4. It is suggested that, because of its relative simplicity, the method is suitable for application to routine procedures.

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The Carotenoids of the Carapace of the Echinoderm *Ophidiaster ophidianus*

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In 1934 Karrer & Benz isolated astacin from the bright pink shells of *Ophidiaster ophidianus*; it is now known (Kuhn & Sörensen, 1938) that astaxanthin is the naturally occurring pigment and that astacin is a manipulative artifact. Karrer & Benz did not report the presence of any other carotenoid. As the importance of the presence of trace carotenoids in understanding the metabolism of carotenoids is increasing, it was desirable to find out if *Ophidiaster ophidianus* accumulated carotenoids other than astaxanthin.

EXPERIMENTAL

Material. The material was collected in Galli of Positano (Naples), and two groups of fourteen animals were examined. The animals were all about 14 cm. in diameter.

Extraction of material. The animals were opened and the internal organs removed completely and discarded. The clean shells were covered with acetone and allowed to stand for 5 min. at room temperature. The acetone was then removed by decantation and the carapaces were re-extracted with further quantities of acetone (usually 3–4 times) until all the pigments were removed. The combined acetone extracts were concentrated at room temperature at the pump, diluted with an equal volume of water and the pigment was extracted with diethyl ether. The ether extract was dried over anhydrous Na_2SO_4 and the solvent then removed under N_2. The residue was redissolved in light petroleum (b.p. 35–50°) and subjected to chromatographic analysis. When it was not possible to examine the extracts immediately, they were stored below −20° in an ice-box.

Chromatographic analysis. Six separate chromatographic analyses were carried out (on portions of the two extracts). The results were identical in each case. The separations were carried out as follows:

(1) A preliminary separation of the pigment mixture was carried out on $CaCO_3$ (British Drug Houses Ltd., A.R. grade), using light petroleum (b.p. 35–50°) containing 4% (v/v) of ethanol as developer. The resulting chromatogram is described in Table 1.

(2) Fraction 1*A* was rechromatographed on alumina (Merck-Brockmann) deactivated by suspending it in methanol for about 30 min., filtering off the methanol and drying overnight at room temperature (Goodwin, 1952*a*). Light petroleum, containing different concentrations of diethyl ether, was used as developer. Eight fractions were obtained as recorded in Table 2. Fractions 2*A* and 2*B* were separated using pure light petroleum and passed straight through the column. On development of the column with light petroleum containing 1·5% (v/v) of diethyl ether,

Table 1. *The first separation of* Ophidiaster *carotenoids*

Adsorbent, $CaCO_3$; developer, light petroleum containing 4% (v/v) ethanol; the fractions are in order of increasing adsorptive power.

Fraction no.	Description	Absorption spectrum maxima in CS_2 (mμ.)
1*A*	Orange-red; passes straight through the column; a mixture (see Table 2)	—
1*B*	Reddish pink	505
1*C*	Orange-red	500
1*D*	Yellow; only present in traces; not examined further	—

fractions 2*C* and 2*D* separated and moved off the column. Fractions 2*E* and 2*F* also separated but were not eluted until the concentration of ether was increased to 12 and 18% (v/v), respectively. The residual, red pigment separated into three fractions (2*G*, 2*H* and 2*I*) when the concentration of ether was further increased. The most strongly adsorbed (2*I*) was eluted only with ethanol.

Quantitative measurements. The various fractions were collected and made up in known volumes of light petroleum and the extinction of the solutions measured at the wavelength of maximal absorption of the pigment concerned. The amount of each pigment present could then be calculated using the $E^{1\%}_{1\,cm.}$ ($\lambda_{max.}$) values recorded by Karrer & Jucker (1949) and Goodwin (1952*b*). In the case of the unidentified carotenoids, $E^{1\%}_{1\,cm.}$ ($\lambda_{max.}$) was assumed to be 2500.

RESULTS

Identification of the pigments

Fraction 1*B* (the major one) is undoubtedly unesterified astaxanthin, as observed by Karrer & Benz (1934). It is hypophasic when partitioned between light petroleum and 90% (v/v) aqueous methanol and exhibits a single, banded absorption spectrum with its maximum at 470 mμ. in light petroleum and at 505 mμ. in CS_2.

Fraction 1*C* is astacin; it is more strongly adsorbed than astaxanthin and has the same shaped curve but with its maximum shifted to slightly lower wavelength (500 mμ. in CS_2) (Goodwin & Srisukh, 1949). It is probably an artifact produced during the extraction procedures.

Fraction 2*B* was proved to be β-carotene by the identity of its absorption spectrum with that of pure β-carotene and the fact that when mixed with an authentic specimen and chromatographed on strong alumina, no separation could be obtained.

