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9. ABSTRACT

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The Biosynthesis of Astaxanthin—XVIII[*1]
The Metabolism of the Carotenoids in the Prawn, *Penaeus japonicus Bate*

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The biosynthesis of astaxanthin in the prawn was further studied using pure carotenoids and preparations obtained from natural sources. Zeaxanthin, obtained from the Chinese lantern, was fed to the prawn, *Penaeus japonicus Bate*. Astaxanthin was biosynthesized from zeaxanthin, thus indicating the existence of a second pathway to astaxanthin in the prawn. Canthaxanthin and astaxanthin from crab waste were respectively metabolized to and absorbed as body astaxanthin in the prawn. Pigmented preparations from corn gluten, alfalfa and *Spirulina* (blue-green alga) were found to increase the body astaxanthin to various degrees.

The pigments of three variants of the marine isopod *Idotea montereyensis* and *I. granulosa* (*Rathke*) were investigated by LEE[1,2] who isolated β-carotene, echinenone, canthaxanthin, 4-hydroxy-4′-keto-β-carotene, lutein, and lutein ester. The metabolic pathway from β-carotene to canthaxanthin was thus suggested as follows:

β-carotene→echinenone→4-hydroxy-4′-β-carotene→canthaxanthin.

GILCHRIST and LEE[3] isolated β-carotene, δ-carotene, echinenone, isocryptoxanthin, canthaxanthin, lutein, zeaxanthin, lutein-5, 8-epoxide, astaxanthin, and 4-hydroxy-4′-keto-β-carotene in *Carcinus maeans*, a crustacea, Decapoda. A metabolic pathway in this animal was proposed as follows:

β-carotene→isocryptoxanthin→echinenone→canthaxanthin→astaxanthin.

DAVIES *et al.*[4] found that California strains of *Artemia salina* converted β-carotene to echinenone and echinenone into canthaxanthin without any apparent intermediates.

HERRING[5,6] studied the carotenoids in *Daphnia magna* fed with *Chlorella pyrenoidosa* and isolted β-carotene, echinenone, canthaxanthin, a keto-carotenoid (probably 3-hydroxy-4-keto-β-carotene), and astaxanthin. He suggested that the animals were able to form echinenone, canthaxanthin, and astxanthin from β-carotene. Alloxanthin has been isolated from the commensal crab, *Pinnotheres pisum* during the study of carotenoids metabolism by CAMBELL[7] and the sand crab, *Emerita analoga* during the study of possible role of caro-

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*1 The previous papers—XVI, XVII. *Comp. Biochem. Physiol.*, in the press.

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tenoids in the reproduction by GILCHRIST and LEE[8], and the origin of this pigment can be traced to the algae consumed by these crabs. Similarly, a common algal pigment, fucoxanthin, was isolated from the barnacles, *Lepas fascicularis* and *L. anacles* and from their faecal pellets (HERRING[9]) and peridinin, a characteristic dinoflagellate carotenoid, has been isolated from *L. fascicularis* (FOX and CROZIER[10]).

The carotenoids in the lobster, *Panulirus japonicus* (KATAYAMA *et al.*[11]), the prawn, *Penaeus japonicus* (KATAYAMA *et al.*[12,13]), and the crab, *Portunus trituberculatus* (KATAYAMA *et al.*)[14] were identified. In these crustacea ingested β-carotene-15, 15′-$^3H_2$ was converted into astaxanthin through isocryptoxanthin, echinenone, canthaxanthin and 3-hydroxy-canthaxanthin respectively (KATAYAMA *et al.*[13-16]).

The present investigation was undertaken to determine the effect on pigmentation of prawn by feeding various carotenoid preparations. These included ^{14}C-labelled β-carotene, synthetic canthaxanthin, zeaxanthin obtained from Chinese lantern, astaxanthin from crab waste, alfalfa, corn gluten and *Spirulina* (a blue-green alga).

Materials and Methods

Feeding tests with alfalfa, corn gluten, Spirulina and canthaxanthin Five aquarium tanks (60×30×30 cm) were used, as described in the previous papers (KATAYAMA *et al.*[10,12-15]). Sea sand was placed at the bottom of each tank at a depth of about 5 cm. The sea water (40 liters) was kept at 25°C, and aerated at a rate of 400 m*l* per minute.

Thirty-five prawn were obtained from a local fish hatchery and were placed in each tank and adapted for five days to a standard artificial diet (KATAYAMA *et al.*[13,16]). Treatments consisted of: the artificial diet (control, tank No. 1), the artificial diet containing 10% alfalfa (tank No. 2), 10% corn gluten (tank No. 3), 10% Spirulina (tank No. 4), and 3% canthaxanthin (tank No. 5).

