The Biosynthesis of Astaxanthin—XV*

The Carotenoids in Chidai, red sea bream, *Evynnis japonica* Tanaka and [the incorporation of labelled astaxanthin from the diet of the red sea bream] to their body astaxanthin

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1. The carotenoids in the red sea bream, Chidai, *Evynnis japonica* Tanaka, have been separated by absorption chromatography, characterized by their absorption spectra, their behavior on the columns, specific chemical tests, and co-chromatography with authentic samples.

2. Canthaxanthin, lutein, astaxanthin and P_E-518 were isolated from the internal organs of the red sea bream. α -Cryptoxanthin, 3,3'-dihydroxy- ϵ -carotene, zeaxanthin, lutein (trace), 3-hydroxy-canthaxanthin and astaxanthin were found in the integuments of the red sea bream.

3. The red sea bream were cultured in the laboratory and for three weeks fed radioactive β -carotene-15,15'-³H₂ and labelled astaxanthin. It was determined that β -carotene was not a major precursor of astaxanthin in the red sea bream, but labelled xanthophylls in the prawn (the contents of astaxanthin were 78% of the total pigments) were absorbed from the diet and deposited in the body of the red sea bream.

It has been shown that the internal organs of the sea bream, Madai, *Chrysophrys major* TEMMINCK and SCHLEGEL, contain β -carotene, echinenone, tunaxanthin¹⁾ (3,3'-dihydroxy- ϵ -carotene)²⁾, lutein, zeaxanthin, and astaxanthin³⁻⁶⁾. α -Carotene, 3,3'-dihydroxy- ϵ -carotene, lutein, zeaxanthin, α -doradexanthin ester⁷⁾ (trace), and astaxanthin were found in the integument of the sea bream³⁻⁶⁾. It was also clarified that in the sea bream, β -carotene was not a major precursor of astaxanthin by feeding β -carotene-15,15'-³H₂, but they could transfer labelled astaxanthin in the food to their body astaxanthin by feeding astaxanthin-15,15'-³H₂⁸⁾. On the other hand, CROZIER⁹⁾ stated that an increase in the proportion of astaxanthin ester of the skin in the Californian sheephead, *Pimelotropon punctum*, was always accompanied by a decrease in the 2,3'-dihydroxy- ϵ -carotene ester and suggested that the precursor of astaxanthin would be 3,3'-dihydroxy- ϵ -carotene ester.

The present investigation was undertaken to isolate the carotenoids in the red sea

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bream, Chidai, *Eyynniss japonica* TANAKA and to delineate the metabolic pathway in this animal from plant carotenoids to astaxanthin. In the internal organs, the existence of canthaxanthin, letein, astaxanthin, and unknown pigment, P_E-518 was confirmed. In the integuments, α -cryptoxanthin, 3,3'-dihydroxy- ϵ -carotene, lutein (trace), zeaxanthin, 3-hydroxycanthaxanthin, and astaxanthin were found. It was also confirmed that in the red sea bream, β -carotene was not a major precursor of astaxanthin, but they could transfer labelled astaxanthin in their food to their body astaxanthin.

Materials and Methods

Fresh red sea bream (about 15 cm in length) were purchased at a local fish hatchery.

Separation and identification of the carotenoids in the red sea bream

1. Purification and identification of the carotenoids in the internal organs of the red sea bream: The internal organs of the red sea bream were collected and extracted repeatedly with acetone until no further pigments were obtained. The acetone solutions were combined, transferred to petroleum ether by the addition of water and washed repeatedly with water to remove traces of acetone. The petroleum ether solution of the pigments was dried over anhydrous sodium sulfate, and concentrated under vacuum to an oil. The pigments were dissolved in about 10 ml of petroleum ether for chromatography on a Microcel-C column. The column was developed with petroleum ether as the developing solvent. Three bands were obtained: Fr-I (lower band, orange), Fr-II (middle band, red), and Fr-III (upper band, dark green).

P₃₉₂: The pigment of Fr-I (lower band) was saponified by dissolving it in 100 ml of absolute ethanol, adding 10 ml of 60% (W/V) aqueous potassium hydroxide and leaving it overnight under nitrogen at room temperature^{7,10}. The saponified pigments were transferred to petroleum ether with water, dried over anhydrous sodium sulfate. The saponified pigments were chromatographed on an alumina column, using 1% acetone in petroleum ether. Only one band was obtained. The purified pigment exhibited absorption maximum at 392 nm in petroleum ether. The structure of this pigment is presently being investigated.

