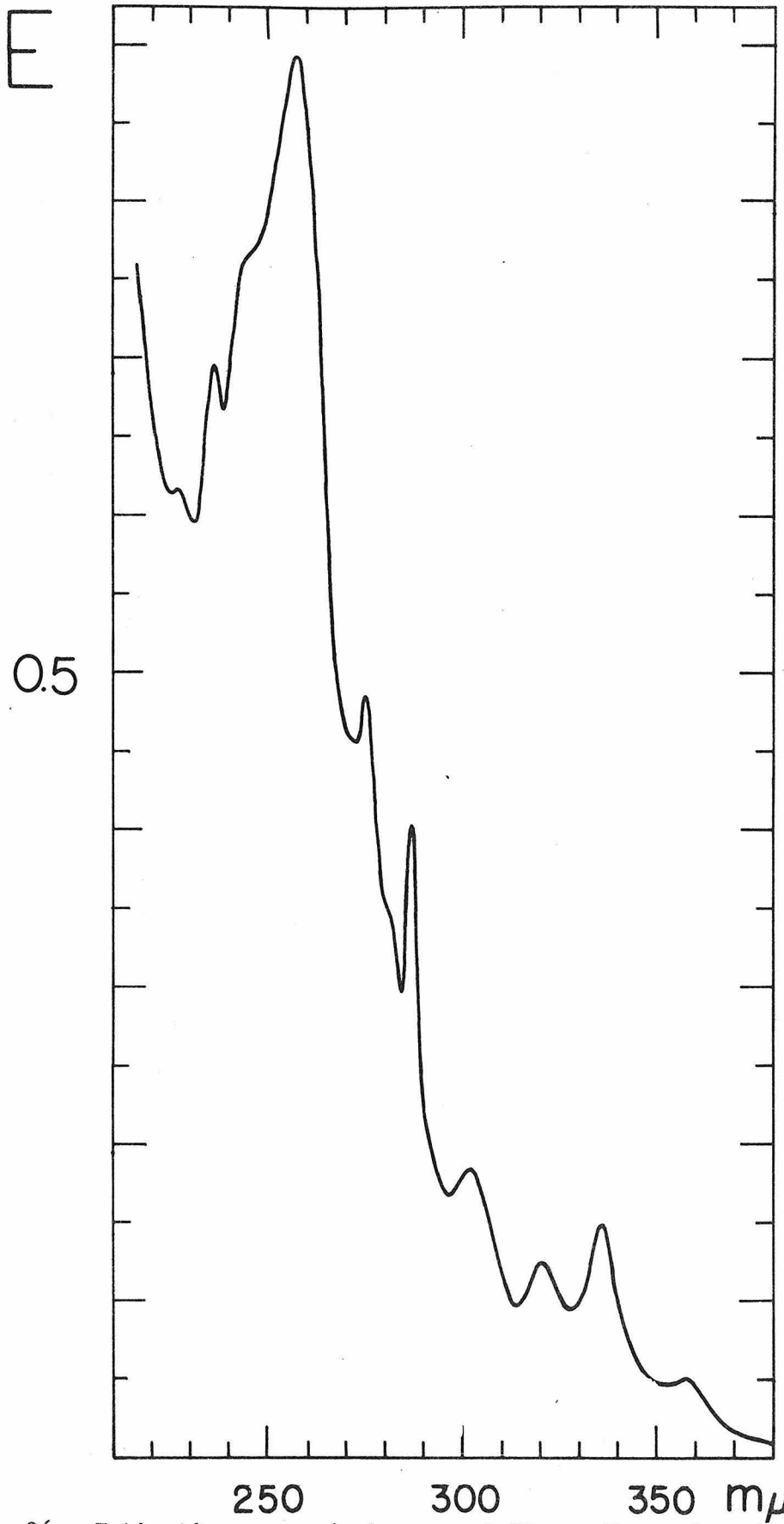


Figure 26. Extinction curve in hexane of fluoranthene fraction ex Thoracophelia.

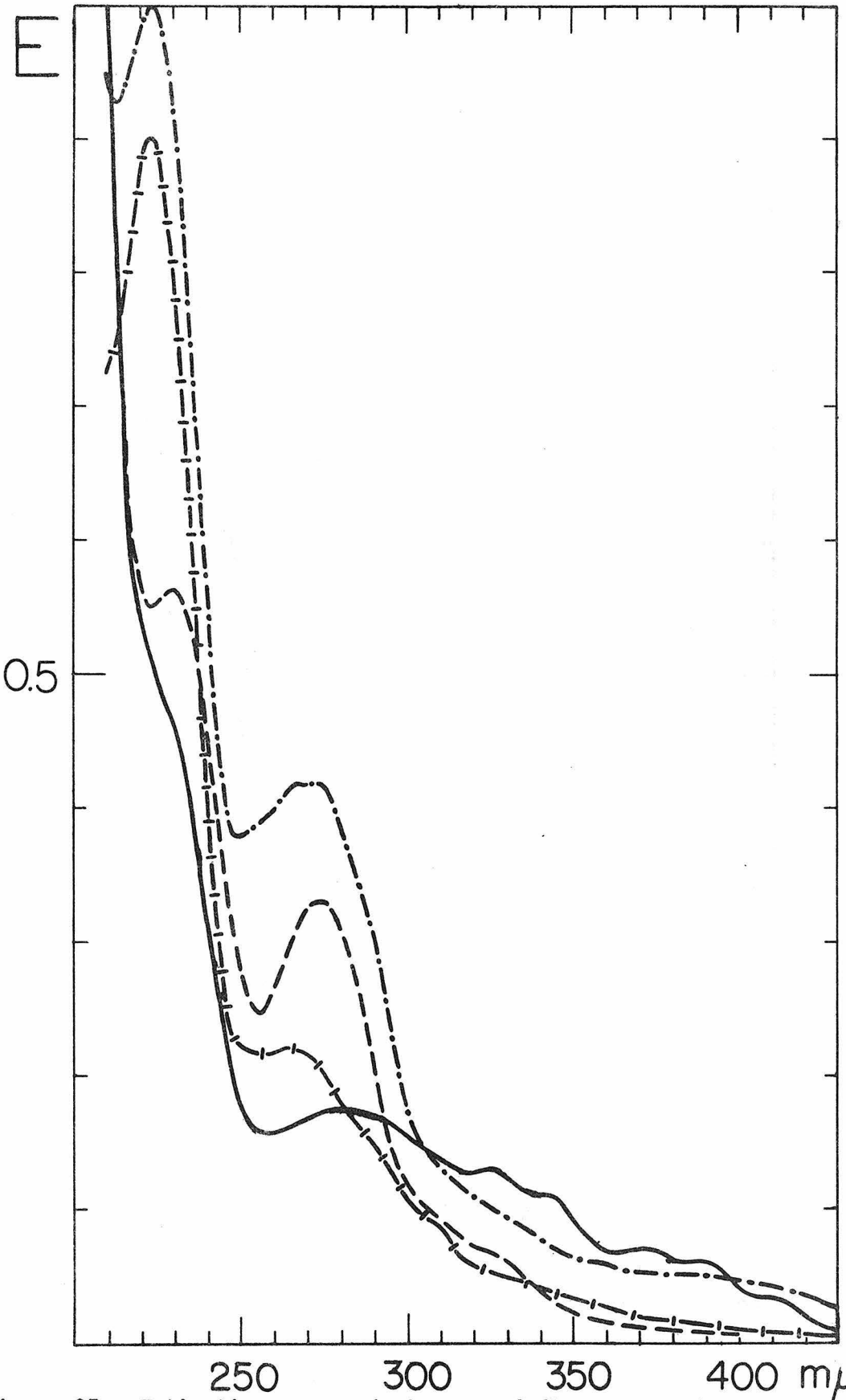


Figure 27. Extinction curves in hexane of fluorescent fractions ex Thoracophelia: _____, fraction IIB2; - - - -, IIB3; -.-.-., IIC2; -'-'-', IID2. Unidentified.

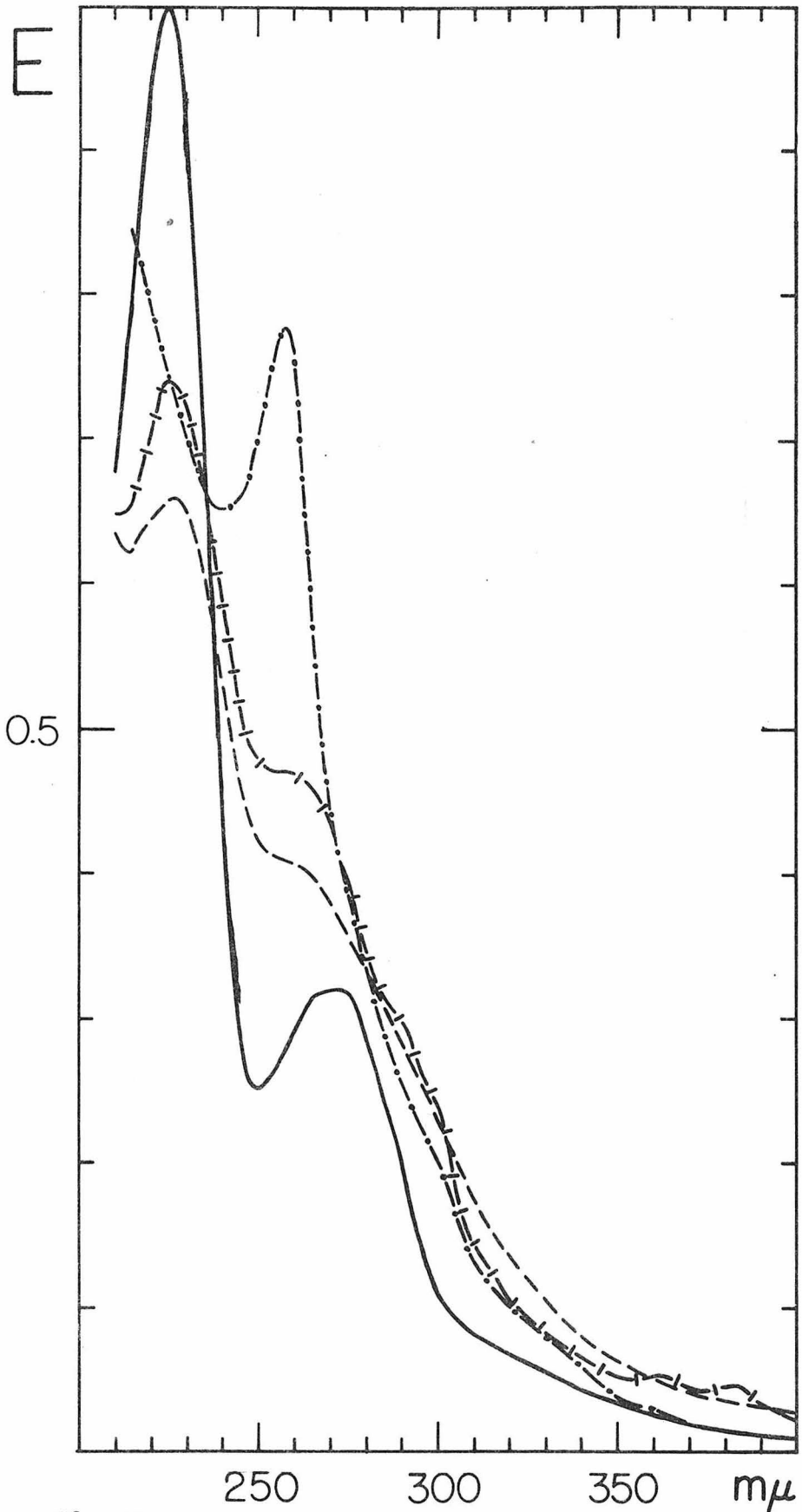


Figure 28. Extinction curves in hexane of fluorescent fractions ex Thoracophelia: - - - -, fraction IIE1; -'-'-', IIE2; -.-.-., IIF; _____, IIIA. Unidentified.

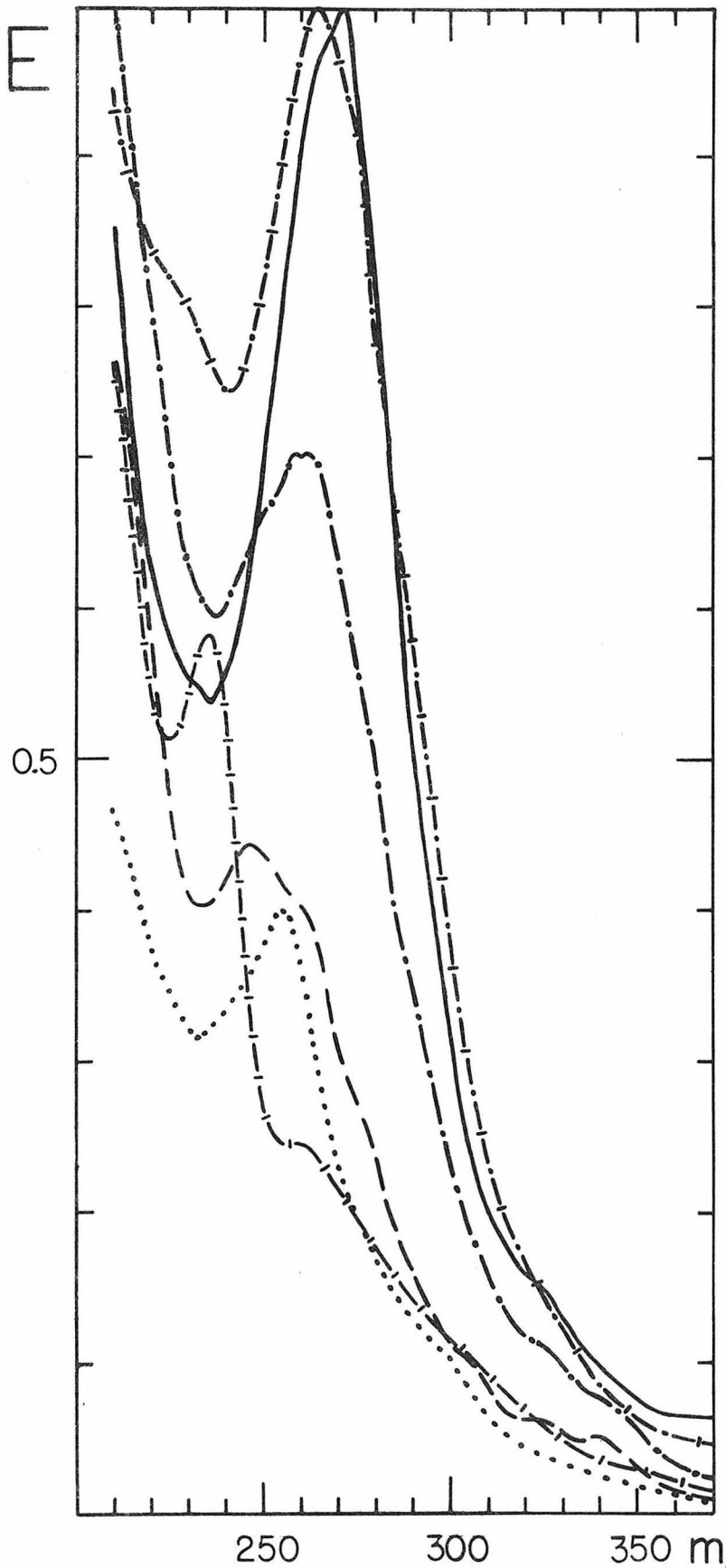


Figure 29. Extinction curves in hexane of fluorescent fractions ex Thoracophelia: -'-.-', fraction IIIB1; _____, IIIB2; -.-.-., IVB1; - - - -, IVB2; -'-'-', V; ....., VI. Unidentified.

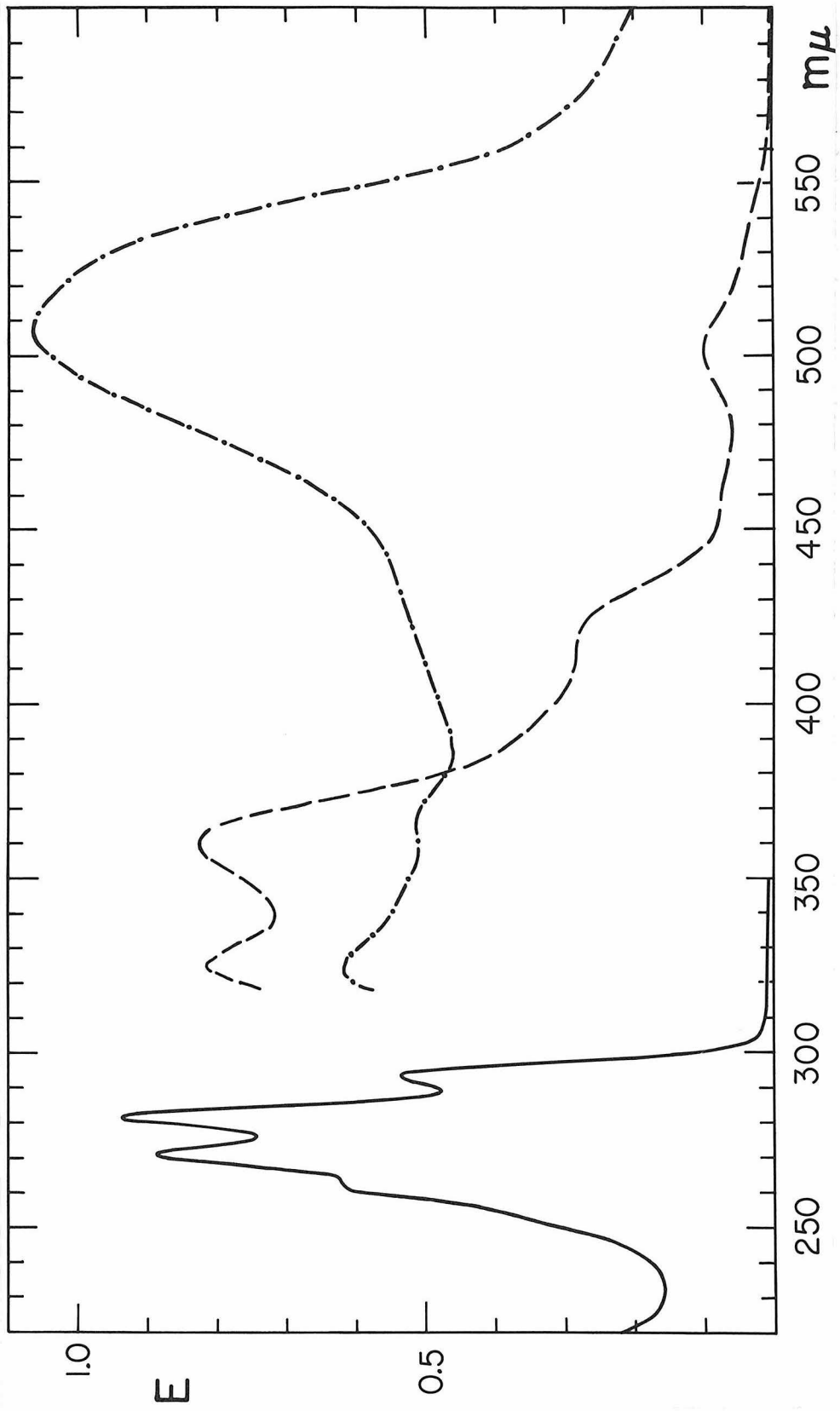


Figure 30. Extinction curves: _____, crystalline sterol isolated from Thoracophelia mucronata, in hexane; - - - -, color reaction of crystalline sterol ex Thoracophelia and antimony trichloride-acetyl chloride reagent; -.-.-., color reaction of commercial 7-dehydrocholesterol with same reagent.

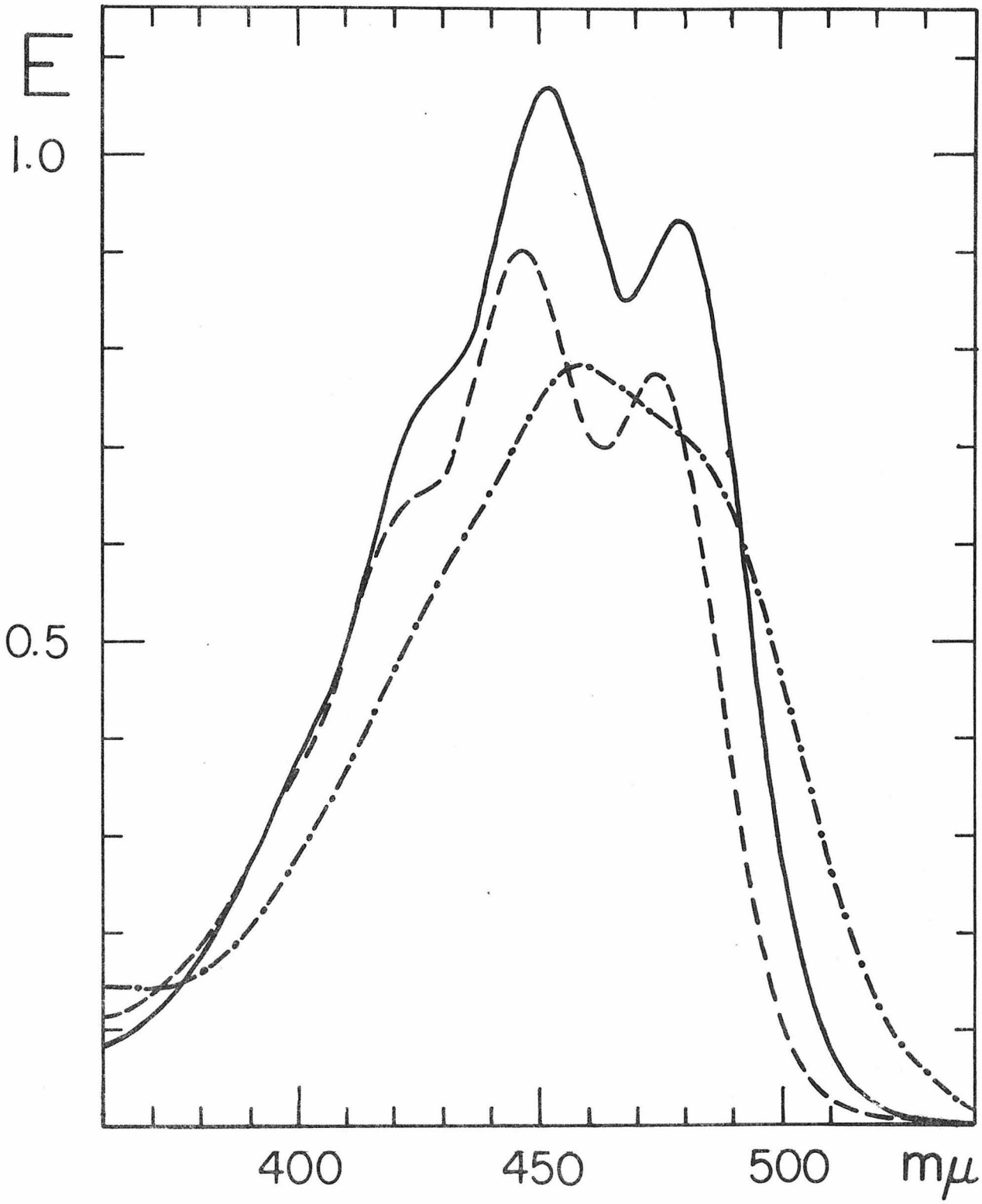


Figure 31. Extinction curves in hexane of pigment fractions ex Thoracophelia: _____, β-carotene; - - - -, neo-β-carotene U; -.-.-., unidentified.

relatively strong fluorescence of its chromatographic zone indicate that this fraction may well have contained a small quantity of a polycyclic aromatic compound.

None of the substances showing spectra such as those observed by Fox and collaborators could be found in the present study.

(e). Fluorescent Substances Obtained from a Deep-Sea Mud Sample.

Investigations by Fox, Updegraff, and Novelli (21) have shown that sediments of the ocean floor contain substantial amounts of carotenoids presumably deposited there centuries ago. Of the pigments present the polyene hydrocarbons (such as β-carotene) appeared to predominate, in contrast to the green marine flora, nearly the whole marine fauna, and the detritus of the sea, all of which show a marked tendency to accumulate oxygenated carotenoids in preference to hydrocarbon pigments. In view of the rather reducing nature of deep-sea mud*, the absence of light, and the observed predominance of the carotenoid hydrocarbons, such a system might also be expected to favor the accumulation of partially hydrogenated carotenoids such as phytofluene, phytoene, and other colorless polyenes.

* The reducing conditions probably prevalent in muds of the ocean floor are evident from the evolution of hydrogen or light hydrocarbon gases and hydrogen sulfide from numerous freshly collected specimens of deep-sea mud (21).

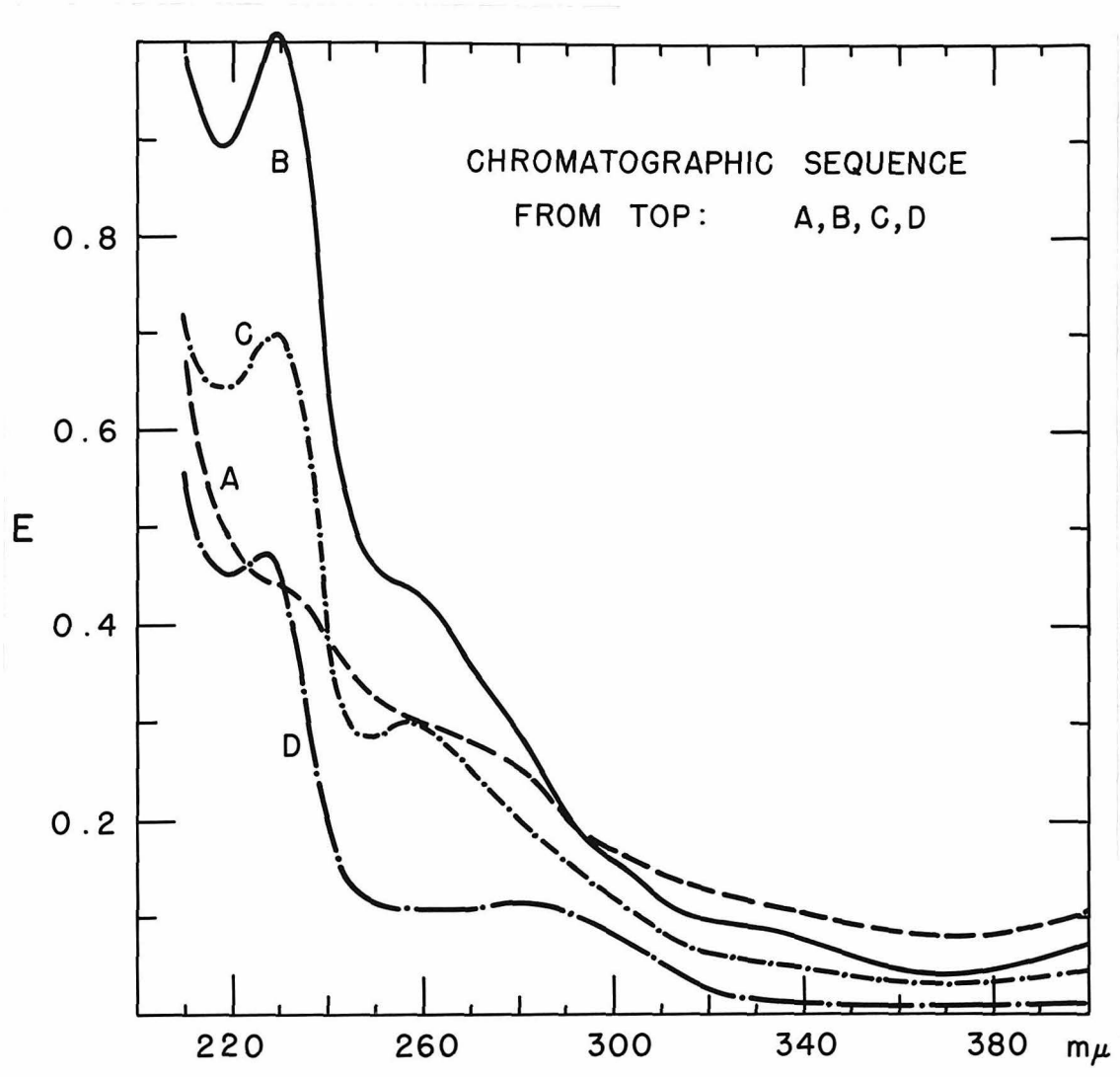


Figure 33. Extinction curves in hexane of some fluorescent compounds contained in a deep-sea mud sample.

