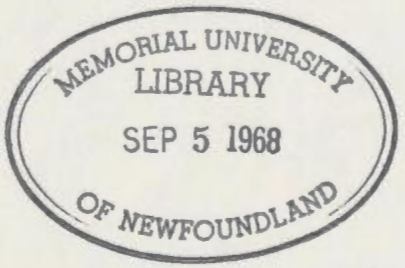
THE CAROTENOID PIGMENTS OF THE HOLOTHURIAN PSOLUS FABRICHII (DÜBEN AND KOREN)

CHRISTOPHER J. DAWSON



THE CAROTENOID PIGMENTS OF THE HOLOTHURIAN PSOLUS FABRICHII (DÜBEN AND KOREN)

A Thesis

by

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Abstract

The carotenoid content of the body wall of the sub-arctic holothurian Psolus fabrichii (Düben and Koren) has been investigated.

The carotenoids were extracted by cold acetone, taken into petroleum ether, and after concentration of this solution were separated by thin layer chromatography on a silica gel H layer, using 10% acetone in petroleum ether as the developing solvent. The ten carotenoids separated were further purified by thin layer chromatography.

As far as possible each carotenoid has been identified by measurement of its light absorption properties, its partition ratio between hexane and 95% methanol, and by investigation of the effects of potassium hydroxide, sodium borohydride, and acidic methanol on the compound.

The direct effect of dietary carotenoids on the carotenoid content of echinoderms has previously been emphasised. It has generally been accepted that the herbivorous diet of holothurians provides mainly carotenes and less polar xanthophylls, and these carotenoids are therefore predominant in the tissues.

The results of this investigation indicate that, though a small part of the dietary carotenoid is stored in the tissues of Psolus fabrichii, the food does not contain measurable amounts of the more abundant body wall carotenoids.

It is proposed that the highly oxidised carotenoids, which compose about 80% of the total carotenoid content might be the intermediates and product of a sequential pathway responsible for the oxidation of dietary β-carotene (and possibly some of its hydroxy derivatives) to astaxanthin, the predominant carotenoid in the organism.

ACKNOWLEDGEMENTS

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INTRODUCTION

The echinoderms have not been well studied with regard to either carotenoid content or metabolism. Of the five living classes, asteroids, ophiuroids, holothurians, echinoids, and crinoids, the first named has received the most attention.

The asteroids and ophiuroids have been classified together as a carnivorous group (1,2,3) containing a preponderance of highly oxygenated carotenoids over carotenes. Work on several species of Pacific coast echinoderms (4) showed carotenoids in all those studied, including four asteroids and three ophiuroids. The asteroids contained xanthophylls in far greater amounts than carotenes, and the ophiuroids showed relatively large amounts of xanthophylls but no detectable carotenes. Further work (5) has confirmed these results in ophiuroids. This difference between the amounts of highly oxidised carotenoids and the hydrocarbon carotenes has been directly related to the carnivorous diet of the group, as their prey is mostly marine animals which themselves contain far more oxygenated than hydrocarbon carotenoids.

The echinoids and holothurians were, at the same time, classified as a herbivorous group. Their diet contained more carotenes and only partially oxidised xanthophylls and thus there were relatively larger amounts of

these compounds in their tissues. In a sand dollar Dendraster excentricus and three species of sea urchins there was generally a higher proportion of carotenes (compared with xanthophylls (4)). Similar results have been obtained for holothurians, and low concentrations of carotenes and free xanthophylls, but no carotenoid acids or xanthophyll esters, have been found in Stichopus parvimensis (4).

More recent work has shown that this classification is less useful than was initially supposed. Thus, although the nature of the carotenoids in ophiuroids has been confirmed, the direct effect of dietary carotenoids has been questioned (5). Unesterified astaxanthin accounts for 80-85% of the carotenoid pigment in the red body wall of the holothurian Cucumaria lubrica (6), and the cake urchin Peronella japonica has been shown to contain astaxanthin (7).

The occurrence of the highly oxygenated carotenoid astaxanthin in representatives of both the holothurians and the echinoids warrants a reconsideration of the dietary characteristics of the respective echinoderm classes.

The study of the pigmentation of the holothurian Psolus fabrichii (Düben and Koren) was begun following reports that at ocean depths of 70 ft. the tentacles, and portions of the body wall, were a bright red colour, though

most red organisms were of a dull colouration at the same depth (8).

There are many reports of luminescence in echinoderms, though this phenomenon has not been noted in any holothurian (9). The little that is known indicates that it is an oxygen-dependent chemi-luminescence caused by the animals themselves and not by symbionts.

Since this work began, red coloured materials have been taken to ocean depths of 40 ft. and little change in colour has been noted, thus there is at present no confirmation of the original reports of this unexpected red colouration.

Initial work on the nature of the pigmentation revealed that the carotenoids present were more oxygenated than those which would be expected in a holothurian, if the strict herbivorous classification was accepted. Little work has been reported on the pigmentation of holothurians in recent years and as, since the work of Fox and Scheer (4), new techniques in carotenoid identification have been developed, it was decided to investigate the nature of the carotenoid pigments in the hope that the results might shed new light on the metabolic potential of holothurians.

The oxidised carotenoids found could be directly related to the dietary intake of this particular species. Psolus fabrichii feeds by the ingestion of plankton which comes into contact with its tentacles. It may be that in

this particular habitat, this organic material is mainly zooplankton, which itself contains relatively large amounts of similar carotenoids.

Alternatively, highly oxidised carotenoids would be found if the organism has the ability to oxidise the carotenes and xanthophylls provided by a herbivorous diet.

It is evident that the ability to oxidise dietary carotenoids is within the metabolic capacity of several classes of invertebrates, and probably some vertebrates (10). The first such metabolic pathway was proposed by De Nicola following work on the carotenoid content of four species of Mediterranean asteroids (11).

It has been known for some time that astaxanthin (I) - as the free molecule, esterified by a fatty acid, or inconjugation with a protein - is a major constituent of asteroid pigments. It was concluded that, in addition to the normal dietary intake of the highly oxidised carotenoids, there were two routes by which dietary β-carotene (II) could be oxidised to astaxanthin (I) (Figure 1).

Cultures of freshwater Anostraca, fed on the alga Chlorella vulgaris have been reared (12). The tissues of the Anostraca contained echinenone(4-keto-β-carotene) (III), canthaxanthin (4,4'-diketo-β-carotene) (IV), astaxanthin (3,3'-dihydroxy-4,4'-diketo-β-carotene) (I), and other hydroxy-keto derivatives of β-carotene. The main algal carotenoids were lutein (3,3'-dihydroxy-α-carotene) (V) and

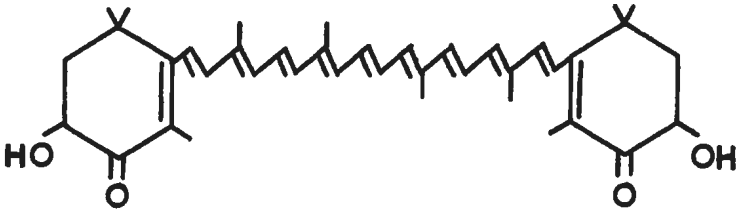
β-carotene (II), and it is therefore evident that part of the dietary carotenoid (probably β-carotene) is oxidised through a sequential pathway to astaxanthin (Figure 2).

Pigments similar to those proposed as intermediates in the Anostraca have also been isolated from two marine isopods Idothea montereyensis and I. granulosa (13,14), and controlled feeding experiments have been used to show that the cladoceran Daphnia magna can also metabolise astaxanthin from ingested β-carotene (15).

These pathways only represent hypotheses since no enzyme studies have been made, nor have the sites of oxidation been located. However the hypotheses are consistent with the information derived from several classes of invertebrates.

In each system β-carotene has been the supposed precursor (though it has been suggested that hydroxy-β-carotenes may fulfil this role in asteroids (3)) for a keto-carotenoid end product which is either canthaxanthin or astaxanthin.

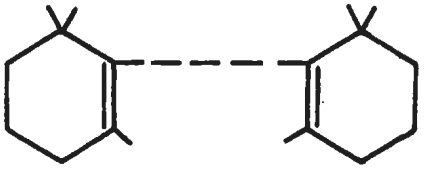
The nature of the intermediates proposed in different organisms seems to indicate that the actual pathways differ slightly. Thus no intermediate 4-hydroxy-β-carotene (isocryptoxanthin) (VI) was found in the isopod Idothea montereyensis (13) and it was suggested that this intermediate might be metabolised so rapidly that it is not present in detectable amounts in the tissues. Thus apparently different pathways may be a result of the different rates of metabolism of the same intermediates in different organisms.