The pigments of Fr-II (middle band, red) were saponified, using the above-mentioned method. The saponified pigments were chromatographed on a Microcel-C column, using 10% acetone in petroleum ether. Three bands were obtained: Fr-II-a (lower band, orange), Fr-II-b (middle band, orange), and Fr-II-c (upper band, red).

Canthaxanthin: The pigment of Fr-II-a (lower band) was repurified on a magnesium oxide column (magnesium oxide: Hyflo-Super-Cel=1:2), using 12% acetone in petroleum ether as the developing solvent. The purified pigment exhibited absorption maxima at 466 nm in petroleum ether and 428, 451, 478 nm after reduction with sodium borohydride in ethanol. The pigment was co-chromatographed on a magnesium oxide column

with pure canthaxanthin obtained from the prawn, *Penaeus japonicus* Bate¹¹⁾, and formed a single zone. These results show this pigment to be canthaxanthin.

Lutein: The pigment of Fr-II-b (middle band) was rechromatographed on a magnesium oxide column (magnesium oxide: Hyflo-Super-Cel=1:2) by using 25% acetone in petroleum ether as the developing solvent. Only one band was obtained. The absorption spectrum showed maxima at 423, 446, 476 nm. The pigment was co-chromatographed on a magnesium oxide column with pure lutein obtained from the alga, *Stigeoclosum* sp.¹²⁾, and formed a single zone. These results show this pigment to be lutein.

Astacin: The pigment of Fr-II-c (upper band) was saponified by using the same method as already mentioned and rechromatographed on a Microcel-C column, using 10% acetone in petroleum ether as the developing solvent. The pigment was repurified on a sugar column by using 0.5% acetone in petroleum ether as the developing solvent. The purified pigment had a maximum at 473 nm in petroleum ether and, after reduction with sodium borohydride in ethanol, the absorption spectrum had a maxima at 427, 451, 477 nm. These characteristics were all identical with those of pure astacin obtained from the lobster, *Panulirus japonicus*¹³⁾.

P_E-518: The pigment of Fr-III (upper band, dark green) was purified on a sugar column, using 1.5% acetone in petroleum ether as the developing solvent. The pigment thus purified had maxima at 488, 518, 552 nm in petroleum ether. After reduction with sodium borohydride in ethanol, the absorption maxima at 435, 461, 490 nm were obtained. Spectral characteristics of P_E-518 obtained from the red sea bream and P₅₁₈ isolated from *Rhodospseudomonas spheroides* by JENSEN¹⁴⁾ are shown in Table 1. The structure of this pigment is under examination.

Table 1. The absorption maxima of P_E-518 and P-518⁹⁾ in visible light recorded in various solvents.

Solvents	P-518 λ_{max} (nm)			P _E -518 λ_{max} (nm)		
Petroleum ether	487.5,	518,	555	487,	518,	550
Ethanol		522			542	
Acetone	495,	528,	559		528	
Ethyl ether					517	

2. Purification and identification of the carotenoids in the integuments of the red sea bream: Dorsal and ventral sections of the integument were collected and extracted repeatedly with acetone in a Waring blender until no further pigments were obtained. The separate acetone solutions of the pigments were combined and transferred to petroleum ether by the addition of water. The petroleum ether solution of the pigments was repeatedly washed with water to remove the trace of acetone, concentrated under vacuum and dried over anhydrous sodium sulfate. The pigments were first chromatographed

on a Microcel-C column, using petroleum ether as the developing solvent. Two bands were obtained: Fr-I (lower band, yellow) and Fr-II (upper band, red).

α -Cryptoxanthin: The pigments of Fr-I (lower band) were saponified by using the same method already mentioned. The saponified pigments were transferred to petroleum ether with water, dried over anhydrous sodium sulfate. The pigments thus obtained were rechromatographed on a magnesium oxide column using 10% acetone in petroleum ether as the developing solvent. Two bands were obtained: Fr-I-A (lower band, orange) and Fr-I-B (upper band, orange). The pigment of Fr-I-A (lower band) was repurified on a magnesium oxide column, using 4% acetone in petroleum ether as the developing solvent. The purified pigment exhibited absorption maxima at 420, 445, 473 nm in petroleum ether. The absorption spectra and the behavior on the column were all in agreement with those exhibited by pure α -cryptoxanthin¹³⁾.

