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Comparative Biochemical Studies on Carotenoids in Aquatic animals*1

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Abstract

The distribution of carotenoids in aquatic animals, crustacean, fresh-water red fish, marine red fish, sea sponge and starfish were confirmed in order to elucidate their metabolic pathways to their main carotenoids.

During identification of these carotenoids, three new carotenoids were isolated and their structural formulae were proposed. Two were isolated from *Tedania digitata* and *Tethya amamensis*, and their structures were proposed to be 3-hydroxy-7, 8-didehydro-β, χ-carotene and 3, 4-didehydro-β, χ-carotene. The other new carotenoid, an astaxanthin-like pigment, was isolated from Onihitode, *Acanthaster planci*, and its structure was established to be 7, 8-didehydro-astaxanthin.

In these aquatic animals, the most abundant pigment is astaxanthin, which is the main substance of their coloration. Therefore, the present investigations were undertaken to pursue the biochemical pathways to astaxanthin through the distributions of carotenoids in tissues and feeding tests applying various kinds of carotenoids.

In these studies, it was found that aquatic animals could be classified into three types based on the biochemical oxidation pattern of carotenoids.

I. Prawn-type carotenoid oxidation: Most of crustacean belong to this type. They can oxidize carotenoids at the 3- and 3′-positions of β-ionone rings with oxo groups at the 4- and 4′-positions, and also at the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions. This implies that they can convert β-carotene, canthaxanthin and zeaxanthin to astaxanthin. Therefore, crustacean should be fed diets supplemented with β-carotene, canthaxanthin, or zeaxanthin for preventing the fading of their coloration.

II. Goldfish-type carotenoid oxidation: Most of fresh-water red fish in Japan belong to this type. They can oxidize carotenoids at the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions, but can not oxidize carotenoids at the 3- and 3′-positions of β-ionone rings with oxo groups at the 4- and 4′-positions. They can convert zeaxanthin and lutein to astaxanthin, but not canthaxanthin and β-carotene. Therefore, these fresh-water red fish should be fed diets mixed with either zeaxanthin or lutein for maintaining or restoring their bright red color.

III. Sea bream-type carotenoid oxidation: Most of marine red fish belong to this group. They cannot oxidize carotenoids at the 3- and 3′-positions of β-ionone rings with oxo groups at the 4- and 4′-positions, and also at the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions. They cannot convert β-carotene, canthaxanthin, or zeaxanthin to astaxanthin. They can only transfer zeaxanthin, lutein, canthaxanthin, and astaxanthin from feed to their own tissues. Therefore, astaxanthin should be supplemented to their diet for maintenance of color or improvement of faded color during culture.

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In porifera, sea sponges could be divided into two groups; one group is unable to oxidize carotenoids as exemplified by *Tethya amamensis*, but the other is capable of oxidation. In the latter group, *Haliclona permollis* can be included, and this sponge was presumed to have the ability to oxidize the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions.

One of the echinoderms, Onihitode, *Acanthaster planci*, appears to belong to organisms with Prawn-type carotenoid oxidation, because its distribution of carotenoids was similar to that of tigar prawn.

In the course of the studies described above, it was found that Micro-Cel C has a certain catalytic hydroxylation of β-carotene and can produce isocryptoxanthin with slight amounts of isozeaxanthin and echinenone. This forewarns investigators engaging in the study of carotenoid chemistry.

Contents

I. Introduction

The beautiful yellow, orange and red pigments found in the skin, shell or exoskeleton of aquatic animals have always attracted biochemists because of their diversity of colors as compared with those present in terrestrial animals. It has been commonly considered that aquatic animals, like all other animals, do not possess any ability to synthesize carotenoids from mevalonic acid, but they can alter alimentary carotenoids by oxidation or deposit them

in their tissues without modification. In common with other animals, these organisms must be provided with carotenoids in their feed, during their cultivation from juveniles for keeping their color in natural.