In order to determine whether phytofluene and/or related polyenes occur in the sediments of the ocean floor, a deep-sea mud sample was tested. The sample consisted of two cores (cylindrical columns) collected from an ocean depth of 216 meters off the coast of Southern California by Dr. D. L. Fox and collaborators of the Scripps Institution of Oceanography. The cores reached a mud depth of 155-160 cm. and therefore ranged in age from fresh material at top to some 960 years at the lowest level of the core (30). The composition of such a mud sample (after drying) included 5.46% total organic matter, 0.32% total organic nitrogen, and 0.15% total lipoid-soluble matter.

The results of the chromatographic analysis of the crude extract of the deep-sea sample showed that the fluorescence of extracts was caused mainly by four substances whose spectra are given in Figure 33. No phytofluene or other similar fluorescent polyene could be detected. Moreover, strongly fluorescent zones indicating polycyclic aromatic compounds were absent from the chromatograms.

f. Fluorescent Substances Obtained from Some Marine Plants.

As mentioned in the Introduction phytofluene occurs widely in land flora, especially in plant organs which produce considerable amounts of carotenoids in the absence of chlorophyll (63).

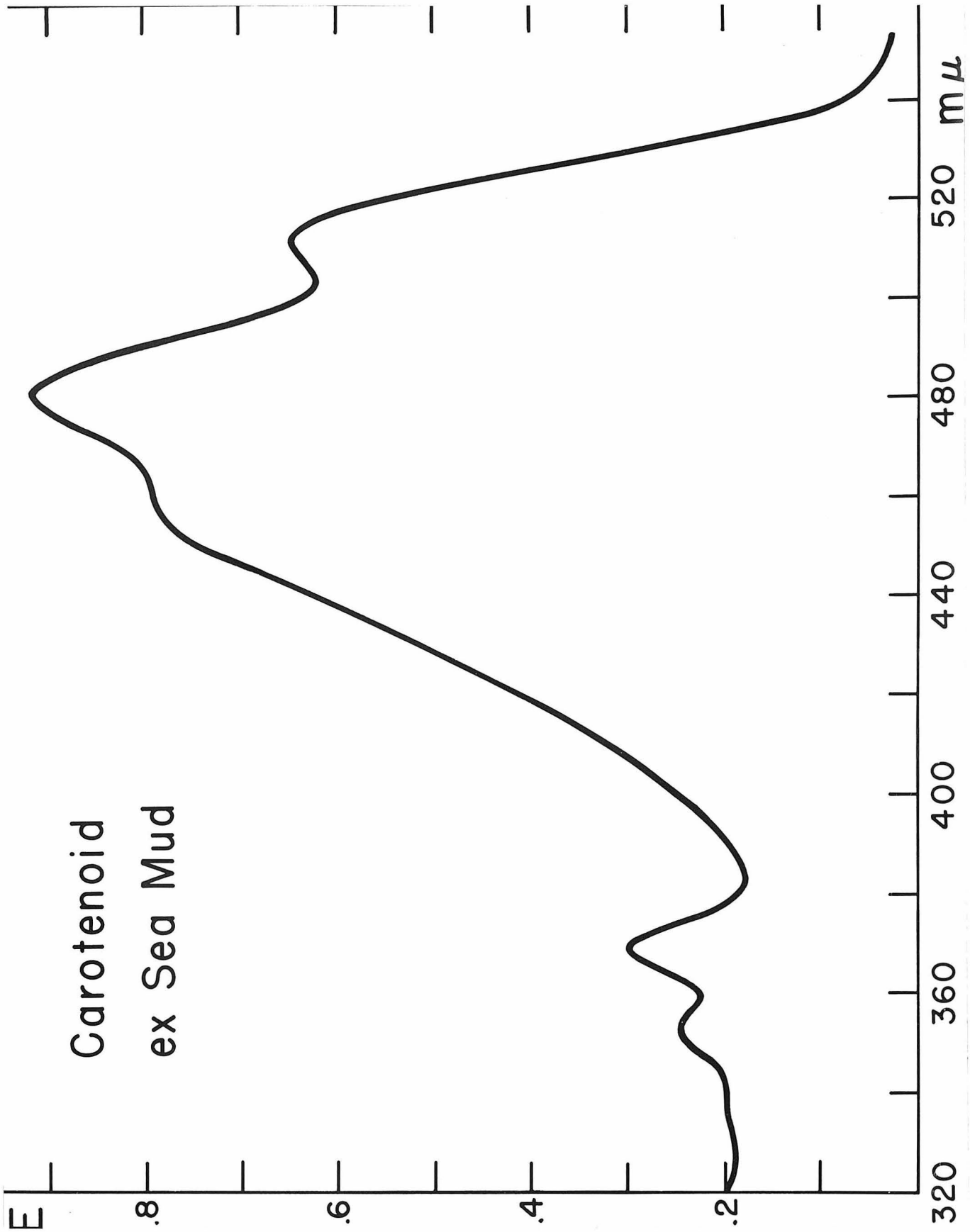


Figure 34. Extinction curve in hexane of an unidentified epiphasic carotenoid contained in a deep-sea mud sample.

Since similar information about the occurrence of phytofluene in plant materials of marine origin was not available, several species of seaweeds collected from the ocean near Corona del Mar, Calif., were tested in this respect. These samples included a green alga, Codium sp.; a brown alga, Cystoseira sp.; two red algae, Coralline algae and Gigartina sp.; and the marine angiosperm, Phyllospadix sp. (also called "mermaid's hair")*. The crude extracts showed the presence of chlorophyll in each instance.

Although the chromatograms contained carotenoid pigment zones as well as several greenish and bluish-gray fluorescent zones, no phytofluene or any similar fluorescent polyenes could be detected. Furthermore, the samples were also found to be free of polycyclic aromatic compounds. Most of the fluorescent fractions obtained displayed spectra resembling those originating from the deep-sea muds.

Two unidentified, fluorescent substances extracted from Cystoseira exhibited a pH-dependent fluorescence; dilute acids increased the fluorescence (intense green) of the solution but dilute alkali quenched it. This phenomenon was reversible. The extinction curves of the two substances mentioned were taken in solutions containing different concentrations of hydrochloric acid.

As seen in Table VI and Figure 32 the extinction curve of one of the substances mentioned (fraction II) varied moderately when

* The results reported for these seaweeds also applied to a sample of the brown alga, Laminaria sp. (68).

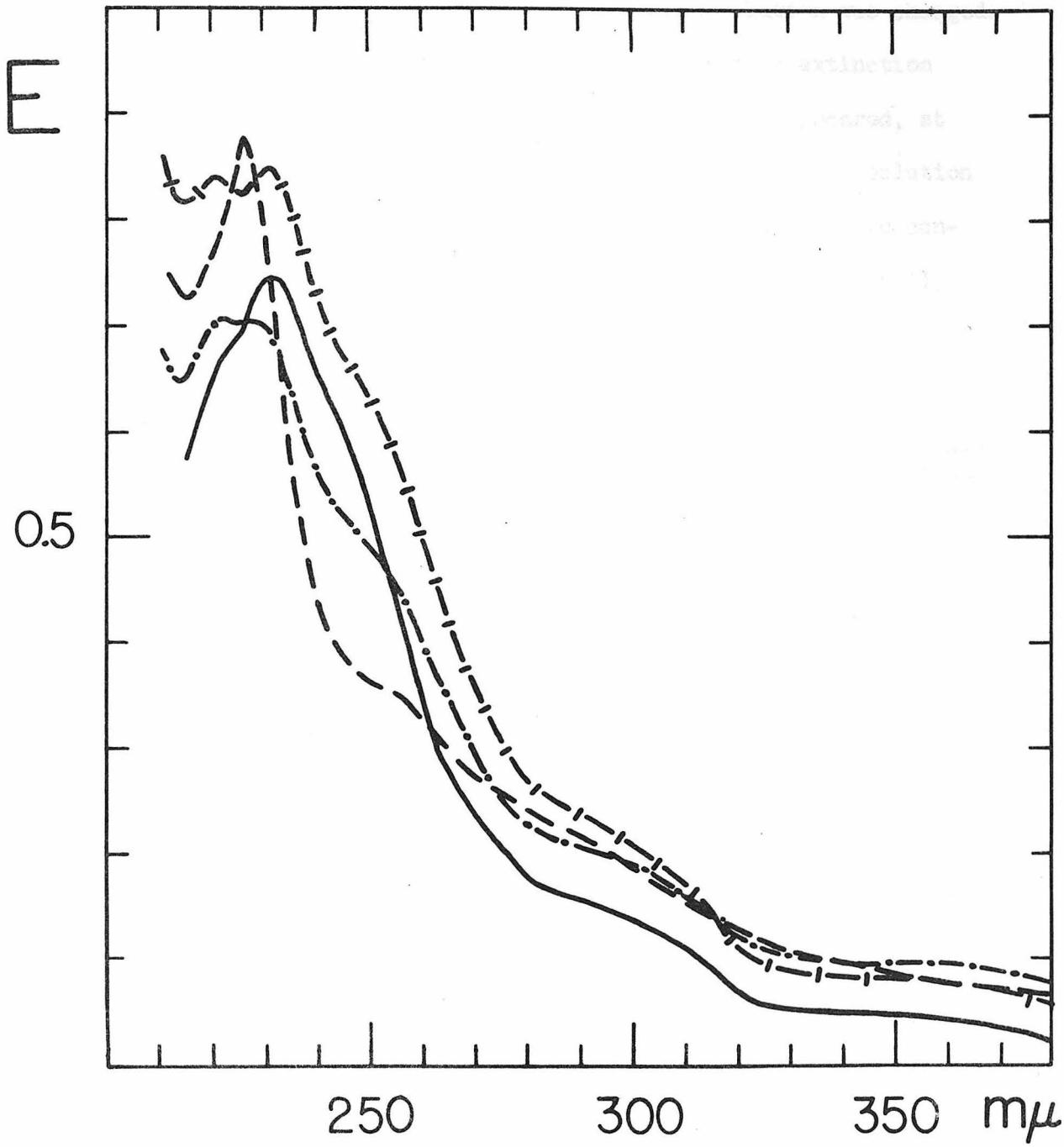


Figure 32. Extinction curves of fluorescent fraction II ex Cystoseira: _____, in ethanol; -'-'-'-, in 50% ethanol; -.-.-.-, in 50% ethanol (also 0.4 F in hydrochloric acid); - - - -, in 50% ethanol (also 3 F in hydrochloric acid). Unidentified.

either the solvent or the acid content of the solution was changed. Thus, in ethanol the preparation displayed a single extinction maximum at 232 mμ., while in 50% ethanol two peaks appeared, at 221 and 231 mμ. The two maxima shown by the 50% ethanol solution appeared to coalesce into one peak as the hydrochloric acid concentration was increased. When the latter was 3F, fraction II showed a sharp extinction maximum at 226 mμ.

Table VI

Influence of Solvent and pH on the Absorption Spectrum of Two Unidentified Fluorescent Substances Obtained from Cystoseira Seaweed

Fraction I

Solution	Conc. of HCl	Solvent % EtOH	Fluorescence	Wave length of extinction max.
A	1.2 F	80	green	----
B	0.6	90	weak bluish	one max.: 226 mμ.
C	3.3	50	green	one max.: 227 mμ.

Fraction II

Solution	Solvent	Conc. of HCl	Wave length of extinction maxima (cf. Figure 32)
D	EtOH:HOH/1:1	0 F	two max.: 221, 231 mμ.
E	"	0.4	broad max.: 222-228 mμ.
F	"	3	single max.: 226 mμ.
G	hexane	0	no definite maxima
H	EtOH	0	one max.: 232 mμ.

g. Discussion of Experimental Results.

(1). Occurrence of Polycyclic Aromatic Compounds in Certain Marine Invertebrates.

The polycyclic aromatic hydrocarbons isolated from Tetraclita barnacles (collected at Corona del Mar) and from Mitella (from La Jolla) as well as the small amounts of some of these compounds detected in Thoracophelia worms evidently do not represent normal metabolic products but appear to have reached them from external sources. This conclusion is supported by the finding that some Tetraclita samples obtained from a different habitat (La Jolla) have been found to be free of polycyclic aromatic compounds.

As is well known, such condensed-ring hydrocarbons can be obtained in large amounts from coal tar or from other products derived from coal and petroleum. A brief description of these sources and the different polycyclic aromatic hydrocarbons which have been obtained from them are given below.

In carbonization or destructive distillation of certain coals (bituminous) at temperatures above 450°, in the absence of oxygen, one of the primary products formed is coal tar. Aromatic hydrocarbons possessing three or more condensed benzene rings which have been proved to be present in such tars include anthracene (and methyl derivatives), phenanthrene (and methyl derivatives), naphthacene (and methyl derivatives), fluoranthene, pyrene, 1,2-benzfluorene, 2,3-benzfluorene, naphtho-2', 3'-1,2-anthracene, 1,2-benz-

naphthacene, chrysene, 1,2-benzanthracene, perylene, 1,2-benzpyrene, 3,4-benzpyrene, and picene (a comprehensive list is given in reference 36).

Polycyclic aromatic hydrocarbons have also been produced from petroleum by passing certain fractions (kerosene and gas oil) through tubes packed with copper catalyst at 630-680° (48). In this manner the following compounds have been obtained: phenanthrene, anthracene (and methyl derivatives), 1,2-benzanthracene, 1,2-benzfluorene, 2,3-benzfluorene, chrysene, 3,4-benzpyrene, and 1,2,7,8-dibenzchrysene.

Another process by which large quantities of polycyclic aromatic hydrocarbons are prepared is the destructive hydrogenation of coal, coal tar, pitch, petroleum, or lignite oils (10, 36). Besides the compounds mentioned, rich amounts of higher molecular weight hydrocarbons such as 1,12-benzperylene and coronene have also been obtained.

In view of the fact that all of the identified polycyclic aromatic hydrocarbons isolated from barnacles are also present in coal tars or in related products, it is very probable that they have originated from tarry materials occurring near the habitat of these animals. Such materials could have been derived from either natural marine tars or pumped bilge tars. We have obtained from the ocean near La Jolla floating pieces of tar studded with small

goose barnacles which had attached themselves onto the surface. It would be of interest to examine such marine tars for the presence of polycyclic aromatic substances.

The fact that both species of barnacles in which the aromatic substances were found, although originating from different habitats, had been growing on pier pilings suggests that the wood-preserving creosotes (coal tar distillates) might have furnished the animals with the compounds in question by means of tarry detritus formed. In this connection it should also be mentioned that a small sample of goose barnacles, collected six months earlier from the same location as the sample studied in the present experiment, yielded two fluorescent fractions whose extinction curves clearly indicated the presence of 3,4-benzpyrene and fluoranthene, respectively.

Since a simple adsorption of tarry products to the exterior of the barnacles is not very likely considering the very great difference in external composition and texture between shelled-barnacles and peduncles, both of which yielded approximately the same compounds, it is reasonable to assume that such materials containing polycyclic aromatic compounds are actually ingested and accumulated inside the organism.

As mentioned earlier, the adult barnacle is a sedentary animal which procures its food by filtering out suspended particles, detritus and plankton, from the ocean. The organic matter in

detritus, which occurs in relatively large amounts in the sea, is in turn derived from decomposing bodies and dead gametes of innumerable plants and animals of all sizes, from animal excrement, and, at certain regions, from sewage, industrial wastes, and other material of land origin. Such marine detritus may exist either in coarsely particulate form or in colloidal dispersion with all intergrading particle sizes represented (19). Thus, finely divided particles of tarry substances would also be taken in by the constant, indiscriminate filtration by barnacles. It would be of interest in this connection to test samples of marine detritus collected from different locations for the presence of polycyclic aromatic hydrocarbons.

In order to define more sharply the occurrence of polycyclic aromatic hydrocarbons in nature, similar investigations should be carried out with other marine invertebrates which depend upon the abundant supplies of finely divided, suspended, adsorbed, or precipitated organic detritus as the primary source of nutrition. Some of these animals are grouped according to feeding habits in Table VII. The "rakers" listed in this table feed by scraping adherent material from rocks, shells, pilings, and other submerged surfaces, while the "grazers" gather finely precipitated loose organic material from the mud surface of the ocean floor with tentacles, arms, or similar structures (19). In our limited studies

of mussels (Mytilus californianus) and some sea stars, only weakly fluorescent extracts were obtained, indicating the probable absence of marked amounts of polycyclic aromatic hydrocarbons. Similarly, no members of this class of compounds could be found in the extracts of the heart urchin Lovenia cordiformis (56) or the sea star Pisaster ochraceus (58), both samples collected near La Jolla, Calif., the former from a depth of 150 fathoms (275 meters).

Table VII

Feeding Habits of Some Marine Invertebrates*

Detritus and Plankton Feeders			Sand and Mud Feeders	
"Rakers or scrapers"	Filtering animals (mucous films)	(setae)	"Grazers"	"Swallowers"
Snails	Clams	Barnacles	Sea cucumbers	Worms
Limpets	Mussels			
Sea urchins	Oysters			
Sand dollars	Tunicates			
	Predators			
	Sea stars			

* Adapted from reference (19).

Since the polycyclic aromatic hydrocarbons are lipoid-soluble substances, their accumulation by certain marine animals (barnacles) may be compared to the accumulation of other fat-soluble compounds, such as the carotenoids which also originate from exogenous sources. One could speculate that the absence of polycyclic aromatic hydrocarbons from some marine invertebrates studied, such as the sea star

and sea urchin (and probably the mussel), is not unreasonable, since the selective accumulation of only certain types of carotenoids by a given species of animal is well known (18). For example, mussels (Mytilus) assimilate only xanthophylls (oxygenated carotenoids) from marine detritus while quantitatively rejecting the carotenes (hydrocarbons). In contrast, the beach worm (Thoracophelia) takes up predominantly β-carotene, while xanthophylls are largely destroyed and rejected. As is well known, in land animals certain species (hens and other birds) are able to accumulate only (or predominantly) oxygenated carotenoids, while other animals (horses and cattle) absorb only the carotenoid hydrocarbons. Some species (swine, sheep, and goats) take up neither kind, while still others (man and frog) accumulate all types of carotenoids without selection (51).

It is unknown to what extent, if at all, barnacles or similar organisms are able to modify the composition of polycyclic aromatic compounds ingested. It is known that many organisms are capable of absorbing and assimilating various hydrocarbons. With respect to polycyclic aromatic hydrocarbons, such investigations have been centered on mammals whose detoxication mechanisms were tested. Thus, 3,4-benzpyrene has been observed to be oxidized (and thereby detoxicated) in the rat to 8-hydroxy-3,4-benzpyrene (slight carcinogenic activity) and to 3,4-benzpyrene-5,8-quinone (non-carcinogenic), both of which may actually inhibit tumor growth (1, 2).

Similarly, 1,2,5,6-dibenzanthracene is detoxicated to yield the non-carcinogenic 4',8'-dihydroxy-1,2,5,6-dibenzanthracene (13, 8, 26).

However, the biological relationship of the polycyclic aromatic hydrocarbons to certain lower forms of life may be completely different from that just mentioned. Many species of bacteria, yeasts, and molds have been observed to be capable of utilizing both aliphatic and aromatic hydrocarbons as the sole source of energy and carbon (for a review of the assimilation of hydrocarbons by micro-organisms, cf. reference 69). In these micro-organisms the oxidation of hydrocarbons is accompanied by oxygen uptake (in aerobes) and the formation of carbon dioxide. The remaining carbon is converted to cell constituents and intermediate degradation products such as organic acids, ketones, alcohols, aldehydes, and modified hydrocarbons. Nitrate-reducing and sulfate-reducing bacteria are also capable of assimilating hydrocarbons.

Those micro-organisms which can utilize polycyclic aromatic hydrocarbons in this manner include certain soil bacteria which are able to assimilate anthracene and phenanthrene and some sulfate-reducing bacteria which are able to assimilate phenanthrene and retene (46, 47). Cultures of certain marine bacteria, when introduced into systems containing no other organic material except

the test hydrocarbon dispersed on ignited sand, have been observed to assimilate not only anthracene, phenanthrene, and 1,2-benz-anthracene but also the strong carcinogen, 1,2,5,6-dibenzanthracene (44) (cf. Table VIII).

Table VIII

Assimilation of Some Polycyclic Aromatic Hydrocarbons by Marine Bacteria*

Hydrocarbon (25 mg.)	CO_2 produced in 4 days at 32°	% of hydrocarbon oxidized
Naphthalene	44.2 mg.	51 %
Anthracene	53.5	64
Phenanthrene	58.5	68
1,2-Benzanthracene	41.2	47
1,2,5,6-Dibenzanthracene	11.6	13

* Adapted from reference (44).

(2). Occurrence of Fluorescent Organic Substances Other than Polycyclic Aromatic Compounds in the Marine Animals, Plants, and Deep-sea Mud Sample Studied.

Although all of the marine materials investigated in the present study contained carotenoid pigments, neither the animal extracts or plant extracts yielded any trace of phytofluene or other closely related, colorless polyenes. These negative findings are in accordance with some previous studies (63). Animal products such as egg yolk, dried milk powder, pig's liver, commercial ox gall concentrate as well as sardine meal, sardine oil, and dog-fish oil,

etc. were all found to be free of phytofluene. In land flora phytofluene does not seem to occur in chlorophyll-rich materials (grass, green leaves, spinach, etc.). This generalization can be probably extended to include marine plants, since all of the chlorophyll-containing seaweed species were found to be free of phytofluene.

In spite of the absence of light and the reducing conditions, no phytofluene could be detected in our deep-sea mud sample. Although the circumstances just mentioned would be expected to decrease the chances for a chemical or photochemical destruction of phytofluene, they by no means could exclude biological degradation of phytofluene by marine bacteria. Investigation of a fresh intertidal ocean mud sample has also shown the absence of phytofluene (39).

The marine materials which were found to be free of both polycyclic aromatic compounds (or contained only traces as in the Thoracophelia) and phytofluene include the Tetraclita barnacles from La Jolla, the Thoracophelia worms, the deep-sea mud sample, and the seaweeds. The fluorescence of their crude extracts was caused by unidentified substances, most of which could not be crystallized, although in a few instances waxy and amorphous appearing solids were obtained. The absorption spectra of all these compounds showed rapidly increasing light absorption with decreasing wave length (below 300 mμ.). In most extinction curves one or two small peaks appeared in the region between 220 and 280 mμ. (in hexane).

h. Some Non-fluorescent By-products Obtained from the Marine Materials Studied.

(1). Carotenoid Pigments.

As mentioned, the chromatograms of all crude extracts obtained from marine animals, plants or muds contained a number of carotenoid pigment zones. Since these pigments were regarded more or less as impurities or contaminants of the fluorescent compounds present, no effort was made to investigate them in detail. However, a few observations may be of some interest.

The chromatographically resolved pigment fractions of Tetraclita (collected from La Jolla) are listed in Table IX and their spectra are shown in Figures 13 and 14. Pigments identified on the basis of spectra included α-carotene, β-carotene, lutein, zeaxanthin, and spirilloxanthin.

The peduncles of the goose barnacle were relatively rich in carotenoids, most of which could be readily extracted with methanol. One such pigment was obtained in the form of purplish-red crystals (m.p. 176-178°) and showed the spectrum as given in Figure 25. Analysis of the crystals yielded the formula $C_{40}H_{56}O_5$. The presence of a relatively large number of oxygen atoms in the molecule was also indicated by the strong adsorption affinity. Furthermore, an ethereal solution of the pigment shaken with 24% hydrochloric acid colored the latter blue. The pigment did not appear to be either astacene (a typical crustacean carotenoid) or fucoxanthin (typical for brown algae) which also gives the color test mentioned.

Epiphasic pigments obtained from the peduncles included

Table IX

List of Some Pigments Obtained from Tetraclita squamosa rubescens (sample collected at La Jolla, Calif.)

Fraction (in chromatographic sequence)	Pigment	Wave lengths of extinction maxima* (in hexane) (mμ.)	Extinction curve (Figure)
IA	Spirilloxanthin	464-5, 493, 527	13 ———
IB	Unidentified	455-6, 482, 515-6	13 --·--
IC	Zeaxanthin	(428), 450, 477	13 -·-·-
ID	Unidentified	(460-4), 483, 515	13 - - -
IE	Lutein	422, 445, 474	13 -'-'-'
IF	Unidentified	464-6 (one max.)	13 -·-'-·
II	Unidentified	456-7 (one max.)	14 - - -
IV	β -Carotene	(428), 451, 478-9	14 ———
VB	α -Carotene	422-3, 445, 474	14 -·-·-

* Wave lengths in parentheses represent points of inflection.

α -carotene, β -carotene, neo-β -carotene U (perhaps formed by stereoisomerization in vitro), spirilloxanthin, and an unidentified carotenoid which did not separate chromatographically from a similar pigment of Tetraclita (fraction II in Table IX and Figure 14). The latter pigment was also obtained from Thoracophelia (fraction IIB1 in Figure 31). Its extinction curve resembled that of a polycis carotenoid, but upon iodine catalysis the maximum was merely

shifted slightly towards shorter wave lengths, while the extinction values at the maximum were diminished.

