The Biosynthesis of Astaxanthin—II.*

The Carotenoids in Benibuna, *Carassius auratus*,
Especially the Existence of a New Keto Carotenoids,
α-doradecin and α-doradexanthin*****

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It is generally accepted that carotenoids cannot be synthesized de novo by any animal. Nicola1,2) found β-carotene, echinenone, cryptoxanthin, zeaxanthin and astaxanthin and also separated small amounts of 3-hydroxy-4-keto-β-carotene with a single peak absorption maximum at 455 mμ, 3,3'-dihydroxy-4-keto-β-carotene with absorption maximum 460 mμ, and large amounts of astaxanthin in Asterina panceri and proposed two alternative sequential pathways for the metabolism of β-carotene to astaxanthin.

Thommen3) investigated the carotenoids in Daphnia and isolated β-carotene, echinenone and canthaxanthin but no astaxanthin, and showed that those animal oxidized β-carotene to canthaxanthin through the step of echinenone.

Krinsky4) found canthaxanthin and echinenone in Artemia salina and postulated the same metabolic pathway.

Bodea et al.5) investigated the pigments of the marine Copepoda Arctodiaptomus salinus (Dady) and found astaxanthin, β-carotene, hydroxy echinenone, a new xanthophyll, crustaxanthin (3,4,3',4'-tetrahydroxy-β-carotene) and three unidentified pigments.

They observed a biochemical correlation between astaxanthin and their minor carotenoids, with hydroxyechinenone and crustaxanthin representing intermediate products with different status of oxidation from β-carotene to astaxanthin.

Lee6,7) studied the pigments of three color variants, red, green, and brown, of the marine isopod Idothea montereyensis and isolated β-carotene, echinenone, canthaxanthin, 4-hydroxy-4'-keto-β-carotene, lutein, and lutein ester, but no astaxanthin. A sequence was thus suggested of β-carotene—echinenone—4-hydroxy-4'-keto-β-carotene—canthaxanthin, another isopod species, Idothea granulosa yielded β-carotene, isocryptoxanthin.


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thin, echinenone, 4-hydroxy-4'-keto-β-carotene, canthaxanthin, isozeaxanthin, and lutein but no astaxanthin. He therefore proposed a metabolic pathway for the conversion of β-carotene to canthaxanthin in this animal as follows: β-carotene→isocryptoxanthin→echinenone→4-hydroxy-4'-keto-β-carotene→canthaxanthin.

Gilchrist and Lee8) investigated the carotenoid metabolism in Carcinus maenas, and isolated β-carotene, δ-carotene, echinenone, isocryptoxanthin, canthaxanthin, lutein, zeaxanthin, lutein-5,8-epoxide, astaxanthin and 4-hydroxy-4'-keto-β-carotene. They also found β-carotene in greatest abundance in the hepatopancreas and in small amounts of the total carotenoid in the epidermis. In contrast, the hepatopancreas contained only small amounts of astaxanthin and traces of the intermediates although these were the dominant pigments in the epidermis. They pointed out that Carcinus maenas is metabolizing β-carotene from its food and transforming it, via a series of intermediates, to astaxanthin. A metabolic pathway was proposed as follows:

β-carotene→isocryptoxanthin→echinenone→4-hydroxy-4'-keto-β-carotene→canthaxanthin→astaxanthin

Chichester9) found that in California Artemia the two step conversion of β-carotene into canthaxanthin was the apparent pathway as follows:

β-carotene

| isocryptoxanthin

| echinenone

| isozeaxanthin

| 4-hydroxy-4'-keto-β-carotene

In the previous report, a new ketocarotenoid was isolated from gold fish (Carassius auratus) which was 3-hydroxy-3',4'-diketo-α-carotene. 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 by measuring infrared spectrum, mass spectrometry, nuclear magnetic resonance spectrometry and absorption maximum of the reduced product with sodium borohydride9).

The carotenoids, β-carotene, lutein, α-doradecin (3-hydroxy-3'4'-diketo-α-carotene) β-doradecin (3-hydroxy-3',4'-diketo-β-carotene), and astacin were isolated from the extracts of Benibuna. It is suggested that astaxanthin ester is synthesized from lutein ester via α- and β-doradexanthin ester by the oxidation of the β-ionone ring, and the isomerization of the α-ionone ring.

Methods

All animals utilized for this investigation were purchased from a local fish hatchery,
sacrificed and their carotenoids were extracted with acetone in a Waring blender. Acetone was redistilled before use, petroleum ether was passed over activated silica. The solid matter was separated by filtration until no further pigment could be obtained. The separate acetone solutions were then combined and the carotenoids were transferred to petroleum ether from acetone by the addition of water, the petroleum ether solution was repeatedly washed until free of acetone, dried over anhydrous sodium sulfate and concentrated under reduced pressure. The absorption spectra of the extracted carotenoids in petroleum ether is shown in Fig. 1.

The carotenoids thus extracted were run on silica-gel thin-layer chromatograms and exhibited $R_f$ values of 0.96, 0.98, indicating that carotenoids existed in the form of esters or carotenes.