After 21 days of feeding, the prawn were sacrificed and the pigments were extracted and purified on the columns by using the same methods as already reported.

Incorporation of ^{14}C-labelled β-carotene into prawn carotenoids

1. Preparation of ^{14}C-labelled β-carotene *Phycomyces blakesleeanus* was grown aerobically in 1 liter Erlenmeyer flasks. Each flask contained 300 m*l* of a liquid growth medium which CHICHESTER *et al.*[17] used to incorporate labelled leucine into carotene by *Phycomyces*, to which the spore suspensions were added. Mevalonic acid-2-^{14}C was added at the rate of 0.2 mc/*l*. The cultures were inoculated on a shaker for one week at room temperature. The mycelial mats were harvested at the point where the mycelium was beginning to turn yellow. The mats were disrupted in acetone in a Waring blender. The homogenate filtered and reblended until the filtrate was colorless. The pigments were transferred to petroleum ether and purified on a magnesium oxide Hyflo-Supercel (1:2 W/W) column. The β-carotene fraction was repurified on an alumina column. The β-

carotene was further purified by thin-layer chromatography. The β-carotene was eluted from the TLC plate and crystallized from petroleum ether and ethanol and dried in a vacuum desiccator.

2. Radioactive β-carotene feeding experiment Radioactive β-carotene was dissolved in soybean oil and added to the artificial diet (KATAYAMA *et al.*[12]). Thirty prawn were cultured for five days on the artificial standard diet and then fed daily 10 g of the artificial diet containing radioactive β-carotene for 21 days. The pigments were extracted and purified as described above.

The purified samples were added to counting vials, dried, dissolved in toluene (5 m*l*) and bleached with U. V. light. The vials were left in the dark over night to allow the U. V.-induced phosphorescence to decay and were assayed after five m*l* of double-strength scintillation solution [0.1 g POPOP (1, 4-bis-2 (5-phenyloxazolyl) -benzene and 1.2 g PPO (2, 5-diphenyloxazolle) in 100 m*l* of toluene] was added and the resultant solution was assayed for ^{14}C activity in a Beckman Scintillation Spectrometer.

Incorporation of zeaxanthin into astaxanthin in prawn Prawn (ca. 7 cm length) were obtained from a local fish hatchery and were separated into two groups. One group was fed the standard artificial diet containing pure zeaxanthin obtained from the Chinese lantern (20 mg/g feed) and the other group on the standard artificial diet for 21 days. A third group was fed β-carotene for comparison. The prawn were collected, extracted with acetone, and the pigments purified as described above. The pigments were identified mainly by their spectral absorption, their behavior on the column, and thin-layer chromatography in comparison with known carotenoids.

. **Carotenoid pigments of the prawn fed astaxanthin obtained from crap waste** Prawn were separated into two groups. One group was fed the standard diet containing 20 mg% of astaxanthin extracted from crab waste; the other group was fed the standard diet as control for 21 days. The pigments were extracted and purified on the columns separately from each group. They were identified by their behavior on the columns, their spectral characteristics and silica-gel thin layer chromatography in comparison with known carotenoids.

Rsults and Disussions

Table 1 lists the result of feeding prawn on an artificial diet supplemented with 10% corn gluten, alfalfa and Spirulina and 3% canthaxanthin. Spirulina, a blue-green alga rich in β-carotene[18] and synthetic canthaxanthin were found to yield the greater concentration of astaxanthin than either corn gluten or alfalfa. These prawn fed *Spirulina* and canthaxanthin were also visibly redder than the other prawn.

Table 2 records the results of feeding prawn ^{14}C-β-carotene. Radioactive carbon from β-carotene can be converted to astaxanthin by the prawn. These results are in accord

Table 1. The contents of astacin in the prawn which were fed corn-gluten, alfalfa, canthaxanthin and *Spirulina*

Diet / Pigment	Artificial diet (control)	Artificial diet containing 10% Corn-gluten	10% Alfalfa	3 mg% Canthaxanthin	10% Spirulina
Conc. of astacin (μg/g body wt.)	3.4	5.1	5.5	10.4	18.4

Table 2. Conversion of ^{14}C-β-carotene into astaxanthin in the prawn.