I

II

The majority of the carotenoids met in this work are derivatives of β-carotene (II). In subsequent formulae this chromophore is abbreviated to II (below).



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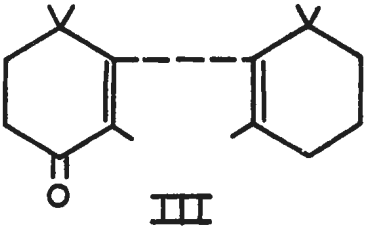

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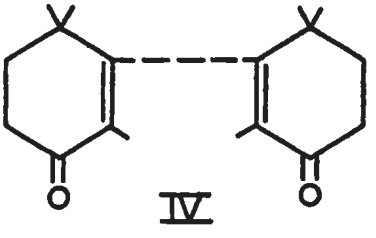

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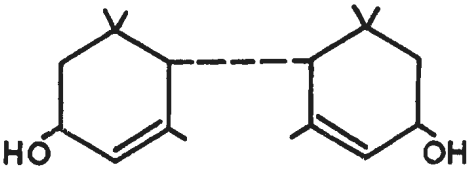

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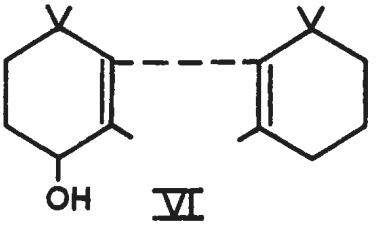


VI

Fig. 1. The proposed pathways for the oxidation of β-carotene to astaxanthin in some species of asteroids (11).

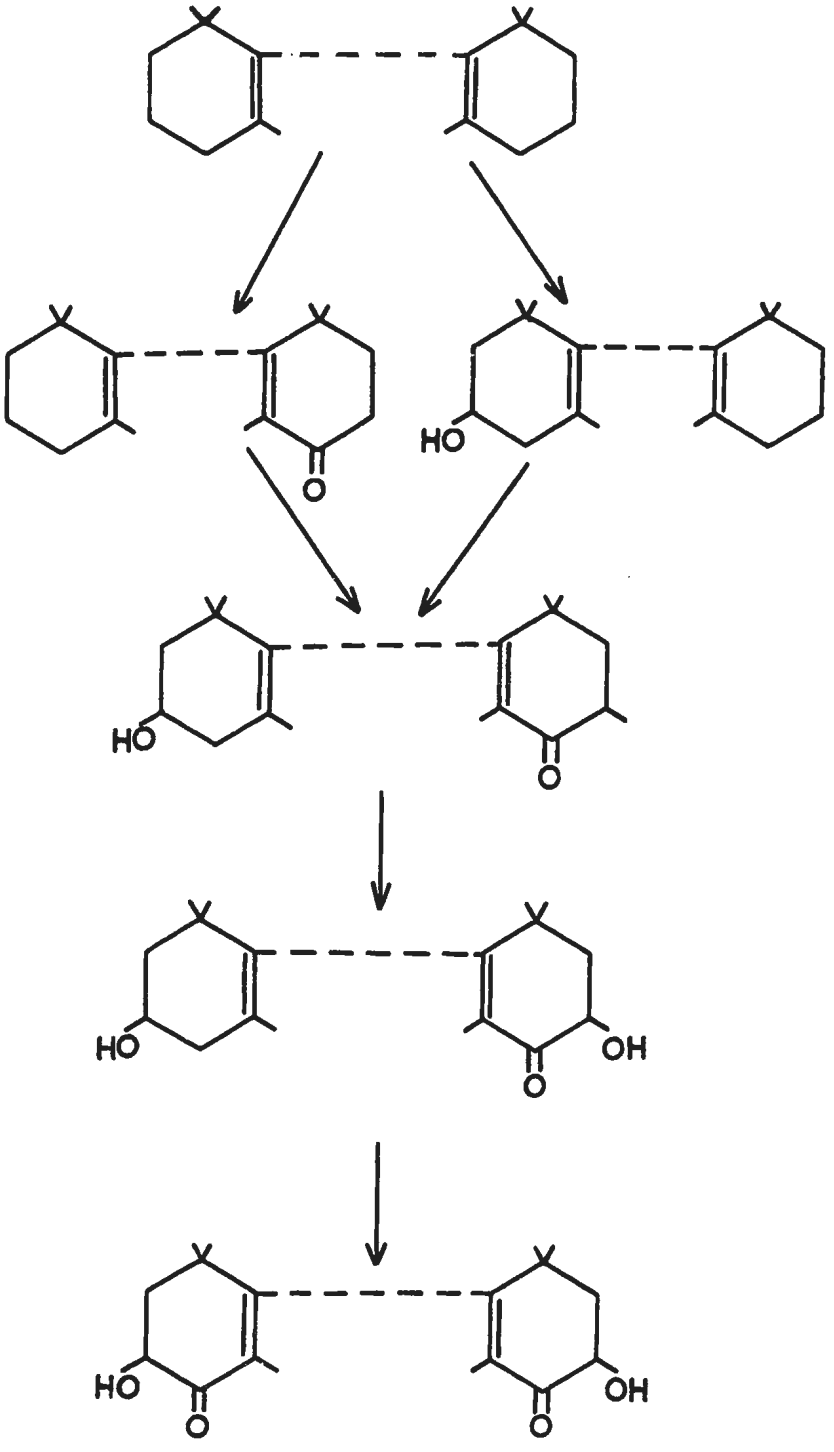
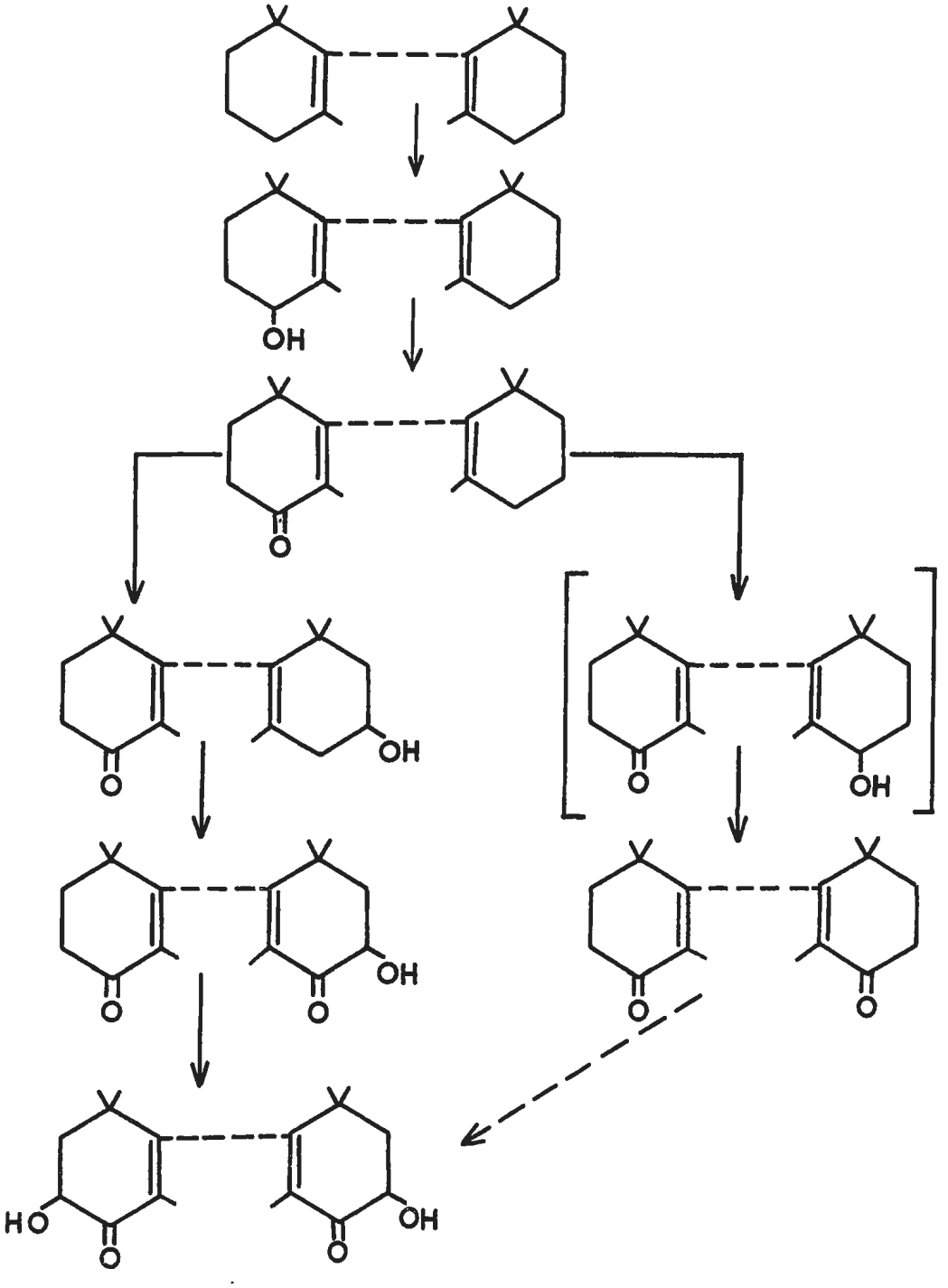


Fig. 2. Possible metabolic pathways in the conversion of ingested β-carotene to astaxanthin in the Anostraca (12). The intermediate 4-keto-4′-hydroxy-β-carotene was not found in the Anostraca.



