The Biosynthesis of Astaxanthin V : Interconvention of the Algal Carotenoids, Stigeoclonium sp. into Fish Carotenoids, Fancy Red Carps

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The Biosynthesis of Astaxanthin—V*

Interconversion of the Algal Carotenoids, *Stigeoclonium* sp. into Fish Carotenoids, Fancy Red Carps

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Abstract

1. The carotenoids of fancy red carps (*Cyprinus carpio Linne*) have separated by absorption chromatography and further characterized by their absorption spectra and behavior on the column chromatography. In some cases melting points were measured and reactions for specific functional groups were performed.

2. The principal carotenoids of the integument were lutein, α-doradexanthin, astaxanthin, and β-doradexanthin and in the internal organs the existence of canthaxanthin, lutein, and zeaxanthin was confirmed.

3. The contents of the stomach of fancy red carps were examined and half-digested algae were found in them, the presence of chlorophylls was also confirmed. In the ponds where the carps were being raised, the algae, *Stigeoclonium* sp, was found. The carotenoids of the algae were separated and the presence of α-carotene, β-carotene, β-zeacarotene, echinenone, canthaxanthin, lutein, zeaxanthin, violaxanthin and neoxanthin was confirmed. The metabolic pathway from the plant carotenoid, into fish carotenoid, was proposed.

Introduction

The carotenoids in algae are reported to have a number of functions in photosynthetic and phototactic organisms. By absorbing light in the region where absorption by chlorophyll is low and transferring this energy to chlorophyll, they increase the capacity of plants and micro-organism to gather light for photosynthesis.[1] Carotenoids protect the cell from photodynamic destruction.[5),3)] It has also been confirmed that carotenoids play a role in the transport of oxygen.[4]

It is generally accepted that fish, like all other animals can not synthesize carotenoids de novo but they can alter alimentary carotenoids and can store the resulted products. The fancy red carps take algae in the pond and metabolize plant carotenoids into fish carotenoids. The present investigation was undertaken to determine the biochemical correlation between algal carotenoids and fish carotenoids.

In the previous report[5),6)], a new keto-carotenoid, 3-hydroxy -3', 4' -diketo-α-

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carotene was isolated. The name α-doradexanthin was proposed for 3,3'-dihydroxy-4'-keto-α-carotene and α-doradecin for 3-hydroxy -3' -4' -diketo-α-carotene. A possible biosynthetic pathway from lutein to astaxanthin was proposed in gold fish.

Experimentals

1. The carotenoids of *Stigeoclonium* sp.

The freshly harvested algae were exhaustively extracted with acetone in a Waring blender. Acetone was redistilled before use. Petroleum ether (CP) was passed over activated silica gel. The deeply colored acetone solution of pigments was diluted with water and the pigments were transferred to petroleum ether in a separatory funnel. This solution was evaporated under reduced pressure, for saponification, 50 ml of 6 % potassium hydroxide in methanol was added, and the pigments were left overnight at room temperature. The pigments were then transferred to petroleum ether by addition of water. The petroleum ether solution of pigments was washed thoroughly with water, dried over anhydrous sodium sulfate, and evaporated to an oil under reduced pressure.

Pigment separation :

The pigments were dissolved in a small volume of petroleum ether and were first chromatographed on a column of Microcel-C. The column was developed with increasing concentration of acetone in petroleum ether (final concentration 10 % acetone in petroleum ether). Five bands were obtained.

Band A pigments from the lower band were rechromatographed on magnesium oxide column (MgO : hyflosupercel = 1 : 2). Three bands were obtained : Band 1, Band 2, and Band 3.

α-Carotene : Band 1 pigment from lower zone was eluted from the column with acetone. The absorption maxima are shown in Fig. 1.

β-Carotene : Band 2 pigment was eluted from the column with acetone. The absorption maxima are shown in Fig. 2.

β-Zeacarotne : Band 3 pigment from upper zone was rechromatographed on alumina column by using 3 % acetone in petroleum ether solution as developing solvent. The absorption maxima and the behavior on the column were all in agreement with those of β-zeacarotene (Fig. 3).