The blood worms contained besides the unidentified pigment just mentioned also β-carotene and neo-β-carotene U (Figure 31).

The deep-sea mud yielded a series of pigments, the main ones being β-carotene, neo-β-carotene U, and an unidentified, epiphasic carotenoid whose extinction curve (Figure 34) was very similar to that of fraction ID (Table IX and Figure 13) of the thatched barnacle.

(2). Colorless By-products.

From the peduncles of Mitella a small quantity of an unidentified, colorless crystalline compound (m.p. 125-126°) was isolated. Its extinction curve (Figure 16) and very weak fluorescence indicated that it did not belong to the polycyclic aromatic class of compounds.

In the course of the investigation of the fluorescent substances present in blood worms, F. J. Petracek obtained in this Laboratory a colorless sterol which was identified on the basis of its absorption spectrum as being either ergosterol or 7-dehydrocholesterol (38). Although these two provitamins D show practically identical extinction curves (25), they can be differentiated by means of the reaction with an antimony trichloride-acetyl chloride reagent (31). Within 5 to 10 minutes after the addition of the reagent, a chloroform solution of ergosterol displays a high extinction maximum at 393 mμ. while one containing 7-dehydrocholesterol gives, instead, a high extinction peak located at 322 mμ.

The failure of the crystalline preparation from blood worms to develop an absorption maximum at 393 mμ. upon treatment with this reagent indicated that the compound could not possibly be ergosterol. However, the extinction curve that resulted contained other maxima located at 360 and 420 mμ. besides the one characteristic for 7-dehydrocholesterol at 324-5 mμ.

Petracek's findings were confirmed in the present study by the isolation of a relatively large amount (300 mg. per kg. of fresh worms) of a similar sterol (spectrum, Figure 30). The latter melted at 137-141° and showed the carbon and hydrocarbon content calculated for 7-dehydrocholesterol. The extinction curve obtained upon treating the preparation with the antimony trichloride reagent differed slightly from that observed by Petracek.

2. EXPERIMENTAL PART.

a. Materials and Methods.

In all of the experimental work reported in this Thesis, certain standard techniques, equipment, and reagents were used repeatedly. In order to eliminate undue repetition of details, a description of these is given in this section which applies to all the work herein, unless specific mention is necessary for a particular case.

Adsorbents. Two brands of calcium hydroxide were used: (1) Shell Brand, Chemical Hydrate, 98% through 325 mesh, Westvaco Chlorine Products, Newark, Calif. (no longer available); (2) Sierra Hydrated Lime, Superfine, United States Lime Products Corp., Los Angeles, Calif. The former brand was used directly for chromatography, but the latter had to be mixed with Celite, No. 545, Johns-Manville Co., in order to obtain a faster rate of filtration. "Calcium hydroxide-celite" refers to a 2:1 mixture (by weight).

The alumina used was Activated Alumina, Grade F, 80-200 mesh, Aluminum Ore Co., East St. Louis, Ill., reground to -200 mesh. "Alumina-calcium hydroxide-celite" refers to a 3:1:1 mixture of alumina, Sierra Hydrated Lime, and celite. "Alumina-silica gel-celite" refers to a 3:1:1 mixture of alumina, silica gel (Davison Silica Gel, specification comm., 924-08-05-215, mesh -200), and celite.

"Silicic acid-celite" refers to a 4:1 mixture of Merck reagent silicic acid and celite.

The calcium carbonate used was Merck Precipitated, U.S.P., brand.

The sugar used was C and H Cane Sugar, Confectioners Powdered.

Solvents. Petroleum ether (Skellysolve B, b.p. 60-70°), hexane (commercial grade), benzene (reagent grade), and acetone (c.p. grade) were used in the chromatographic work. The hexane and acetone were freed of traces of fluorescent impurities by simple distillation. Acetone (c.p. grade) was used for all elutions; in some instances the eluent consisted of acetone containing 5-25% (by volume) methanol (commercial grade) or ethanol (commercial grade, 95%). For crystallizations reagent grade solvents (benzene, chloroform, absolute methanol, absolute ethanol) and redistilled hexane were used. For spectral work reagent grade solvents (benzene, chloroform, absolute ethanol) and a purified hexane was used. The latter was prepared by treating Phillips commercial grade hexane or Eastman practical grade hexane repeatedly with fuming sulfuric acid until its optical density (after washing and a final distillation) was close to that of distilled water.

General notes. For separation of extracts from the ground starting materials a basket centrifuge was used (diameter, 20 cm.; lined with cloth or filter paper; International Centrifuge, type SB, size 2). Evaporations and concentrations were performed in vacuo

(water aspirator) while a stream of dry nitrogen or carbon dioxide bubbled slowly through the solution. Sintered glass funnels were used for elutions. After the addition of hexane or/and benzene to the eluates (filtrates), the acetone was washed out with water in a continuous apparatus (32). All crystallizations were carried out in centrifuge tubes. Crystalline preparations were dried first by evaporating the solvent *in vacuo* (water aspirator) with a stream of dry nitrogen and then heating at refluxing acetone temperature *in vacuo* (0.1 mm.) for 1-2 hours in the Abderhalden drying pistol. All melting points are corrected and were taken in an electrically heated Berl block using sealed capillary tubes. For the carotenoid sample *ex Mitella* the capillary tube was sealed under vacuum (0.1 mm.).

Chromatograms. The chromatograms were observed either in the light of a General Electric Photoflood No. 1 bulb using a molded Corning filter (No. 5840; 1/4 in. thick) or in the light of a General Electric AH-4 lamp (high pressure mercury arc) equipped with a blue-purple fluorescence filter, transmitting 300-500 mμ. Other operations were carried out in weak daylight.

In the chromatograms the figures on the left refer to the width of the zones in mm.; "fl." denotes fluorescent or fluorescence.

The composition of the chromatographic developers are given as either volume-percent or volume-ratio.

Spectral measurements. Visual absorption maxima were taken

in a Loewe-Schumm evaluating grating spectroscope, Zeiss, light filter BG-7. When the position of the maxima of a given solution had been determined, a few drops of a dilute iodine solution (2-5 ug.) were then added to the spectroscopic cell, and the readings were repeated after a few minutes.

All extinction curves were taken in a Beckman photoelectric spectrophotometer, Model DU. Measurements were made with the sensitivity knob set midway between its most clockwise and most counterclockwise positions.

Microanalyses. The author is indebted to Professor A. J. Haagen-Smit and Mr. G. Swinehart for microanalyses.

b. Thatched Barnacle (Tetraclita squamosa rubescens).

(1) Thatched Barnacles from Corona del Mar, California.

(a) Extraction.

Two and a half kilograms of the thatched barnacle were collected from wooden pilings surrounded by clear sea water near the Kerckhoff Marine Laboratory in Corona del Mar, California. The fresh material was kept overnight in methanol, which became very intensely blue-fluorescent. After decantation of the methanol the whole animals were ground up with two liters of 1:1 petroleum ether-methanol in a large mortar. The suspension was shaken mechanically for 30 minutes and filtered through a Buchner funnel, after which a similar extraction was repeated five times, using petroleum ether-methanol mixtures in the volume proportions, 2:1, 2:1, 3:1, 3:1, and 3:1. Water was cautiously added to the combined filtrates in 20-liter separatory funnels; the aqueous layer was drained off and repeatedly re-extracted with petroleum ether. All the petroleum ether extracts were combined with a similar extract of the methanol used initially to cover the animals, then washed methanol-free, dried*, concentrated in vacuo to 2 liters, and finally saponified by standing in broad Erlenmeyer flasks over a layer of 20% methanolic KOH for three hours. The petroleum ether extract changed from a dark greenish-black to a dark orange upon saponification but retained the intense blue fluorescence. Water was added slowly to the mixture in order to transfer the

* All petroleum ether or hexane solutions mentioned in the experimental parts were dried with anhydrous sodium sulfate.

fluorescent substances into the petroleum ether layer. The aqueous phase was drained off, and the petroleum ether layer washed alkali-free. The dried extract was filtered through a 30-mm. packed layer of celite and concentrated in vacuo to 200 ml.

(b) Resolution of extracted material.

The crude extract was developed on two 30 x 8 cm. columns of calcium hydroxide-celite with petroleum ether until the bottom fluorescent zone had almost reached the cotton plug:

Chromatogram No. 1

30	brownish; several weakly fl. zones, including also an orange and a red streak in the lower 7 mm. part	Section I
30	almost colorless; several minor bluish-gray fl. zones	Section II
30	strong purplish blue fl. (25 mm.), followed by intense yellow fl. (5 mm., also a very pale yellow color)	Section III
70	strong bluish-gray fl., including an orange pigment in its lower 15 mm. part	Section IV
110	colorless; upper 40 mm., purplish fl.; next 40 mm., very bright bluish fl.; lowest 10 mm., violet fl.	Section V

Sections I-V were separated by cutting, eluted with acetone-ethanol (3:1), and transferred with water into petroleum ether. The aqueous layers from the transfers were exhaustively extracted with fresh petroleum ether; all extracts of the same zone were combined and concentrated. The aqueous phases from Sections I-III also had to be extracted with chloroform. The chloroform solutions obtained were evaporated completely and the dry residues were taken up with petroleum ether.

Section I.

Section I (red solution) was developed with 1% acetone-petroleum ether on calcium hydroxide-celite (30 x 8 cm. column):

Chromatogram No. 2

35	light brown
10	orange
2	almost colorless; yellowish fl.
10	pink
3	pinkish red
15	almost colorless; weak blue to bluish gray fl.
20	colorless; 4-5 narrow blue, violet, and yellow fl. zones
200	colorless; non-fl.

Neither pigments nor fluorescent zones from this section were investigated further.

Section II.

Section II was developed on a 30 x 8 cm. calcium hydroxide-celite column with petroleum ether containing 0.5% acetone:

Chromatogram No. 3

15	pale pink; non-fl.	
90	colorless; blue-gray fl.	Section IIA
5	very pale yellow; strong blue-gray fl.	IIB
4	very pale yellow; yellow fl.	
13	blue-violet fl. (colorless from here to the bottom of the column)	IIC

6 2	yellow fl. blue-violet fl.	IID
6	yellow fl.	IIE
4 10	blue-violet fl. weak blue-gray fl.	IIF
105	non-fl.	

Sections IIA-IIF were separated by cutting, eluted with acetone, and transferred into petroleum ether. These sections were rechromatographed under similar conditions (developers, 2-5% acetone in petroleum ether), each yielding a complex chromatogram containing many poorly differentiated blue, blue-violet, bluish-green, greenish-yellow, and yellow fluorescent zones which were not investigated further.

Section III.

The petroleum ether solution (80 ml.) was developed with the same solvent on a 30 x 8 cm. column of calcium hydroxide-celite:

Chromatogram No. 4

140	almost colorless; moderate blue-gray fl. in lower 105 mm. of the zone	
40	pale yellow; blue-gray fl. trailing into strong yellow fl.	IIIA
20	strong blue-violet fl. containing a 5-mm. pale orange zone	IIIB
4	pale yellow; non-fl.	
15	very pale yellow; strong blue fl.	IIIC
80	colorless; several weak fl. zones	

Section IIIA.

The petroleum ether solution of the 40-mm. zone was resolved on a similar column by developing with 1% acetone-petroleum ether:

Chromatogram No. 5

30	non-fl.
150	yellow fl.
17	two bright blue fl. zones
93	several minor fl. zones

Crystalline polycyclic aromatic compound A (unidentified). The petroleum ether solution of the 150-mm. fraction was developed on alumina-calcium hydroxide-celite (30 x 8 cm. column) with benzene-petroleum ether (1:1). The intensely yellowish fluorescent upper main zone (25 mm.) was rechromatographed three times, first on alumina-calcium hydroxide-celite and then twice on calcium hydroxide-celite (developer, 10% acetone in petroleum ether). Finally, the petroleum ether solution was evaporated, and the residue was recrystallized from hexane in a 1-ml. centrifuge tube. Yield, 0.5-1 mg. After one more recrystallization from benzene the crystals, washed with hexane and dried, melted at 149-153° (decomp.). Spectrum, Figure 1 (full line). The golden yellow crystals showed moderate yellow fluorescence; however, in hexane solution or in adsorbates intense yellowish-green fluorescence appeared.

1,12-Benzperylene. After transfer into petroleum ether, the 17-mm. fraction was evaporated completely, and the residue was recrystallized from hexane (benzene actually is the better solvent for this purpose) in a 1-ml. centrifuge tube. The pale yellow, rectangular plates were washed with hexane and dried. Yield, 0.5-1 mg.; spectra, Figure 2. M.p. 271-274° (decomp.); mixed with 1,12-benzperylene ex perylene (see p. 95), m.p. 278-279°. The crystalline preparation showed intense greenish fluorescence in the solid state and a strong blue-violet fluorescence in adsorbates or in hexane solution. This fraction did not separate on the column from an authentic 1,12-benzperylene sample and has been identified with the latter.

Sections IIIB and IIIC.

Sections IIIB and IIIC were separately developed on calcium hydroxide-celite (30 x 8 cm. columns) with 0.5% acetone in petroleum ether. Both chromatograms showed as the main component a strong blue fluorescent zone (width, 90-95 mm.). After transfer into petroleum ether the two main fractions were combined and rechromatographed on alumina-calcium hydroxide-celite (30 x 8 cm.; developer, benzene-petroleum ether 1:1). The fairly homogeneous, blue fluorescent zone observed was transferred to petroleum ether and evaporated completely. However, the oil-residue did not crystallize (not even after standing at 4° for one year).

Section IV.

This petroleum ether solution was resolved by developing with the same solvent on calcium-hydroxide-celite (30 x 8 cm. column):

Chromatogram No. 6

60	colorless; a greenish fl. streak	
140	intense bright blue fl.; yellow pigment in lower part of the zone	IVA
30	pale orange; blue fl.	IVB
20	colorless; blue fl.	
20	pale yellow; bluish green-white fl.	IVC (cf.p.87)
30	colorless; non-fl.	

Section IVA.

The petroleum ether solution of the 140-mm. zone was developed on alumina-calcium hydroxide-celite (30 x 8 cm.) with benzene-petroleum ether (1:3):

Chromatogram No. 7

40	several weakly fl. zones and pigments	
50	weak blue-violet fl. (colorless from here to bottom)	
120	intense bluish-white fl. (lower edge blue-violet fl.)	IVA1
95	non-fl.	
	filtrate, pale yellow; blue fl.	IVA2

3,4-Benzpyrene. The 120-mm. zone (IVA1) combined with a similar one (IVB1) obtained from rechromatographing section IVB (cf. Chromatogram No. 9) was transferred into petroleum ether* and

* The polycyclic aromatic hydrocarbons studied can be transferred more readily from acetone into 1:1 petroleum ether-benzene than into petroleum ether or hexane alone.

filtered through two silicic acid-celite (4:1) columns (30 x 8 cm.) which retained a weak yellowish-green fluorescent impurity. The almost colorless, intensely blue fluorescent filtrate (8 liters) was concentrated in vacuo to almost dryness, yielding a suspension of yellowish crystals with yellow-green fluorescence. The slurry was transferred to a 10 ml. centrifuge tube and evaporated to 4 ml. with a stream of nitrogen. The crystals were washed several times with cold petroleum ether and dried. Yield, 50 mg. Recrystallization of this crude product from warm hexane yielded 20 mg. of pale yellow square plates and needles which were washed with cold hexane and dried in vacuo at 60° for 1 hour. M.p. 120-130°; spectrum, Figure 3.

Analysis. Calculated for $C_{20}H_{12}$: C, 95.20; H, 4.80; mol.-weight, 252. Found: C, 95.00; H, 5.03; mol.-weight, 232 (in camphor) and 252 (in exaltone).

Ten milligrams of the crystalline preparation were sent to Dr. Shimkin for carcinogenic tests on mice. Experimental details for this assay are described in reference (43).

In order to obtain an authentic reference sample of the highest purity 3,4-benzpyrene (Eastman Kodak, white label) was chromatographed on alumina-calcium hydroxide-celite (developer, 1:3 benzene-hexane), whereby several minor zones were eliminated. The purified substance wasrecrystallized from benzene-methanol; m.p. 176-177°. The fluorescence of this sample appeared to be slightly more violet than that of the natural product. Spectra, Figure 4. The two

preparations did not separate on the column.

A coupling of both samples with p-nitrobenzenediazonium chloride was carried out (see p. 91). Both dye solutions were then purified by developing with benzene on alumina-calcium hydroxide-celite. The two purified dyes did not separate from each other in the mixed chromatogram test. They showed identical spectral maxima, although the respective extinction values did not strictly coincide (Figure 5).

On treatment with conc. sulfuric acid 3,4-benzpyrene gave a dark red solution with bluish gray fluorescence, while the natural product formed a dark purplish red solution with a deep blue fluorescence.

Chrysene. The blue-fluorescent filtrate IVA2 was combined with a similar filtrate (IVB2) obtained by rechromatographing section IVB (cf. Chromatogram No. 9). The solution was then concentrated in vacuo from 8 liters to 200 ml. and developed on alumina-calcium hydroxide-celite (27 x 5.8 cm. column) with 5% benzene in petroleum ether:

Chromatogram No. 8

50	several pigments
90	blue-violet fl. (colorless from here to the bottom of the column)
36	five fl. zones (greenish, bluish, and violet fl.)
85	non-fl.

The 90-mm. zone was rechromatographed under similar conditions. The petroleum ether solution of the purified fraction was evaporated almost to dryness in a 2-ml. centrifuge tube, yielding blue fluorescent colorless crystals. The latter were recrystallized from warm hexane and then from benzene-ethanol by first covering the crystals with ethanol, warming the suspension at 70-80°, and then adding just enough benzene to dissolve the solid. On cooling colorless irregular plates were obtained which were washed several times with cold hexane and dried. Yield, 1.5 mg.; m.p. 250-253°. Spectrum, Figure 6.

Analysis. Calculated for $C_{18}H_{12}$: mol.-weight, 228. Found: 224 (in camphor).

A pure, authentic chrysene sample was prepared from a crude commercial sample (Sigma Chemical Co.) by repeated chromatographic adsorptions on alumina-calcium hydroxide-celite in order to remove several colored and strongly fluorescent impurities. A final recrystallization of the purified material from benzene yielded colorless chrysene melting at 255-256°; mixed with the natural product, m.p. 254-255°. Comparison of the spectra of the two preparations, Figure 6.

Section IVB.

The petroleum ether solution of the 30-mm. orange zone (combined with section VA) was developed on a 30 x 8 cm. column of alumina-calcium hydroxide-celite with benzene petroleum ether (1:4):

Chromatogram No. 9

60	yellow-orange fl. streak above an orange pigment	
20	colorless; intense blue fl. with blue-violet fl. near lower edge	IVB1
80	non-fl. (colorless from here to the bottom of the column)	
85	blue-violet fl.	IVB2
15	non-fl.	
40	bluish white fl.	

Development of the column was continued until the 85-mm. and 40-mm. fluorescent zones had been washed into the filtrate (IVB2). The 20-mm. zone was added to fraction IVA1; the filtrate was combined with fraction IVA2.

Section V.

The petroleum ether solution was developed on two alumina-calcium hydroxide-celite columns (30 x 8 cm.) with the same solvent:

Chromatogram No. 10

35	several fl. zones; 20-mm. orange pigment	
10	violet-blue fl. (colorless from here to bottom)	VA
8	non-fl.	
70	intense greenish-blue fl.	VB
10	non-fl.	
20	bluish-violet fl.	VC
30	non-fl.	
35	bluish-violet fl.	VD
80	dull yellow-brown fl.	

Fluoranthene. The petroleum ether solution of the 70-mm. zone (VB) was developed on alumina-calcium hydroxide-celite (30 x 8 cm.) with 0.5% acetone in petroleum ether. The 20-mm. main fluorescent zone (combined with a similar zone obtained from rechromatographing section IVC) was developed on a similar column with 5% benzene in petroleum ether. The latter column yielded a somewhat hetergeneous, intensely fluorescent 30-mm. zone, the eluate of which was transferred into petroleum ether and concentrated in vacuo to 5 ml. After standing at 4° for several days, almost colorless crystals appeared which were recrystallized from hexane; yield, 29 mg. Upon recrystallization from abs. ethanol, long colorless, strongly fluorescent (light blue) needles were obtained which were washed once with cold ethanol, then twice with cold hexane, and dried. M.p. 106-107°; spectrum, Figure 7.

Analysis. Calculated for $C_{16}H_{10}$: C, 95.01; H, 4.99; mol.-weight, 202. Found: C, 95.10; H, 5.08; mol.-weight, 203 (in exaltone).

Commercial fluoranthene (Eastman Kodak, white label), purified by chromatographic adsorption and recrystallization from ethanol, melted at 110-110.5°; mixed with the natural product, m.p. 108-109°. A mixed chromatogram test showed no separation. Spectra, Figure 7.

Crystalline polycyclic aromatic compound B (unidentified). The petroleum ether solution of the 20-mm. zone (VC) was developed on alumina-calcium hydroxide-celite (30 x 8 cm.) with 5% benzene in

petroleum ether. Of the two differentiated, blue-violet fluorescent zones, the lower 20-mm. broad more violet fluorescent one was added to section VD. The top 25-mm. zone was rechromatographed as described and yielded then a single zone, the eluate of which, after transfer into petroleum ether, was evaporated completely. Yellow, oily crystals appeared which, when recrystallized from hexane, yielded 1.5 mg. of pale yellow, brilliantly green fluorescent plates that sublimed in vacuo without alteration. M.p. 178-184°; mol. weight, found: 184 (in exaltone). Spectrum, Figure 8.

Anthracene and Phenanthrene. The petroleum ether solution of the 35-mm. zone (VD) was developed on a 30 x 8 cm. column of alumina-calcium hydroxide-celite with the same solvent and the content of the 62-mm. zone, after transfer into petroleum ether, was evaporated to a small volume. Colorless, strongly blue-violet fluorescent crystals appeared (23 mg.) which yielded colorless plates when recrystallized from hexane. M.p. unsharp over the range, 120-145°. Spectra, Figure 9. The sample did not separate from commercial anthracene (Eastman Kodak, white label) in the mixed chromatogram test.

Analysis. Calculated for $C_{14}H_{10}$: C, 94.33; H, 5.66; mol.-weight, 178. Found: C, 94.52; H, 5.78; mol.-weight, 181 (in exaltone).

In order to resolve this crystalline anthracene-phenanthrene mixture, a solution of 10 mg. in 5 ml. of benzene was exposed to intense sunlight in a 5 ml. pyrex volumetric flask for two days (7).

The liquid turned brownish yellow and deposited crude dianthracene crystals which became colorless when washed on the filter with benzene and then with alcohol; m.p. of two samples, 236-239° and 238-241°. The dianthracene from the natural product was reconverted to anthracene by melting in sealed tubes in an electrically heated Berl block. The hexane solution of the cooled melt was developed on alumina-calcium hydroxide-celite (18 x 1.9 cm.) hexane. The main, 25-mm. blue-violet fluorescent zone was transferred into 5 ml. of optical hexane, and its spectral curve was taken. From the reading at 375 mμ. it was estimated that this solution contained 0.2 mg. of anthracene. For comparison pure anthracene was prepared from a commercial sample by dimerization in sunlight, melting the dimer in a sealed tube, chromatography of the product, and recrystallization from benzene-ethanol (m.p. of dianthracene, 247-248°; m.p. of anthracene, 217-218°). Spectra, Figure 10.

In order to identify the phenanthrene, the filtrate of the insolated solution was evaporated, and the petroleum ether solution of the dry residue was developed with the same solvent on a 18 x 1.9 cm. column of alumina-calcium hydroxide-celite until approximately 50 ml. of chromatographic filtrate were collected. The lower half of the column was cut off, eluted, and transferred into benzene-hexane (1:1). The solvents were evaporated, and the hexane solution of the dry residue was tested in the spectrophotometer. Since some slight maxima, typical for anthracene, still appeared in the

extinction curve, the insolation and all subsequent operations were repeated. Yield, after sublimation in vacuo, 1.2 mg. For a comparison commercial phenanthrene was purified in a similar manner (insolation, etc.); m.p. 98.5-99°. The natural product melted at 88-91°, and a mixture with the commercial sample, at 96-97°. Spectra, Figure 11.

(c) Determination of specific and molecular extinction curves.

The specific and molecular extinction curves for the crystalline substances isolated from the Tetraclita squamosa rubescens sample from Corona del Mar, viz. 1,12-benzperylene, 3,4-benzpyrene, chrysene, fluoranthene, anthracene-phenanthrene mixture, and two unidentified compounds, were obtained by single determinations in each case using optical hexane. The amounts used varied from 0.3 to 2 mg. and were weighed on a microbalance.

The molecular extinction curves of purified commercial 3,4-benzpyrene, chrysene, fluoranthene, anthracene, and phenanthrene were obtained by averaging the determinations of the absorption spectrum of two independently weighed samples of each compound dissolved in known volumes of optical hexane. The samples used, varying from 10-20 mg., were weighed on the semi-microbalance. For 1,12-benzperylene, however, only one determination was carried out using 2.028 mg. of the substance synthesized from perylene (see p. 95). The wave lengths and molecular extinction coefficients at the maxima for

these reference compounds in hexane solution are listed in Table XX

The molecular extinction values of the natural product were always lower than those of the corresponding authentic sample. In order to obtain graphical comparison with the extinction curves of standard samples, the extinction heights were equated at certain maxima and all other points correlated. The maxima used for this purpose were: coronene, 341 mμ;* 1,12-benzperylene, 299 mμ.; 3,4-benzpyrene, 363 mμ.; chrysene, 267.5 mμ.; fluoranthene, 287 mμ. for the Tetraclita sample and 341 mμ. for the Mitella preparation; anthracene, 375 mμ.; phenanthrene, 292 mμ.; and unidentified crystalline compound E in Figure 1, 250 mμ.

(d) Coupling of 3,4-benzpyrene with diazonium solution.

The coupling reaction of 3,4-benzpyrene with p-nitrobenzenediazonium chloride was carried out according to Fieser and Campbell (16). After the purple color had developed, the reaction mixture was resolved by chromatography.

A solution of 12 mg. of p-nitroaniline (Eastman Kodak, white label) in 4 drops of conc. hydrochloric acid plus 10 drops of glacial acetic acid was diazotized at 0-5° by adding a solution of 21 mg. of sodium nitrite in two drops of dist. water. After diluting this solution with 2 ml. of acetic acid, the p-nitrobenzenediazonium chloride solution was added to a solution of 3,4-benzpyrene (36 mg. in 7.5 ml. of glacial acetic acid). Five minutes after mixing, the

* The molecular extinction coefficient at 341 mμ. for coronene (in chloroform solution) was taken from reference (37).