The carotenoids were initially separated on a column of aluminum oxide (grade 2,
WAKO PURE CHEMICALS Co. Ltd., for chromatographic use) by using 0.5%-4.5% acetone in petroleum ether in order to remove oil from the carotenoids. Three resulting bands were eluted with acetone (Fig. 2) and retransferred to petroleum ether. The petroleum ether solution of each band was repeatedly washed with water to remove traces of acetone and evaporated to dryness under high vacuum.

Fr. I was rechromatographed on magnesium oxide: Hyflo-supercel (1:2) by using petroleum ether as developing solvent. (Fig. 3) Two zones, Fr. I-A, Fr. I-B were obtained.

Fr. I-A was only oil and Fr. I-B was identified as \( \beta \)-carotene: The absorption spectrum was identical to that of pure \( \beta \)-carotene. Fr. II+III was separated by chromatography on a column of Microcel-C (JOHN MANVILLE).

The pigment solution was placed on the column in petroleum ether and developed with 2.2% acetone in petroleum ether as developing solvent. The four resulting bands were eluted with acetone (Fig. 4) and retransferred to petroleum ether. The petroleum ether solution of each band was repeatedly washed with water to remove traces of acetone and evaporated to dryness under high vacuum.

The carotenoids of each band were saponified separately by dissolving it in 100 ml of absolute ethyl alcohol, adding 10 cc of 60% (w/v) aqueous potassium hydroxide and leaving it overnight under nitrogen at room temperature. The saponified pigments were transferred to petroleum ether with water, dried with anhydrous sodium sulfate, and chromatographed as follows.

**Band 1.** The carotenoids were rechromatographed on magnesium oxide: hyflo-supercel (1:2) using 25% acetone in petroleum ether as developing solvent and separated into two bands.

Band 1-A was identified as lutein: The absorption spectrum of the lower band was identical with that of lutein.

Band 1-B was identified as zeaxanthin: The absorption spectrum of the upper band was identical with that of zeaxanthin. The amount of zeaxanthin was very little.

**Band 2.** This carotenoid was rechromatographed on a magnesium oxide: hyflo-supercel (1:2) column, using 25% 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 with water. The pigment thus obtained was rechromatographed on a Microcel-C column, using 10% acetone in petroleum ether. The band was cut from the column and eluted, washed with water, and concentrated under vacuum. The purified pigment was then rechromatographed on a dired, powdered sugar column using 2% acetone in petroleum ether as a developing solvent.

The isolated pigment in petroleum ether exhibited the same absorption maximum of a new keto carotenoid, α-doradecin which was reported, the structure is 3-hydroxy-3',4'-diketo-α-carotene\(^9\)) (Fig. 5 and 6).

**Band 3.** The saponified pigment was rechromatographed on a magnesium oxide column (MgO: hyflosupercel=1:2), using 30% acetone in petroleum ether as developing solvent, and on Microcel-C column, using 10% acetone in petroleum ether as developing solvent. The pigment of the main zone showed \(\lambda_{\text{max}} 461 \text{ m}\mu\) in petroleum ether, 465 m\(\mu\) in ethyl alcohol, after reduction, \(\lambda_{\text{max}} 428, 451, 479 \text{ m}\mu\). These values were identical with that for hydroxy steroidalene\(^2\)) and dehydroxy adonixanthin\(^11\)). The amount of this pigment was little and would thus be 3-hydroxy-3',4'-diketo-β-carotene\(^9\)).

**Band 4.** Astacin: Before saponification this pigment was purified repeatedly on a Microcel-C column by 2% acetone in petroleum ether. In petroleum ether this pigment exhibited an absorption maximum at 468 m\(\mu\), after reduction \(\lambda_{\text{max}} 425, 451, 471 \text{ m}\mu\) (Fig. 7). The pigment was transferred to ethyl ether, after saponification the solution was acidified by the addition of glacial acetic acid and washed with water to remove acetic-
acid. The pigments were rechromatographed on a dried, powdered sugar column. The zone was eluted with acetone and transferred to petroleum ether. \( \lambda \) max 472 m\( \mu \) in petroleum ether. Those results were identical with that of astaxanthin and astacin which were found in gold fish.9)

**Results and Discussion**

The existences of \( \beta \)-carotene, lutein, zeaxanthin, \( \alpha \)-doradecin, (3-hydroxy-3',4'-diketo-\( \alpha \)-carotene), \( \beta \)-doradecin (3-hydroxy-3',4'-diketo-\( \beta \)-carotene) and astacin were confirmed in Benibuna. All of these carotenoids exist in the form of esters except \( \beta \)-carotene.

In the previous report, it was suggested that two keto carotenoids might be involved in the metabolic pathway from plant carotenoids, lutein ester, to fish carotenoid astaxanthin ester in gold fish. In Benibuna, the results were identical to those of gold fish as schematized in Fig. 8.

**Summary**

1. The presence of \( \beta \)-carotene, zeaxanthin, lutein, \( \alpha \)-doradecin, \( \beta \)-doradecin and astacin was confirmed in Benibuna and all of those carotenoids except \( \beta \)-carotene exist in the form of esters.

2. A possible biosynthetic pathway from lutein ester to astaxanthin ester is proposed in Benibuna and it is identical with that of gold fish.

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References