	Specific activity (cpm/mg)	Total count
β-Carotene in the food	820000	1230000
Astaxanthin	16000	2988

Table 3. Incorporation of β-carotene and zeaxanthin into astaxanthin in the prawn.

Pigment fed 20 mg/g	Astaxanthin isolated (μg/g body wt.)
None	3.3
β-Carotene	21.0
Zeaxanthin	55.0

Table 4. Transformation of the astaxanthin in the diet, obtained from the crab waste into body astaxanthin in the prawn

	Amounts of astaxanthin (μg/g body wt.)
None (control)	1.0
Astaxanthin obtained from the crab waste 20 mg% in the diet	1.8

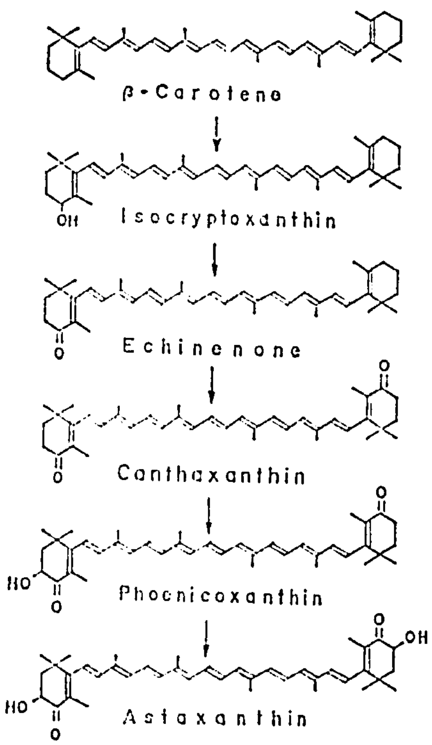

Fig. 1

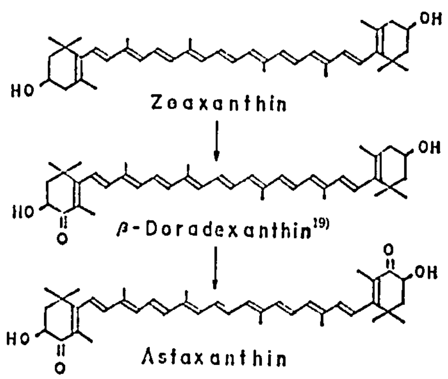

Fig. 2

Fig. 1. The metabolic pathway from β-carotene to astaxanthin in prawn, crab, and lobster.

Fig. 2. The metabolic pathway from zeaxanthin to astaxanthin in prawn.

with the feedings that *Spirulina* preparations rich in β-carotene and β-carotene labelled with ^{3}H (KATAYAMA *et al.*[12]) are readily incorporated into astaxanthin.

Table 3 shows that zeaxanthin is somewhat more effective as a pigment source than β-carotene. Figure 1 shows a diagram that has been proposed (KATAYAMA *et al.*[12,14,15]) for the conversion of β-carotene to astaxanthin. It is apparent that zeaxanthin most represents a second pathway to astaxanthin with β-doradexanthin as an intermediate (Fig. 2).

It can also be seen from Table 4 that crab waste astaxanthin can be utilized directly to body astaxanthin in the prawn.

In the previous papers[10,12-16], the astaxanthin in prawn, crab and lobster was labelled when they were fed the artificial diet containing $^{3}H_2$-β-carotene, and it was proposed that β-carotene was converted to astaxanthin through the steps of isocryptoxanthin, echinenone, canthaxanthin and 3-hydroxy-canthaxanthin. Similar results were obtained by feeding ^{14}C-β-carotene to prawn.

Recently the existence of β-doradexanthin[19] was reported by MATSUNO *et al.*[20] and Simposon[21] in red crab, and these authors proposed that zeaxanthin might be converted to astaxanthin via β-doradexanthin. It was confirmed that in prawn there is another metabolic pathway from zeaxanthin to astaxanthin by feeding the standard artificial diet containing 20 mg/g of pure zeaxanthin obtained from the Chinese lantern.

Consumer acceptance of prawn on the Japanese market is largely based on color. From a practical point of view this color must be supplied in the diet either as astaxanthin or as a carotenoid that can be converted to astaxanthin by the prawn. The results reported in this paper show that pure pigments such as β-carotene, zeaxanthin and canthaxanthin can be converted to astaxanthin. In actual practice it will probably be necessary to utilize pigments from microbiological sources such as *Spirulina* where the prawn can convert various pigments to astaxanthin. Preformed astaxanthin can also be derived from waste materials such as crab waste, and be deposited without change.

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