Table XX

Molecular Extinction Coefficients of Some Polycyclic Aromatic Hydrocarbons* in Hexane solution at the Wave Lengths of the Maxima

Wave length (mμ.)	$E^{mol.}_{1\,cm.} \times 10^{-4}$	Wave length (mμ.)	$E^{mol.}_{1\,cm.} \times 10^{-4}$	Wave length (mμ.)	$E^{mol.}_{1\,cm.} \times 10^{-4}$
3,4-Benzpyrene (m.p. 176-177°)		Chrysene (m.p. 255-256°)		Fluoranthene (m.p. 110-110.5°)	
220	2.67	221.5	3.49	215.5	3.08
226.5	2.95	241.5	2.24	235.5	4.945
254.5	4.19	258	8.025	245.5	1.49
265	4.99	267.5	15.13	252	1.21
272	3.15	282	1.26	258.5	1.03
283.5	4.74	293.5	1.25	262.5	1.09
296	6.085	305.5	1.37	271.5	1.21
312	0.333	319	1.355	276	2.46
330.5	0.523	343.5	0.0509	281.5	1.87
346	1.31	350.5	0.0307	287	4.42
363	2.615	352	0.0317	308.5	0.376
380	2.715	360.5	0.0518	323	0.638
383	3.06			341	0.822
393	0.428			358.5	0.876
402	0.4065				
Anthracene (m.p. 217-218°)		Phenanthrene (m.p. 98.5-99°)		1,12-Benzperylene (m.p. 279-280°)	
220.5-		220	2.46	222.5	5.11
221	1.19	244	5.26	253	1.54
246	1.10	250.5-		276	2.38
252	20.80	251	6.78	288	3.94
309	0.124	274	1.43	299	5.32
323	0.287	280.5	1.12	312.5	0.439
338.5	0.561	292	1.39	329	0.600
355.5	0.847	308	0.0176	344.5-	
370	0.393	315	0.0203	345	0.911
375	0.838	322.5	0.0232	362	1.98
		329.5	0.0255	383	2.525
		338	0.0227	405	0.0198
		345.5	0.0220		

* Commercial products except for 1,12-benzperylene which was prepared from perylene (9). The samples used in the determination of these data were purified by both chromatography and recrystallization.

solution turned red-purple; after standing for four hours at room temperature 25 ml. of benzene were added to the very dark solution. Water was cautiously introduced to drive the purple dye into the benzene layer, which was then washed thoroughly with water, dried with sodium sulfate and filtered. It was diluted with an equal volume of hexane and developed with benzene on a 30 x 8 cm. column of alumina-calcium hydroxide-celite:

Chromatogram No. 11

10	four colored streaks (red-purple and red-orange)	
5	faint purple	
7	almost colorless	
10	purple	
50	almost colorless	
40	dark purple	Fraction I
40	colorless	
30	pale yellow; intense greenish-yellow fl.	II
25	colorless	
35	pale yellow; intense greenish-yellow fl.	III
20	colorless Filtrate, containing unreacted 3,4-benzpyrene as well as two minor fl. zones	

5-p-Nitrobenzeneazo-3,4-benzpyrene. A small aliquot of the benzene solution of fraction I was evaporated completely, and the residue was taken up in optical hexane for the spectral curve determination; maxima: (260 mμ., inflection), 268-269, 327-328, 340, 398, and 508 mμ. (cf. Figures 5 and 22). The main part of fraction I

was also evaporated completely, and the solid residue was recrystallized from chloroform-hexane, forming long dark purple-black blades which were dried in vacuo at 110°. M.p. 248-249°.

Analysis. Calculated for $C_{26}H_{15}O_2N_3$: C, 77.80; H, 3.76. Found (after correcting for 2.8% ash): C, 79.47; H, 4.01.

Unidentified 3,4-benzpyrene derivative. A small aliquot of II (benzene solution) was transferred into optical hexane; maxima: 267, 288, 300, 393 mμ. The remaining solution was evaporated completely, and the red residue was recrystallized from benzene-hexane. Yield, 0.5-1 mg.; m.p. 226-229° (decomp.). This orange colored crystalline preparation showed red-orange fluorescence in the solid state. When dissolved, it exhibited intense greenish-yellow fluorescence in benzene, a moderate blue fluorescence in hexane, and no fluorescence in acetone.

Analysis. Found: mol.-weight, 508 (in camphor).

Unidentified 3,4-benzpyrene derivative. A small aliquot of III (pale yellow benzene solution) was transferred into optical hexane; maxima: 259, 268, 290, 302, 353, 371, 391.5 mμ. Evaporation of the main solution yielded a yellow-brown oil. The addition of a few drops of benzene and hexane caused the formation of yellow crystals which were washed with hexane and recrystallized from benzene-hexane. Yield, 3 mg.; m.p. 196-199° (decomp.). The crystalline preparation fluoresced a yellow-green, while the fluorescence of its solutions exhibited the same dependence on solvent as mentioned for fraction II.

Analysis. Found (after correcting for 0.45% ash): C, 84.07; H, 4.35.

For the diazo-coupling reaction with the 3,4-benzpyrene preparations obtained from the Tetraclita squamosa rubescens and Mitella polymerus one ml. of the diazonium solution (see above) was added to a solution of 0.5 mg. of test sample in 5 drops of glacial acetic acid. A parallel experiment with authentic 3,4-benzpyrene (0.5 mg. in 5 drops of glacial acetic acid) was carried out simultaneously. The red-purple color generally appeared within 2 to 4 hours. After 10 hours the reaction mixture was transferred into benzene, and the dried benzene solution was developed on alumina-calcium hydroxide-celite (18 x 1.9 cm.) with the same solvent. From either the reference or the test sample (from either species of barnacles) solution only a single, violet zone migrated in the column*, although several brightly colored streaks remained strongly adsorbed at the very top (cf. Chromatogram No. 11). The violet zone was transferred into benzene, and an aliquot of this solution was transferred into optical hexane. The extinction curve was determined immediately. Since in the visible region the extinction values of the dye decreased relatively rapidly, the readings were taken starting at 680 mμ. and proceeding down to 210 mμ.

(e) Preparation of 1,12-benzperylene.

An authentic sample of 1,12-benzperylene was synthesized from

* No greenish yellow fluorescent zones were observed on the column from these small scale coupling experiments (cf. Chromatogram No. 11).

perylene by the method of Clar (9) as follows.

One-hundred mg. of perylene (Delta Chemical Co.) and 120 mg. of maleic anhydride (Eastman Kodak, white label) were dissolved in 2 ml. of nitrobenzene (Eastman Kodak, white label). The dark red solution was refluxed for 90 minutes and yielded, on cooling, a dark orange solid which was filtered off with suction and washed with ether. Yield, 56.5 mg.

The perylene-maleic anhydride adduct obtained above was ground together with 0.4 g. of soda-lime, giving a pale red-brown powder. This mixture was transferred into a sublimation apparatus and heated (brass turnings) without vacuum up to 350°. The perylene that sublimed out was washed from the cold finger with benzene, and the temperature was again raised to 350°. The system was then evacuated (0.1 mm.) and kept at 410° for three and a half hours. The yellow sublimate was dissolved in benzene and added to a benzene extract of the dark brown sublimation residue. This solution was diluted with an equal volume of hexane and developed on alumina-calcium hydroxide-celite (30 x 8 cm. column) with 1:1 benzene-hexane:

Chromatogram No. 12

25	several weakly fl. zones
73	non-fl.
45	intense violet-blue fl. (1,12-benzperylene)
45	non-fl.
60	intense greenish-blue fl. (unchanged perylene; about 16 mg. recovered)
45	non-fl.

The benzene solution of the 45-mm. violet-blue fluorescent zone was evaporated completely. The residue was then recrystallized from xylene, yielding 6.5 mg. of pale greenish-yellow crystals. Recrystallization from xylene-hexane yielded rectangular and square plates. After cooling at 0° the intensely green fluorescent crystals were washed with hexane and dried in vacuo at 60°. M.p. 279-280°. Spectrum, Figures 2 and 19.

(2) Thatched Barnacles from La Jolla, California.

(a) Extraction

Nine kilograms (approximately 1400 individuals) of the thatched barnacle Tetraclita squamosa rubescens were collected from rocks near La Jolla, Calif. and stored under methanol at 4° until they were worked up. After decantation of the dark green methanol, the animals were crushed with small rocks in a mechanical ball mill. The crushed material was collected under fresh methanol and extracted with hexane-methanol mixtures (1:1, 2:1, 3:1) by shaking mechanically for 30 minutes. The extracts were separated by centrifuging the mixtures through a filter paper lined basket, and the methanol was removed from the combined filtrates by cautiously adding water. The methanol used in storing the starting material was also extracted with hexane (addition of water, etc.), and this hexane solution was added to the main extract. The total extract was washed with water, dried, concentrated in vacuo to 500 ml., and saponified twice by standing over 20%

methanolic KOH overnight. The layers were separated by cautious addition of water. The hexane solution, upon washing alkali-free and drying, appeared orange-brown and showed moderately strong fluorescence in ultraviolet light.

(b) Resolution of extracted material.

The crude extract mentioned (75 ml.) was developed with hexane on a 27 x 5.8 cm. column of calcium hydroxide-celite:

Chromatogram No. 13

16	five pigment streaks (mostly pink)	Section I
40	pale yellow-brown (containing colorless solid)	
15	pale orange and pink zones	
2	dark red-brown	Section II
2	two pigment streaks (yellow, orange)	
24	five pale orange zones	Section III
85	yellow-orange	Section IV
40	light yellow	Section V
30	colorless	
	Filtrate, colorless; blue-fl.	Section VI

Sections I-V were separated by cutting, eluted with acetone, and transferred into hexane. Section VI was concentrated to 25 ml.

Section I.

This was developed on calcium hydroxide-celite in a 20 x 3.8 cm. column with 15% acetone in hexane:

Chromatogram No. 14

5	three pale pigments (pink, red-orange, pink)	
7	two dark pink zones	Fraction IA
4	two pale red-orange zones	
3	colorless	
6	pale red-orange	IB
9	colorless except for 2 mm. pale yellow-orange	
4	pale yellow-orange	IC
5	colorless	
10	pale red-brown	ID
35	colorless	
12	very faint red-brown	
12	yellow	IE
50	colorless	
10	pale red	IF
20	colorless	

Fractions IA-IF were transferred into hexane. Spectral curves for unidentified pigment fractions IB-IF, Figure 13:

(IB, --.--.; IC, -.-.-.;ID, - - -; IE, -'-'-'; IF, -.-'-.).

Fraction IA was rechromatographed on calcium hydroxide-celite (18 x 1.9 cm.; developer, benzene):

Chromatogram No. 15

6	yellow-gray
3	pink and orange zones
2	yellow-gray
11	deep red
2	colorless
2	pale orange
18	two adjacent pink zones
120	colorless

The spectral curve of the orange hexane solution (purplish tint) of the 11-mm. broad, deep red zone corresponded to that of spirilloxanthin (Figure 13, full line).

Section II.

Section II was rechromatographed on calcium hydroxide-celite (20 x 3.8 cm.; developer, 3% acetone in hexane):

Chromatogram No. 16

38	almost colorless
7	pinkish red
3	pink
11	pinkish red
140	colorless except for two pale pigments

The 7-mm. and 11-mm. zones were quite similar in color but appeared to be separated from each other by the 3-mm. pink section. The 7-mm. zone was discarded, while the 11-mm. one was transferred into hexane. Spectrum, Figure 14 (dashed line); unidentified pigment.

Section III.

Section III was developed on alumina-calcium hydroxide-celite (20 x 3.8 cm.) with 20-30% benzene in hexane:

Chromatogram No. 17

3	yellow	
1	pink; yellow-green fl.	III A
2	colorless; yellow-green fl.	
3	red-orange	
8	almost colorless	
6	pale orange	
7	colorless; weak blue fl.	III B
170	colorless, top half of this zone; orange, lower half	

Fractions III A and III B were rechromatographed under similar conditions (18 x 1.9 cm. columns). Fraction A fluoresced green when adsorbed on alumina, but its acetone or benzene solution was non-fluorescent. Spectra, Figure 12 (IIIA, ...; IIIB, -'-'-'); both fractions remained unidentified.

Section IV.

Section IV was developed on a 20 x 3.8 cm. column of calcium hydroxide-celite with 1% acetone in hexane. The main, yellow-orange zone recovered from the new column exhibited the β-carotene spectrum (Figure 14, full line).

Section V.

Section V was developed on a 20 x 3.8 cm. column of alumina-calcium hydroxide-celite with hexane:

Chromatogram No. 18

1	orange	
1	yellow; yellow fl.	VA
47	colorless; non-fl.	
14	three orange zones	VB
108	colorless; non-fl.	
2	colorless; blue fl.	VC
10	colorless; non-fl.	

The fluorescent fractions VA and VC were rechromatographed on alumina-calcium hydroxide-celite (18 x 1.9 cm.; developers, benzene-hexane 1:4 and 1:20, respectively). Spectra, Figure 12 (VA, --.--.; VC, _____); unidentified. Fraction VB was rechromatographed repeatedly on calcium hydroxide-celite (developer, hexane) until a homogeneous yellow zone was obtained. The spectral curve of this pigment corresponded to that of α -carotene (Figure 14, -.-.-.). In the partition test with hexane/95% methanol, this compound showed epiphasic behavior.

Section VI.

Section VI was developed on alumina-calcium hydroxide-celite (20 x 3.8 cm.) with benzene-hexane (1:4):

Chromatogram No. 19

30	non-fl.	
6	weak violet-blue fl.	VI A
32	non-fl.	
5	blue fl.	VI B
125	non-fl.	

The spectra for the two fluorescent zones from this column are given in Figure 12 (VI A, -.-.-., unidentified; VI B, - - -, unidentified).

c. Goose Barnacle (Mitella polymerus).

(1) Extraction.

In order to obtain crystalline preparations of polycyclic aromatic substances a large scale extraction was carried out using 14 kg. of peduncles (weighed saturated with aqueous methanol) of goose barnacles from La Jolla, Calif. The intact animals were growing on and among mussels on the pier pilings at the Scripps Institution of Oceanography and, after collection, were stored under methanol at 4° until they were worked up.

The capitulum of each barnacle was cut off by hand from the peduncle. The total peduncle portion of the starting material was first passed through an electrical chopper and then ground up in Waring blendors with fresh methanol which extracted practically all the pigment from the tissues. This methanol extract was readily

separated from the gray, fibrous material by filtration in a large basket centrifuge. The orange-brown filtrate was then extracted with benzene (containing 25% hexane to diminish emulsification) in 20-liter separatory funnels by the cautious addition of water which immediately formed a colorless, milky lower phase that was drained off and discarded. The upper benzene phase was washed free of methanol, dried, and concentrated in vacuo to 250 ml. (dark brown, oily liquid). This concentrate was subsequently chromatographed without saponification (extract M).

The fibrous residue of the peduncles remaining after removal of the methanol by filtration was extracted in the usual manner three times with hexane-methanol mixtures (1:1, 2:1, 3:1). The hexane extract was washed with water, dried, concentrated in vacuo to 500 ml. and saponified twice by standing over 20% methanolic KOH overnight. All of the pigment in the dark red alkaline phase was transferred into fresh hexane by adding water. During this transfer the addition of benzene and acetone to the methanol solution before introduction of water diminished emulsion-formation. The total hexane extract was washed alkali-free and concentrated to 250 ml. (extract H).

(2) Resolution of extract H.

The orange-brown crude extract was developed on calcium hydroxide-celite in a 30 x 8 cm. column with 2% acetone in hexane. After 1.5 liters of developer had passed through, the chromatogram assumed the following appearance:

Chromatogram No. 20

5	pinkish red	Section I
4	pale red	
130	colorless; two weakly fl. zones	
10	pale brownish red; including a 3-mm. broad white fl. part	
5	colorless	Section II
2	pale yellow-orange	
130	colorless; containing bluish fl. (15-mm.) followed by grayish fl. (10-mm.)	Section III
	Second filtrate, orange; blue fl. zone	Section IV
	First filtrate, yellow; strong blue fl. (well separated from the zone of the "second filtrate" on column)	Section V

Sections I, II and III were eluted with acetone containing 20% ethanol and transferred into hexane. Sections IV and V were washed free of acetone and concentrated in vacuo to 50 ml. and 75 ml., respectively.

Section I.

This section was chromatographed on calcium carbonate (20 x 3.8 cm.; developer, 0.5% acetone in hexane). This column was pervaded with a weak grayish fluorescence and contained several minor pigments which were discarded. The main pink zone (50-mm.) was transferred into hexane. The spectral curve of this orange solution corresponded to that of spirilloxanthin (cf. full line curve in Figure 13).

Section II.

Section II was developed on a 20 x 3.8 cm. column of calcium hydroxide-celite with 3% acetone in hexane and yielded as the main zone a 3-mm. red-brown pigment (pervaded with a bluish white fluorescence) which was adsorbed below a broad, pale reddish-brown zone (discarded; it may have resulted from tailing of the 3-mm. zone). The main pigment, possessing a single extinction maximum at 458 mμ., resembled a poly-*cis* carotenoid. However, catalyzing the solution with iodine and exposing to light for 10 minutes merely shifted the maximum to 454 mμ. with an accompanying small decrease in the extinction values. This pigment is probably identical with a similar one from the thatched barnacle (Chromatogram No. 16 and Figure 14, - - -) and from the blood worm (Chromatogram No. 38 and Figure 31, -.-.-.). (Cf. footnote on p. 109.)

Section III.

This section (75 ml.) was rechromatographed on alumina-calcium hydroxide-celite (24 x 4.8 cm.; developer, benzene-hexane 1:3). The chromatogram contained a dirty-yellow fluorescent zone at the very top (discarded) and a 2-mm. broad, intensely blue fluorescent zone near the middle of the column length. After rechromatography (under similar conditions) the spectral curve of this zone in hexane solution exhibited the 3,4-benzpyrene maxima in the region, 320-440 mμ.; however, a contaminant showing maxima at 265 and 272 mμ. obscured the absorption peaks of 3,4-benzpyrene in the region, 210-320 mμ.

Section IV.

Section IV was adsorbed on alumina-calcium hydroxide-celite (24 x 4.8 cm.; developer, benzene-hexane 1:3). From the resulting chromatogram the 27-mm. broad top part containing several narrow fluorescent zones was discarded. Below it there appeared an 80-mm. zone which included pale orange, orange, and yellow-orange pigments. This colored zone, combined with a similar pigment obtained from section V, was resolved by repeated chromatographic adsorption on calcium hydroxide-celite (developer, 2% acetone in hexane) into three homogeneous pigment fractions that gave the spectra of neo-β-carotene U, β-carotene, and α-carotene, respectively.

Section V.

This section was developed on a 24 x 4.8 cm. column of alumina-calcium hydroxide-celite with hexane. The top 45-mm. pigment section (pervaded with a weak bluish-gray fluorescence) was added to section IV. The rest of the colorless column consisted of an 82-mm. non-fluorescent interzone, a 4-mm. zone showing intense bluish fluorescence, and a 95-mm. non-fluorescent part. The 4-mm. zone mentioned was rechromatographed (18 x 1.9 cm.). The fluorescence of this zone did not resemble that of fluoranthene. Moreover, the spectrum did not contain any of maxima typical for fluoranthene but, instead, showed a rapid increase in extinction in going from 400 to 210 mμ. with two small maxima at 257 and 231-232 mμ. (unidentified fraction).

(3) Resolution of extract M.

The first attempts to chromatograph this crude extract only resulted in columns cracking soon after the extract had been introduced. The adsorbent after extrusion of the column and crushing with a spatula became gel-like and sticky. After elution and transfer into benzene-hexane 1:1 the total extract was concentrated in vacuo to almost dryness; then hexane was added, and the solution was again evaporated almost completely. The residue was dissolved in 240 ml. of hexane and divided into four equal portions. Each aliquot was diluted with an equal volume of hexane and chromatographed on a 30 x 8 cm. calcium carbonate column (containing a 25-mm. layer of powdered sugar packed on top in order to retain the gummy material). After partial development with 2% acetone in hexane the following chromatogram resulted:

Chromatogram No. 21

25	colorless (sugar)	
1	orange streak	Section I
70	several pink zones	
120	almost colorless interzone	Section II
20	several brownish-red zones	
5	colorless interzone	
55	several red zones	Section III
	Filtrate, orange-brown; bluish fl.	

The chromatogram - containing a large number of pigment zones - was very colorful, but due to its complexity it was not described

above in detail. Developing was continued until all the pigments of Section III had washed into the orange-brown filtrate. Corresponding sections from the four columns were combined, eluted with acetone, and transferred into hexane. No further work (with one exception) was done on the predominantly hypophasic pigments of the goose barnacle other than submitting Sections I, II, and III to an additional chromatographic resolution. The one exception mentioned refers to the main pigment in section III which was subsequently crystallized and analyzed for C and H as described below. On the following pages are described in detail these three chromatograms, since they seem to offer a suitable basis for an intended investigation of the goose barnacle pigments*.

Section I (50 ml.)

One 30 x 8 cm. column of powdered sugar; developer, 0.5% acetone in hexane:

* In an independent small-scale investigation carried out earlier in which no distinction had been made between a "methanol extract" and a "hexane (or petroleum ether) extract" as it was done here, after saponification of the crude petroleum ether extract of the peduncles, the alkaline phase became very dark red. Adding water and fresh petroleum ether to the latter precipitated a red solid and formed a red emulsion. Upon acidification with dil. sulfuric acid the pigments could be transferred into petroleum ether. After chromatographic resolution on powdered sugar this solution yielded five pigment fractions whose extinction curves showed a single, broad, and relatively symmetrical peak; maxima in hexane: two fractions, 470-474 mμ.; the third, 478 mμ.; the fourth, 478-481 mμ.; and the fifth, 498 mμ. (the latter in pyridine). The main petroleum ether extract was resolved chromatographically (calcium hydroxide-celite) into neo-β-carotene U, β-carotene, and an unidentified pigment whose spectral curve contained a single maximum at 458-459 mμ., in hexane. This pigment was predominantly epiphasic toward hexane/95% methanol; it did not separate on the column from a similar fraction (Figure 14, dashed line) obtained from the thatched barnacle collected near La Jolla.

Chromatogram No. 22

140	several green and pale pigment zones
35	pinkish red
5	almost colorless
5	orange-brown
5	almost colorless
11	red-orange
2	orange, red lower edge
45	colorless; bluish fl.
	Filtrate, bluish fl.

Section II (75 ml.)

One 30 x 8 cm. column of calcium carbonate; developer, 4% acetone in hexane:

Chromatogram No. 23

30	three green zones (sugar layer)
5	almost colorless
5	orange-brown
60	two green zones with pink background
10	orange
2	almost colorless
3	yellow-orange
5	almost colorless
10	yellow to orange zone
1	colorless
2	pale orange
1	colorless
40	brownish red
25	colorless
18	brownish red
3	almost colorless
30	brownish red
40	colorless
	Filtrate, yellow; blue fl.

Section III (75 ml.)

Two 30 x 8 cm. columns of calcium carbonate; developer, 2% acetone in hexane:

Chromatogram No. 24

25	green (sugar layer)
20	four red-brown zones
17	almost colorless (faint zones)
5	pale red-brown
40	faint red-brown
5	faint green
25	faint red-brown
3	faint green
5	red-orange
3	faint green
6	orange
2	almost colorless
2	red-brown
3	colorless
10	dark red-brown (main pigment zone)
2	yellow
2	pink
6	colorless
2	yellow
5	colorless
8	orange-brown
2	colorless
4	faint orange
6	colorless
8	faint yellow
10	almost colorless
5	pale orange-brown
25	colorless; blue fl. Section III F
	Filtrate, yellow; blue fl.

Unidentified crystalline pigment. The hexane solution of the main pigment zone of Section III was concentrated in vacuo to 20 ml. and developed on calcium carbonate (30 x 8 cm.) with 2% acetone in hexane:

Chromatogram No. 25

40	five to six orange to red-brown zones
5	colorless
10	brownish red
20	colorless
27	three orange zones
2	almost colorless
13	brownish red
3	colorless
70	brownish red
40	almost colorless
12	four narrow pigment zones
30	colorless; containing 5-mm. bluish-green fl. zone

The 70-mm. brownish-red zone was eluted with acetone containing 1-5% methanol. Transfer into benzene-hexane 1:1 gave an orange-red solution which was rechromatographed on calcium carbonate (30 x 8 cm.; developer, 4% acetone in hexane) and freed of traces of other pigments. After evaporating the benzene-hexane solution to dryness, the red to reddish-black solid residue was transferred with chloroform into a 5-ml. centrifuge tube. Subsequent evaporation under a nitrogen stream gave a dark red oil with some red crystals on the walls; on the dropwise addition of hexane a red solid precipitated which was washed twice with cold hexane. It was recrystallized by dissolving in a minimum amount of warm benzene and adding hexane dropwise until crystals began to form. After cooling, the purplish-red (leaf-shaped plates and aggregations were washed in the centrifuge tube four times with cold hexane and dried in vacuo (0.1 mm.) at 57°. Yield, 14 mg.; m.p. 176-178°. Spectrum, Figure 25.

Analysis. Found: C, 77.27; H, 8.45. Calculated for $C_{40}H_{56}O_5$: C, 77.88; H, 9.15.

The pigment was slightly soluble in hexane, fairly soluble in acetone or methanol, and readily soluble in benzene, chloroform, and pyridine. Its hexane solution was orange-yellow, while the ethanol or pyridine solution was bright orange-red (brownish tint in the ethanol solution and violet tint in the pyridine solution).

In the partition test with hexane/95% methanol the pigment was found to be hypophasic.

On shaking an ether solution with 24% aqueous hydrochloric acid the hypophase turned a blue color with a greenish tint, while the epiphase remained orange. When the blue hypophase was removed and the epiphase was shaken with fresh acid, the lower phase again became blue. This could be repeated three more times, the last treatment giving only a slightly bluish aqueous layer below the faint yellow-orange ether phase.

Polycyclic aromatic compounds.

Section IIIF which included most of the fluorescent substances of extract M was transferred into 100 ml. of hexane containing 10-20% benzene. The orange-red solution was chromatographed on calcium hydroxide-celite (30 x 8 cm. column) with 2% acetone in hexane:

Chromatogram No. 26

1 12	colorless pinkish red	Fraction F1
30	pale yellow; weak bluish and greenish fl. zones	F2
10	almost colorless, non-fl. interzone	
25	almost colorless; bluish white fl.	F3
57	bluish gray fl. (30-mm.); red-orange (12-mm.); blue fl. (15-mm.)	F4
15	colorless; non-fl. interzone	
30	colorless; bluish violet fl.	F5
45 7 10 30	green-gray fl.; yellow pigment in lower 15-mm. part colorless, non-fl. interzone colorless; weak violet fl. colorless, non-fl. section	F6
	Filtrate, colorless; non-fl.	

Fractions F2-F6 were eluted with acetone (strongly fluorescent eluates) and transferred into hexane containing 20-40% benzene.

Fraction F2.