Echinenone : Band B pigments were extracted from the column with acetone and were purified on magnesium oxide column (MgO : hyflosupercel=1 : 2) by using 4 % acetone in petroleum ether as developing solvent. The absorption spectrum indicates a single absorption maximum in petroleum ether and suggests a keto group in conjugation with double bond system, after reduction λ max 426, 450, 476 mμ (Fig. 4).

Canthaxanthin : Band C pigments were purified on magnesium oxide column (MgO : hyflosupercel = 1 : 2). The column was developed with 12 % acetone in petroleum ether. The absorption spectrum shows a single absorption maximum

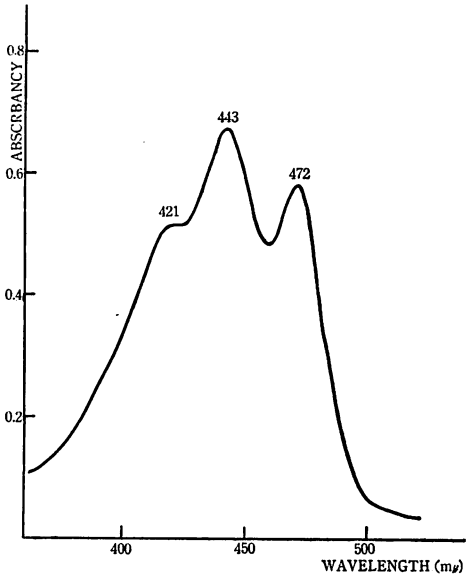


Fig. 1. Absorption spectrum of α-carotene obtained from *Stigeoclonium* sp. in the ponds where fancy red carps are being raised.

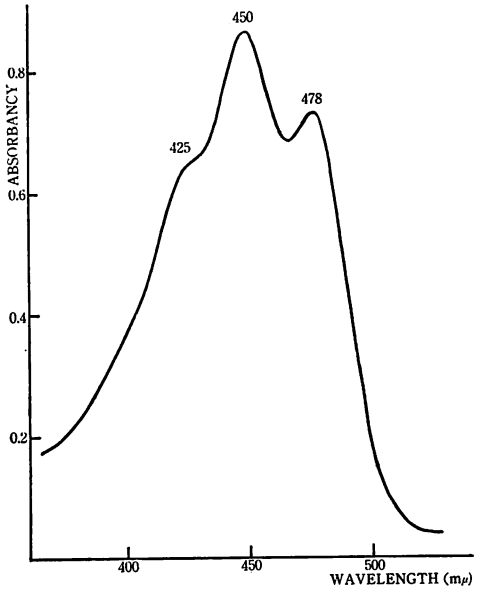


Fig. 2. The absorption spectrum of β-carotene obtained from *Stigeoclonium* sp. in petroleum ether.

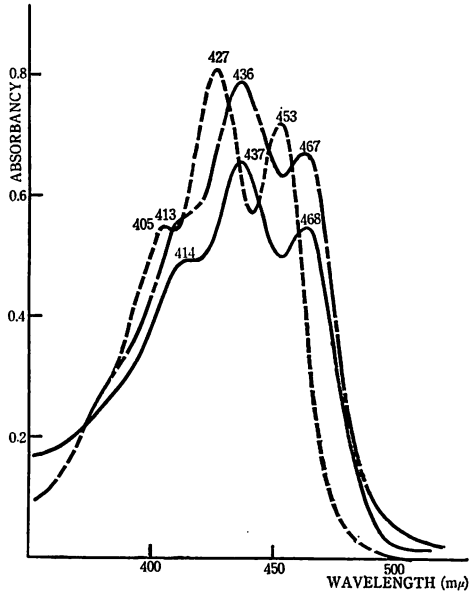


Fig. 3. The absorption spectrum of β-zeacarotene obtained from *Stigeoclonium* sp.
Dotted curve : in petroleum sther.
Dashed curve: in chloroform.
Solid curve: in benzene.