Function of carotenoids in invertebrates.

Despite the essential nature of carotenoids in the metabolism of vertebrates and micro-organisms, the function of these pigments in invertebrates is not yet understood.

Work on the nutritional requirements of insects has yielded conflicting results, for carotenoid free diets created no apparent deficiency, other than the expected pigmentary ones, in the clothes moth Tineola bissellia (16), the cockroach Blatella germanica (17) and the grasshopper Melanoplus bivittatus (18). In most other insects studied there was some little effect on their physiology but the species remained completely viable for several generations. The parasite Agria affinis, however, required vitamin A acetate for normal growth and fertility (19).

The shore crab Carcinus maenas (20,21) has been kept for three months on a carotenoid free diet with no adverse effects, and studies on the isopod Oniscus asellus (22), and the anostracans Artemia salina (23) and Daphnia magna (15) indicate that there seems to be no measureable requirement for vitamin A under normal conditions. Extremely small amounts may be needed for visual purposes in some invertebrates, as shown to be necessary in the decapod crustacea (24,25).

Investigations on the total concentration of carotenoid pigments during the development of the eggs of the lobster (26) and the echinoderm Paracentrotus lividus (27)

have revealed little depletion of pigment, though there seems to be a relative increase in astaxanthin at the expense of β-carotene in the developing eggs of the locust (28).

Vevers (29) has summarized the present knowledge of the relationship between carotenoids and photosensitivity in asteroids. Some study is being given to the action spectrum of the photokinetic responses of the sand star Astropecten in the hope of finding the responsible pigments.

Thus it is known that many invertebrates contain relatively large amounts of carotenoids which are often actively metabolised, but in general there is no apparent requirement for either carotenoids or related compounds, nor does the carotenoid fulfil any obvious secondary function.

Experimental

Specimens of Psolus fabrichii were collected from the sea bed near Bay Bulls, a small port on the eastern coast of the Avalon Peninsula of Newfoundland. The animals were kept frozen at -20° until required.

They were defrosted by leaving at room temperature for several hours and scrubbed to remove algae from the surface. As the surface was extremely rough, care had to be taken to remove all the algae without scraping off too much of the coloured ectodermal layer of Psolus. The specimens were dissected to separate the body wall, the tentacles, and the gonads, and the parts not required immediately were refrozen. In this investigation only the body wall carotenoids have been investigated thoroughly.

Extraction of the carotenoid pigments.

The parts to be extracted were cut into small pieces and homogenised with cold acetone (about 5°) in a Waring blendor. The blendor was used at low speeds for short periods of time to avoid excessive heating of the contents. The acetone extract was decanted off, and the procedure repeated until all the colour was removed from the tissue (usually three or four successive extractions).

The acetone extract was centrifuged to remove suspended material and then diluted with three times its own volume of saturated brine. This solution was shaken with one-third its volume of petroleum ether (b.p. 35-50°), and most of the carotenoid was extracted into this layer.

After separation of the two layers the aqueous acetone layer was shaken with more petroleum ether to complete the extraction of the pigment. It was found that all the pigment could not be removed by this treatment, and a strong yellow fluorescence under ultra-violet light suggested that this solution still contained either pterins or flavins. This solution was therefore retained.

The petroleum ether layers were combined and dried over anhydrous sodium sulphate for 1 h. The drying agent was filtered off and the solution was allowed to stand overnight at -20^{o} when the precipitated protein was filtered off.

As the presence of lipids would interfere with the subsequent thin layer chromatography (TLC) of the extract these were removed by the method of Fox and Hopkins (10,30). Magnesium oxide was added, a small amount at a time, with continuous agitation until the carotenoid pigments were adsorbed. The cloudy liquor was discarded and the magnesium oxide was washed several times with fresh petroleum ether. The carotenoid pigments were eluted by a solution of 10% methanol in petroleum ether. A small part of the pigment could not be removed by this solvent and a little acetic acid was added to effect complete desorption. The solution was washed with water and dried over anhydrous sodium sulphate. After concentration in vacuo at 30^{o} the concentrated solution was subjected to TLC on a silica gel H (Merck, acc. to Stahl) layer.

The silica gel was applied to a 20 x 20 cm. plate by pouring a slurry in chloroform (previously shaken several times with half its volume of water and dried over calcium chloride). A layer applied in this way was as effective in its separation of the carotenoid pigments as one applied by a commercial applicator.

The petroleum ether solution was applied to the layer by means of a narrow bore dropping pipette or a thin walled capillary tube. The plate was developed in the dark, at room temperature, in a Desaga tank, using a solution of 10% acetone in petroleum ether (b.p. 35-50^{o}), and the solvent was allowed to travel about 15cm. up the plate.

The ten coloured zones were separately scraped off the plate, and to each was added a solution of 10% acetone in petroleum ether. Each was 'gassed' with nitrogen, covered by a waxed film and kept in the dark at 0^{o} until a sufficient amount of the pigment was collected.

Further purification of each carotenoid pigment.

The pigment to be purified was eluted from the silica gel by petroleum ether containing sufficient acetone for complete desorption. The solution was washed with water to remove the acetone, and dried over anhydrous sodium sulphate. Following concentration of the solution in vacuo at 30^{o}, the pigment was rechromatographed on a silica gel H layer using petroleum ether containing various amounts of acetone as the developing solvent. The less polar pigments were separated

from pigments of similar polarity using a solution containing 5 - 10% acetone, whereas the more polar compounds required up to 30% acetone to effect satisfactory separation.

This procedure was repeated until the pigment was seen to move as a single band.

Identification of a carotenoid pigments.

The identification of carotenoids isolated from natural sources requires the application of several standard techniques. To avoid unnecessary repetition, a description of the methods used, and of the use of the results in the elucidation of the carotenoid structure is given first. When experimental work on the individual carotenoids is described the brief title refers to the application and results of one of these experiments.

Determination of the light absorption spectrum.

The visible absorption spectrum of each carotenoid was recorded using either the Unicam S.P. 800 or the Perkin-Elmer Model 202 recording spectrophotometer, both instruments being regularly calibrated against a holmium oxide filter. Whenever sufficient carotenoid was available the spectrum was recorded in four solvents, hexane, chloroform, pyridine, and carbon disulphide (Baker 'Analyzed" Reagent), which were chosen because the spectra of most known carotenoids have been recorded in either one or more of them.

The recording of the light absorption spectrum is important for two reasons. Firstly, once the structure of an isolated carotenoid has been indicated from other measurements, a comparison of the shape and position of its absorption can be made with an authentic sample.

Secondly, when the nature of the compound is not immediately evident, the ultra-violet and visible light absorption properties provide the best indication of the chromophoric system present. The wavelengths of the absorption maxima increase with the number of conjugated double bonds, but the actual positions of the maxima are dependent on various structural features and the solvent used. The principal light absorption maxima of some standard polyenes in the carotenoid series are shown in Table I (31).

Many carotenoids exhibit light absorption properties which do not correspond exactly with the data given in Table I, and these departures are often of great help in making structural assignments. The presence of cyclic end groups of the type found in β-carotene (II), and the conjugation of the polyene system with another chromophore (-CHO,-COOH,-COOMe, C=O, aryl) alters the wavelength at which the principal absorption occurs.

Although lycopene, γ-carotene and β-carotene formally possess the same chromophore the positions of their absorption maxima differ significantly. The presence of the cyclic system

leads to a lack of planarity in the molecule which limits the overlap of the π-orbitals associated with the ring double bond and the polyene chain, and produces the observed shift of the light absorption maxima to shorter wavelengths (about 12 mμ for each end cyclised) together with a partial loss of fine structure (Table II).