Fraction F2 - including in addition to the original zone all the "a" zones from chromatograms of fractions F3 to F6 (see below) as well as the filtrates from Chromatograms No. 23 and 25 - was developed with benzene on alumina-calcium hydroxide-celite (24 x 4.8 cm.):

Chromatogram No. 27

27	brownish with orange and pink zones; greenish fl.	
3	colorless	
2	yellow-orange	
4	colorless	
5	yellow; yellowish-green fl.	
20	colorless	
30	pinkish red	
50	colorless; non-fl.	
25	colorless; violet-blue fl.	F2a
10	colorless; weakly fl.	
25	colorless; bluish gray to blue fl.	F2b
30	colorless; non-fl.	

Crystalline polycyclic aromatic compound C (unidentified). The 25-mm. zone F2a was rechromatographed on alumina-calcium hydroxide-celite (20 x 3.8 cm.; developer, benzene). The 45-mm. homogeneous, blue fluorescent zone obtained was transferred into benzene-hexane (1:1) and evaporated to dryness in a 0.5-ml. centrifuge tube. After standing at 4° for one month some yellow crystals were noted in the brownish oil. The latter was cautiously pipetted out; the minute amount of crystals was washed twice with cold hexane, dissolved in warm benzene, and transferred into a clean tube after centrifuging down traces of insoluble contaminants. After evaporating this solution completely the addition of one drop of hexane to the residue caused formation of yellow crystals. The yellow-orange mother liquor was removed, and the crystals were washed three times with cold hexane,

and dried. These yellow crystals fluoresced a yellowish green color. The sample was dissolved in optical hexane for the spectral curve determination (Figure 17). Although the curve resembled, in part, that of both 3,4-benzpyrene and 1,12-benzperylene, mixed chromatogram tests showed clear separations in each instance, i.e., compound C was much more strongly adsorbed than the reference samples.

Fraction F2b.

The 25-mm. bluish-gray to blue fluorescent zone (color of fluorescence resembled that of fluoranthene) was developed on alumina-calcium hydroxide-celite (20 x 3.8 cm.) with benzene-hexane (1:1):

Chromatogram No. 28

18	several weakly fl. zones	
12	greenish fl.	
35	non-fl.	
16	blue fl.	F2b1
2	weaker fl.	
12	blue fl.	F2b2
8	greenish yellow fl.	F2b3

Crystalline polycyclic aromatic compound D (unidentified). The 16-mm. zone F2b1 was transferred into benzene-hexane (1:1) which was then evaporated to dryness in a 0.5-ml. centrifuge tube. The solid material that appeared on standing at 4° was washed once with cold hexane and recrystallized from 2-3 drops of warm hexane. After two

washings with hexane and drying, the total yield of yellow, greenish fluorescent crystals was dissolved in optical hexane for the determination of the spectral curve (Figure 16).

Coronene. The 8-mm. greenish-yellow fluorescent zone (F2b3) was transferred into a 1:1 mixture of benzene and hexane and crystallized from hexane. The pale yellow slender needles fluoresced green in the solid state. In hexane solution the fluorescence was green, and in benzene solution, bluish-green. The spectral curves in benzene and chloroform agreed with those reported in the literature for coronene (10, 37); cf. Figure 18 and Table III.

Fraction 2b2 was crystallized in a similar manner and gave a very small amount of crystalline product. The hexane solution of the crystals showed essentially the same extinction curve as that of coronene.

No authentic sample of coronene was available for a mixed chromatogram test.

Fraction F3.

This fraction (40 ml.) was developed on a 24 x 4.8 cm. column of alumina-calcium hydroxide-celite with benzene-hexane (1:1):

Chromatogram No. 29

35	orange-red streak; several fl. zones	F3a
110	weak violet fl.	
20	yellowish fl.	F3b
36	blue fl.	F3c
60	olive drab fl.	

Crystalline polycyclic aromatic compound E (unidentified). The 20-mm. zone (F3b) was chromatographed on alumina-calcium hydroxide-celite (24 x 4.8 cm.; developer, benzene-hexane 1:1). The main, greenish-yellow fluorescent zone (23 mm.) in the middle part of the new column was separated from a weak violet-blue fluorescent zone located immediately below it by cutting and transferred into 1:1 benzene-hexane. This solution, when evaporated to dryness in a 0.5-ml. centrifuge tube, yielded a yellow solid which was washed with cold hexane and recrystallized from benzene-hexane. Yield, 0.1-0.5 mg. of golden yellow crystals; m.p. 157-160° (decomp.).

This crystalline preparation is probably identical with the corresponding, unidentified crystalline compound A found in the Tetraclita squamosa rubescens (ex Corona del Mar.) They are similar in the following respects: color and fluorescence in the solid, dissolved, and adsorbed state; adsorption behavior on alumina; melting point; and spectrum (Figure 1). Mixed m.p. of the two substances, 155-159° (unsharp). No separation took place in the mixed chromatogram test.

1,12-Benzperylene. The 36-mm. blue fluorescent zone (F3c) was rechromatographed on alumina-calcium hydroxide-celite (24 x 4.8 cm.; developer, 1:1 benzene-hexane). The resulting main violet-blue fluorescent zone (43 mm.) was separated from a weak yellowish fluorescent zone immediately above it and transferred into benzene-

hexane (1:1). This solution was evaporated to dryness in a 0.5-ml. centrifuge tube. The yellow solid residue was washed once with cold hexane and recrystallized by dissolving in benzene and slowly evaporating the solution until yellow crystals formed. Fluorescence, greenish-yellow. The sample was washed with cold hexane three times and dried. Yield, 0.1-0.5 mg. M.p. 271-275.5° (decomp.); mixed with synthetic 1,12-benzperylene, m.p. 278-279°. Spectra, Figure 19. A mixed chromatogram test with 1,12-benzperylene showed no separation.

Fraction F4.

Fraction F4 (40 ml.) was developed on a 24 x 4.8 cm. column of alumina-calcium hydroxide-celite with benzene-hexane (1:3):

Chromatogram No. 30

18	orange and red pigments; several fl. zones	F4a
15	non-fl. (column colorless from this point to bottom)	
4	blue fl.	F4b
70	weak violet fl.	
35	intense blue fl. with grayish green fl. near top of zone	F4c
100	olive drab fl.	

Fraction F4b. The benzene-hexane (1:1) solution of the 4-mm. zone (F4b) was rechromatographed on alumina-calcium hydroxide-celite (20 x 3.8 cm.; developer, the same solvent mixture). The several fluorescent top zones were discarded, and an 18-mm. broad

strongly blue fluorescent zone halfway down the column was cut out*. The extinction curve of the latter was almost identical with that of compound H (fraction F5b). A mixed chromatogram test with the two fractions gave no separation.

Fraction F4c.

This fraction was developed on alumina-calcium hydroxide-celite with benzene-hexane 1:3 (24 x 4.8 cm.):

Chromatogram No. 31

12	weak fl.	
17	yellowish-green fl. (discarded)	
65	non-fl.	
20	bluish-violet fl.	F4c1
10	dull grayish-yellow fl.	F4c2
20	intense blue fl.	F4c3
100	non-fl.	

The three zones F4c1-F4c3 were separated carefully by cutting, eluted with acetone, and transferred into benzene-hexane (1:1).

Crystalline polycyclic aromatic compound F (unidentified). The benzene-hexane solution of F4c1, when evaporated almost to dryness

* No fluorescence was discernible in the remaining 90-mm., but its eluate fluoresced weakly. Evaporating the hexane solution to dryness gave a small amount of colorless solid which, after washing once with cold hexane, was recrystallized from benzene-hexane. M.p. 125-126° (clear melt). Spectrum, Figure 16; unidentified. This crystalline preparation which fluoresced only very weakly (bluish-gray) probably does not belong to the class of polycyclic aromatic compounds.

in a 0.5 ml. centrifuge tube and kept at 4°, gave a yellow solid which was washed with cold methanol after pipetting out the yellowish mother liquor. It was recrystallized by dissolving it in a minimum amount of warm 1:1 mixture of hexane and ethanol and cooling. Almost colorless, greenish fluorescent crystals were obtained after washing once with cold ethanol, then twice with cold hexane, and drying. Yield, 0.1-0.5 mg.; m.p. 199-204° (decomp.). Spectrum, Figure 20.

Crystalline polycyclic aromatic compound G(unidentified). The benzene-hexane solution of F4c2 was evaporated to dryness in a 0.5 ml. centrifuge tube. The small amount of yellow solid that formed after standing at 4° was washed with cold hexane, then recrystallized from warm hexane, and washed twice with cold hexane. Pale yellow crystals with greenish yellow fluorescence were obtained. Spectrum, Figure 20.

3,4-Benzpyrene. Upon evaporating the benzene-hexane solution of the 20-mm. intensely blue fluorescent zone F4c3 to a small volume in a 2-ml. centrifuge tube, yellow crystals appeared. They were washed at 0° with cold abs. methanol and dissolved in benzene which was then transferred to a 1 ml. centrifuge tube and evaporated. The residue was covered with a few drops of methanol, and just enough benzene was added to 60° to dissolve the solid. The yellow crystals which formed upon cooling were washed twice with cold methanol and dried. Yield, 1 mg.; m.p. 132-141° (unsharp). The crystalline preparation did not separate on the column from an authentic 3,4-

benzpyrene sample. Spectra, Figure 21.

A coupling of p-nitrobenzenediazonium chloride with both authentic 3,4-benzpyrene and these crystals was carried out. The two dye solutions were purified by developing on alumina-calcium hydroxide-celite with benzene. They did not separate in the mixed chromatogram test and yielded very similar extinction curves (Figure 22).

Fraction F5.

This fraction (50 ml.) was developed on alumina-calcium hydroxide-celite (24 x 4.8 cm.) with a benzene-hexane mixture (1:3):

Chromatogram No. 32

25	several red and orange zones; some fl. zones	F5a
3	non-fl. (colorless from this point to bottom of the column)	
8	intense blue fl.	F5b
140	non-fl.	
20	weak violet fl.	F5c
45	non-fl.	

Crystalline polycyclic aromatic compound H (unidentified). The 8-mm. zone (F5b) was rechromatographed on alumina-calcium hydroxide-celite (20 x 3.8 cm.; developer, 1:1 benzene-hexane). After long development the resulting 32-mm. broad zone (intense blue fluorescence) was transferred into benzene-hexane (1:1). Upon evaporation in a 1-ml. centrifuge tube, reddish-brown crystals were obtained which

were only slightly soluble in hexane. A recrystallization from benzene-hexane gave almost colorless, long needles which were washed with cold hexane and dried. Yield, 1-2 mg. A second recrystallization produced colorless crystals (fluorescence, violet) which melted sharply at 107°. Spectrum, Figure 17. This crystalline preparation is more strongly adsorbed on alumina-calcium hydroxide-celite than is 3,4-benzpyrene; when developed with benzene-hexane (1:1), the two substances are separated easily.

Chrysene. The 1:1 benzene-hexane solution of the 20-mm. zone (F5c) was evaporated almost to dryness in a 0.5-ml. centrifuge tube. The yellow crystals were washed once with cold hexane and recrystallized from benzene-ethanol. Colorless, rather irregular plates; fluorescence, violet-blue. The sample was washed three times with cold hexane and dried. Yield, 0.1-0.5 mg. M.p. 253-254°; mixed with authentic chrysene, m.p. 254°. Spectra, Figure 23. The natural product did not separate from chrysene in the mixed chromatogram test.

Fraction F6.

Fraction F6 (50 ml.) was developed on alumina-calcium hydroxide-celite (24 x 4.8 cm.) with hexane containing 5% benzene:

Chromatogram No. 33

25	several fl. zones	F6a
55	four fl. zones, intermixed with pigments	
6	non-fl. (column colorless from this point downward)	
7	strong greenish-blue fl.	F6b
85	non-fl.	
4	violet fl.	F6c
20	non-fl.	

Fluoranthene. The 7-mm. zone F6b was rechromatographed on alumina-calcium hydroxide-celite (24 x 4.8 cm.; developer, 5% benzene in hexane):

Chromatogram No. 34

4	non-fl.
5	greenish fl. (discarded)
145	non-fl.
25	greenish-blue fl.
60	weak violet fl. (discarded)

The fluorescence of the 25-mm. zone and its spectral curve in hexane solution indicated that it contained fluoranthene. However, preliminary attempts to crystallize this fraction failed, including seeding with a trace of authentic fluoranthene. Consequently, the fluorescent oil was then heated in a sublimation apparatus in vacuo (0.1-mm.) at 110-115° for 30 minutes. Fluorescent, liquid droplets

condensed on the cold finger, while a less fluorescent, oily residue remained in the outer tube. Condensate and residue were dissolved in hexane separately and transferred into 0.5 ml. centrifuge tubes. Upon complete evaporation and standing overnight at 4° crystals formed only in the fraction obtained from the cold finger. They were washed rapidly with small amounts of cold hexane and recrystallized from the same solvent. Spectrum, Figure 24.

The presence of some contaminants in this preparation was evident from its spectrum as compared to fluoranthene; furthermore, it was observed that ethanol precipitated some white solid - different from fluoranthene - from the hexane solution.

Fraction F6c. Attempted crystallization of the 4-mm. violet fluorescent zone of the F6 column was unsuccessful. The spectral curve of the oil in hexane solution exhibited a steady increase in extinction as the wave-length decreased from 400 to 210 mμ. None of the extinction maxima characteristic for anthracene or phenanthrene were present, although the sharply rising curve was interrupted by small peaks located at 255-256 mμ., 228-232 mμ., and 212 mμ. (unidentified).

d. Beach Worm ("Blood worm")(Thoracophelia mucronata).

(1) Extraction.

Eleven kgs. of the worms (including some accompanying sand) were collected at the Scripps Institution of Oceanography,

La Jolla* and kept under acetone (8 liters) at 4° until they were worked up. The procedure for the extraction was essentially that followed by Petracek (38).

After decantation of the acetone the material was ground in Waring blendors to a fine slurry with fresh acetone. The residue obtained from centrifuging this suspension through a basket lined with filter paper was extracted in the usual manner three times with hexane-acetone mixtures (1:1, 2:1, 3:1). Filtration of the acetone suspension was difficult, but the subsequent hexane-acetone extraction mixtures filtered readily. All hexane-acetone filtrates were combined, and the lower of the two phases, formed following the addition of 1-2 l. of benzene and cautious addition of an excess of water, was discarded. The acetone originally used to cover the worms (dark greenish-brown) together with the acetone employed in the blendors was extracted with two liters of a 1:1

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* "Collection of marine bloodworms is done in the following way: Since they are small, buried in the damp sand for depths of from 2 to 12 in. (depending upon physical conditions), and required in great numbers, the sand in which they are living is shovelled into a screening device. For this purpose, we have found it useful to employ a plastic screen, 18 meshes per in., backed by a coarse galvanized wire screen or so-called 'hardware cloth', to provide adequate support. These screens are in turn fastened to a cedar frame 36 in. long x 16 in. wide x 4 in. deep, which is supported above the sand at a height of from 1 to 3 ft. for operational success and convenience. The mass of material on the screen is washed copiously with sea water to remove the sand, which passes readily through the meshes. Contaminating organisms such as the sand crab Emerita analoga, the sand flea Orchestia traskiana, and bits of marine plant detritus, are removed by hand either from the screen or from the worms, placed in a bucket and rapidly swirled in fresh sea water, bringing the contaminants to the top of the mass." (17).

benzene-hexane mixture (addition of water, etc.), and the benzene-hexane solution was combined with the main extract. After washing, the hexane extract (containing benzene) was concentrated in vacuo to 1 liter (brownish-black with strong bluish-gray fluorescence) and saponified with 0.5 liter of 20% methanolic KOH overnight. The mixture was then separated in the usual manner, and the hexane solution recovered concentrated to 200 ml. (dark brown). At this point 800 ml. of acetone were added, and the large quantity of yellow-brown solids that precipitated was filtered off with suction and discarded*. The filtrate was washed free of acetone, dried, and filtered through a layer of celite with suction. The yellow-orange filtrate was still slightly turbid, but after concentrating in vacuo to 100 ml. it became a clear, dark red, and strongly fluorescent (blue).

(2) Resolution of extracted material.

The crude extract (oily liquid) was developed on a 30 x 8 cm. column of alumina-calcium hydroxide-celite with benzene-hexane (1:3):

* This apparently amorphous solid dissolved in water to form a soapy, alkaline solution which gave a white precipitate following the addition of dilute hydrochloric acid. According to Petracek, the substance precipitated from the crude hexane extract of blood worms by acetone is a salt of a weak acid (38).

Chromatogram No. 35

10	colorless (sugar layer)**	
48	yellow, orange, red zones; light brown to violet fl. background	Section I
7	yellow-green fl. with yellow-orange streak	Section II
55	orange; bluish fl.	Section III
180	colorless; very weak fl. at bottom	Section IV
	second filtrate, colorless; blue fl.	Section V
	first filtrate, colorless; blue fl.	Section VI

Sections I-IV were eluted with acetone separately and transferred into hexane; V and VI were concentrated in vacuo to 40 ml. each.

7-Dehydrocholesterol (?).

When Section I was concentrated in vacuo to a small volume, a large quantity of long, colorless needles appeared. When the liquid remaining reached 25-30 ml., the solution and suspended crystals were transferred into a 50-ml. centrifuge tube. After cooling at 0° the orange-red mother liquor was pipetted out, and the crystals were washed in the centrifuge tube once with cold hexane and several times with ethanol and then methanol. The crude, yellowish

** The top sugar layer was used to retard the migration of gummy materials which would have split the column of packed adsorbent (cf. Chromatogram No. 21).

preparation was recrystallized twice by dissolving the crystals in hot ethanol and then adding dist. water, until just before the turbidity point. Upon cooling irregular, colorless plates appeared which were repeatedly washed with cold ethanol-water 1:1 and water. The almost colorless crystals were dried in vacuo (0.1 mm.) at 80°. Yield, 1.57 g. Spectral curve, Figure 30 (full line).

Analysis. Calculated for $C_{27}H_{44}O$ (7-dehydrocholesterol): C, 84.31; H, 11.53. Found: (a) sample dried at 80° in vacuo (0.1-mm.) for two hours, C, 83.34; H, 12.07; (b) sample dried at 80° in vacuo (0.1-mm.) for 18 hours (m.p. 137-140°), C, 84.48; H, 11.53.

Fifty milliliters of hexane was added to the orange-red mother liquor from the original crystallization of the sterol; the solution was washed free of ethanol, dried, and developed on a 27 x 5.8 cm. column of calcium hydroxide-celite with 4% acetone in hexane:

Chromatogram No. 36

3	greenish fl. (discarded)
160	yellowish-brown; weak violet-blue fl. (discarded)
45	several pigment and fl. zones
55	colorless; non-fl.

The eluate (transferred into hexane) of the lower 100-mm. of the column (containing the sterol) was combined with Section II and chromatographed on alumina (see Chromatogram No. 37). The top section (IIA) of the new column was transferred into hexane and combined with the mother liquors (also transferred into hexane) of

recrystallizations carried out previously with the sterol. This total solution was then evaporated to dryness in a 50 ml. centrifuge tube. The residue was dissolved in hot ethanol, and the sterol was precipitated from the solution by adding water. This yellowish preparation was recrystallized by first covering the solid with a little acetone and then adding dropwise chloroform to the slurry (kept in a 60-80° water bath) until a clear solution resulted. With slow cooling colorless crystals appeared in feather-like clumps*. After standing at 0° for 30 minutes the yellow mother liquor was removed, and the crystals were stirred repeatedly in the centrifuge tube with cold fresh portions of methanol until the wash liquid became completely colorless (6-8 washings). The colorless crystals were dried in vacuo (0.1-mm.) at 80° for 8 hours. Yield, 1.35 g.; m.p. 139-141°. Total yield of crystalline 7-dehydrocholesterol from 11 kg. of blood worms, 2.93 g.

Adding conc. sulfuric acid carefully to a chloroform solution of the crystalline preparation produced a dark red color (brownish tincture) in both layers. On treating a chloroform-acetic anhydride solution of the substance with conc. sulfuric acid, the chloroform layer rapidly changed from a light blue to a dark bluish green, while the acid layer assumed a rose red color (black interface between the two layers).

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*Chloroform-acetone was found to be superior to ethanol-water as a solvent pair for recrystallizing this substance.

The reaction with antimony trichloride-acetyl chloride reagent (composition, 18 g. antimony trichloride plus 3 ml. acetyl chloride per 100 ml. chloroform solution) was carried out with the natural sterol and crude commercial 7-dehydrocholesterol (Delta Chemical Co.) as follows (31). One ml. of the chloroform solution of the sterol (1 mg. of substance per ml. of solution) was diluted to 10 ml. with the antimony trichloride-acetyl chloride reagent in a volumetric flask. Five minutes after rapid mixing the spectral curve was determined for the region 318-600 mμ. (beginning readings at 318 mμ. and proceeding to higher wavelengths; total time, 30 minutes). The blank for the spectrophotometer was prepared by diluting 1 ml. of chloroform to 10 ml. with the reagent. Spectra, Figure 30.

Several unsuccessful attempts were made to prepare a 3,5-dinitrobenzoate of the natural product (49).

Section II.

Section II was developed on alumina-calcium hydroxide-celite (24 x 4.8 cm.) with benzene-hexane (1:1):

Chromatogram No. 37

5	colorless; green fl.	
2 50	yellow and orange streaks pale orange	IIA
3	orange-red; blue-green fl.	IIB
10 10 30	pale orange colorless; weak blue fl. very pale yellow	IIC
27	colorless; violet-blue fl.	IID
90	colorless; weak violet fl.	IIE
	Filtrate, yellow; blue fl.	IIF

The six fractions (IIA-IIF) were submitted to further chromatographic resolution until relatively homogeneous zones were obtained. Attempts to crystallize the purified fluorescent fractions were, however, unsuccessful.

Fraction IIB. One 20 x 3.8 column of alumina-calcium hydroxide-celite; developer, benzene-hexane (1:1):

Chromatogram No. 38

38	minor pigment and several minor fl. zones	
12	red	IIB1
20	colorless; blue-greenish gray fl.	IIB2
35	weak bluish-gray fl.; orange pigment in the 15-mm. top section of zone	IIB3

Fraction IIB1. The 12-mm. red zone following further purification by developing on calcium hydroxide-celite with 2% acetone in hexane yielded the spectral curve given in Figure 31 (-.-.-.). Unidentified pigment.

Fraction IIB2. Evaporation of the hexane solution gave an orange oil which could not be crystallized. The oil (diluted with hexane) was chromatographed on alumina-calcium hydroxide-celite (18 x 1.9 cm.; developer, benzene). The 26-mm. strong blue-green fluorescent zone that appeared yielded the spectral curve in Figure 27 (full line). Unidentified fluorescent fraction.

Fraction IIB3. Evaporation of the hexane solution to dryness also gave a non-crystallizable, orange oil. Chromatography under

similar conditions as just mentioned produced a 50-mm. greenish-gray fluorescent zone. Spectrum, Figure 27 (dashed line). Unidentified.

Fraction IIC. One 20 x 3.8 cm. column of alumina-calcium hydroxide-celite: developer, benzene-hexane (1:1):

Chromatogram No. 39

15	minor pigment and fl. zones	
20	orange; weak blue fl.	IIC1
15	pale yellow; weak blue fl.	
15	colorless; weak violet fl.	IIC2

Fraction IIC1. The oil obtained by evaporation of the hexane solution to dryness was diluted with hexane and developed on alumina-calcium hydroxide-celite (18 x 1.9 cm.) with benzene. Two zones were recovered - a 45-mm. broad one showing weak blue-greenish-gray fluorescence (IIC1a) and adsorbed below it, a 25-mm. zone with a faint violet-blue fluorescence (IIC1b). The former yielded a spectral curve similar to IIB2, while IIC1b exhibited a non-characteristic spectrum.

Fraction IIC2. The oil was diluted with hexane and chromatographed under similar conditions as fraction IIC1. The zone recovered contained a 10-mm. of yellowish-gray fluorescence together with 5-mm. of weak violet fluorescence. Spectrum, Figure 27, (-.-.-.); unidentified.

Fraction IID. One 20 x 3.8 cm. column of alumina-calcium hydroxide-celite; developer, benzene-hexane (1:1):

Chromatogram No. 40

25	colorless
10	very pale yellow-orange
20	very pale yellow-orange; blue fl.
10	very pale yellow-orange
150	colorless

The non-crystallizable oil from the 20-mm. zone was diluted with hexane and developed on alumina-calcium hydroxide-celite (18 x 1.9 cm.; developer, 1:3 benzene-hexane). Two adjacent zones resulted - a 35-mm. grayish-blue fluorescent zone (IID1) and a 30-mm. blue fluorescent zone (IID2) adsorbed below it. Spectral curve of IID2, Figure 27 (-'-'-'). The extinction curve of IID1 possessed only a small maximum at 264-265 mμ. Neither fluorescent fraction could be unidentified.

Fraction IIE. One 20 x 3.8 cm. column of alumina-calcium hydroxide-celite; developer, benzene-hexane (1:3):

Chromatogram No. 41

15	non-fl.
45	blue fl.; lower 10-mm. part, blue-violet fl.
140	non-fl.

The non-crystallizable oil obtained from the 45-mm. zone was rechromatographed under similar conditions (18 x 1.9 cm.). The zones cut out from the new chromatogram included a 30-mm. weak blue to blue-white fluorescent zone (IIE1) adsorbed above a 10-mm. blue-violet fluorescent zone (IIE2). Spectra, Figure 28 (IIE1, - - -;

IIE2, -'-'-'); unidentified. The curve of IIE2 contained small peaks at 383 and 362 mμ. which may have been caused by a trace of some polycyclic aromatic compound present. However, after further chromatographic purification the extinction curve remained essentially unaltered.

Fraction IIF. One 20 x 3.8 cm. column of alumina-calcium hydroxide-celite; developer, benzene-hexane (1:3):

Chromatogram No. 42

30	almost colorless; minor fl. zones
30	pale orange
140	colorless
	Filtrate, blue-violet fl.

Evaporating the filtrate to dryness yielded only a non-crystallizable oil which, when diluted with hexane and developed on alumina-calcium hydroxide -celite (18 x 1.9 cm.; developer, 1:9 benzene-hexane), formed a 15-mm. broad weakly grayish-blue fluorescent zone. Spectral curve, Figure 28 (-.-.-.); unidentified.

Section III.

Section III (25 ml.) was developed on a 24 x 4.8 cm. column of alumina-calcium hydroxide-celite with benzene-hexane (1:3):

Chromatogram No. 43

30	colorless; non-fl.	
8	colorless; blue fl.	IIIA
20	pale orange; blue-violet fl.	IIIB
65	orange; bluish fl.	IIIC
120	colorless	

The three fractions IIIA-IIIC were submitted to further chromatographic purification (see below). Attempts to crystallize the relatively homogeneous fluorescent zones obtained were, however, unsuccessful.