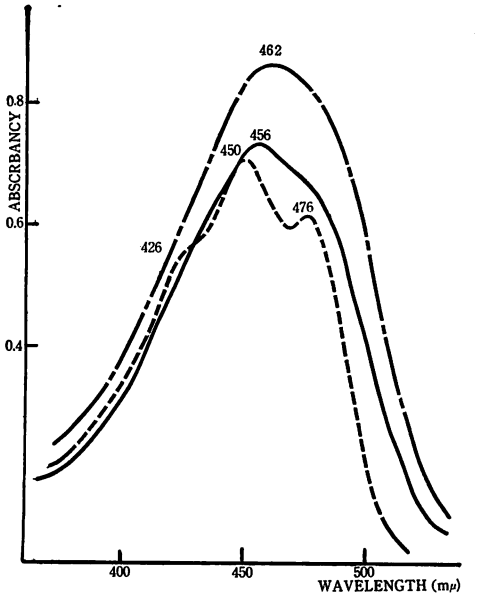


Fig. 4. The absorption spectrum of echinenone obtained from *Stigeoclonium* sp.
Solid curve : in petroleum ether.
Dashed curve : in 95% ethanol.
Dotted curve : the products of sodium borohydride reduction in 95% ethanol.

in petroleum ether, after reduction with sodium borohydride λ max 427, 451, 478 mμ. These results were identical with those of pure canthaxanthin (Fig. 5).

Band D pigments were rechromatographed on magnesium oxide column (MgO : hyflosupercel = 1 : 2). The column was developed with 25 % acetone in petroleum ether and two zones were obtained : Band 1 and Band 2.

Lutein : Band 1 pigment from lower zone was eluted wih acetone. The absorption maxima are shown in Fig. 6.

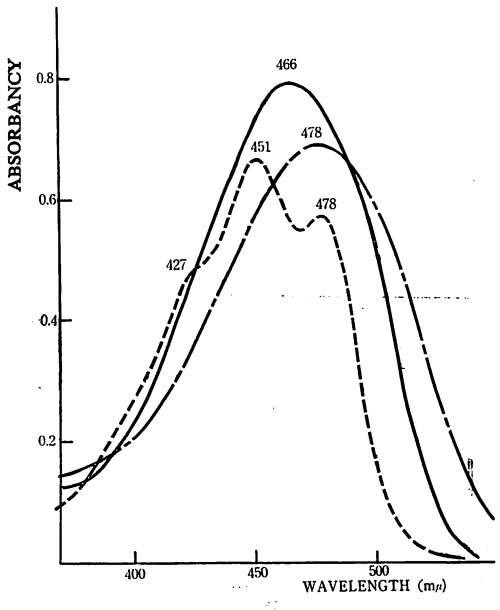


Fig. 5. The absorption spectrum of canthaxanthin obtained from *Stigeoclonium* sp.
Solid curve : in petroleum ether.
Dashed curve : in 95% ethanol.
Dotted curve: the products of sodium borohydride reduction in 96% ethanol.

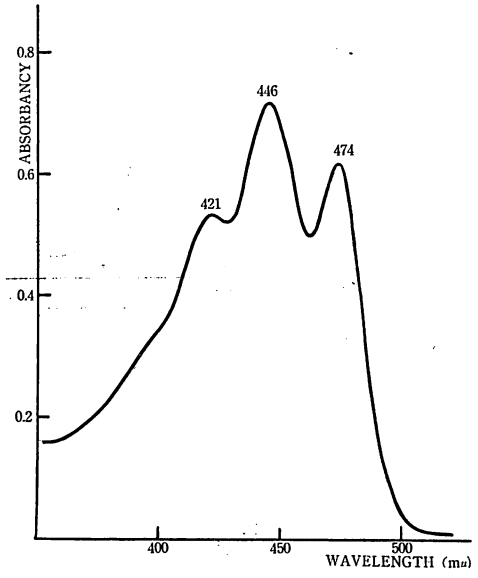


Fig. 6. The adsorption spectrum of lutein obtained from *Stigeoclonium* sp.