The conjugation of the polyene chain with another chromophore will result in a shift of the absorption maxima to higher wavelengths, often with a complete loss in fine structure. Thus when the chromophore of β-carotene is in conjugation with a keto group at the 4-position of the β-ionone ring (4-keto-β-carotene (III)),the single absorption maximum is centred at 457 mμ. in hexane and has only a slight shoulder at 472 mμ. Substitution of both the 4 and 4' positions of the β-carotene chromophore (4,4'-diketo-β-carotene (IV))results in a perfectly symmetrical light absorption curve centred at 460 mμ. in hexane. This single peaked symmetrical absorption curve is characteristic of the β-carotene chromophore with the 4,4'-diketo substitution pattern. Thus astaxanthin (3,3'-dihydroxy-4,4'diketo-β-carotene (I), λ max. 467 mμ.) has a similar absorption curve.

Determination of the partition ratio.

The partition ratio of each carotenoid extracted in sufficient amounts was measured between hexane and 95% methanol using the method of Petracek and Zechmeister (32).

Table I. Visible light absorption properties of some isoprenoid hydrocarbons with acyclic chromophores (31).

Polyene	No. of conjugated double bonds	Principal light absorption maxima (mμ) for all-trans isomer in hexane
Phytoene	3	276, 286, 298
Phytofluene	5	331, 347, 366
ζ -carotene	7	380, 401, 425
α-zeacarotene	8	398, 421, 449
Neurosporene	9	416, 444, 470
δ-carotene	10	431, 456, 487
Lycopene	11	443, 472, 504
3',3'-dehydro-δ-carotene	12	456, 481, 515

Table II. The effect of the cyclisation of the polyene chromophore on the visible light absorption properties in petroleum ether (31).

Carotene	Structure	Wavelength of principal absorption bands (mμ) in petroleum ether
Lycopene	No cyclisation	447, 475.5, 506
δ-carotene	One β-ionone end group	431, 456, 487
β-carotene	Two β-ionone end groups	- 451, 482

Clean magnesium turnings (5 gm.) and resublimed iodine (0.5 gm.) were heated under reflux with commercial methanol (75 ml.) until the iodine disappeared and all the magnesium was converted into the methoxide. Commercial methanol (900 ml.) was added and the mixture was boiled for 30 min. under reflux, after which time the solution was distilled, under anhydrous conditions, to yield absolute methanol, the first 25 ml. of the distillate being discarded (33). The absolute methanol was diluted to 95% by the addition of deionised water.

Hexane (Baker 'Analyzed' Reagent) was dried over anhydrous sodium sulphate.

A solution of the pigment was made up in either hexane (previously saturated with 95% methanol) or 95% methanol (previously saturated with hexane) the more effective solvent for the particular carotenoid being chosen, and the absorbance of this solution was determined at the wavelength of maximum absorbance.

An equal volume of the second solvent was added and the mixture was slowly inverted 20 times in a stoppered cylinder to ensure complete partitioning of the solute. The absorbance of the pigment in the same solvent layer and the same wavelength was determined, and the partition ratio of the pigment was calculated.

The value of the partition ratio gives an indication of what polar groups might be attached to the non-polar hydrocarbon skeleton (Table III).

Table III. Partition ratios of β-carotene derivatives with different polar functional groups (32).

	Polar Functional Groups	Partition Ratio
β-carotene (II)	None	100/0
Echinenone (III)	monoketo	93/7
Canthaxanthin (IV)	diketo	50/50
Cryptoxanthin (3-hydroxy-β-carotene)	monohydroxy	82/18
Isocryptoxanthin (VI) (4-hydroxy-β-carotene)	monohydroxy	86/14
Zeaxanthin (3,3'-dihydroxy-β-carotene)	dihydroxy	11/89
Isozeaxanthin (4,4'dihydroxy-β-carotene)	dihydroxy	22/78
4-keto-4'hydroxy-β-carotene	monohydroxy monoketo	34/76
Zeaxanthin dipalmitate	diester (long chain acid)	100/0
Isozeaxanthin diacetate	diester (short chain acid)	86/14

Treatment with alcoholic potassium hydroxide solution

A portion of each pigment was dissolved in as little absolute ethanol as possible and to every 10 ml. of this solution was added 1 ml. of a solution of potassium hydroxide (6 gm.) in water (10 ml.). The alkaline mixture was left in the dark, at room temperature for several hours.

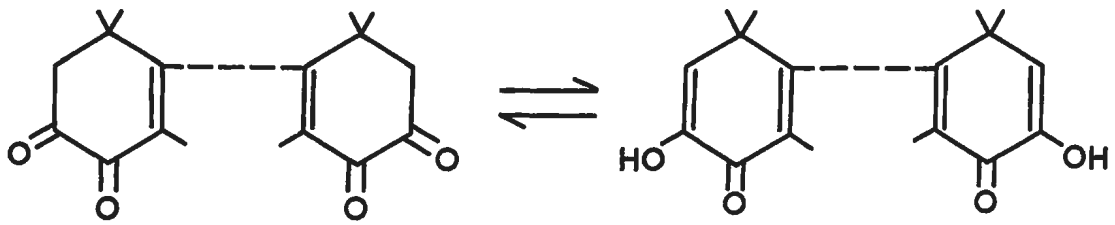
The solution was diluted with three times its volume of saturated brine, and petroleum ether (b.p. 35-50°) was added (ca. one volume to three of the alkaline solution). The mixture was shaken gently and the two phases allowed to separate, when most of the carotenoid was retained in the upper petroleum ether layer. The extraction was repeated to remove all the pigment from the aqueous ethanol layer. The petroleum ether solution was washed with water until the washings were no longer alkaline, and dried over anhydrous sodium sulphate.

The treated portion of the pigment was compared with the original isolated compound in order to detect any change in the light absorption spectrum, the partition ratio, or the behaviour on a thin layer chromatogram.

The treatment has an effect on the properties of two types of carotenoids; esters of xanthophylls and those with the 3-hydroxy-4-keto substitution pattern on a β-ionone ring.

Xanthophylls frequently occur as esters of both long and short chain acids. Hydrolysis of the ester bond liberates the free xanthophyll and while there is no change in the light absorption spectrum, there is a marked change in the partition ratio, the free xanthophyll being more polar than the ester. This difference in polarity is also shown qualitatively by the lower Rf value on the same thin layer plate as the ester.

Those carotenoids with the 3-hydroxy-4-keto substitution on the β-ionone ring are oxidised by this treatment to the corresponding 3,4-diketo compounds. Thus astaxanthin (I) is oxidised to astacene (3,3';4,4'-tetra keto-β-carotene) (VII). The 3,4-diketo group is capable of keto-enol tautomerism and in the case of astacene the enol form is more stable. The acidic nature of the enol hydroxyl group is illustrated by the formation of red insoluble potassium salts at the interphase between the petroleum ether and aqueous layers during the extraction of the pigment from the saponification mixture.

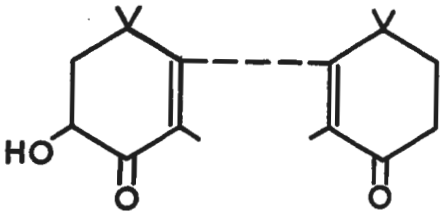


KETO ENOL

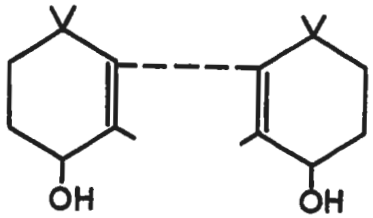
VII

Guaraxanthin (believed to be 7,8-dihydro-astaxanthin) (VIII) which was extracted from the Scarlet Ibis (34) behaves in a similar manner, also forming insoluble potassium salts. Other carotenoids with the same structure, such as phoenixoxanthin (IX) (3,3'-dihydroxy -4-keto-β-carotene), are also oxidised to the corresponding 3,4-diketo compounds but are reported not to form insoluble potassium salts (10,34).

VIII



IX



X

As the oxidation of the 3-hydroxyl group to a keto group also increases the effective length of the chromophore there is a slight change in the position of the light absorption curve. The presence of a keto group instead of a hydroxyl group decreases the polarity of the compound and both the partition ratio and the thin layer behaviour change (34) (Table IV).

Table IV. The light absorption spectra and partition ratios of three carotenoids with the 3-hydroxy-4-keto substituted β-ionone ring, and of their oxidation products (34).

	Phoenicoxanthin (IX)	Astaxanthin (I)	Guaraxanthin (VIII)
Absorption maximum (mμ):			
hexane	462	467	455
chloroform	479	483	---
Partition ratio	29/71	9/91	37/63
Alkali treated product	Neutral	Acid	Acid
Absorption maximum (mμ):			
hexane	467-468	474	462
chloroform	484	486-488	482
pyridine	---	490	482
Partition ratio	36/64	25/75	35/65

Reduction of the carbonyl groups.