In Japan many kinds of aquatic animals are being cultured and their annual production amount to more than one hundred thousand tones: these species are rainbow trout, *Salmo gairdnerii irideus* (GIBBON), red sea bream, *Pagrus major* TEMMINCK and SCHLEGEL, crimson sea bream, *Evynnis japonica* TANAKA, tiger prawn, *Penaeus japonicus* BATE, spiny lobster, *Panulirus japonicus*, goldfish, *Carassius auratus*, carp, *Cyprinus carpio* LINNE, fancy red carp, *Cyprinus carpio* LINNE *v.*

The Japanese have been traditionally used red sea bream, tiger prawn and spiny lobster for such ceremonies as a wedding ceremony, a new year ceremony and so on. If their proper reddish colors are faded, their values must be almost lost. The Japanese are a folk of eating much and many fish and discriminate their freshness by their colors. Therefore, the colors of fish can not be neglected. Those aquatic animals only with high market prices are now being cultured on the basis of economical balance. If their bright red colors are lost, their prices will be seriously discounted and these discounts can not support any more these fish cultures as enterprises. By accumulating the knowledges of aqui-culture through the present cultural management, those necessary technology can be attained for increasing production of marine foods really needed by human being. The present studies are, thus, very important to maintain the knowledge sources of aqui-culture for developing these cultures toward the real object.

The present studies concern the sense of the Japanese to color of aquatic organisms which can not be neglected in situ. The demand is now being concentrated to the maintenance of their proper color through cultivations. Since these aquatic animals are devoid of the capability of synthesizing these bright red color pigments, the necessary pigments must be supplied in their diets.

In these aquatic animals, the most abundant pigment is astaxanthin which is the main causal substance of their coloration.

For this reason, the present investigations were undertaken for pursuing the biochemical pathway of carotenoids through their distribution of carotenoids in tissues and the feeding tests applying various kinds of carotenoids or those in natural resources.

Through these studies, the present author found the facts that there are three types of biochemical oxidation of carotenoids:

i) Prawn-Type carotenoid oxidation
ii) Goldfish-Type carotenoid oxidation
iii) Sea bream-Type carotenoid oxidation

From these metabolic pathways of carotenoids, the maintenance of coloration of crustacean such as tiger prawn and spiny lobster was concluded to be realized by supplying β-carotene, canthaxanthin, zeaxanthin or astaxanthin through their diets. The maintenance of coloration of fresh-water red fish should be attained by supplying zeaxanthin, lutein or astaxanthin with their diets. However, the maintenance of coloration of marine red fish was clarified to be achieved only by supplying astaxanthin through their diets.

On the way of studying the biotic alteration of relating carotenoids toward astaxanthin in these important aquatic animals, it became interesting to elucidate the metabolic route of the carotenoids in such members of the aquatic animals as starfish or sea sponges in which the existence of astaxanthin had been reported.

The present author isolated astaxanthin which had been considered as a main carotenoid in Onihitode, *Acanthaster planci,* but in the reality, this carotenoid was a new carotenoid, 7, 8-didehydroastaxanthin, and proposed the metabolic pathway toward this carotenoid from the other carotenoids.

The present author also isolated two kinds of new carotenoids from sea sponges, tedaniaxanthin from *Tedania digitata* SCHMIDT and tethyatene from *Tethya amamensis* FHIELE, and proposed their structures to be 3-hydroxy-7, 8-didehydro-β, χ-carotene and 3, 4-didehydro-β, χ-carotene, respectively. On the basis of the distribution of carotenoids in four species of sea sponge, the metabolic routes to their main carotenoids were proposed.

For developing the carotenoid chemistry, the separation and the purification of carotenoids are extremely important and various kinds of adsorbents are, thus, applied for column chromatography. Micro-Cel C is one of the most important adsorbents and many investigators utilize this as adsorbent. Through the present studies, it was found that the catalytic action of Micro-Cel C induces hydroxylation of β-carotene and produces mainly isocryptoxanthin and slight amounts of isozeaxanthin and echinenone. This forewarns the investigators in this field unless erroneous conclusions should be drawn by using Micro-Cel C.