Zeaxanthin : Band 2 pigments from upper zone were extracted with acetone from the column. Its absorption maxima are shown in Fig. 7.

Violaxanthin: Band E pigments were purified on the column of Microcel-C by using 8 % acetone in petroleum ether as developing solvent. The pigments thus purified were eluted from column with acetone. The absorption maxima are shown in Table 1.

Neoxanthin : Band F pigments were rechomatographed on the column of Microcel-C by using 10 % acetone in petroleum ether. The zone thus obtained was eluted from the column with acetone. The absorption maxima are shown in Table 2.

In Stigeoclonium sp., which grows in the pond, the presence of α-carotene, ß-carotene, ß-zeacarotene, echinenone, canthaxanthin, lutein, zeaxanthin, violaxanthin and neoxanthin was confirmed. Lutein ester was most abundant in this plant carotenoids.

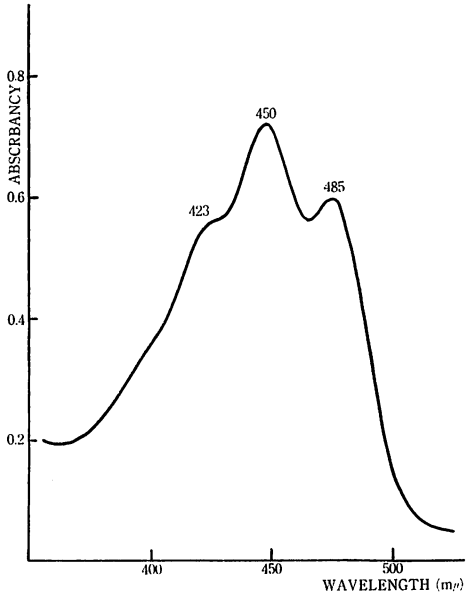


Fig. 7. The absorption spectrum of zeaxanthin obtained from *Stigeoclonium* sp.

Table 1. Spectral characteristics of Band D.

pigment	λmax in ethyl ether	λ max in ethyl ether (after addition of HCl)	shift of maior peak
Band C	422, 442, 470	382, 403, 428	39
pure violaxanthin	417, 440, 469	381, 403, 429	37

Table 2. Spectral characteristics of Band E.

pigment	λmar in ethyl ether	λmax in ethyl ether (after addition of HCl)	shift of major peak
Band D	417, 442, 470	399, 424, 448	18
pure neoxanthin	417, 440, 468	400, 423, 450	17

2. Carotenoids in fancy red carps :

A. Carotenoids in the integument of fancy red carps : Fancy red carps for this investigation were purchased from a local fish hachery, sacrified and extracted three to four times with acetone until the extract was colorless. The acetone extracts were combined, diluted with water and the pigments were transferred

to petroleum ether. The petroleum ether solution of pigments was evaporated under reduced pressure. The absorption spectra of the crude carotenoids in the fancy red carps is shown in Fig. 8.

Pigment separation :

The pigments were first chromatographed on column of Microcel-C. The column was developed with 1.5 % acetone in petroleum ether and three bands were obtained : Band A, Band B, and Band C.

Lutein : Band A pigmenrs were seponified and were run on Magnesium oxde mixed with celite (1 : 2 w/w). The column was developed with 25 % acetone in petroleum ether. It was identified by this feature, as well as by the shape and position of the absorption maxima (Fig. 9).

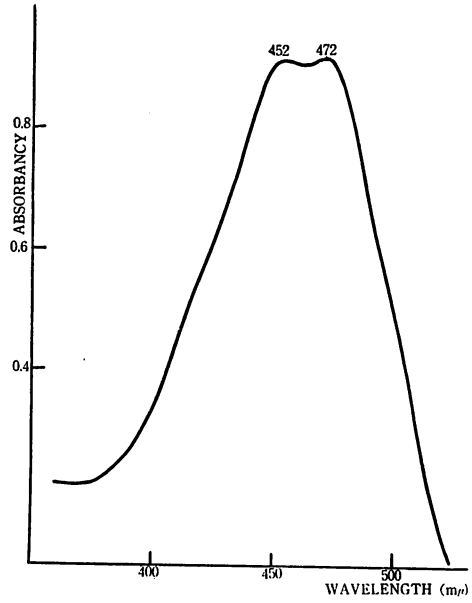


Fig. 8. The absorption spectrum of the crude carotenoids obtained from fancy red carps in petroleum ether.