Fraction IIIA. One 20 x 3.8 cm. column of alumina-calcium hydroxide-celite; developer, benzene-hexane (1:3):

Chromatogram No. 44

30	non-fl.	
8	blue-gray fl.	
8	dark zone	recovered
10	blue-gray fl.	
154	non-fl.	

The non-crystallizable, orange oil obtained from the 26-mm. section of the column just described was developed on the same adsorbent in a 18 x 1.9 cm. column with 1:4 benzene-hexane. The recovered zone consisted of 52-mm. of light blue fluorescence containing a 6-mm. dark region in the center of the zone. Spectral curve, Figure 28 (full line); unidentified.

Fraction IIIB. Column and developer as given for IIIA:

Chromatogram No. 45

45	non-fl.; includes a minor orange zone near top
70	pale orange; blue fl.
85	colorless; non-fl.

The red oil obtained by evaporating the solvent from the hexane solution of the 70-mm. zone was diluted with hexane and developed on the same adsorbent (18 x 1.9 cm.) with benzene-hexane (1:4).

The new chromatogram contained a 50-mm. yellow-orange, strong blue to blue-violet fluorescent zone (IIIB1) adsorbed above a 10-mm. orange zone (IIIB2) whose fluorescence (blue) seems to be quenched by the pigment. Spectra, Figure 29 (IIIB1, -'-.-'; IIIB2, ——); unidentified.

Fraction IIIC. One 20 x 3.8 cm. column of alumina-silica gel-celite; developer, benzene-hexane (1:3). No separation of the orange pigment from the blue fluorescence was obtained in this column. The recovered zone was then developed on calcium hydroxide-celite (18 x 1.9 cm.) with 2% acetone in hexane, yielding a 25-mm. yellow-orange zone (IIIC1) well separated from a 30-mm. orange zone (IIIC2) below it. Their spectra in Figure 31 (IIIC1, - - -; IIIC2, ——) indicate that these pigments were neo-β-carotene U and β-carotene, respectively.

Section IV.

This section (25 ml.) was developed on a 20 x 3.8 cm. column of alumina-silica gel-celite with 5% benzene in hexane:

Chromatogram No. 46

25	orange, pale yellow-orange zones; blue fl. (discarded)	IVA
60	bluish fl.; colorless from this point to bottom of the column	IVB
15	weak fl. interzone	
15	blue-greenish gray fl. (sharply defined)	IVC
100	non-fl.	

Fraction IVB. The non-crystallizable, yellow-orange oil was diluted with hexane and developed on alumina-calcium hydroxide-celite (18 x 1.9 cm.; developer, 5% benzene in hexane). The chromatogram contained a 17-mm. violet fluorescent zone (IVB1) adsorbed right above an 18-mm. broad blue-fluorescent zone (IVB2). Spectra, Figure 29 (IVB1, -.-.-.; IVB2, - - -); unidentified.

Fraction IVC. The addition of ethanol to the oil obtained from evaporation of the hexane solution to dryness precipitated a gelatinous solid which was discarded. The mother liquor was transferred into hexane and developed on an 18 x 1.9 cm. column of alumina-calcium hydroxide-celite with 5% benzene in hexane, yielding a homogeneous, greenish-blue fluorescent zone (21-mm.). The fluorescence of this fraction both in adsorbates and in solutions resembled that of fluoranthene. Spectral curve, Figure 26; maxima in hexane at 227, 236, 257, 275, 287, 302, 320.5, 336, 358 mμ. (maxima corresponding to those of fluoranthene are underlined). This fraction did not separate from an authentic chromatographically purified fluoranthene sample in the mixed chromatogram test.

Section V.

Section V was developed on a 24 x 4.8 cm. column of alumina-silica gel-celite with benzene-hexane (1:9). A 15-mm. broad blue fluorescent zone remained at the top of the column. It was transferred into hexane, and on evaporation the solution yielded an oil

from which ethanol precipitated some gelatinous solid. The latter was recrystallized from benzene-ethanol, and after washing with ethanol and after drying the pale yellow, greenish-blue fluorescent preparation was dissolved in optical hexane. Spectrum, Figure 29 (-'-'-'); unidentified.

Section VI.

This section was developed on a 20 x 3.8 cm. column of alumina-calcium hydroxide-celite with 5% benzene in hexane:

Chromatogram No. 47

25	non-fl.
60	weak bluish-gray to bluish-violet fl.
120	non-fl.

The 60-mm. zone yielded a colorless, waxy solid following the evaporation of its hexane solution to dryness in a 0.5-ml. centrifuge tube. Adding ethanol slowly to the hexane solution of the solid substance (kept at 0°) resulted in an apparently crystalline product. Recrystallization from benzene-methanol, chloroform-ethanol, ethanol-water, or pyridine-water gave a gelatinous solid on cooling at 0°. With hydroxylic solvents there was a strong tendency for the substance to precipitate out as an oil. In the solid state the preparation fluoresced blue; its hexane solution fluoresced a blue-violet. Spectral curve, Figure 29 (dotted line); unidentified.

e. Marine Plants.

(1) Extraction.

Five species of marine seaweeds were collected near Corona del

Mar, Calif. in amounts varying from one to five kilograms (weighed free of excess liquid). Each sample was worked up as follows. The seaweed was ground up with methanol in a Waring blendor; after filtration the solutes in the filtrate were transferred by addition of water into petroleum ether, while the residue was extracted three times with petroleum ether-methanol mixtures (1:1, 2:1, 3:1). After removal from the solid material in the basket centrifuge (or Buchner funnel), water was added cautiously until two liquid phases separated. All petroleum ether extracts were then combined, dried, and saponified by standing over 20% methanolic KOH overnight. The alkali was eliminated by washing, and the dried extract was concentrated in vacuo to 75 ml.

(2) Resolution of extracted Material.

The crude extract of each seaweed was chromatographed on a 30 x 8 cm. column of calcium hydroxide-celite. In each chromatogram the section of the column from the β-carotene zone to the bottom of the column appeared to be pervaded with a blue to blue-gray fluorescence, while the section above this orange zone contained yellowish-green and greenish-gray fluorescent zones, intermixed with pigment zones and streaks. The fluorescent sections from the whole column were combined and rechromatographed under similar conditions. The individual fluorescent zones (in some cases after further chromatographic purification) were examined in the spectrophotometer.

The chromatogram of each seaweed extract is described below.

Coralline algae.

Starting material, 2.57 kg. Two columns; developer, petroleum ether:

Chromatogram No. 48

6	three minor pigments
18	pink
2	almost colorless
3	pinkish red
8	almost colorless
2	faint yellow
7	colorless
7	faint reddish-brown
2	almost colorless
2	red-brown
25	colorless
4	orange
9	colorless
26	orange (probably β-carotene)
1	colorless
11	pale yellow
8	colorless
5	pale yellow
20	colorless
22	yellow (probably α-carotene)
45	colorless
5	very pale yellow
15	colorless

Five different fluorescent zones were obtained. Each yielded a spectral curve (unidentified) rising sharply in extinction with decreasing wavelength and containing one small maximum near 260 mμ. (in hexane). Phytofluene or a similar polyene was absent.

Gigartina sp.

Starting material, 0.82 kg. Two columns; developer, 0.5%

acetone in petroleum ether:

Chromatogram No. 49

1	red-orange
2	pink; yellowish fl.
23	colorless; fl.
15	pale red
30	almost colorless; fl.
20	orange (probably β -carotene)
2	colorless
10	pale orange
20	colorless; fl.
10	pale yellow
15	colorless; weak fl.
23	yellow (probably α -carotene)
27	colorless; fl.
8	pale yellow
60	colorless; fl.

Seven fluorescent zones were cut out. All except two fractions (which exhibited an additional small maximum at 229 mμ.) gave extinction curves similar to those obtained from the Coralline algae.

Cystoseira sp.

Starting material, 0.91 kg. One column; developer, petroleum ether containing 2 to 5% acetone:

Chromatogram No. 50

10	very pale orange
25	colorless
10	very pale yellow
22	colorless; orange fl.
5	very pale orange
6	colorless
20	pale orange
39	almost colorless; bluish and greenish fl. zones
30	orange (probably β -carotene)
2	colorless

14 pale orange
18 colorless
12 pale yellow
12 colorless
18 yellow (probably α -carotene)
20 colorless

When the 39-mm. zone was rechromatographed, no fluorescent zones appeared. However, the aqueous alcohol phase from transferring the eluate of the 39-mm. zone into petroleum ether fluoresced green. On closer examination it was found that addition of acids (dil. HCl, H_2SO_4, HNO_3, or dil. and glacial acetic acid) increased this fluorescence, while dilute alkali decreased it. Moreover, the fluorescence thus quenched by alkali could be restored by the addition of acid and quenched again by the addition of a base. A small aliquot evaporated completely and taken up in petroleum ether showed no particular fluorescence but when shaken with acidified ethanol, the latter fluoresced with the bright green color observed previously. The total aqueous alcohol solution was exhaustively extracted with petroleum ether until the lower phase, while still slightly fluorescent, did not increase in fluorescence on acidification. The concentrated colorless petroleum ether extract was developed on calcium hydroxide-celite (24 x 4.8 cm.) with 2% acetone in petroleum ether:

Chromatogram No. 51

70	non-fl.	
7	bright greenish fl.	Fraction I
20	non-fl.	
10	bright greenish fl.	Fraction II
10	light bluish fl.	
120	non-fl.	

Since fractions I and II showed the dependence of fluorescence on pH mentioned above, their spectra were determined in solutions containing known amounts of dil. hydrochloric acid. The following solutions were prepared.

Fraction I. A portion of the petroleum ether solution of the 7-mm. zone was evaporated to dryness in a 10-ml. volumetric flask, and the residue was dissolved in 2 ml. of 6 N HCl plus 8 ml. of abs. ethanol to give solution A. Five ml. of A were diluted to 10 ml. with ethanol to give B, while 5 ml. of 6 N HCl were added to the remaining 5 ml. of A to C. The extinction curves of solutions B and C were similar, each containing a single maximum at 226 and 227 mμ., respectively (cf. Table VI).

Fraction II. A portion of the petroleum ether solution of this fraction was evaporated completely, and the residue was re-dissolved in ca. 20 ml. of abs. ethanol. To four 5 ml. aliquots of this solution were added, respectively, 5 ml. of dist. water (D),

5 ml. of 0.755 N HCl (E), 5 ml. of 6 N HCl (F), nothing (G). These solutions stood overnight at 4° before the spectral curves were taken. The extinction curve of a fresh ethanol solution (H) was also taken immediately after preparation of the solution. The spectrum of fraction II was modified slightly when either the pH or the composition of the solution was varied (cf. Figure 32 and Table VI).

Attempts to crystallize either fraction I or II (both unidentified) were unsuccessful.

Codium sp.

Starting material, 2.70 kg. Two columns; developer, 2% acetone in petroleum ether:

Chromatogram No. 52

3	orange
3	yellow
10	pale orange
5	pale pink
12	almost colorless
4	pale orange
3	almost colorless
18	pale pink
3	almost colorless
5	orange
19	almost colorless; fl.
10	yellow; fl.
1	almost colorless
10	orange (probably β-carotene)
8	colorless
10	pale orange
5	almost colorless
11	yellow
3	colorless
16	yellow-orange (probably α-carotene)
8	colorless
5	yellow
5	colorless
8	yellow
52	colorless

Several green and blue-fluorescent zones were observed in this chromatogram, but these fractions did not give characteristic absorption curves in the near ultra-violet.

Phyllospadix sp. ("Mermaid's Hair").

Starting material, 4.20 kg. Three columns; developer, 2% acetone in petroleum ether:

Chromatogram No. 53

8	three green and brown zones
4	red
3	pale yellow-brown
6	pink
2	pale yellow-brown
2	pink
2	almost colorless
17	three narrow yellow-orange and yellow zones
38	almost colorless
5	orange
3	colorless
30	orange-yellow
14	almost colorless; bluish fl.
50	dark orange (probably β-carotene)
1	colorless
15	yellow-orange
10	colorless; bluish fl.
10	yellow-orange
57	colorless; bluish fl.

Eight fluorescent fractions were obtained. Six of them gave extinction curves similar to those from the Coralline algae (maxima, in hexane: three fractions, 263 mμ.; the fourth, 229 mμ.; the fifth, 255 mμ.; and the sixth, 256 mμ.). (Two further fractions yielded non-characteristic curves.)

f. Deep-sea Mud Sample.

(1) Extraction.

The starting material consisted of two mud cores (total weight, 15 kg.) obtained off the coast of Southern California at the following locations: lat. 32°37.84' N, long. 117°22.18' W; and lat. 32°37.72' N, long. 117°22.10' W.

The mud sample was stirred with methanol, and upon sedimentation of the suspension the supernatant liquid was siphoned off. After repeating these operations, the combined solutions were extracted thoroughly with petroleum ether. The remaining mud was extracted four times with mixtures of petroleum ether-methanol (1:1, 2:1, 3:1, and 3:1). The solutes were transferred into petroleum ether by the addition of water. The combined extracts were washed with water, dried, concentrated in vacuo to 1 liter and kept over a layer of 20% methanolic KOH for a night. After washing out the alkali, the orange-brown solution was dried and concentrated to 150 ml.

(2) Resolution of extracted material.

The crude extract was developed on a 30 x 8 cm. column of calcium hydroxide-celite with petroleum ether containing 2% acetone:

Chromatogram No. 54

18	brownish	
3	two red streaks	Section I
19	several minor pigments; several fl. zones	
17	colorless; weak fl.	
10	pale orange (neo- β -carotene U)	
10	colorless	
13	orange (all-trans- β -carotene)	Section II
2	colorless; weak blue-gray fl.	
10	very pale orange	
10	almost colorless; weak blue-gray fl.	
50	pale yellow (probably α -carotene)	
115	colorless; non-fl.	
	Filtrate, blue-fl.	Section III

Section I.

The petroleum ether solution was developed on calcium hydroxide-celite (27 x 5.8 cm.) with petroleum ether containing 2.5% acetone:

Chromatogram No. 55

1	colorless	
1	pinkish red	
5	almost colorless	
3	pinkish red	IA
10	very pale red-orange; greenish fl.	
110	six minor pigments	
30	pale yellow-orange	IB
60	colorless but including a 10-mm. pale yellow zone	

The 30-mm. zone (IB), separated from other pigments above and below by colorless interzones, yielded an extinction curve corresponding to that of neo-β-carotene U; maxima, in petroleum ether: 446-447, 473 mμ. (cf. Figure 31, dashed line).

The 13-mm. section (IA) was rechromatographed under similar conditions (24 x 4.8 cm.; developer, petroleum ether containing 3% acetone):

Chromatogram No. 56

15	colorless
4	red
8	almost colorless
5	pale pink
3	colorless; greenish-yellow fl.
4	colorless
2	yellow orange
200	colorless

The 4 mm. broad red zone yielded, after chromatographic adsorption on calcium carbonate (developer, benzene petroleum ether 1:3), the spectral curve represented in Figure 34. In a partition between petroleum ether and 95% aqueous methanol, the pigment remained completely in the epiphase.

The 3-mm. fluorescent zone gave a non-characteristic extinction curve (Figure 33, curve A).

Section II.

The petroleum ether solution was developed on calcium hydroxide-celite (27 x 5.8 cm.) with petroleum ether containing 2% acetone:

Chromatogram No. 57

145	colorless; non-fl.	
25	orange	IIA
3	colorless; weak blue-gray fl.	IIB
15	pale orange	
5	colorless; weak blue-gray fl.	IIC
18	two pale yellow zones	
40	colorless; non-fl.	

The 25-mm. orange zone (IIA) was identified as all-trans- β -carotene on the basis of its extinction curve, its partition behavior in the system, petroleum ether/95% methanol, and a mixed chromatogram test in which it did not separate from an authentic sample of β -carotene.

The two blue-gray fluorescent zones (IIB and IIC) were each rechromatographed under similar conditions. Spectra, Figure 33 (IIB, curve B and IIC, curve C); unidentified.

Section III.

The fluorescent filtrate was washed free of acetone and concentrated to 10 ml. Spectrum, Figure 33, curve D; unidentified.

B. AN INVESTIGATION OF FLUORESCENT EXTRACTS OBTAINED FROM SOME NON-MARINE MATERIALS (PEAT MOSS, HUMUS, AND GARDEN SOIL).

1. THEORETICAL PART.

From a sample of dark-colored arable soil, Kern in Switzerland obtained an intensely fluorescent benzene extract from which he isolated, after chromatographic resolution, several crystalline polycyclic aromatic hydrocarbons (29). One of these could be identified as chrysene, while the other two compounds (yellow crystals with yellow and green fluorescence) remained unidentified. In order to obtain some information as to the possible origin of these compounds Kern also investigated samples of peat and wood tar. No polynuclear compounds were found in the former material, but the wood tar yielded chrysene, in addition to two other fluorescent substances which were similar to, though not identical with, those he obtained from the soil.

The extinction curve of one of these unidentified hydrocarbons (m.p. 171°; mol.-weight, 283) isolated from the soil sample by Kern resembles closely that of the 3,4-benzpyrene preparation obtained from the Tetraclita barnacles in the present study. Kern's preparation could well have been an impure, naturally occurring sample of this carcinogenic hydrocarbon, analogous to the crystalline substance extracted from barnacles.

In the investigation of organic compounds occurring in fossil materials Blumer (also working in Switzerland) succeeded in isolating several hydrocarbons and pigments by chromatographic analysis of

fossil sea lilies (Millericrus sp.) originating from the lower malm of the Bernese Jura(3). The hydrocarbons he obtained are of interest because they included a non-fluorescent naphthalene derivative (m.p. 121°) and an intensely yellow-green fluorescent crystalline preparation (m.p. 326-330°, decomp.) which was identified on the basis of its spectrum as an alkyl derivative of 1,12-benzperylene.

In the present series of experiments some starting materials similar to those studied by the authors mentioned, such as peat moss, humus, and garden soil, were tested for organic fluorescent compounds. Although the crude extracts of these materials fluoresced strongly in ultra-violet light, no polycyclic aromatic compounds could be detected upon chromatographic resolution of the extracts.

Peat Moss. The sample studied originated from a commercial preparation of moss (Sphagnum sp.) which had been stripped from the surface of the bog and dried solely by exposure to sun and wind (12). As is well known, the uses of Sphagnum moss, e.g., as surgical dressing, is based mainly upon its capacity for absorbing water. However, it has also been suggested that Sphagnum might have antiseptic properties, since intact logs, portions of ancient Viking ships, and even human bodies (after century-old submersion) have been removed from bog water well-preserved (22, 45).

The extract of the peat moss sample yielded upon chromatographic fractionation two unidentified, fluorescent substances whose spectra

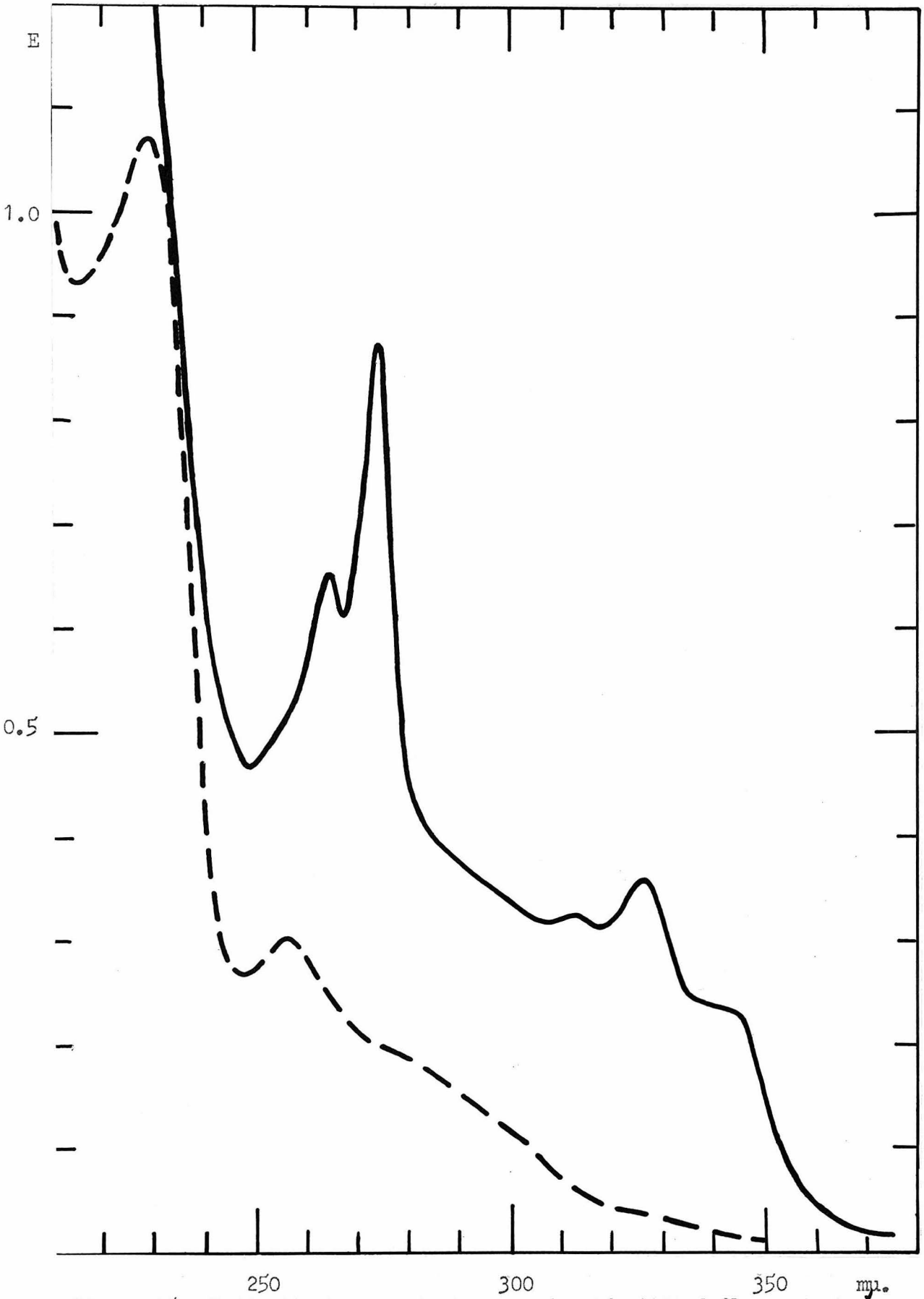

Figure 35. Extinction curves in hexane of unidentified fluorescent fractions ex Sphagnum peat moss: _____, fraction I; - - - -, II.

are shown in Figure 35. The extinction curve represented by the dashed line is similar to some of the fluorescent fractions obtained from marine seaweeds or deep-sea mud. The fraction showing the full line curve was the main fluorescent zone in the chromatogram of the crude extract. Because of its well-defined spectrum this substance should be investigated more closely.

Humus. The starting material consisted of a sample of oak-leaf mold (oak leaves which had been decomposed by being kept outdoors in a moist condition for a year).

The intensely blue fluorescent humus extract could be resolved into several fluorescent substances, two of whose spectra (unidentified) are shown in Figure 36. The general features of one of these extinction curves (full line; fraction IA) resembled that of a polyene type such as phytofluene or phytoene. However, the substance did not give the blue color reaction in the acid-earth test for polyenes and, unlike phytofluene, varied in fluorescence with both solvent and pH of the solution.

Garden Soil. The sample of soil studied yielded a strongly blue fluorescent extract. However, the chromatographically homogeneous fractions which resulted did not show characteristic absorption spectra.

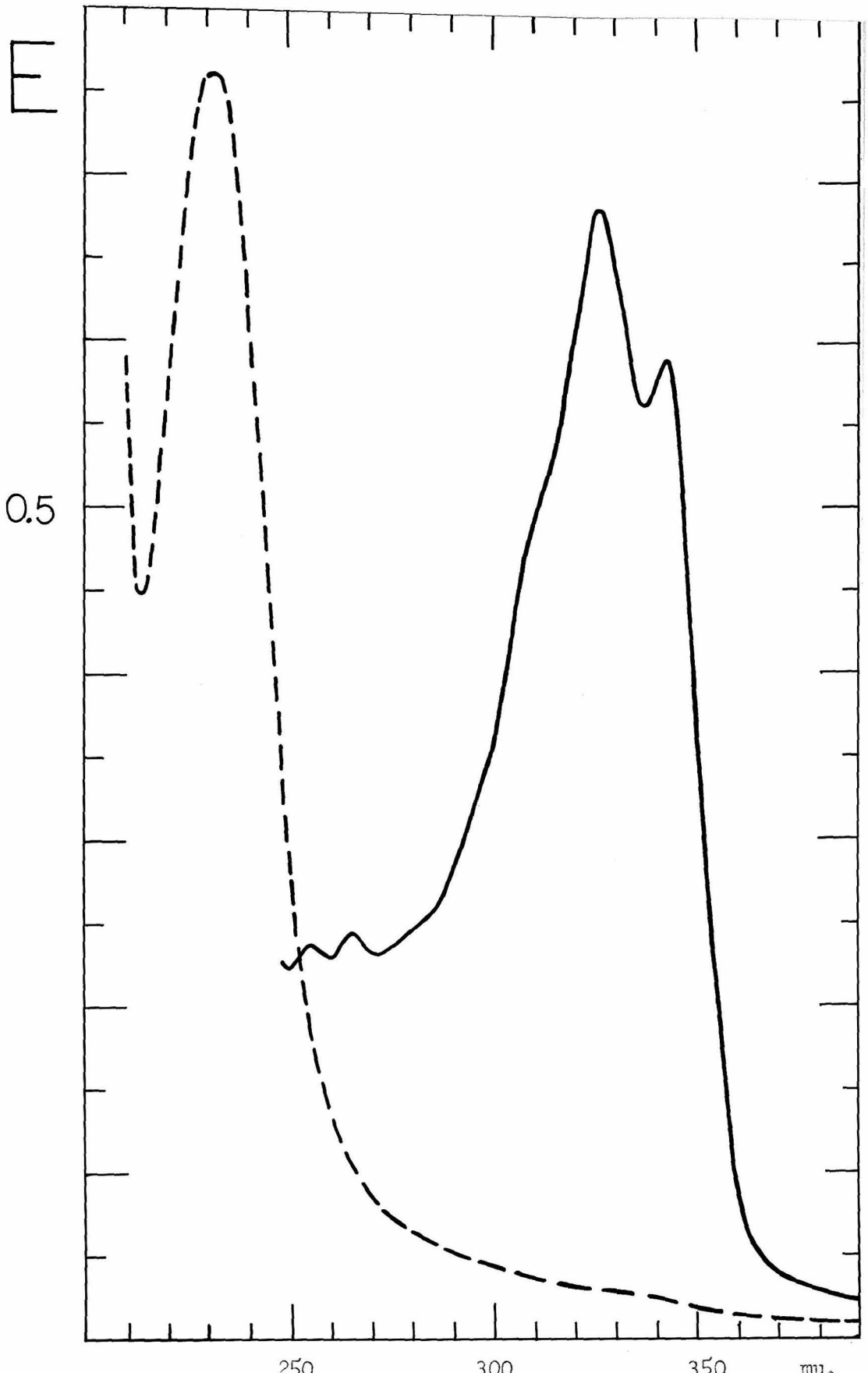

Figure 36. Extinction curves in hexane of unidentified fluorescent fractions ex humus (oak leaf mold): _____, fraction IA; - - - -, II.