II. Extraction, purification and identification of carotenoids from aquatic animals

Methods of isolation of carotenoids and their reaction have been reviewed by JENSEN[1], the spectroscopic methods by VETTER[2], the total syntheses by MAYER and ISLER[3], enzymes of biosynthesizing carotenoids by BRITTON and GOODWIN[4] and identifications of carotenoids by DAVIES[5]. It is the author's intention to cover some of these methods that are pertinent to the subject, since carotenoids occur in a variety of aquatic animals and no method of the extraction can be applied satisfactory to the others.

II–1. Extraction of carotenoids

In the present study, fresh samples from a local fish hatchery or market were separated into integuments with fins in the case of fish or carapaces in the case of Crustacea, internal organs and the other parts, and were subjected to the extraction in repeated way with acetone by using Waring blender until these tissues became colorless. Acetone extracts of the pigments were collected in a separating funnel and petroleum ether was added to acetone extracts. By adding water, the pigments were transferred from acetone aqueous layer to petroleum ether layer and acetone aqueous layer was discarded. The petroleum ether layer was washed with newly added water to remove trace of acetone according to the procedure shown in Fig. 1. The petroleum ether solution of these pigments was concentrated under vacuum and dehydrated with sodium sulphate.

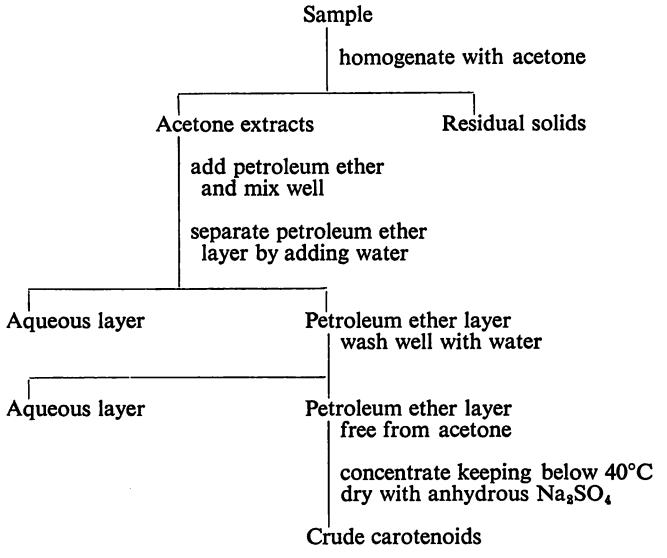


Fig. 1. Extraction of the carotenoids.

II–2. Saponification of the extracts

Saponification of the extracts is essential for separating carotenoids from unwanted lipids in the extracts and for purification of carotenoids from aquatic animals with tissues containing abundant oil. If such carotenoids as astaxanthin, astaxanthin ester and fucoxanthin are contained in samples, the saponification should be avoided, because of the tendency of denaturing the original carotenoids.

For saponifying the extracts in this study, sufficient amount of absolute ethanol was added to the pigment extracts for dissolving them completely, then 60% (W/V) aqueous potassium hydroxide was added at the rate of 1 ml to every 10 ml of the ethanol solution. The alkaline mixture was left in the dark at room temperature under nitrogen for 12 hrs. Three volumes of water and one of petroleum ether were prepared in a 1 liter separating funnel, then the alkaline solution of this pigments was so added to separating funnel as not to exceed more than 10 ml at one time and mixed gently, but not vigorously and allowed to stand until two layers are clearly separated. Then the carotenoids were recovered from the upper layer of petroleum ether. If the separation of two layers could not occur, two or three drops of absolute ethanol or a slight amount of solid sodium chloride should be added to resolve the offensive emulsion. The extraction with petroleum ether was repeated twice, then the petroleum ether extract was washed gently with water to make free of alkali. The washing was repeated until the washing waste indicated no longer alkali to phenolphthalein. The petroleum ether extract was concentrated under vacuum and dehydrated with anhydrous sodium sulphate by leaving it an hour or more.