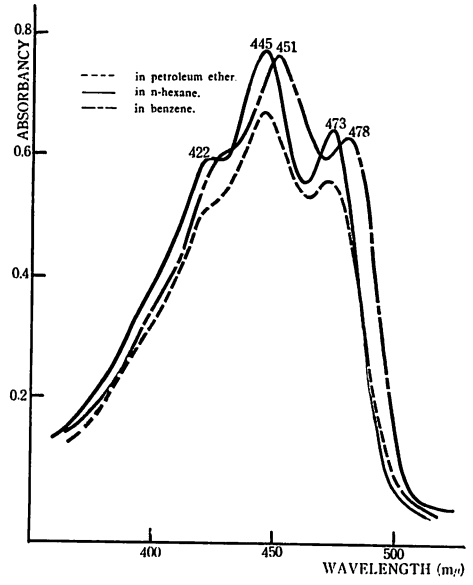


Fig. 9. The absorption spectrum of lutein obtained from fancy red carps.

α-Doradecin : Band B pigments were saponified, the saponified pigments were chromatographed on a magnesium oxide column (MgO: hyflosupercel=1 : 2), using 30 % acetone in petroleum ether as developing solvent in order to remove any trace of lutein. The band was eluted with 10 % acetic acid in ethyl ether and transferred to petroleum ether and rechromatographed on a Microcel-C column, using 10 % acetone in petroleum ether. The band was cut out from the column and eluted with 10 % acetic acid in ethyl ether. The pigments thus purified were rechromatographed on a dried powdered sugar column, using 2 % acetone in petroleum ether as developing solvent.

The purified pigments in petroleum ether exhibited the same absorption max-

ima of a new keto carotenoid which was isolated from gold fish[5]. (Fig. 9). Its structure is 3-hydroxy-3', 4'-diketo α-carotene the isolated pigment was crystallized from n-hexane. Its melting point was 154-156°C.

The pigment was reduced by the addition of sodium borohydride to a solution of the pigment in 95% ethyl alcohol. The solution was left under nitrogen in the refrigerator for an hour. The absorption spectrum of the reduced pigments agreed with that of lutein. (Fig. 10). Those values were identical with those of α-doradecin, 3-hydroxy,-3', 4'-diketo α-carotene[5),6),7)].

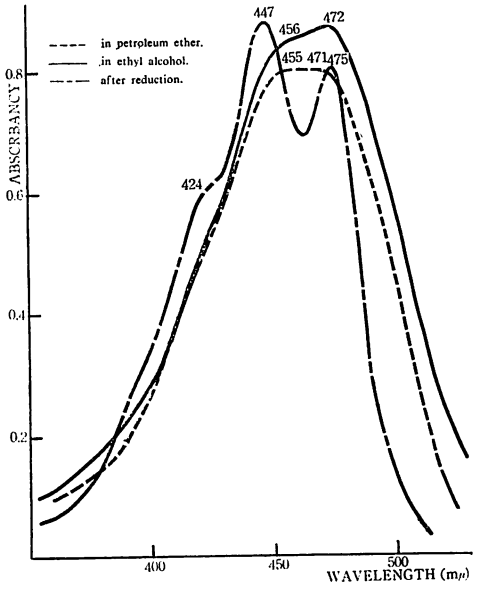


Fig. 10. The absorption spectrum of anew keto-carotenoid, α-doradecin obtained from fancy red carps.

ß-Doradexanthin : Band C pigments were saponified, transferred to petroleum ether by addition of water and dried with sodium sulfate. The pigments were chromatographed on Microcel-C column and developed with 10% acetone in petroleum ether. The pigments were rechromatographed on a dried powdered sugar column by using 2% acetone in petroleum ether as developing solvent. The absorption spectrum in petroleum ether solution, the behavior on the column and the absorption spectrum of the reduced products were identical with those of B-doradecin obtained from gold fish[5].