2. EXPERIMENTAL PART.

a. Peat Moss.

(1) Extraction.

Six hundred and fifty grams of peat moss (Sphagnum sp.) were dehydrated by standing in methanol overnight at 4°. After filtering the suspension with suction, the residue was extracted with petroleum ether-methanol (2:1) by mechanical shaking for 15 minutes. The filtered liquid separated into two phases upon addition of water. The lower, yellowish aqueous phase was discarded, while the petroleum ether layer, combined with a petroleum ether extract of the methanol filtrate obtained from the dehydration, was washed methanol-free, dried over calcium chloride, and concentrated in vacuo to 200 ml.

(2) Resolution of extracted material.

The crude, dark-brown extract was filtered through a packed layer of celite and then developed on a 30 x 8 cm. column of calcium hydroxide with petroleum ether containing 0.5% acetone:

Chromatogram No. 58

10	brown; including a fl. streak	
40	almost colorless; weak fl.	
5	bright fl. (colorless from this point to bottom of column)	Section I
245	non-fl. Filtrate, fl.	Section II

Section I. The petroleum ether solution of the 5-mm. zone was developed on calcium hydroxide (18 x 1.9 cm.) with the same solvent, and the main 15-mm. zone (strong greenish-blue fluorescence) from the new column was rechromatographed under similar conditions. The petroleum ether solution of the purified fraction fluoresced a

blue color and yielded the extinction curve represented in Figure 35 (full line). Unidentified fraction.

Section II. The filtrate, after washing out the acetone and drying, was added to the petroleum ether solution of the 245-mm. section. The combined solution was developed on alumina-celite 3:1 (20 x 3.8 cm.) with 0% to 6% acetone in petroleum ether. The main zone, whose fluorescence became weaker as it migrated down the column, was transferred into petroleum ether and filtered through an 18 x 1.9 cm. column of silicic acid-celite (4:1). This solution (violet-blue fluorescence), after saponification over 20% methanolic KOH, yielded the spectral curve given in Figure 35 (dashed line). Unidentified fraction.

b. Humus.

(1) Extraction.

Eight kilograms of humus (oak leaf mold) were kept under methanol at 4° for several days. After filtering the suspension in a basket centrifuge, the residue was extracted four times with petroleum ether-methanol mixtures (1:1, 2:1, 3:1, 3:1). The extraction liquids were centrifuged from the solid humus, combined, and treated with an excess of water. The lower aqueous phase was re-extracted with petroleum ether. The methanol used to cover the starting material was similarly extracted with petroleum ether. All extracts were combined, washed methanol-free, dried, and saponified over 20% methanolic KOH for a night. The dark orange, strongly blue fluorescent petroleum ether layer was washed alkali-free, dried,

and concentrated in vacuo to 200 ml.

(2) Resolution of extracted material.

The crude extract when poured on top of a calcium hydroxide-celite column split the latter in half lengthwise because of the presence of gummy material. After eluting with acetone-ethanol (1:1) and transferring into petroleum ether, the extract (red) was concentrated to 50 ml. and developed on a 30 x 8 cm. column of calcium hydroxide-celite with the same solvent until most of the fluorescent zones had passed into the filtrate. A 35-mm. zone with yellow-orange fluorescence remaining at the top of the column was discarded, but the petroleum ether extract of the eluate of the entire column below this zone was added to the 4 liters of chromatographic filtrate. The combined solution was concentrated to 75 ml. and developed with petroleum ether on two 30 x 8 cm. columns of calcium-hydroxide-celite:

Chromatogram No. 59

3	yellow fl.	
72	mixture of fl. zones, mostly blue-gray fl.	
10	intense blue-green fl.	Section I
80	mixture of fl. zones	
20	blue-gray fl.	Section II
70	non-fl.	
10	blue-gray fl.	Section III
30	non-fl.	

Section I.

The petroleum ether solution of the 10-mm. zone was developed on a 30 x 8-cm. column of calcium hydroxide-celite with the same solvent:

Chromatogram No. 60

10	yellow fl.	
65	gray-blue fl.	
40	blue-green fl.	Fraction IA
5	non-fl.	
15	blue-gray fl.	IB
8	non-fl.	
8	blue-gray fl.	IC
9	gray	
130	non-fl.	

Fraction IA. The 40-mm. zone was submitted to two adsorptions on calcium hydroxide-celite (developer, 0.5% acetone in petroleum ether). The purified fraction was then saponified over 20% methanolic KOH and chromatographed once more under similar conditions. Spectrum, Figure 36 (full line); unidentified.

A colorless, fluorescent solid (0.3 mg.) melting at about 53-55° was obtained from this fraction by evaporating the solution completely and recrystallizing the residue twice from benzene-methanol. The preparation failed to give a blue color in the acid-earth (Super-filtrol) test for polyenes.

The following observations were made with the mother liquor from the crystallization mentioned above. When partitioned between equal volumes of hexane and 95% methanol, the ratio of concentration in the epiphase to that in the hypophase was 2:1 for fraction IA. Petroleum ether and acetone solutions were only weakly fluorescent, while methanol solutions fluoresced a blue-green color. Moreover, the fluorescence of the methanol solution became an intense green in the presence of dil. hydrochloric acid. The subsequent addition of dil. alkali to the acidified solution quenched the fluorescence and also precipitated a colorless, amorphous solid which could be dissolved in petroleum ether. Evaporating this solution completely and taking up the residue with acidified methanol again gave an intensely green fluorescent solution.

Fractions IB and IC. These two fluorescent fractions yielded extinction curves which showed a steady increase in extinction with decreasing wavelength. However, fraction IC did possess a small maximum at 260-262 mμ. (in hexane).

Section II.

The petroleum ether solution was developed on a 30 x 8 cm. column of calcium hydroxide-celite with the same solvent. A 170-mm. zone with faint blue-gray fluorescence appeared which yielded the spectral curve in Figure 36, (dashed line). Unidentified fraction.

Section III.

The petroleum ether solution was chromatographed on calcium hydroxide-celite. The broad fluorescent zone passed rapidly into the filtrate, imparting to it a deep blue fluorescence. However, this solution did not give a characteristic extinction curve.

c. Garden Soil Sample.

(1) Extraction.

A sample of garden soil (6 kg.) obtained from the California Institute of Technology farm near Arcadia, Calif., was stirred with methanol and allowed to stand overnight at 4°. After filtration in a basket centrifuge the solutes extracted by the methanol were transferred into petroleum ether, while the soil was extracted four more times with petroleum ether-methanol mixtures (1:1, 2:1, 3:1, 3:1). Following the addition of water and separation of the aqueous methanol layers all petroleum ether extracts were combined, dried, and saponified by standing over 20% methanolic KOH for three hours. The orange extract was washed free of alkali, dried, and concentrated in vacuo to 250 ml.

(2) Resolution of extracted material.

The crude extract (with strong blue fluorescence) was developed on calcium hydroxide-celite (30 x 8 cm.) with 2% acetone in petroleum ether:

Chromatogram No. 61

3	yellow-brown	
12	almost colorless	
1	brown	
35	several fl. zones (colorless from here to bottom)	Section I
15	non-fl.	
20	blue-violet fl.	Section II
55	non-fl.	
30	a blue-green fl. and a blue-gray fl. zone	Section III
75	non-fl.	
15	weak blue-gray fl.	Section IV
30	non-fl.	
	Filtrate, blue-violet fl.	

Sections I, II, III, and IV were rechromatographed separately under similar conditions.

Section I yielded a blue-violet and several greenish-gray fluorescent zones, none of which, however, gave a characteristic extinction curve. Sections II and III each gave a bluish-gray fluorescent zone. Only the extinction curve of the zone from section III showed a small maximum at 263 mμ. (in hexane). Two weak bluish-gray fluorescent zones were obtained from section IV. Their extinctions curves showed a single small peak at 260 and 225 mμ., respectively (in hexane). All fractions were unidentified.

II. STEREOISOMERIZATION OF PRO-γ-CAROTENE, PROLYCOPENE, AND LYCOPENE IN CHICKENS AND HENS.

A. THEORETICAL PART.

Previous biological studies on naturally occurring polycis carotenoids, as well as on artificially prepared cis compounds, were designed to determine the quantitative relationships in vitamin A potency between these isomers and the corresponding all-trans form (for a review of this field, cf. reference 53). The very small daily doses (micrograms) involved in these assays were insufficient for furnishing information as to what extent cis-trans isomerizations of carotenoids occurred in the rat or chick. The present investigation was undertaken in order to obtain some information on the degree of stability of polycis carotenoids, both from a chemical and a stereochemical viewpoint, when administered in relatively large amounts over a period of several days to hens and chickens which had been depleted of carotenoids.

The compounds which were studied in this connection included pro-γ-carotene, prolycopene, and all-trans-lycopene (the latter for a comparison). Pro-γ-carotene and prolycopene belong to the class of naturally occurring polycis carotenoids (33,54,59,67) in which many of the conjugated double bonds not sterically hindered for cis-trans isomerism are in the cis configuration (57). Thus, pro-γ-carotene is considered to be 3,5,7,9,11-penta-cis-γ-carotene, while prolycopene is probably 1,3,5,7,9,11-hexa-cis-lycopene (52). In the vitamin A assays mentioned, pro-γ-carotene

has been shown to exhibit a high activity in both the rat and the chick (23,60), whereas prolycopene and all-*trans*-lycopene are inert in this respect (14,15).

Although various stereochemical forms of the carotenoids can be produced *in vitro* from any single member of the set, less is known as to the biological interconversion of such isomers. Kemmerer and Fraps studied the steric rearrangement of small quantities (0.1-0.5 mg.) of neo-β-carotene U (probably 3-mono-*cis*-β-carotene*) in the rat (28). In one experiment these authors recovered 37.5% of the pigment administered from the stomach and intestines, after allowing a Wesson oil solution of neo-β-carotene U to remain in the digestive tract of the rat for 6 hours. The recovered pigments included 58.2% of unchanged neo-β-carotene U, 17.7% of all-*trans*-β-carotene, 11.5% of neo-β-carotene B (probably a central-peripheral di-*cis*-β-carotene), and 12.6% of an isomer called "impurity A". In another experiment in which a similar solution was fed to three rats for 3 days, the authors recovered 21% of the pigment administered. The recovered mixture included 35.8% neo-β-carotene U, 45.4% all-*trans*-β-carotene, 8.4% neo-β-carotene B, and 10.4% "impurity A".

The general methods of investigation employed in the present study of the chemical stability and stereochemical transformations

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* The probable stereochemical configurations for the *cis*- carotenoids mentioned in this Thesis are taken from reference (52).

of pro-γ-carotene, prolycopene, and all-trans-lycopene in an animal organism are briefly summarized as follows. Relatively large quantities of the test carotenoids (dissolved in Wesson oil) were administered to depleted chickens and hens for three days (cf. Table XIX)*. During the feeding period the feces of the birds were collected, and at the end of three days they were sacrificed. The livers, gut walls, ovaries, gizzards, and body fat were removed, and crude extracts of these tissues, the saline washings of the gut interiors, and the feces were prepared and resolved chromatographically. The individual pigment fractions (well-differentiated on the column) were examined in the Beckman spectrophotometer in order to estimate quantitatively the amounts recovered and then diagnosed as to their stereochemical configuration by visual observation of their spectra before and after iodine catalysis of the solutions. Only those pigments which gave the maxima of the stereoisomeric equilibrium mixture upon iodine catalysis were accepted as belonging to the stereoisomeric set under investigation.

Tables X-XIV, XVII and XVIII contain the results of the chromatographic analysis of the carotenoids recovered from the feces, gut washings, and various tissues after the feeding of pro-γ-carotene, prolycopene, and all-trans-lycopene to chickens and hens. As shown by these tables, most of the pigment administered was destroyed.

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* The depletion of the experimental animals, the administration of the carotenoids, and the collection of the starting materials (feces, gut washings, and tissues) were performed by Professor H. J. Deuel, Jr. and Dr. J. Ganguly of the University of Southern California.

Table X

Stereoisomeric γ-Carotenes Recovered After the Administration of a Total of 9 mg. Pro-γ-carotene to 2 Young Chickens

Pigment	Carotenoids Recovered From feces	From tissues & gut washings	Total
Unchanged pro-γ-carotene	0.857 mg.	0.139 mg.	0.996 mg.
All-trans-γ-carotene formed	.340	.065	.405
Neo-γ-carotenes formed	.294	.054	.348
A polycis-γ-carotene formed	.128	.024	.152
Unidentified altered pigments	.037	----	.037
Total recovered	1.656	0.282	1.938
(Total isomerized)	(0.762)	(0.143)	(0.905)

Of the total pro-γ-carotene fed only 21.5% could be recovered from the feces and tissues in form of pigments (Table X). Forty-seven percent of this recovered pigment had bio-isomerized to different configurations. The isomers thus formed included not only all-trans-γ-carotene and some neo-γ-carotenes but also a new polycis-γ-carotene which showed weaker adsorption affinity and shorter wave length extinction maxima than did pro-γ-carotene (Figure 37). This polycis-γ-carotene was observed in the liver, gut washings, and feces (cf. Table XI).

Table XI

Stereoisomeric γ-Carotenes Recovered After the Administration of a Total of 9 mg. Pro-γ-carotene to 2 Young Chickens

Fraction (in chromatographic sequence)	Wave Lengths of Extinction Maxima (mμ.) Beckman spectrophotometer Solvent: hexane	Evaluating grating spectroscope Before iodine catalysis	Upon iodine catalysis	Pigment	Amount (mg.)
From feces:					
F1 (top)	401-3, 424-5, 449	453 (blurred)	No change	Unidentified	0.012
F2	402, 426, 446	Indistinct	No change	Unidentified	0.025
F3	436, 460, 491	461, 492	Small decrease in wave length	γ-Carotene	.340
F4	456-8, 485	456, 490.5	Small increase in wave length	Neo-γ-carotene	.170
F5	431, 454, 481-2	457.5, 489.5	Small increase in wave length	Neo-γ-carotenes	.124
F6	434-5, 456-8	461 (unclear)	459.5, 490	Pro-γ-carotene	.857
F7	428-9, 448-50	457 "	459, 490.5	Polycis-γ-carotene	.128
From livers:					
L1 (top)	436, 460, 490	460, 492.5	Small decrease in wave length	γ-Carotene	0.017
L2	436, 461, 491	461, 493.5	Small decrease in wave length	γ-Carotene	.036
L3	458, 486	459, 492	Small increase in wave length	Neo-γ-carotene	.020
L4	432-3, 453-4, 481	457, 487.5	Small increase in wave length	Neo-γ-carotenes	.030
L5	434, 457	461 (blurred)	459.5, 491	Pro-γ-carotene	.113
L6	428, 448	456.5 "	458.5, 491.5	Polycis-γ-carotene	.022

Table XI (continued)

Fraction (in chromatographic sequence)	Wave Lengths of Extinction Maxima (mμ.) Beckman spectrophotometer Solvent: hexane	Evaluating grating spectroscope Before iodine catalysis	Upon iodine catalysis	Pigment	Amount (mg.)
From gut walls:					
G1 (top)	436, 461, 491			γ-Carotene	0.005
G2	458, 487-8			Neo-γ-carotene	.004
G3	434, 456	461 (unclear)	460, 492	Pro-γ-carotene	.016
From gut washings:					
GW1 (top)	437-8, 460, 489-90	458, 493	Small decrease in wave length	γ-Carotene	0.007
GW2	434, 457	458.5 (blurred)	458, 491	Pro-γ-carotene	.010
GW3	429, 450-2 (after iodine: 434, 459, 488-9)			Polycis-γ-carotene	.002

Table XII

Stereoisomeric Lycopenes Recovered After the Administration of a Total of 15 mg. Prolycopene to 2 Mature Hens

Pigment	Carotenoids Recovered From feces	From tissues & gut washings	Total
Unchanged prolycopene	0.445 mg.	0.363 mg.	0.808 mg.
All-trans-lycopene formed	.712	.042	.754
Neolycopenes formed	.562	.027	.589
Polycis-lycopenes formed	.611	.194	.805
Unidentified	0.050	0.033	0.083
Total recovered	2.380	0.659	3.039
(Total isomerized)	(1.885)	(0.263)	(2.148)

In the prolycopene experiment it was possible to account for only 20.3% of the carotenoid administered; 71% of the recovered pigments possessed configurations other than that of prolycopene (Table XII). They included newly-formed polycis-lycopenes in addition to all-trans-lycopene and some neolycopenes. Four polycis-lycopenes were obtained from the feces. One of these exhibited weaker adsorption affinity (toward calcium hydroxide) and shorter wave length extinction maxima than prolycopene, while the other three polycis compounds displayed the opposite behavior in both respects (Figure 38). Some of these products were also present in the liver, ovaries, gut walls, and gut washings, besides traces of further polycis members of this set (cf. Table XIII).

Table XIII

Stereoisomeric Lycopenes Recovered After the Administration of a Total of 15 mg. Prolycopene to 2 Mature Hens

Fraction (in chromatographic sequence)	Wave Lengths of Extinction Maxima (mμ.) Beckman spectrophotometer Solvent: hexane	Evaluating grating spectroscope Before iodine catalysis	Evaluating grating spectroscope Upon iodine catalysis	Pigment	Amount (mg.)
From feces:					
F1	429, 452-3			Unidentified	0.050
F2	444-5, 468-9, 500-1	470.5, 502	Small decrease in wave length	Lycopene	.079
F3	444-5, 470, 501-2	470, 502.5	Small decrease in wave length	Lycopene	.633
F4	440, 465-6, 496	464.5, 498	Small increase in wave length	Neolycopene A	.501
F5	458-9, 485-7	459.5, 493.5	Small increase in wave length	Neolycopene	.061
F6	446, 470	447, 474.5	469.5, 500.5	A polycis-lycopene	.296
F7	442-4, 465-7	472 (unclear)	469.5, 499.5	A polycis-lycopene	.121
F8	440-1, 462-4	467.5 "	470, 500	A polycis-lycopene	.098
F9	438	470 "	471, 501	Prolycopene	.445
F10	413, 432-3	462 "	468, 499	A polycis-lycopene	.096
From livers:					
L1	444-5, 471, 500-1	471, 501.5	Small decrease in wave length	Lycopene	0.030
L3	442-4, 466-7	472.5 (unclear)	470.5, 500	A polycis-lycopene	.020
L4	438	465 "	469, 500	Prolycopene	.205
L5	410-1, 430	463.5 "	469, 501	A polycis-lycopene	.024

Table XIII (continued)

Fraction (in chromatographic sequence)	Wave Lengths of Extinction Maxima (mμ.) Beckman spectrophotometer Solvent: hexane	Evaluating grating spectroscope Before iodine catalysis	Upon iodine catalysis	Pigment	Amount (mg.)
From ovaries:					
O1	444, 467-8, 496	470.5, 500.5	469, 500	Lycopene	0.005
O2	440-1, 463-4, 492-3	466, 496.5	Small increase in wave length	Neolycopene	.027
O3	446, 471	475 (unclear)	469, 500.5	A polycis-lycopene	.042
O4	440, 463	469 "	470, 500.5	Polycis-lycopenes	.070
O5	438	467 "	469, 500	Prolycopene	.117
O6	430-1	463.5 "	469, 500	A polycis-lycopene	.028
From gut walls:					
G1	445, 471, 498	Indistinct	Indistinct	Unidentified	0.006
G2	447-8, 472	478 (unclear)	Indistinct	Unidentified	.005
G3	444, 470-1	475 "	470, 498	A polycis-lycopene	.007
G4	439	472 "	470, 500	Prolycopene	.033
From gut washings:					
GW1	443-4, 468-9, 499	470.5, 502	Small decrease in wave length	Lycopene	0.007
GW2	448 (after iodine: 467, 494)			A polycis-lycopene	.003
GW3	438	Indistinct	468, 500	Prolycopene	.008
From gizzard:					
S1	402, 426, 448			Unidentified	0.003
S2	444, 472	477 (blurred)	No change	Unidentified	.007
S3	423, 444, 472	477 "	"	Unidentified	.005
S4	443-4, 472			Unidentified	.004
S5	442-4, 470-1			Unidentified	.003

Figure 37.

Molecular extinction coefficients of pigments recovered from chick feces after feeding pro-γ-carotene: _____, unchanged pro-γ-carotene; - - -, a polycis-γ-carotene formed. Solvent, petroleum ether (b.p. 60-70°).

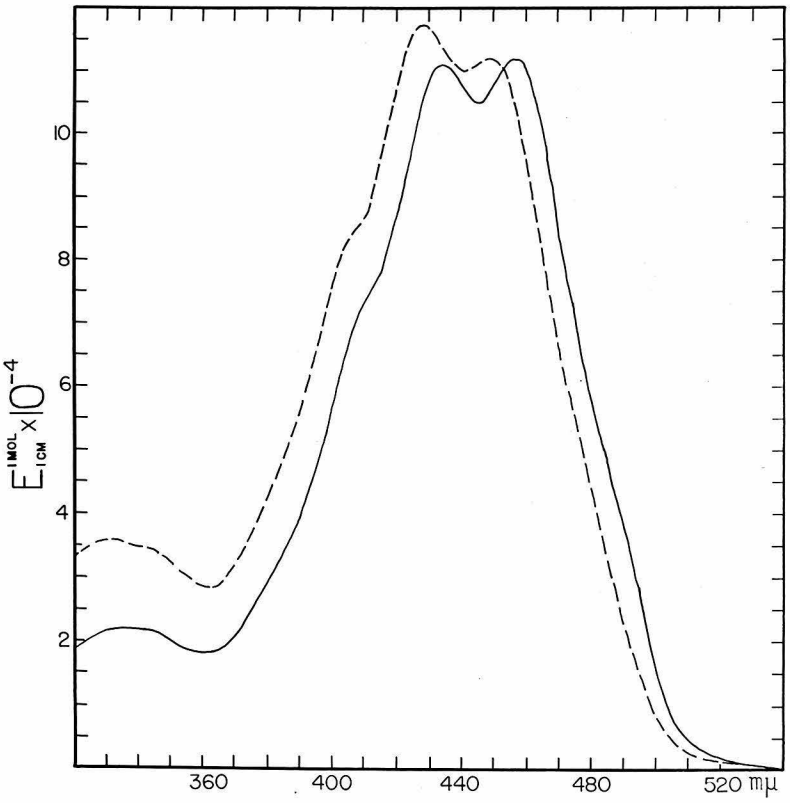
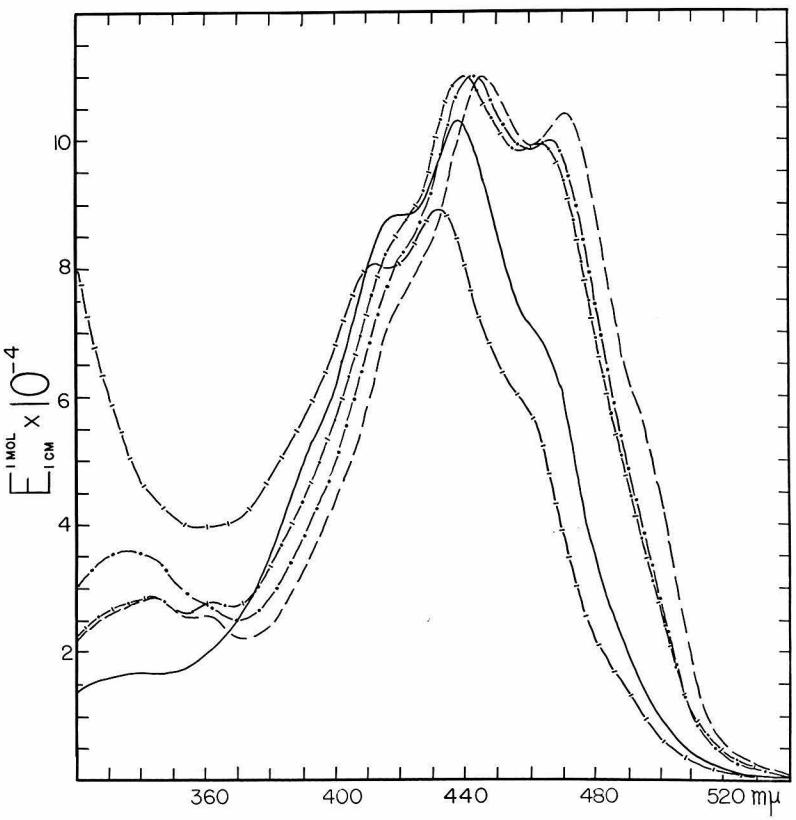


Figure 38.

Molecular extinction coefficients of pigments recovered from hen feces after feeding prolycopene: _____ unchanged prolycopene; - - -, a polycis-lycopene (F6); -.-.-., a polycis-lycopene (F7); -'-.-', a polycis-lycopene (F8); -'-'-'-, a polycis-lycopene (F10). Solvent, petroleum ether (b.p. 60-70°).



A remarkable feature of the bio-stereoisomerizations observed in the chickens and hens is that they do not proceed merely from the polycis forms fed to the corresponding all-trans (and neo-) isomers. Since trans→cis rearrangements in carotenoid molecules cause a shift of the extinction maxima toward shorter wave lengths, (52, 57) the bio-stereoisomerization studied yielded, evidently, a polycis isomer containing more double bonds in the cis configuration than the pigment administered.

Table XIV

Stereoisomeric Lycopenes Recovered After the Administration of a Total of 15 mg. All-trans-lycopene to 2 Mature Hens

Pigment	Carotenoids Recovered From feces	From tissues & gut washings	Total
Unchanged all-trans-lycopene	3.375 mg.	0.112 mg.	3.487 mg.
Neolycopenes	0.909	----	0.909
Unidentified	----	.126	.126
Total recovered	4.284	0.238	4.522

The results of the present investigation are compared in Tables XV and XVI with those of in vitro stereoisomerizations of pro-γ-carotene (62, 67) and prolycopene (33, 57) as reported by Zechmeister and collaborators. With the former compound, by refluxing a petroleum ether solution or melting the crystals, one to two polycis-γ-carotenes with shorter wave length maxima and weaker adsorption

Table XV

Cis-trans Isomerizations of Pro-γ-carotene*

	Method Used	Stereoisomeric γ-carotenes Formed
(1)	Standing in presence of catalytic amounts of iodine for 90 seconds at room temperature (solvent, petroleum ether).	All-trans-γ-carotene, neo-γ-carotenes, pro-γ-carotene.
(2)	Refluxing a petroleum ether solution for 30 min.	All-trans-γ-carotene, pro-γ-carotene (predominant product), a polycis-γ-carotene.
(3)	Melting crystals in sealed tube filled with CO_2 at 130-135° for 20 min.	All-trans-γ-carotene, neo-γ isomers, pro-γ-carotene, a neo-isomer, a polycis-γ-carotene.
(4)	Melting crystals in sealed tube at 130° for 1 min.	All-trans-γ-carotene, neo-isomers, pro-γ-carotene, polycis-γ-carotenes ("all-cis" and "penta-cis").
(5)	Feeding Wesson oil solution of pro-γ-carotene to chickens for 3 days.	All-trans-γ-carotene, neo-isomers, pro-γ-carotene, a polycis-γ-carotene.