II–3. Purification of carotenoids

The most important technique of all in the separation, purification and identification of the carotenoids is that of the column chromatography. In this work, the petroleum ether extract was applied on the top of the column and column was eluted at first with non-polar solvent, successively with those gradually increasing in polarity. In this way, the pigments were successively eluted from their proper bands on the column. In general, various kinds of adsorbents can be used for separating and purifying carotenoids. The adsorbents used for the column chromatography in this study are listed below in the order of the increase in the capability of adsorbing carotenoids.

Starch < Sucrose < $CaCO_3$ < Al_2O_3 (deactivated) < Micro-Cel C < Silica gel < MgO < Al_2O_3

Starch and sucrose were used for separating polar carotenoids such as astacene, phoenicoxanthone and tedanin, $CaCO_3$, Al_2O_3 (deactivated) and Micro-Cel C for intermediate polar carotenoids such as zeaxanthin and α-doradecin, and Silica gel, MgO and Al_2O_3 for non polar carotenoids such as α-carotene, β-carotene, γ-carotene and ε-carotene.

Special caution concerning hydroxylation of β-carotene due to Micro-Cel C.

The adsorbents are useful for the separation and the purification of carotenoids, but there can be troublesome on the denaturation of carotenoids provided that no caution was paid for selecting these adsorbents to special carotenoids. The author found hydroxylation of β-carotene occurring on column chromatography. Table 1 indicates the conversion of β-carotene on columns packed with various kinds of adsorbents.

This table shows apparently that the column of Micro-Cel C converts β-carotene to re-

Table 1. Conversion of β-carotene by contacting with various kinds of adsorbents.

Carotenoids induced \ Adsorbents	Amount of carotenoid (μg)						
	Silica gel	Kiesel-guhr	Celite	Hyflo-supercel	MgO	Alumina	Micro-Cel C
Original amount of β-carotene	1,564	1,300	1,300	1,828	1,828	1,524	1,591
After exposure to adsorbent:							
β-carotene	1,099	880	1,048	1,808	1,468	982	819
Mutatochrome	6	9	11				24
Isocryptoxanthin	6	11	10				537
Mixture with epoxides predominating (R_f values lower than that of isocryptoxanthin)	11	3	3				69
Mixture with epoxides predominating (R_f values equal or higher than that of isocryptoxanthin)				5	16	4	

markable amount of isocryptoxanthin with some mutatochrome and the other carotenoids. The columns of silica gel, kieselguhr and celite also convert β-carotene to isocryptoxanthin and the other carotenoids, but their amounts are negligible. Isocryptoxanthin was not detected on Hyflosupercel, MgO and alumina.