The pigment was dissolved in 95% ethanol and a few small crystals of sodium borohydride were added. The solution was left under nitrogen for 1 h. at room temperature, after which time the excess sodium borohydride was destroyed by the addition of absolute methanol. The pigment was extracted into petroleum ether and the solution was dried by anhydrous sodium sulphate. The properties of the reduced product were compared with those of the isolated pigment.

This treatment reduces any carbonyl group present to the corresponding alcohol, this change being accompanied by an increase in the hypophasic nature of the pigment in the partition test, and by a change in the Rf value on TLC.

If the carbonyl group is in conjugation with the polyene chromophore there is also a change in the shape and position of the light absorption spectrum. A shift of 8mμ. to lower wavelengths characterises the reduction of one keto group conjugated to the polyene chromophore through a β-ionone ring, whereas the reduction of two such groups gives a similar shift of about 12 mμ.

From the change in the partition ratio on reduction the number of carbonyl groups can often be estimated, and the number of these in conjugation with the polyene chromophore through the β-ionone ring system can be deduced from the change in the position of the light absorption maximum. The spectrum of a reduced product is virtually that of the parent

carotene, thus though echinenone (III) and canthaxanthin (IV) are reduced to different xanthophylls the products have almost identical absorption spectra, which in turn are almost identical with that of the parent hydrocarbon , β-carotene (II).

Methylation of allylic hydroxy groups.

A small amount of the carotenoid was dissolved in methanol, and 6N hydrochloric acid (3 drops) was added. The solution was allowed to stand at room temperature for ½ h. and the pigment was extracted into petroleum ether. A methylated product is less polar than the original compound, and this change is detectable by the greatly increased Rf value when run on the same silica gel H thin layer as the original xanthophyll. As only allylic hydroxyl groups are methylated by this treatment, such groups can be detected in either the isolated pigment or in its reduction product.

The successful methylation of a reduced keto-carotenoid indicates that the hydroxl group (and hence the original keto carbonyl group) is in the allylic position, whereas when the methylation of such a reduced product is not possible by this method, the original keto group must occupy a non-allylic position.

Detection of 5,6-epoxy groups

0.1 N hydrochloric acid (several drops) was added to a chloroform solution of each carotenoid extracted and the mixture was allowed to stand at room temperature for 3 min.

A shift in the position of the light absorption maximum of 18 to 25 mμ. to shorter wavelength indicates the presence of one 5,6-epoxy group in the molecule, whereas a similar shift of 40 mμ. indicates the presence of two such groups.

No epoxy group was detected in any of the carotenoid pigments of Psolus fabrichii.

Thin layer chromatographic behaviour

Whenever possible after tentative identification, each pigment was compared with an authentic sample of that pigment by thin layer chromatography. If a particular carotenoid was not identified, or if an authentic sample was not available, the pigment was compared with carotenoids showing similar behaviour on thin layers.

A mixture of α and β-carotenes were purchased from Nutritional Biochemicals Company, samples of echinenone and canthaxanthin were donated by Hoffmann-La Roche, Basel, and a sample of astacene was kindly donated by Professor B.C.L. Weedon, Queen Mary College, University of London. Reduction of part of the echinenone and canthaxanthin by sodium borohydride in ethanol was used to provide samples of isocryptoxanthin (4-hydroxy-β-carotene) (VI) and isozeaxanthin (4,4'-dihydroxy-β-carotene) (X).

The stationary phase was usually silica gel H, though magnesium hydrogen orthophosphate (British Drug Houses, Laboratory Reagent) was also used occasionally. The layer was applied to either a 20 x 20 cm. or a 5 x 20 cm. glass plate by means of a Desaga applicator, which was used to spread a slurry, made by mixing silica gel H (25 gm.) with deionised water (25g.) to a layer of 250 μ. thickness. The standard developing solvent was a mixture of 5% to 30% acetone in petroleum ether (b.p. 35-50°), though benzene was sometimes substituted for the petroleum ether.

To compare two compounds, a concentrated solution of each was made in petroleum ether and a spot of each, together with a spot of a mixture of the two, was applied to a layer by means of a thin walled capillary tube. The chromatogram was developed in a Desaga tank until the solvent front had ascended about 15 cm. The solvent system was chosen so that the compounds had Rf values of between 0.4 and 0.6.

Two compounds were considered by be identical only if the separate spots had the same Rf values and the mixed spot could not be resolved, in at least two different systems. The Rf value is characteristic of the pigment on one particular plate, for even when donditions were standardised, the Rf value of a carotenoid varied slightly on different plates.

RESULTS

Total carotenoid content

The carotenoid pigments were extracted from a known weight of tissue, and dissolved in dry hexane. The total volume of the solution was measured, and the extinction of an aliquot of the solution was determined at 468 mμ. (the light absorption maximum).

The carotenoid content of the extract was calculated using the formula: $x = \frac{E\ y}{100\ E^{1\%}_{1cm}}$

where E = extinction in y ml.
x = weight dissolved
$E^{1\%}_{1cm}$ = extinction coefficient of of a 1% solution in a 1 cm. light path.

A value of 2400 was taken for $E^{1\%}_{1cm}$ and no allowance was made for the different extinctions of the carotenoids at 468 mμ. The total carotenoid content was 8.7 mg. / 100 gm. of the body wall.

Composition of the carotenoid extract

Initial separation by TLC revealed the presence of 10 different carotenoid fractions. The colour on a silica gel thin layer, the absorption maxima in petroleum ether and the percentage of each is shown in Table V.

Table V. Preliminary data derived for the 10 carotenoids separated by TLC from the acetone extract of Psolus fabrichii.

Fraction	Amount as % of total	Colour on silica gel	Absorption maxima in petroleum ether (mμ)	Rf values on silica gel H / 10% acetone in petroleum ether
1	1.0	orange	~422,447,473	.9
2	0.5	yellow	451	.57
3	1.0	yellow	~405,428,457	.54
4	2.0	red	466	.51
5	4.0	orange	454	.43
6	4.0	red	464	.35
7	9.0	yellow	407,431,459	.32
8	25.0	orange/red	460	.19
9	7.5	orange	466	.08
10	42.0	red	468	.04

~ indicates a shoulder.

Fraction 1

Absorption maxima (mμ):

hexane	425,447,472
Partition ratio	100/0
	The pigment was not identical with an authentic sample of β-carotene, as a mixed spot of the two separated slightly on silica gel H, with petroleum ether containing 10% acetone.

As the light absorption spectrum was virtually identical with that of β-carotene, fraction 1 was presumed to be either a mono cis-isomer or a mixture of cis-isomers of β-carotene, arising from the isomerisation of the naturally occurring all-trans form during the isolation procedure.

Fraction 2

Absorption maximum (mμ):

hexane	451

The pigment was present in such small amounts that it could not be unequivocally identified. The position and shape of the absorption spectrum, and the position on the initial thin layer suggests that it is a mono-keto compound with the keto group in conjugation with the polyene chromophore.

Fraction 3

Absorption maxima (mμ):	
hexane	~405,430,457 (Figure 3)
pyridine	~417,446,473
carbon disulphide	~425,458,485
Partition ratio:	94/4
Alkali treatment:	No effect
Reduction product	Absorption spectrum unchanged. Two products formed.

Rf values on silica gel H, 20% in petroleum ether.

echinenone (III)	.78
isocryptoxanthin (VI)	.66
reduced 3	.62
reduced 3	.60

Acid methanol — No effect

As the absorption spectrum is similar to that of fraction 7, the possible nature of the chromophore is discussed later.

Fraction 4

Absorption maximum (mμ):	Symmetrical
hexane	467
pyridine	487
carbon disulphide	499
Partition ratio:	100/0

Alkali treated product	Forms red potassium salts
Absorption maximum (mμ):	Symmetrical
hexane	471
pyridine	491
carbon disulphide	504
Partition ratio:	28/72
TLC	Inseparable from the alkali treated products of fractions 6 and 10, and from authentic astacene (VIII).

It was concluded that fraction 4 was a di-ester of astaxanthin (I) with a long chain (high molecular weight) acid.

Fraction 5

Initial work indicated that this compound might be echinenone (III), and comparison was made with an authentic sample of that compound (Table VI). The results indicated that fraction 5 and echinenone were identical.

Fraction 6

The light absorption properties were virtually identical with those of fraction 4, as was the effect of alkali and of sodium borohydride on the alkali oxidised product. This product could not be separated, by TLC, from authentic astacene and from similar alkali oxidised products obtained from fractions 4 and 10.

Fraction 6 differed from fraction 4 only in that it was more hypophasic in the partition test, having a partition ratio of 85/15. It was concluded that fraction 6 was an mono ester of astaxanthin.