Astacin : After having saponified Band D pigments, they were transferred to petroleum ether with water and dried with sodium sulfate. The pigments thus obtained in petroleum solution were placed on a column of Microcel-C and developed with 10% acetone in petroleum ether. Only one band was obtained. After eluting out with acetic acid in ethyl ether, the pigments were rechromatographed on a dried powdered sugar column. The zone was eluted with acetone and transferred to petroleum ether. λ max 472 mμ in petroleum ether. After reduction with sodium borohydride in 95% ethyl alcohol at 5°C, the absorption spectrum of the reduced pigment agreed with that of β-carotene. The isolated, purified pigment was crystallized from n-hexane and ethyl ether solution m. p. 242-244°C. Those values were identical with those of astacin obtained from gold fish[5], (Fig 11).

B. Carotenoids in the internal organs : The internal organs were collected and extracted repeatedly with acetone in a Waring blender. The acetone solution of the pigments was diluted with water and extracted with petroleum ether. The petroleum ether solution of pigments was repeatedly washed with water. The

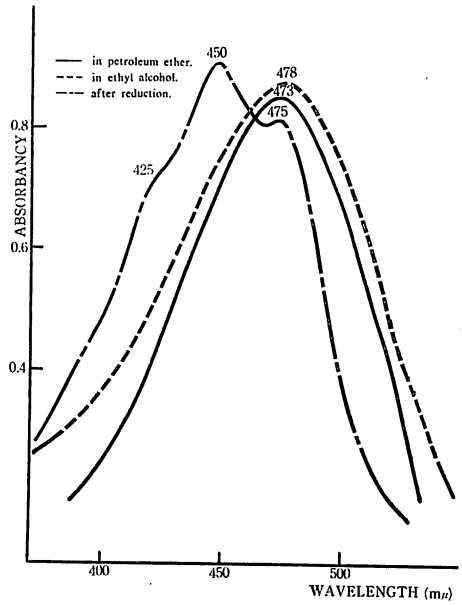


Fig. 11. The absorption spectrum of astacin obtained from fancy red carps.

petroleum ether solution was concentrated under vacuum and dried over sodium sulfate. The absorption spectrum of the crude carotenoids in petroleum ether is shown in Fig. 12.

The petroleum ether solution of pigments was chromatographed on Microcel-C column by using 2 % acetone in petroleum ether as developing solvent. Two bands were obtained : Band-I and Band-II.

Canthaxanthin : The pigments of Band-I were saponified by disolving them in

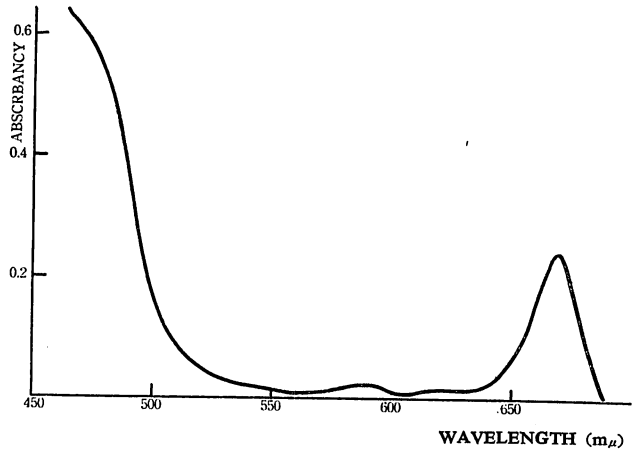


Fig. 12. The absorption spectrum of the crude carotenoid from the internal organs of fancy red carps.

20 cc of absolute ethanol, adding 2 cc of 60 % (w/v) aqueous potassium hydroxide solution and leaving it overnight at room temperature. The saponified pigments were transferred to petroleum ether by addition of water.