Fraction 2*E* is cryptoxanthin. Only two pigments have absorption spectra very similar to that of β-carotene, cryptoxanthin and zeaxanthin. In the phase test with 90% (v/v) aqueous methanol, the former is epiphasic and the latter hypophasic (Karrer & Jucker, 1949). Fraction 2*E* is also epiphasic and, furthermore, is equally distributed between the two phases when partitioned between light petroleum and 95% (v/v) aqueous methanol, a further characteristic test for cryptoxanthin. Finally, a comparison of the adsorptive power of 2*E* with that of cryptoxanthin and zeaxanthin (Goodwin, 1952*b*) shows 2*E* to be the same as that of cryptoxanthin.

From its position on the column (Table 2) and its absorption spectrum, fraction 2*A* appears to be a *cis*-isomer of β-carotene, probably *neo*-β-carotene B (Zechmeister, 1944). α-Carotene, which would have about the same adsorptive power, appears to be ruled out because of the lack of definition of the absorption spectrum of 2*A* (Fig. 1).

Fig. 1 also illustrates the absorption spectrum of 2*C*, the maxima of which are characteristic of γ-carotene; its position on the column is also characteristic of γ-carotene (Goodwin, 1952*a*). Because of the very small amounts of this pigment which are in *Ophidiaster*, it has not been possible to confirm completely its identity with γ-carotene; the slight doubt which remains is due to the fact that it has never been possible to get a specimen of 2*C* with the same shape of absorption curve as γ-carotene.

Table 2. *Chromatographic separation of fraction* 1*A of Table* 1

Adsorbent, deactivated alumina; developer, light petroleum containing diethyl ether; the fractions are in order of increasing adsorptive power. Figures in brackets denote inflexions.

Fraction no.	Description	Absorption spectrum maxima in light petroleum (mμ.)	Ether required to separate pigment from that named above (%, v/v)
2*A*	Yellow, diffuse	(420), 442, 472	0
2*B*	Orange, diffuse	(420), 448, 476	0
2*C*	Rose	(430), 460, 485	1·5
2*D*	Yellow	448	1·5
2*E*	Orange-yellow	(420), 448, 475	12
2*F*	Yellow	455	18
2*G*	Red-pink	460	24
2*H*	Red-pink	470	50
2*I*	Red-pink	470	Ethanol

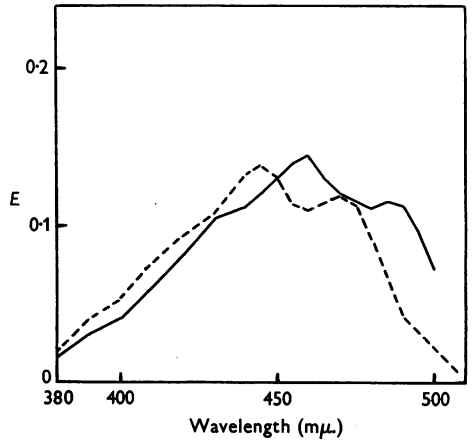


Fig. 1. Absorption spectrum of pigments. 2*A*, - - - -; and 2*C*, ——. Solent, light petroleum (b.p. 35–50°).

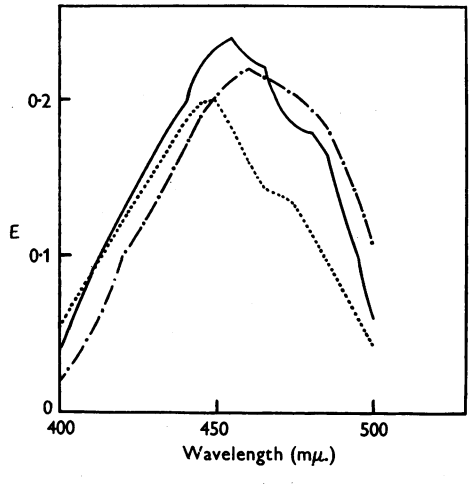


Fig. 2. Absorption spectra of pigments. 2*D*, ...; 2*F*, ——; and 2*G*, ·–·–· Solvent, light petroleum (b.p. 35–50°).

Fraction 2*D* (Fig. 2) is not identifiable with any known pigment; its spectrum suggests the presence of a keto group, but its weak adsorptive power seems to preclude this. A very similar pigment is widely distributed in berries (Goodwin, 1953*a*).

Fractions 2*F* and 2*G* appear to be new pigments for a survey of the literature (Goodwin, 1952*c*) did not reveal any carotenoid with the properties of these two pigments. From the fact that both have essentially single-banded spectra (Fig. 2) they are probably keto-carotenoids. Fractions 2*H* and 2*I* are traces of astaxanthin and astacin, respectively, carried through during the first fractionation.

Quantitative experiments

Four experiments were carried out to determine the relative distribution of the various pigments in *O. ophidianus*; the mean results are given in Table 3. It will be seen that in agreement with the findings of Karrer & Benz (1934), astaxanthin is the major component.

Table 3. *The quantitative distribution of carotenoids in the carapaces of* Ophidiaster ophidianus

(*neo*-β-Carotene B and γ-carotene have not been unequivocally identified. The traces of astacin produced during manipulation have been ignored.)