* Data for (1), (2), and (3) were taken from reference (67) and that for (4), from reference (62).

affinity than the starting substance were obtained. In the case of prolycopene, either a brief contact of a solution with iodine catalyst or melting the crystals produced several polycis-lycopenes which were located both above and below the unchanged starting compound and showed longer and shorter wave length maxima, respectively, than prolycopene. In all these stereoisomerizations the all-trans compound as well as neo-isomers was also formed.

Table XVI

Cis-trans Isomerizations of Prolycopene*

Method Used	Stereoisomeric Lycopenes Formed
(1) Standing in presence of catalytic amounts of iodine for 2 min. (solvent, petroleum ether).	All-trans-lycopene, neolycopenes, several polycis-lycopenes, prolycopene, a polycis-lycopene.
(2) Same as (1) except standing period increased to 30 min.	All-trans-lycopene, neolycopenes.
(3) Melting crystals in sealed tube at 115° for 5 min.	All-trans-lycopene, a polycis-lycopene ("crystallizable isomer"), prolycopene, a polycis-lycopene ("all-cis-lycopene").
(4) Feeding Wesson oil solution of prolycopene to hens for 3 days.	All-trans-lycopene, neolycopenes, several polycis-lycopenes, prolycopene, a polycis-lycopene.

* Data for (1) and (2) were taken from reference (33) and that for (3), from reference (57).

In contrast to the feeding experiments using polycis compounds, only unchanged starting material and neo-isomers could be recovered from the feces and tissues after administering all-trans-lycopene (15 mg.) to 2 hens (cf. Table XVII). The recovery of total pigment was 30%, of which 20% consisted of stereoisomerized products (Table XIV). In general, polycis compounds have not been observed when the all-trans- form of carotenoids was isomerized by various in vitro methods (52).

In the course of the present investigation, the chromatograms of a few crude extracts have been observed to contain several adjacent

Table XVII

Stereoisomeric Lycopenes Recovered After the Administration of a Total of 15 mg. All-trans-lycopene to 2 Mature Hens.

Fraction (In chromatographic sequence)	Wave Lengths of Extinction Maxima (mμ.) Beckman spectrophotometer Solvent: hexane	Evaluating grating spectroscope Before iodine catalysis	Upon iodine catalysis	Pigment	Amount (mg.)
From feces:					
F1	445, 470, 502			Lycopene	0.025
F2	444-5, 471, 503	473, 504.3	469.5, 501	Lycopene	1.860
F3	445, 472, 503	473, 504.4	501.4	Lycopene	1.490
F4	439-40, 465-6, 496	467.5, 500.1	501.5	Neolycopenes	0.909
From livers:					
L1*				Lycopene	0.050
L2	444, 470, 500			Lycopene	.049
L3	446, 472-3 (after iodine: 445, 470)			Unidentified	.046
From gut walls:					
G1	424-5, 447-8, 470			Unidentified	0.015
G2	428-9, 448, 476			Unidentified	.065
From gut washings:					
GW1	444, 471, 502			Lycopene	0.013

* Fraction L1 was lost during washing operations. On the column it formed a red-orange zone which by comparison with the feces chromatogram was assumed to be all-trans-lycopene and estimated to be about 0.05 mg.

zones which showed the spectrum of the corresponding all-trans form of the pigment administered. In the pro-γ-carotene experiment fractions L1 and L2 (Table XI) each yielded the extinction curve for all-trans-γ-carotene, while fractions F2 and F3 from the pro-lycopene experiment (Table XIII) and fractions F1, F2, and F3 from the lycopene experiment (Table XVII) were all found to show the spectrum of all-trans-lycopene.

In order to investigate this phenomenon more closely, an unusually large dose of all-trans-lycopene (192 mg.) was given to 8 hens, and the feces collected during the 12-day feeding period were examined. As seen in Table XVIII two well-defined zones (F1 and F2) were obtained which exhibited the spectrum of all-trans-lycopene.

Table XVIII

Pigments Recovered After Administering 192 mg. of All-trans-lycopene to 8 Hens

Fraction	Visually observed maxima		Pigment	Amount (mg.)
	Fresh hexane solution	After iodine catalysis		
F1	445, 473.5, 504.5 mu.	Small decrease in wave length	All-trans-lycopene	3.23
F2	444.5, 473, 505	Small decrease in wave length	All-trans-lycopene	59.4
F3	438, 466.5, 499	(440), 468.5, 501.5	Neolycopene A	25.5

When F2 (Table XVIII) was rechromatographed, again two adjacent red-orange zones resulted, both of which gave the extinction curve for

all-trans-lycopene. The lower (and larger) one of these two zones was definitely identified as all-trans-lycopene by analysis of the crystals prepared from it and by the non-separation of the pigment from pure lycopene (obtained from commercial tomato paste) in the mixed chromatogram test.

Analysis of the crystals obtained from the smaller and more strongly adsorbed zone F1 (Table XVIII) suggested that this pigment could be an oxygenated lycopene. However, in mixed chromatogram tests no separation from authentic lycopene occurred. Moreover, the crystalline preparation did not appear to separate even from the lycopene sample (F2) recovered from the feces, in contrast to its behavior in the chromatogram of the crude extract.

The occurrence in the chromatograms mentioned of several closely located pigments exhibiting the spectrum of the all-trans carotenoid is similar to some so far unexplained observations reported in the literature for all-trans-γ-carotene (in connection with its isolation from Mimulus longiflorus) (66) and for all-trans-lycopene (obtained from prolycopene by iodine catalysis) (33).

It is possible that the appearance of these neighboring "all-trans-lycopene" and "all-trans-γ-carotene" zones is caused by the presence of small amounts of isomeric or structurally slightly different compounds in which the chromophoric system of conjugated double bonds is the same as that in the all-trans carotenoid (e.g.,

saturation of the isopropylidene group or groups in the molecule with hydrogen) (24).

Summary.

When relatively large amounts of the polycis compounds, pro-γ-carotene and prolycopene, were administered to chickens and hens which had been previously depleted of carotenoids, about 80% of the pigment was destroyed, while the rest was recovered mainly from the feces. The bio-isomerization that occurred yielded the all-trans and some neo-forms of the carotenoid administered besides polycis isomers, some of which possessed more cis double bonds than the starting material.

Some observations on the behavior of all-trans-lycopene in hens were also made.

B. EXPERIMENTAL PART.

1. Starting Materials.

Pro-γ-carotene, prolycopene, and all-trans lycopene* were administered in separate experiments to New Hampshire red chickens which had been maintained in weak diffuse light on a carotenoid-free diet for a minimum depletion period of two weeks (70). The requisite doses were prepared by thoroughly mixing Wesson oil solutions of the carotenoid samples with the carotenoid-free food (cf. Table XIX).

The stools were collected each morning and preserved under methanol in a deep-freeze (-29°). The birds were anesthetized with pentobarbital sodium and sacrificed; the liver, body fat, gizzard, and ovaries were removed and immediately frozen. In the case of the intestine, the gut walls were frozen after the contents had first been removed by washing them with 3 x 20 ml. of saline (intestinal washings were analyzed separately). All manipulations were performed in the dark.

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* Analytically pure and chromatographically homogeneous crystalline carotenoid samples were used. The polycis compounds were prepared from the ripe berries of Pyracantha augustifolia, while the all-trans-lycopene was obtained from commercial tomato paste.

Table XIX

The Administration of Pro-γ-carotene, Prolycopene, and All-trans-lycopene to Chickens and Hens

Compound Administered	Total Amount Fed	Number of Animals	Feeding Period
Pro-γ-carotene	9 mg.	2 young chickens	3 days
Prolycopene	15 mg.	2 mature hens	3 days
All-trans-lycopene	15 mg.	2 mature hens	3 days
All-trans-lycopene	192 mg.	8 mature hens	12 days

2. Extraction.

Corresponding organs, tissues, and materials from all the animals of a given experiment were combined before extraction.

After filtration (Buchner funnel) the feces sample and the residue from the saline gut washings were dehydrated with methanol. The filtrates, combined with the methanol used for the dehydrations, were extracted with petroleum ether (or hexane). The feces and gut washings were extracted separately exhaustively with petroleum ether (or hexane)-methanol mixtures*. After washing out the methanol all corresponding petroleum ether extracts were combined, dried, and saponified by standing over 20% methanolic KOH.

* The ratio of petroleum ether to methanol varied as follows for successive extractions: 1:1, 2:1, 3:1. For all extractions after the third one, the ratio of petroleum ether to methanol was kept constant at 3:1.

The liver, gut walls, and gizzard were cut into small pieces which were then ground up under methanol in Waring blendors. The filtrates were treated with petroleum ether and water, while the solid residues were reextracted with petroleum ether-methanol mixtures (2-3 extractions). After the addition of water the aqueous methanol layers were discarded, and all corresponding petroleum ether extracts were combined, dried, and saponified.

The body fat and ovaries were cut into small pieces which were then allowed to stand under 10-15% methanolic KOH overnight. After filtration the alkaline filtrates were treated with petroleum ether and water. The solid residues (the ovary residue had to be also ground with sand) were extracted once with 1:1 petroleum ether-methanol, and following the addition of water the petroleum ether extracts were washed and dried. In all three experiments the extracts of the body fat were colorless and were not investigated.

The saponified extracts mentioned above were all washed free of alkali, dried, and concentrated in vacuo to 25-50 ml.

3. Resolution of Extracted Material.

The crude extracts were chromatographed on calcium hydroxide-celite. Only the chromatograms of the feces extracts will be described below.

a. Pro-γ-carotene assay.

Feces extract, one 20 x 3.8 cm. column; developer, 4% acetone in petroleum ether:

Chromatogram No. 62

1	orange	Fraction F1	unidentified
6	pale yellow	F2	unidentified
44	colorless		
5	pale yellow-orange		
2	colorless	F3	all-trans-γ-carotene
9	dark orange		
3	colorless		
10	pale red-orange	F4	neo-γ-carotene
20	several very faint zones		
5	colorless	F5	neo-γ-carotenes
4	pale yellow		
3	almost colorless		
20	orange-yellow	F6	unchanged pro-γ-carotene
2	colorless		
15	pale yellow		
3	almost colorless	F7	a polycis-γ-carotene
3	very pale yellow		
25	colorless		

Extracts of liver, gut walls, and gut washings gave similar chromatograms.

b. Prolycopene assay.

Feces extract, one 27 x 4.8 cm. column; developer, 4% acetone in petroleum ether:

Chromatogram No. 63

2	colorless		
1	orange-brown	Fraction F1	unidentified

2 2	colorless red	F2	all-*trans*-lycopene
3	almost colorless		
11	red-orange	F3	all-*trans*-lycopene
23	pale red-orange	F4	neolycopene A
2	pale pink	F5	neolycopene
8	colorless		
3	dark orange	F6	a poly*cis*-lycopene
1 2	colorless red-orange	F7	a poly*cis*-lycopene
8 23	two yellow zones almost colorless (faint yellow)	F8	poly*cis*-lycopenes
12	orange	F9	unchanged prolycopene
20	colorless		
8	pale yellow	F10	a poly*cis*-lycopene
90	colorless		

Extracts of the liver, gut walls, gizzard, and ovaries gave only slightly different chromatograms.

c. All-*trans*-lycopene assay (with 15 mg.).

Feces extract, one 27 x 4.8 cm. column; developer, 15% acetone in petroleum ether:

Chromatogram No. 64

3 8	dark red-brown almost colorless		
2 3	yellow red-orange	Fraction F1	all-*trans*-lycopene

10	faint pink		
25	red-orange	F2	all-trans-lycopene
40	pinkish red	F3	all-trans-lycopene
16 4	orange-brown pale orange-brown	F4	neolycopenes
105	colorless		

The extracts of the liver, gut walls, and gut washings gave similar chromatograms. Extracts of ovaries, gizzard, and blood (blood plasma of heart) were chromatographed on 12 x 0.9 cm. columns. In each instance a yellow fraction was washed down rapidly into the filtrate; the blood extract also yielded a small reddish zone on the column (probably lycopene).

Small amounts of fractions F2 and F3 were obtained crystalline from benzene-methanol. Neither preparations separated from pure lycopene (ex tomato paste) on the column.

d. All-trans-lycopene assay (with 192 mg.).

Feces extract, one 30 x 8 cm. column; developer, 15% acetone-hexane:

Chromatogram No. 65

5 25	yellow-brown very faint pink		
17	red-brown	Fraction F1	all-trans-lycopene
120	light pink, increasing in intensity to red-brown	F2	all-trans-lycopene

44 2	pale orange to orange-brown yellow	F3	neolycopenes
35 10 20	colorless very pale yellow colorless		

Fraction F1. The 17-mm. zone was developed on calcium hydroxide-celite (20 x 3.5 cm. column) with benzene:

Chromatogram No. 66

15	brown
6	gray
7	pale red
35	colorless
50	dark red-orange
1	gray
5	pale orange
15	colorless
20	orange
10	very faint red
25	colorless

The 50-mm. dark red-orange zone was transferred into benzene, and after evaporating the solution completely the residue was recrystallized from chloroform-ethanol. The crystals were washed once with ethanol and a second time with methanol and dried in vacuo at 60°. Yield, 1.1 mg.

Analysis. Calculated for $C_{40}H_{56}O$: C, 86.67; H, 10.22. Found (after correcting for 5.6% ash): C, 86.52; H, 10.03.

This preparation did not appear to separate from pure lycopene on the column (developer, benzene).

All-trans-lycopene. The 120-mm. zone (F2) was developed with benzene on a 30 x 8 cm. column of calcium hydroxide-celite:

Chromatogram No. 67

25	brownish gray
35	almost colorless
30	orange-red
23	orange-red
95	red-orange
35	orange-brown
35	colorless

The 30-mm. and 23-mm. orange-red zones were well defined, although not separated by a colorless interzone. In contrast, the boundary between the 23-mm. and 95-mm. zones was barely discernible.

The benzene solution of the 95-mm. zone was evaporated, and the residue was recrystallized from benzene-methanol and then from chloroform-ethanol.

Analysis. Calculated for $C_{40}H_{56}$: C, 89.48; H, 10.52. Found (after correcting for 0.8% ash): C, 89.92, 89.90; H, 10.65, 10.07.

This sample did not separate from pure lycopene on the column.

The crystalline preparations obtained from F1 and F2 were submitted to mixed chromatogram tests. When developed with benzene on calcium hydroxide-celite no separation took place. With the same adsorbent and 20% acetone-hexane as developer, an apparent separation was initially discerned. The column was extruded at this point, and the red zones were carefully separated by cutting. The middle one which appeared to be the interzone was discarded. However, when the top and bottom zones were combined and again chromatographed, only

one homogeneous zone resulted.

4. Identification and Quantitative Estimation.

The spectra of the well-differentiated pigment fractions obtained from the chromatograms mentioned were observed both in the visual spectroscope and photoelectric spectrophotometer. Only those pigments which yielded, upon iodine catalysis in light, the well-known maxima of the stereoisomeric equilibrium mixture were accepted as belonging to the stereoisomeric set under investigation.

The quantity of pigment present in each fraction was calculated from the extinction reading at the main maximum in hexane (cf. Table XXI).

The molecular extinction coefficients of γ-carotene, pro-γ-carotene, lycopene, neolycopene A, and prolycopene were taken from reference (57), while those for poly<u>cis</u>-lycopene V and α-carotene were obtained from (59) and (61), respectively.

Table XXI

The Calculation of Amounts of Pigments Recovered After Feeding Pro-γ-carotene, Prolycopene, and All-trans-lycopene to Chickens and Hens

Experiment	Fractions* (Solvent, petroleum ether, b.p. 60-70°)	Calculated as	Using $E^{mol.}_{max.\ 1cm.} \times 10^{-4}$ (Solvent, hexane)	
Pro-γ-carotene	F1-F5, L1-L4, G1, G2, GW1	γ-carotene	461-2 mu.	14.6
	F6, F7, L5, L6, G3, GW2, GW3	pro-γ-carotene	457	11.2
Prolycopene	F2, F3, L1, O1, GW1	lycopene	472-3	18.6
	F4, F5, O2	neolycopene A	465	12.2
	F9, L4, O5, G4, GW3	prolycopene	438	10.3
	F6, F7, F8, L3, O3, O4, G3, GW2	polycis-lycopenes	441-6	11.0**
	F10, L5, O6	polycis-lycopene V	431-2	8.9
	F1, G1, G2, S1-S5	α-carotene	445-6	14.6
Lycopene (15 mg.)	F1, F2, F3, L2, GW1	lycopene	472-3	18.6
	F4	neolycopene A	465	12.2
	L3, G1, G2	α-carotene	445-6	14.6
Lycopene (192 mg.)	F1, F2	lycopene	472-3	18.6
	F3	neolycopene A	465	12.2

* F refers to feces; L, liver; G, gut walls; GW, gut washings; O, ovaries; and S, gizzard.

** Average value for the following polycis-lycopenes: "crystallizable isomer" (57), polycis-lycopenes I, II, and III (59).

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Propositions submitted by B. K. Koe

1. Nitrosyl chloride reacts with carbon-carbon double bonds, in many cases forming crystalline adducts of unsaturated compounds. (Nitrosyl Chloride, by L. J. Beckham, W. A. Fessler, and M. A. Kise: Chem. Rev. 48, 319 (1951)). For example, it has been used widely for the characterization of terpenes (pinene, dipentene, etc.). I propose that nitrosyl chloride be used for the preparation of crystalline derivatives of colorless polyenes such as phytofluene and phytoene, which have not been obtained in crystalline form so far.

2. Wendler, Roseblum, and Tishler oxidized β-carotene to vitamin A aldehyde (30% yield) with hydrogen peroxide-osmium tetroxide (J. Am. Chem. Soc. 72, 234 (1950)). I propose that this reagent be used for oxidative degradation studies of phytofluene and phytoene to confirm the proposed structures of these two compounds (L. Zechmeister and A. Sandoval: J. Am. Chem. Soc. 68, 197 (1946); J. W. Porter and F. P. Zscheile: Arch. Biochem. 10, 547 (1946); J. W. Porter and R. E. Lincoln, ibid. 27, 390 (1950)).

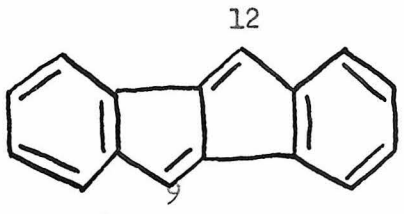
3. Boron trifluoride etherate reacts with solutions of carotenoids to give a dark blue complex. I propose that the nature of the complex formed be investigated at low temperatures in a moisture-free and oxygen-free system with gaseous boron trifluoride.

4. When carotenoids are treated with "acidic" reagents (electron acceptors) such as antimony trichloride (Carr-Price reagent), boron trifluoride, conc. sulfuric acid, etc., the resulting

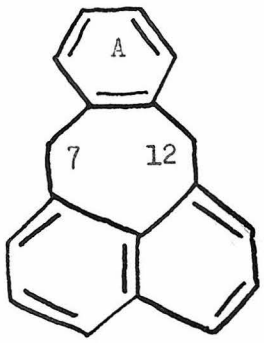
bathochromic effect could be explained by assuming that the reagent attacks one end of the conjugation system and causes a positive charge to resonate along the chain of conjugated double bonds (Remick, Electronic Interpretations of Organic Chemistry, 1943, p. 183); G. N. Lewis and G. T. Seaborg: J. Am. Chem. Soc. 61, 1886 (1939)). I propose that electron-donating reagents and free radicals should also cause a bathochromic effect in carotenoids and similar polyenes.

5. According to M. L. Quafe (J. Am. Chem. Soc. 66, 308 (1944)), γ-tocopherol, but not β-tocopherol, couples with p-nitrobenzenediazonium chloride to give an azo dye. I propose an explanation involving hyperconjugation of the methyl group with the benzene ring, thereby facilitating electrophilic substitution at the position p to that methyl group.

6. Phenanthrene is formed as the main product by irradiating a cyclohexane solution of trans-stilbene with ultraviolet light (C. O. Parker and P. E. Spoerri, Nature 166, 603 (1950)). I propose that the possible formation of $\Delta^{9,11}$-diphensuccindadiene from the irradiation of 1,4-diphenylbutadiene with ultraviolet light be investigated.



$\Delta^{9,11}$- diphensuccindadiene



7,12-dihydropleiadene

7. The aromatic hydrocarbon pleiadene, a polycyclic heptadiene derivative, has not yet been reported, although 7,12-dihydropleiadene as well as pleiadenedione are known (L. F. Fieser and M. Fieser: J. Am. Chem. Soc. 55, 3010 (1933); Elsevier's Encyclopedia of Organic Compounds, Vol. 14). I propose that the strong tendency of ring A to assume a Kekule' structure would favor the formation of a 7,12-diradical structure in the dehydrogenation of 7,12-dihydropleiadene, whereby loss by polymerization would occur.

8. Certain marine bacteria are able to assimilate polycyclic aromatic compounds, including 1,2,5,6-dibenzanthracene (F. D. Sisler and C. E. Zobell: Science 106, 521 (1947)). I propose that the course of assimilation of this compound and of other carcinogenic, polycyclic aromatic hydrocarbons be studied by isolation and characterization of the intermediate products.

9. Recently, Petracek and Zechmeister (J. Am. Chem. Soc. 74, 184 (1952)) found that the native form of phytofluene in fresh tomatoes is a photosensitive cis isomer. It would be of interest to determine the stereochemical configuration of phytofluene occurring in other land plants. Furthermore, it should be investigated whether or not mutations induced in microorganisms which normally produce phytofluene may affect the stereochemical configuration of the phytofluene (and/or other polyenes) formed (J. Bonner, A. Sandoval, Y. W. Tang, and L. Zechmeister: Arch. Biochem. 10, 113 (1946)).

10. Nodular callus growths from carrot tap-root fragments grown on nutrient medium develop green, purple, and brown pigments; in contrast, on media containing indole-3-acetic acid such tissue cultures show a yellowish-brown to gold color. A third type of tissue characterized by thallus-like organs is formed in cultures grown on media containing the carcinogens, 3,4-benzpyrene, 1,2,5,6-dibenzanthracene, or methyl cholanthrene (M. Levine: Am. J. Botany 37, 445 (1950)). I propose that a chromatographic resolution and comparative investigation of the pigments and, possibly, colorless polyenes, contained in these three types of tissue be carried out.

11. Two structures have been proposed for the compound germanium phenyl (Ge_6Ph_6) which is obtained by treating phenyltrichloro-germane with potassium in boiling xylene in a carbon dioxide atmosphere (R. Schwarz and M. Schmeisser: Ber. 69, 579 (1936); N. V. Sidgwick: Chemical Elements and their Compounds, Oxford, 1950, Vol. I., p. 575-6). In the solid state the compound is colorless and is assigned a ring structure with aromatic resonance, while in solutions a diradical structure (open-chain) has been suggested because of the intense yellow color and the uptake of 8 atoms of halogen per germanium phenyl molecule. Solutions of germanium phenyl should therefore be paramagnetic. I propose that magnetic measurements of solutions of this substance be carried out in order to confirm the diradical structure.

12. Recent evidence has shown that displacement reactions of the silanes with nucleophilic reagents very probably proceed through a transition complex containing a pentacovalent silicon atom (C. G. Swain, R. M. Esteve, and R. H. Jones: J. Am. Chem. Soc. 71, 965 (1949); H. Gilman and G. E. Dunn: ibid. 72, 2178 (1950); M. S. Newman, R. A. Craig, and A. B. Garrett: ibid. 71, 869 (1949)). With electrophilic reagents two reaction mechanisms are possible. One of these (a) is similar to that just mentioned and may also involve a pentacovalent silicon atom, while the second mechanism (b) may involve a siliconium ion as an intermediate (L. H. Sommer, R. E. Strien, and F. C. Whitmore: J. Am. Chem. Soc. 71, 3056 (1949); L. H. Sommer and J. Rockett: ibid. 73, 5130 (1951)). I propose that these displacement reactions be studied with optically active silanes (asymmetric silicon atom) in order to determine which reaction path is followed. Those reactions in which there is a nucleophilic attack on silicon should be accompanied by a Walden inversion, while reactions involving a siliconium ion should result in a product with appreciable racemization (A. E. Remick, Electronic Interpretations of Organic Chemistry, 1943, p. 389-394).

13. In the cleavage of the silanes $(CH_3)_3Si$-R by anhydrous hydrogen chloride in glacial acetic acid to give trimethylchlorosilane and RH, the following order of ease of removal of groups (R) was observed: p-thienyl > p-anisole and p-dimethylaminophenyl > p-tolyl > phenyl > p-chlorophenyl > benzyl and β-phenylethyl (H. Gilman

and F. J. Marshall: J. Am. Chem. Soc. 71, 2066 (1949)). I propose that the mechanism of this reaction consists of an initial attachment of the electrophilic reagent (proton or H_2OAc^+) to the carbon (of the R group) bonded to silicon. The products are then formed either by a nucleophilic displacement with Cl^- or by formation of a siliconium ion which reacts with Cl^- to give the trimethylchlorosilane. The cleavage reactions observed with dibenzothienylsilanes and some substituted naphthylsilanes (H. Gilman et. al.: J. Am. Chem. Soc. 71, 2066 (1949); 72, 2629 (1950); 72, 4884 (1950); 73, 5887 (1951). can be explained by the mechanism proposed.

14. Bickel observed that β-alkoxy-2-chlorochalcones undergo exchange of the alkoxy groups when treated with potassium hydroxide and an alcohol containing a different alkyl residue but he did not regard this reaction as an ester interchange (C. L. Bickel: J. Am. Chem. Soc. 71, 336 (1949)). I propose that the mechanism of this interconversion of β-alkoxy derivatives of 2-chlorochalcones is analogous to that of trans-esterification or alcoholysis of esters and should also take place in the presence of those catalysts that are ordinarily used in such reactions (e.g. p-toluenesulfonic acid)(L. P. Hammett: Physical Organic Chemistry, 1940, p. 356; L. Farkas, O. Schachter, and B. H. Vromen: J. Am. Chem. Soc. 71, 1991 (1949)).