The pigments thus obtained were chromatographed on magnesium oxide column (MgO : hyflosupercel = 1 : 2), using 12 % acetone in petroleum ether as developing solvent and were purified again on Microcel-C column using 3 % acetone in petroleum ether as developing solvent. The pigment thus purified exhibited absorption maximum at 466 mμ in petroleum ether and after reduction with sodium borohydride, λ max, 425, 451, 474 mμ (Fig. 13). Those values were identical with those of canthaxanthin.

Lutein and Zeaxanthin : The pigments of Band-II were saponified and were chromatographed on magnesium oxide column by using 25 % acetone in petroleum ether as developing solvent. Two Bands were obtained : yellow pigments from lower band were eluted out from the column with acetone. The absorption spectrum in petroleum ether and the behavior on the column were in agreement with lutein (Fig. 14). Orange pigments from upper band were eluted out from the column with acetone. The absorption spectrum in petroleum ether was identical with pure zeaxanthin (Fig. 15).

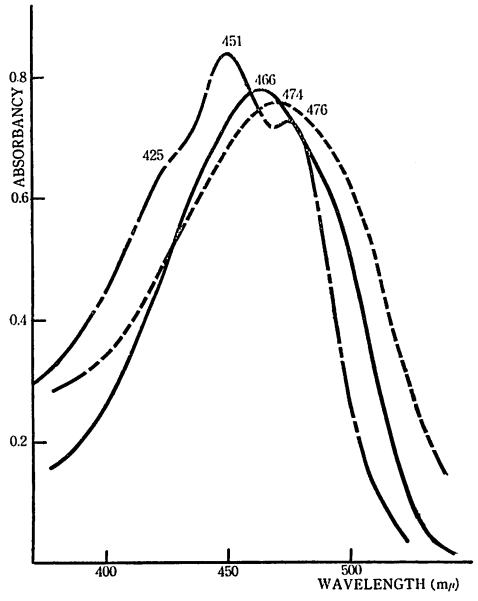


Fig. 13. The absorption spectrum of canthaxanthin obtained from the internal organs of fancy red carps.
Solid curve : in petroleum ether.
Dotted curve : in 95 % ethanol.
Dashed curve : the products of sodium borohydride reduction in 95% ethanol.

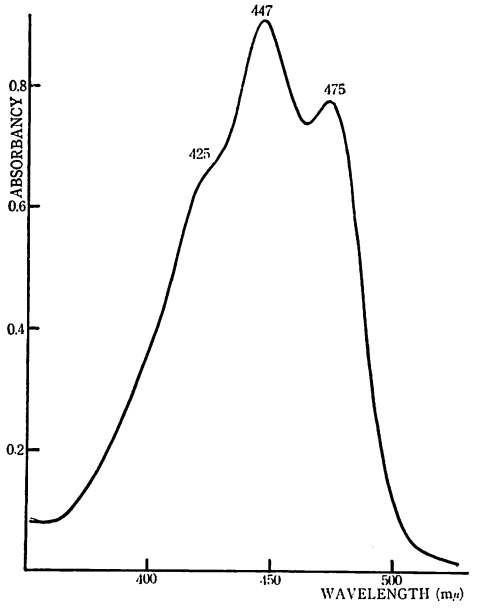


Fig. 14. The absorption spectrum of lutein obtained from the internal organs of fancy red carps.