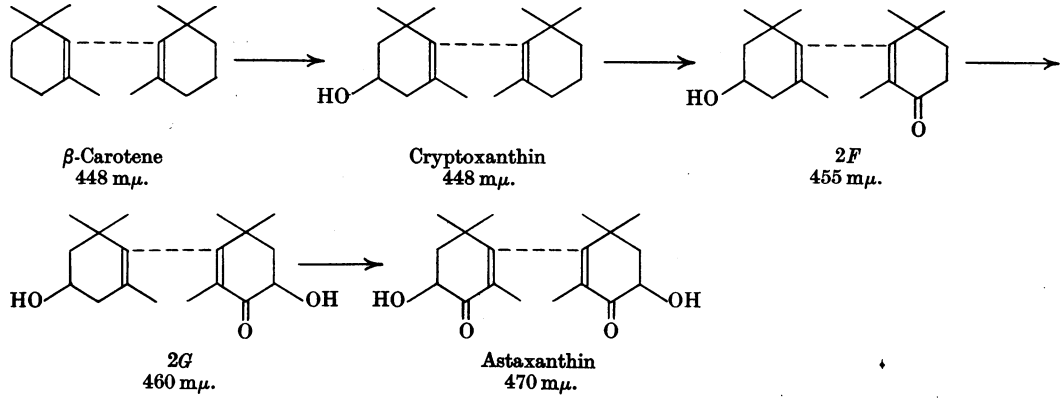
Carotenoid	Amount (% of total pigments listed)
neo-β-Carotene B	6·0
β-Carotene	6·8
γ-Carotene	0·8
2*D*	3·3
Cryptoxanthin	11·0
2*F*	4·8
2*G*	10·0
Astaxanthin	57·3

DISCUSSION

This investigation shows the importance of re-examining animal tissues for carotenoids in the light of modern information. Although astaxanthin, the only pigment identified by Karrer & Benz (1934), is by far the major component, there are small but important amounts of seven other pigments. Of special interest are two, 2*F* and 2*G*, which appear to be entirely new. Not only are these new, but tentative structures can be assigned to them which would suggest that they might be intermediates in the synthesis of astaxanthin from β-carotene.

Considering the absorption spectra maxima of β-carotene and astaxanthin, introduction of two 3-hydroxy-4-keto groupings results in a shift in wavelength of 22 mμ., i.e. from 448 to >470 mμ. The introduction of one such grouping would result in a shift of 11 mμ., i.e. about 5 mμ. more than if the substitution were a simple 4-keto substitution, e.g. echinenone, 4-oxo-β-carotene, $\lambda_{max.}$ 452 mμ. (Goodwin, 1953*b*). The wavelength (458–460 mμ.) given by Goodwin & Taha (1950) is too high.

Fraction 2*F*, which has a very similar spectrum to that of echinenone, but which is more strongly adsorbed, might be 3-hydroxy-4′-oxo-β-carotene (455 mμ.); addition of a hydroxy group at 3′ would shift this band to about 460 mμ. (2*G*), and the introduction of a keto group at position 4 (i.e. next to an isolated —OH group) would increase $\lambda_{max.}$ by 10 to that of astaxanthin. Thus a possible scheme of synthesis of astaxanthin from β-carotene could be represented as shown on p. 558.



β-Carotene 448 mμ. | Cryptoxanthin 448 mμ. | 2F 455 mμ.

2G 460 mμ. | Astaxanthin 470 mμ.

Much further work is required before this scheme can be fully established or otherwise, but it is important to point out that β-carotene has been shown to be almost certainly a precursor of astaxanthin in locust eggs (Goodwin & Srisukh, 1949).

This work on *O. ophidianus* emphasizes the importance of the study of the minor carotenoid components in living tissues.

SUMMARY

1. The presence of astaxanthin in the shell of *Ophidiaster ophidianus* is confirmed, and that of β-carotene and cryptoxanthin demonstrated. *neo*-β-Carotene B and γ-carotene are also probably present.

2. Three new pigments are reported: two are probably keto-carotenoids.

3. The quantitative distribution of carotenoids in the shell of *O. ophidianus* is recorded.

4. A possible biosynthetic route from β-carotene to astaxanthin is discussed.

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The Metabolism of Collagen and other Proteins of the Skin of Rabbits

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It has been shown with the aid of [α-^{14}C]glycine that, in the adult rat, collagen from a wide variety of sources is metabolically almost inert, if compared with proteins such as those of plasma or of liver (Neuberger, Perrone & Slack, 1951; Neuberger & Slack, 1953). Results indicating a relatively slow turnover of collagen in guinea pigs (Robertson, 1952) have also been reported. However, in the growing rat, labelled glycine is incorporated into collagen at a fairly fast rate (Neuberger *et al.* 1951) and the use of this amino acid thus seemed to afford a convenient tool to examine the mechanism by which this fibrous protein is formed.

It was reasonable to assume that insoluble collagen is derived from a more soluble precursor and the presence of soluble, collagen-like proteins in connective tissue supports such a hypothesis. It was first observed by Zachariades (1900) that the tendon of the rat tail, if suspended in very dilute formic acid or acetic acid, swelled markedly and

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