HSU et al.[48] showed that β-carotene could be converted to isocryptoxanthin, echinenone or isozeaxanthin in goldfish, however, the conversion of β-carotene to these carotenoids seemed suspicious, because no report certified the existence of these carotenoids in goldfish was provided without using the column of Micro-Cel C. Therefore, the present author examined the hydroxylation of β-carotene by making β-carotene to contact with Micro-Cel C in petroleum ether solution. There were produced several hydroxy-, epoxy- and keto-carotenoids. The first product had the absorbance at λ_{max} 404, 425 and 450 nm in petroleum ether, characteristic of mutatochrome, and was adsorbed at above position of echinenone on the silica gel sheets. The absorption spectrum was not affected by the addition of dilute HCl to the ethanol solution of this pigment, but this pigment on the chromatogram turned from yellow to deep bleu when exposed to HCl gas. The second product, a ketocarotenoid was co-chromatographed with authentic echinenone and exhibited an asymmetric peak with the maximum at 453 nm and a shoulder at 474 nm in petroleum ether. The reduction of the pigment with $NaBH_4$ changed the color from orange to yellow and transformed the single peak in the original spectrum to three peaks at λ_{max} 424, 447 and 475 nm. The reduced compound was identified as isocryptoxanthin. The third product, the major constituent of the products, was identified as isocryptoxanthin through its absorbance at λ_{max} 424, 447 and 475 nm in petroleum ether and the co-chromatography with the authentic isocryptoxanthin. The fourth product showed absorbance at λ_{max} 437, 463 and 490 nm in petroleum ether and move on silica gel sheets with the solvent front of benzene containing methanol at 3%, showing that this carotenoid is devoid of any substituents. These properties are consistent with a certain dehydro-β-carotene. The fifth product, which had the absorbance at λ_{max} 404, 425 and 450 nm, appeared to be 4-hydroxy-5′, 8′-epoxy-β-carotene. The sixth product was the characteristic spectrum with the absorbance at λ_{max} 425, 447 and 474 nm in petroleum ether. The R_f value on the co-chromatography was identical with that of authentic isozeaxanthin.

As shown in Table 2, it is clear that β-carotene decreased outstandingly by a 1 hr-contact with Micro-Cel C and converted mainly to isocryptoxanthin and some to mutatochrome, echinenone, isozeaxanthin and the other carotenoids under the condition of increase in Micro-Cel C in petroleum ether solution of β-carotene. The effect of time length of exposuring β-carotene to Micro-Cel C was not shown clearly because of invariable yields of isocryptoxanthin regardless of extending the contact period.

This tells us that the caution should be taken for choosing these adsorbents by confirming the existence of such a catalytic action as that of Micro-Cel C, otherwise we may have such misunderstanding as HSU's results. It is the first time for the present author to propose the mechanism of the hydroxylation reaction of β-carotene through a Micro-Cel C column as shown in Fig. 2. Generally, the adsorbents should be chosen according to the type of carotenoids to be separated. For avoiding such kinds of mutation of carotenoids as mentioned

Table 2. Conversion of β-carotene when contacted with Micro-Cel C in various amounts[a] and contacting times[b].

Carotenoids induced \ Adsorbents	Amount of carotenoid (μg)					
	10 g Micro-Cel	20 g Micro-Cel	30 g Micro-Cel	15 minutes	30 minutes	1 hour
Original β-carotene	1,093	1,093	1,093	1,077	1,077	1,077
After exposure to Micro-Cel C:						
β-carotene	531	59	25	510	418	262
Mutatochrome, Echinenone	20	28	34	17	24	30
Isocryptoxanthin	246	437	714	234	304	314
Dehydro-β-carotene, Isozeaxanthin, 4-OH-5′, 8′-epoxy-β-carotene	35	43	52	43	45	69
% yield of isocryptoxanthin	23	40	65	22	28	29

a: Mixtures consisting of an equal amount of β-carotene, the specified amount of Micro-Cel C, and enough petroleum ether to cover were allowed to stand for 15 min. The carotenoids were then extracted and separated on a MgO-Hyflosupercel column with 25% acetone in petroleum ether as solvent. The values listed are the averages of two trials.

b: Mixtures consisting of 1077 μg of β-carotene, 10 g of Micro-Cel C, and 50 ml of petroleum ether were allowed to stand for the specified time period. The carotenoids were then extracted and separated as described above.