Table VI. The data derived for fraction 5 and for authentic echinenone. Data marked * recorded for echinenone by Petracek and Zechmeister (32).

	fraction 5	Echinenone
Absorption maximum (mμ):	Single peak with shoulder	Single peak with shoulder
hexane	458 (Figure 4)	457
chloroform	473	474
pyridine	476	478
carbon disulphide	492	491
Partition ratio	94/6	93/7*
TLC	Mixed spot not resolved by (a) 10% acetone in petroleum ether or (b) 10% acetone in benzene, both on silica gel H.	
Alkali treatment	No effect	No effect
Reduced product	Yellow ethanolic solution	Yellow ethanolic solution
Absorption maximum (mμ):		
hexane	~421,447,474 (Figure 4)	~423,448,475
Partition ratio	87/13	86/14*
Acid methanol	Allylic hydroxyl present.	Allylic hydroxyl present.
	Both the reduced products and the methylated reduced products were inseparable.	

Fraction 7

Absorption maxima (mμ):	
hexane	406,430,459 (Figure 3)
chloroform	415,440,470
pyridine	422,446,476
carbon disulphide	433,457,489
Partition ratio	58/42
Alkali treatment	No effect
Reduced products	Two products formed each with the same absorption spectrum as fraction 7.
Partition ratios	32/68 and 24/76
Acid methanol	No allylic hydroxyl groups present.
TLC	Rf values on silica gel H / 15% acetone in petroleum ether.

z. isocryptoxanthin (VI)	.76
fraction 7	.71
canthaxanthin (IV)	.69
reduced 7	.64
reduced 7	.61
isozeaxanthin (X)	.60

The absorption maxima in hexane correspond to a conjugated system of 8½ double bonds. As there is no carbonyl group in conjugation with the polyene system (no change of absorption spectrum on treatment with sodium borohydride), this corresponds to 8 conjugated double bonds in conjugation with a β-ionone ring. The fine structure of the spectrum is evidence

for the lack of conjugated carbonyl group, and also suggests that both ends of the polyene chain are not cyclised.

The only compound reported with this structure is β-zeacarotene (7',8'-dihydro-γ-carotene) (XI) a compound first isolated from maize (35) and later synthesised (36).

XI

It is probable that fraction 3 is a mono-keto and fraction 7 a di-keto derivative of this carotene. The position of the keto groups is not known, though it is apparent that the 4-position of the β-ionone ring is not so substituted. No explanation can be given for the formation of two reduction products from both fractions 3 and 7.

The presence of non-allylic keto groups has not been reported very often, though such compounds might be expected to exist, and Krinsky and Lenhoff proposed such groups in the keto-pirardixanthins which they extracted from Hydra pirardi (37).

Fraction 8

Preliminary work indicated a similarity to canthaxanthin (4,4'-diketo-β-carotene) (IV), and a comparison was made with an authentic sample of this compound (Table VII).

The results showed that Fraction 8 was identical with canthaxanthin.

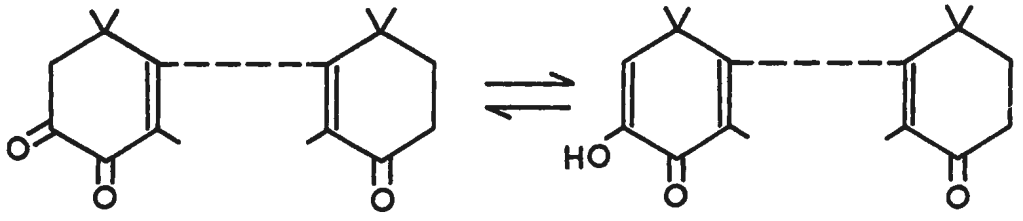
Table VII. The properties of fraction 8, an authentic sample of canthaxanthin, and canthaxanthin extracted from flamingoes (10). Data marked * was measured by Petracek and Zechmeister (32).

	Fraction 8	Canthax-anthin	Canthax-anthin from flamingoes
Absorption maximum (mμ)	Symmetrical	Symmetrical	Symmetrical
hexane	460.5	461	460-462
chloroform	478	478	475
pyridine	482.5	482	Not reported
carbon disulphide	496.5	496	Not reported
Partition ratio	52/48	50/50 *	50/50
Alkali treatment	No effect	No effect	No effect
TLC	Mixed spot not resolved on silica gel.		---
Reduction product			
Absorption maximum (mμ)			
hexane	~423,448,474	~422,448,474	β-carotene type
Partition ratio	23/77	22/78 *	22/78
TLC	Mixed spot could not be resolved on silica gel H.		---
Acid methanol	Allylic OH present	Allylic OH present	Allylic OH present

Fraction 9

The symmetrical absorption curve, and its position, indicate a 4,4'-diketo derivative of β-carotene. The decrease in polarity, and the shift of the absorption spectrum to longer wavelengths on alkali treatment suggest that at least one 3-hydroxy-4-keto substituted β-ionone ring is oxidised by this treatment. The compound is intermediate in thin layer behaviour between canthaxanthin (V) and astaxanthin (I). This data suggests that fraction 9 is 3-hydroxy-4,4'diketo-β-carotene (IX), a compound which has been isolated from the tissues of three flamingo species, and named phoenicoxanthin (10).

The pigment of Psolus fabrichii differs from phoenicoxanthin in that it gave insoluble red/orange potassium salts on alkali treatment, whereas the oxidation product of phoenicoxanthin (phoeniconone (XII)) is reported to be neutral (10,34). The conditions needed for the salt formation seemed to be rather critical and were not completely reproducible.



XII

The properties of fraction 9 are compared with those of phoenicoxanthin in Table VII.

Table VII. The properties of fraction 9 and of pheonicoxanthin (34).

	Fraction 9	Phoenicoxanthin
Absorption maximum (mμ):	Symmetrical	Symmetrical
hexane	463	462
chloroform	479	479
pyridine	482	---
carbon disulphide	497	---
Partition ratio	27/73	29/71
Alkali treated product	Acid. Forms red/ orange salts.	Neutral. No salts formed.
Absorption maximum (mμ):		
hexane	466	467-468
chloroform	484	484
pyridine	486	---
carbon disulphide	500	---
Partition ratio	36/64	36/64
Reduced product		
Absorption maximum (mμ):		
hexane	~425,448,476	Like β-carotene
Partition ratio	8/92	4/96 to 8/92
Acid methanol	Allylic OH present	Allylic OH present

Fraction 10

The pigment was oxidised to an acidic compound which formed red, insoluble potasium salts. This product was compared with an authentic sample of astacene (VII) (Table VIII).

The properties of the isolated pigment were almost identical with those reported for astaxanthin by Fox and Hopkins (34) (Table IX).

Table VIII. The properties of the alkali oxidised product from fraction 10 and of an authentic sample of astacene.

	Alkali oxidised product of fraction 10	Astacene
Absorption maximum (mμ):	Symmetrical	Symmetrical
hexane	471	472
chloroform	488	488
pyridine	492	492
carbon disulphide	507	508
Partition ratio	23/77 to 28/72	24/76
TLC	Mixed spot not resolved on (a) silica gel H : 20% acetone in petroleum ether (b) silica gel H :20% acetone in benzene (c) magnesium orthophosphate :20% acetone in petroleum ether	
Reduced product		
Absorption maximum (mμ):		
hexane	~425,448,476 (Figure 5)	~425,448,476
Partition ratio	8/92	5/95 to 9/91
Acid methanol	Allylic hydroxyl present	Allylic hydroxyl present

Table IX. The properties of fraction 10 and of astaxanthin extracted from the Scarlet Ibis. (34).

	Fraction 10	Astaxanthin from the Scarlet Ibis
Absorption maximum (mμ):	Symmetrical	Symmetrical
hexane	468	467
chloroform	482	483
pyridine	484	Not reported
carbon disulphide	500	Not reported
Partition ratio	9/91	9/91
Alkali treated product	Forms red salts	Forms red salts
Absorption maximum (mμ):	Symmetrical	Symmetrical
hexane	471(Figure 5)	474
chloroform	488	486-488
pyridine	492	490
carbon disulphide	507	Not reported
Partition ratio	23/77 to 28/72	25/75
Reduction product		
Absorption maxima (mμ):		
hexane	~425,448,476 (Figure 5)	~423,448,475
Partition ratio	8/92	5/95 to 9/91
Acid methanol	Allylic hydroxyl present	Allylic hydroxyl present

It was thus confirmed that fraction 10 was unesterified astaxanthin.