The pigment of Fr-I-B (upper band) was repurified on a magnesium oxide column, using 14% acetone in petroleum ether as the developing solvent. Two bands were obtained: Fr-I-b-I (lower band, yellow) and Fr-I-B-2 (upper band, orange).

3,3'-Dihydroxy- ϵ -carotene: The pigment of Fr-I-B-1 (lower band) was rechromatographed on a magnesium oxide column, using 10% acetone in petroleum ether as the developing solvent. Only one band was obtained. The pigment thus purified showed absorption maxima at 415, 438, 468 nm in petroleum ether. The pigment was co-chromatographed on a magnesium oxide column with pure 3,3'-dihydroxy- ϵ -carotene obtained from the sea bream, *Chrysophrys major* TEMMINCK and SCHLEGEL⁶⁾, and formed a single zone.

Lutein and zeaxanthin: The pigments of Fr-I-B-2 (upper band) were repurified on a magnesium oxide column, using 25% acetone in petroleum ether as the developing solvent. Two bands were obtained: Fr-I-B-2-a (lower band, orange) and Fr-I-B-2-b (upper band, orange). The pigment of Fr-I-B-2-a (lower band) exhibited the absorption maxima at 420, 446, 475 nm in petroleum ether. The absorption spectra and the behavior on the column were all identical with pure lutein obtained from the red carp, *Cyprinus carpio* LINNE¹⁵⁾. Only trace amounts of lutein were obtained.

The pigment of Fr-I-B-2-b (upper band) showed the absorption maxima at 426, 450, 477 nm in petroleum ether and was co-chromatographed on a magnesium oxide column with pure zeaxanthin obtained from the red carp, *Cyprinus carpio* LINNE¹⁵⁾.

This pigments of Fr-II were saponified by using the same method as previously mentioned. The saponified pigments were rechromatographed on a Microcel-C column, using 12% acetone in petroleum ether as the developing solvent. Two bands were obtained: Fr-II-a (orange, lower band) and Fr-II-b (pink, upper band).

Phoeniconone¹⁶⁾ (3-keto-canthaxanthin): The pigment of Fr-II-a (lower band) was rechromatographed on a sugar column by using 0.5% acetone in petroleum ether as the

developing solvent. Only one band was obtained. The absorption spectrum indicated a single absorption maximum at 468 nm in petroleum ether. The pigment thus purified was co-chromatographed on a sugar column with pure pheniconone obtained from the prawn, *Penaeus japonicus* BATE¹⁷⁾ and formed a single zone. After reduction with sodium borohydride in ethanol, maxima at 425, 451, 487 nm were obtained. These results were consistent with the pigment of Fr-II-a being pheniconone.

Astacin: The pigment of Fr-II-b (upper band) were repurified on a sugar column, using 1.0% acetone in petroleum ether as the developing solvent. Only one band was obtained. The purified pigment showed a single absorption maximum at 474 nm in petroleum ether. This pigment was co-chromatographed on a sugar column with pure astacin obtained from the gold fish, *Carassius auratus*⁷⁾, and formed a single zone. After reduction with sodium borohydride in ethanol, maxima at 426, 451, 478 nm were obtained. These results show that the pigment of Fr-II-b was astacin.

Incorporation of radioactivity from β -carotene-15,15'- $^3\text{H}_2$ into astaxanthin and the transformation of labelled astaxanthin from the diet into body astaxanthin

1. Incorporation of β -carotene-15,15'- $^3\text{H}_2$ into astaxanthin in the red sea bream: 2.7 mg of β -carotene-15,15'- $^3\text{H}_2$ (specific activity: 160 uCi/mg) were dissolved in 26.0 g of unpurified soy bean oil and mixed with 300 g of the modified artificial standard diet previously reported^{8,15,17,18)}. The diet containing β -carotene-15,15'- $^3\text{H}_2$ was stuffed into vinyliden chloride film, steamed for 30 minutes and kept in a refrigerator until use.