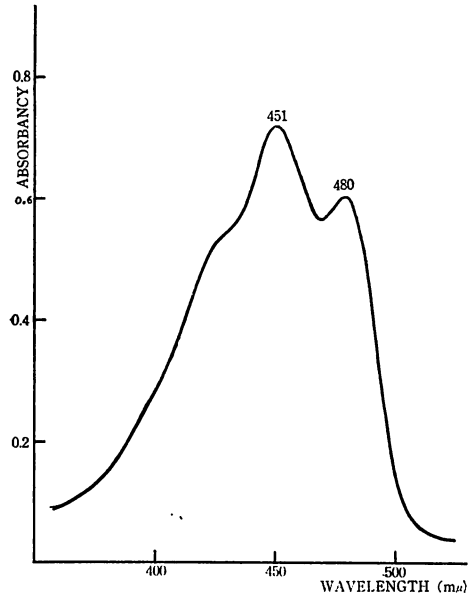
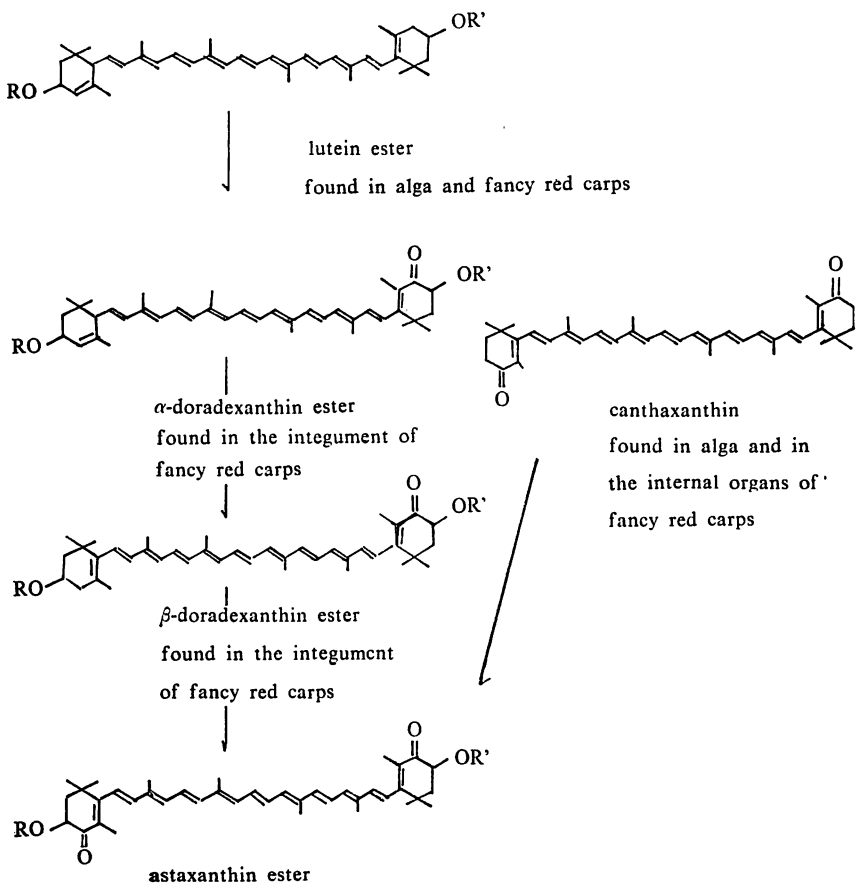


Fig. 15. The absorption spectrum of zeaxanthin obtained from the internal organs of fancy red carps.

Discussion

In *stigeoclonium* sp. that grows in the pond, the presence of α-carotene, ß-carotene, ß-zeacarotene, echinenone, canthaxanthin, lutein, zeaxanthin, violaxanthin and neoxanthin was found. Lutein ester was most abundant in these algal carotenoids (about 40 %). It is interesting to note that lutein ester, canthaxanthin would be the precursors in the metabolic pathway from plant carotenoids (lutein ester, canthaxanthin) to fish carotenoide (astaxanthin) as follows. This metabolic pathway from lutein ester to astaxanthin ester was proposed in gold fish[5]. Hata *et al*[8] stated that ß-carotene might be a precursor of astaxanthin in gold fish.

It is also very interesting to point out the existence of ß-zeacarotene. Davis *et al*[9] cited kinetic evidence that ß-carotene is formed via ß-zeacarotene in Phycomyces. Chichester and Co-workers[10] found that the level of ß-zeacarotene increased in *Rhodotorula* when the yeast was either cultured at 5° or treated with ß-ionone. ß-Zeacarotene has been found in yellow corn by shimpson *et al*[11]. The existence of echinenone has also been confirmed in one kind of *Diatomaceae*[12] growing in the tunk which prawns were being raised, and there might be the close biochemical correlation between the carotenoids in prawns[13] and those in algae.



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