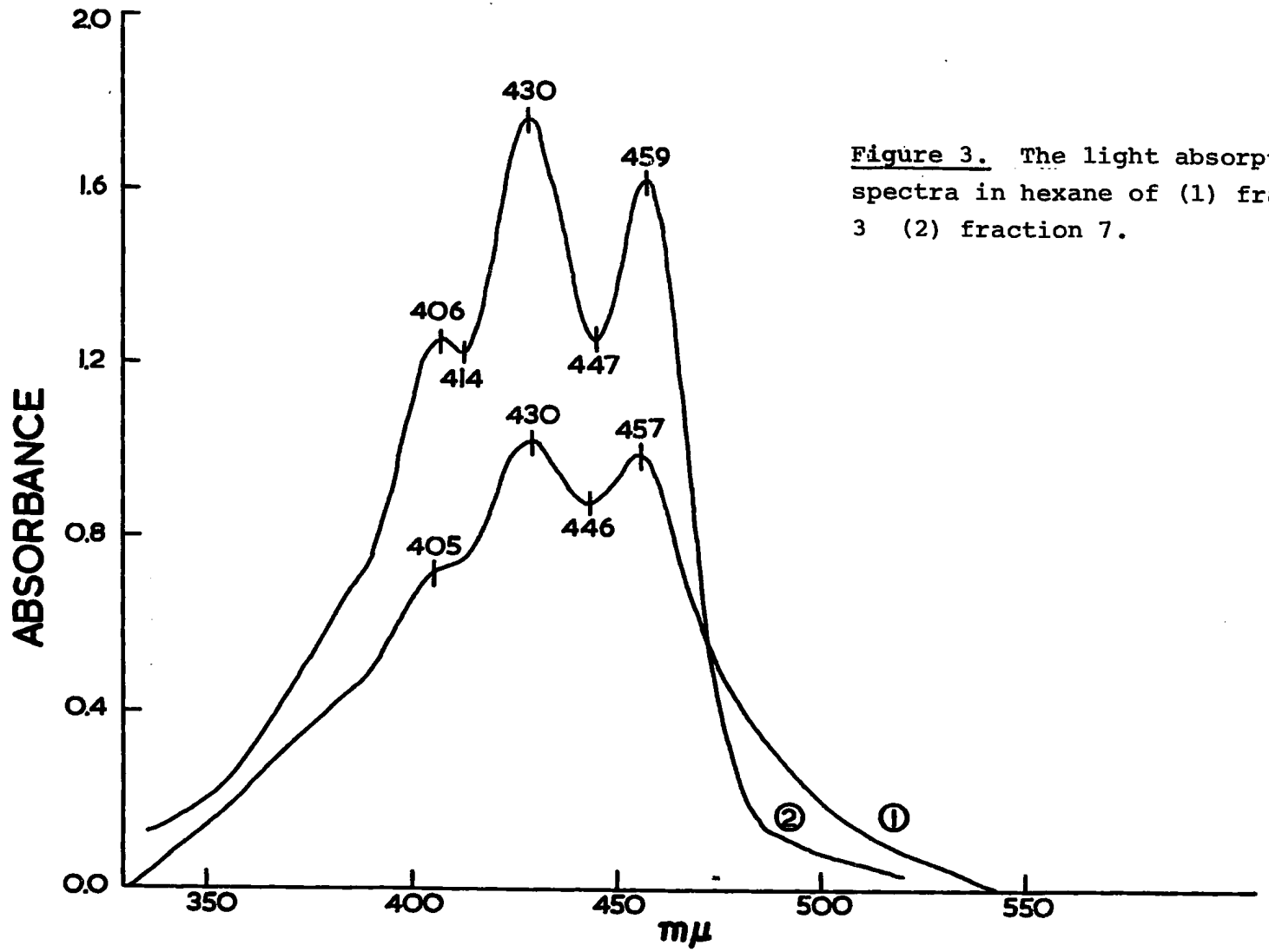


Figure 3. The light absorption spectra in hexane of (1) fraction 3 (2) fraction 7.

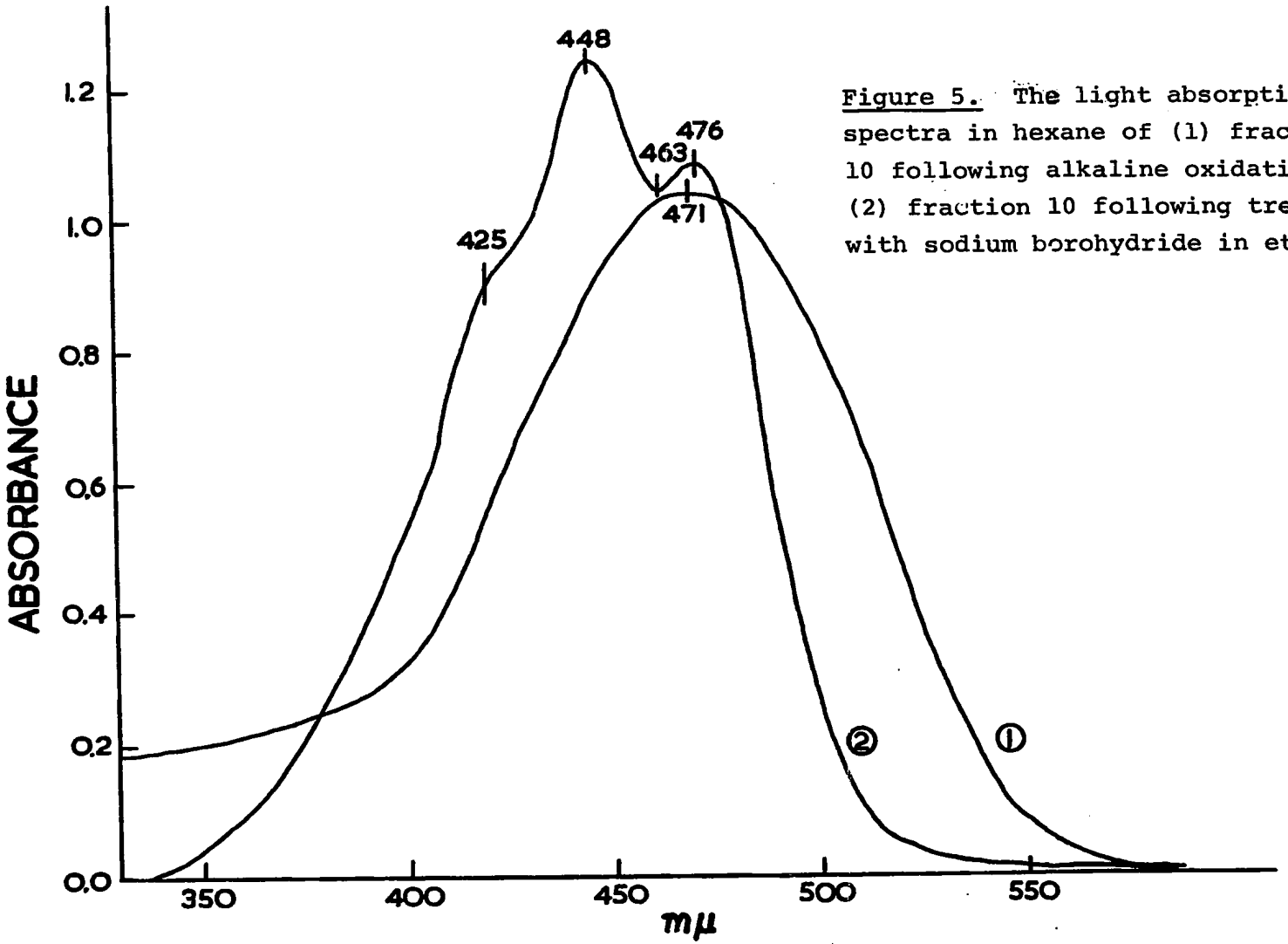


Figure 5. The light absorption spectra in hexane of (1) fraction 10 following alkaline oxidation (2) fraction 10 following treatment with sodium borohydride in ethanol.

Discussion

A preliminary analysis of the carotenoids in the gut contents of several specimens revealed relatively large amounts of β-carotene and smaller amounts of mono- and di-hydroxy-β-carotene derivatives, but no polar keto-carotenoids were detected. These findings indicate that the dietary carotenoids are an indirect source of the body wall keto-carotenoids, and that a sequential oxidation pathway, similar to that proposed in other invertebrates, is responsible for the conversion of dietary β-carotene (and possibly some of its hydroxy derivatives) into astaxanthin.

In view of the nature of the carotenoids in the gut contents, it is interesting to note that no hydroxy-β-carotene derivatives could be detected in the body wall. It seems that either these compounds are not absorbed through the gut wall, or more likely that they are rapidly oxidised to mono- and di-keto-β-carotene derivatives in the tissues.

It is also apparent that oxidation of the dietary β-carotene, if this does occur, does not take place through mono- and di-hydroxy compounds, as has been proposed in other invertebrates, or if it does, these intermediates are metabolised very rapidly and are present in undetectable amounts.