Each of five red sea bream was cultured in a tank 80×120 cm, covered with 10 cm of sea sand. The red sea bream were fed 6 g of the modified, artificial diet containing β -carotene-15,15'- $^3\text{H}_2$ every day for three weeks. The carotenoids in the red sea bream were extracted and the astaxanthin fraction was separated on a Microcel-C column. This fraction was saponified and purified on the powdered sugar column. The astacin thus purified was added to a counting vial and assayed for ^3H activity, using methods as previously reported^{17,19,20)}. The following results were obtained (Table 2). These results show that β -carotene is not a precursor of astaxanthin in the red sea bream.

Table 2. Incorporation of β -carotene-15,15'- $^3\text{H}_2$ into astaxanthin in the red sea bream, *Evynnis japonica* Tanaka.

Pigments in the red sea bream	Radioactivity cpm/mg pigment
Astacin in the integuments of the red sea bream	520

2. Preparation of labelled carotenoids and transformation of labelled carotenoids into the red sea bream: It was previously shown¹⁷⁾ that when the prawn was fed β -carotene-15,15'- $^3\text{H}_2$, labelled β -carotene as well as echinenone, canthaxanthin, pheniconone and astacin were isolated.

In the present experiment 200 prawn were cultured in the laboratory and fed the standard diet¹⁷⁾. Beta-carotene-15-15'-³H₂ was added to the diet and the prawn were fed this diet for three weeks. These prawn were in turn fed to the red sea bream every day for three weeks. The carotenoids from the latter animals were extracted and purified. Pure astacin was obtained, bleached and assayed for radioactivity as reported earlier^{17,19,20)}. The results (Table 3) show that the red sea bream can absorb the carotenoids from the prawn (astaxanthin is the main carotenoid, its contents are 78% of the total pigments¹¹⁾) and deposit labelled astaxanthin from its dietary pigments.

Table 3. Transformation of labelled carotenoids in the diet to their body astaxanthin in the red sea bream, *Evynnis japonica* TANAKA.

Astacin	Radioactivity cpm/mg pigment
In the integuments of the red sea bream	79,000
Prawn that had been fed on β -carotene-15,15'- ³ H ₂ every day for three weeks	450,000

Results and Discussions

The carotenoid pigments of the internal organs in the red sea bream are listed in Table 4 in the order in which they were eluted from the columns. The relative amount of each pigment is given as a percentage of the total

Table 4. The spectral characteristics and relative abundances of the carotenoids in the internal organs of the red sea bream, *Evynnis japonica* TANAKA.

Pigment	Spectral characteristics					Relative abundances (%)
	λ_{max} (nm) in Petroleum ether		λ_{max} (nm) in ethanol after reduction		λ_{max} (nm) in chloroform	
Canthaxanthin	466		428, 451, 478		482	8
Lutein	423, 446, 476				428, 456, 487	2
Astacin	473		427, 451, 477			50
P _E -518	488, 518, 555		435, 461, 490			40

The carotenoids isolated from the integument are also listed in Table 5 in the order in which they were eluted from the columns. The relative amount of each pigment is given as a percentage of the total.

For three weeks the red sea bream were cultured in the laboratory and fed the modified artificial diet containing β -carotene-15,15'-³H₂ (group 1) and labelled carotenoids in the prawn (the contents of astaxanthin are 78% of the total pigments¹¹⁾) (group 2). It was shown that β -carotene was not a major precursor of astaxanthin in the red sea bream, but other labelled carotenoids in the prawn were transferred or transformed to body astaxanthin, though CROZIER⁹⁾ reported that 3,3'-dihydroxy- ϵ -carotene would be a pre-

Table 5. The spectral characteristics and relative abundances of the carotenoids in the integuments of the red sea bream, *Eyynnys japonica* TANAKA.

Pigments	Spectral characteristics			Relative abundances (%)
	λ_{max} (nm) in Petroleum ether	λ_{max} (nm) in ethanol after reduction	λ_{max} (nm) in Chloroform	
α -Cryptoxanthin	420, 445, 473		428, 453, 481	1
3,3'-Dihydroxy- ϵ -carotene	415, 438, 468		423, 448, 478	7
Lutein	420, 446, 475		428, 456, 487	trace
Zeaxanthin	426, 450, 477		429, 462, 494	1
3-Keto-canthaxanthin	468	425, 451, 487		1
Astacin	474	426, 451, 478		88

cursor of astaxanthin in the California sheephead, *Pimelometopon pulchrum*. The red sea bream belongs to SEA BREEM-FORM from the point of view of the "Biosynthesis of Astaxanthin"¹⁹⁾ in the aquatic animals.

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