Location and nature of the hypothetical oxidation pathway

As the viscera are less coloured than the body wall and the tentacles, it is probable that the absorbed dietary carotenoids are transported to the ectodermal layers and then rapidly oxidised to the red carotenoid canthaxanthin (V).

The rapid oxidation of the dietary carotenoids would explain the absence of detectable amounts of hydroxy-β-carotene content in the body wall. The presence of relatively large amounts of canthaxanthin (V) and astaxanthin (I) suggests that the metabolic steps between canthaxanthin and astaxanthin esters are relatively slow.

The possible metabolic steps are shown in Figure 6.

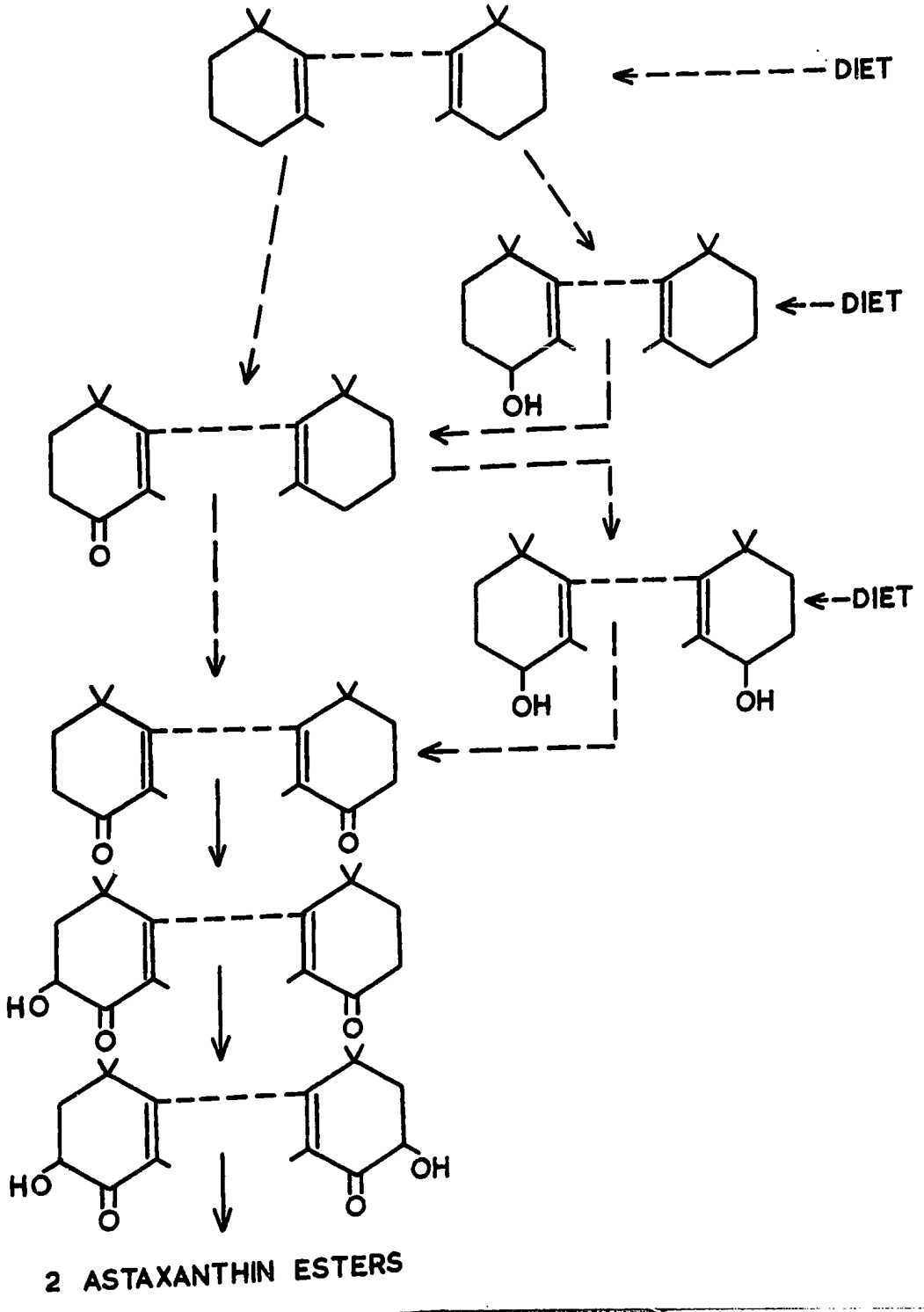
Classification of echinoderms and particularly holothurians

Although β-carotene, and the two unidentified carotenoids (fractions 3 and 7) have been isolated from both the body wall and the gut contents of Psolus fabrichii, it appears that the major part of the body wall carotenoids may be metabolised by the animal from dietary precursors.

Thus, though traditionally the direct effect of the diet on the carotenoid content of echinoderms has been emphasized, it seems that two factors, the food and the genetically controlled ability to oxidise part of the dietary carotenoids, may be of importance.

It is possibly of significance that the two holothurians in which astaxanthin has been confirmed (Cucumaria lubrica(6) and Psolus fabrichii) are both members of the Order Dendrochirota. These organisms are omnivorous, feeding by the ingestion of organic material which adheres to the tentacles as they are waved through the water. Thus Pearse (38) found both small animals (copepods and ostracods) and plant material in the stomach of Thyone briareus.

Fig. 6. Possible metabolic pathways for the conversion of ingested β-carotene (and its hydroxy derivatives) to astaxanthin in Psolus fabrichii.



The Aspidochirota, the other Order of holothurians which has been investigated with regard to carotenoid content (4,40,41), feed on bottom material, with subsequent digestion of the organic content, and expulsion of the non-digestible fraction.

The carotenoid content of some of the holothurians studies is shown in Table X.

There is no definite evidence that the highly oxidised carotenoids found in the body wall are not supplied by the diet, and it is possible either that the gut contents investigated were not typical with regard to carotenoid content or that the organism does concentrate small amounts of keto-compounds obtained from the diet, the more abundant dietary carotenes and xanthophylls being eliminated from the organism.

However, though the source of the keto-carotenoids is not at present determined, the fact that such compounds are found in the tissues in such large amounts is conclusive evidence that all holothurians cannot be classified together into one herbivorous, carotene containing class of echinoderms.

Table X. The occurrence of carotenoids in some holothurians.

Order	Holothurian	Carotenes + Echineone	Hydroxy carotenes	Keto-carotenoids
Dendrochirota	Cucumaria elongata (39)	+	?	
	Cucumaria lubrica (6)	+	+	+
	Phyllophorus lucidus (39)		+	
	Psolus fabrichii	+		+
Aspidochirota	Holothuria brunneas (40)		Unresolved mixtures	
	Holothuria nigra (40)		Unresolved mixtures	
	Holothuria poli (40)		Unresolved mixtures	
	Holothuria tubulosa (40)		Unresolved mixtures	
	Mesothuria intestinalis (4)		+	
	Stichopus parvimensis (4)	+	+	

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Addenda

p. 10 Reference to work on Astropecten. D.L. Fox and T.S. Hopkins. Physiology of Echinodermata. Edited R.A. Boolootian. Interscience publishers (1966).

p. 16 The β-ionone end group, a term widely used in the field of carotenoid chemistry, refers to the 2,6,6-trimethyl-1-cyclohexen-1-yl group, this being a feature of the compound 3-buten-2-one,4-(2,6,6-trimethyl-1-cyclohexen-1-yl) or β-ionone.

p. 28 Reference to the formula for the calculation of the carotenoid content of the extract is:-

B.H. Davies. Chemistry and Biochemistry of Plant Pigments. Edited T.W. Goodwin. Academic Press (1965)

Eight specimens (678 g.) were dissected and the carotenoids extracted from the body walls (200g. wet weight).

The total carotenoid content of the extract was determined, and following the separation by TLC, the percentage of each pigment present was calculated. The recovery of the pigments from the TLC plate was 96% of the carotenoids in the original extract.

Losses were due partly to oxidation of the carotenoids on the thin layer plate, but mainly to incomplete elution from the silica gel phase.

p. 35 The two reduced products from fraction7 did not appear to be due to incomplete reduction, as repeated treatment with sodium borohydride did not alter the relative proportions of the two (about 50/50).

The possibility that the two are isomers, one having a cis configuration about a double bond cannot be overlooked.

p. 42 The section entitled "Classification of echinoderms and particularly holothurians" is retitled "Carotenoids in relation to holothurian biology".

Errata

p. 12 The reference to the removal of lipids attributed to Fox and Hopkins (10,30) should read Fox *et al.* (10,30).

p. 19 Table III. The heading of column 3 should read:- Partition ratio. Hexane/95 % methanol.