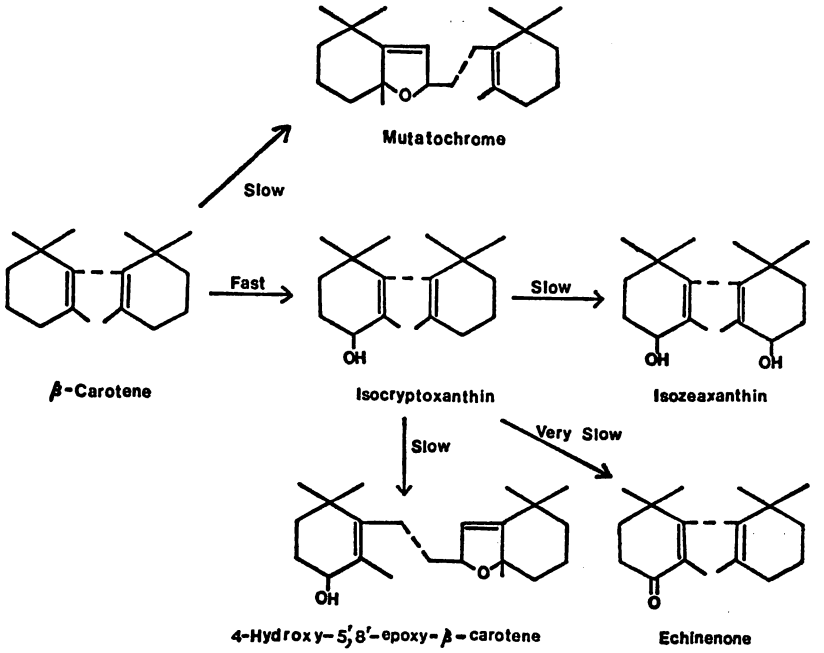


Fig. 2. Proposed reactions of β-carotene on Micro-Cel C.

above, MgO column was used for preliminary separations of carotenoids in this study.

II–4. Identification of carotenoids

Identification of carotenoids was based on the nature on elution from column and the characteristic colors, the visible absorption spectra, the special chemical tests, the partition coefficients and the co-chromatography with authentic carotenoids.

a. Nature on elution from column and characteristic color.

When carotenoid mixture is subjected to column chromatography using MgO, alumina, Micro-Cel C or sucrose as a stationary phase and petroleum ether-acetone as a mobile phase, individual carotenoids initiate to move making bands on the column, then these are eluted from the column. The pattern of elution of these carotenoids are dependent on the acetone content in petroleum ether as shown in Table 3. Therefore, the nature of carotenoids on elution from columns can be applicable to identifying them with their characteristic color.

In this study, these two natures of carotenoids were utilized for their identification.

Table 3. Acetone content (v/v%) in eluant** for carotenoid column chromatography.

Carotenoids	Adsorbents				Characteristic color on adsorbents
	MgO*	Alumina	Micro-Cel C	Sucrose	
α-Carotene	0	0	0		Yellow
β-Carotene	0–1	0–1			Yellow-Orange
β-Zeacarotene		0–1			Yellow
Echinenone	4–5	4	5		Pink
Isocryptoxanthin	5–6				Yellow-Orange
Cryptoxanthin	5–6		6		Yellow-Orange
Canthaxanthin	12		7–10		Pink
4-Hydroxy-echinenone	15				Pink
3, 3′-Dihydroxy-ε-carotene	15	15	8		Yellow
Lutein	10–15		10		Yellow
Zeaxanthin	20–30		10		Yellow-Orange
Isozeaxanthin	20–30		8		Yellow-Orange
Violaxanthin	20–30		8		Yellow
Neoxanthin			10		Yellow
α-Doradecin	20–30		10	0	Pink-Red
β-Doradecin	25–30		10	0	Pink-Red
Phoeniconone	30		10	0–1	Pink-Red
Astacene	30			0–2	Pink-Red
7, 8, 7′, 8′-Tetradehydroastaxanthin	20–30			0	Pink-Red
7, 8-Didehydroastaxanthin	20–30			0	Pink-Red

* MgO: Hyflosupercel (1 : 2 W/W)

** petroleum ether

b. Characteristic absorption spectra

The wave length of characteristic absorption bands of carotenoids shift toward the region of longer wave length, when the number of conjugated double bonds in the molecule increases. Similarly, their absorption bands also move depending on the kinds of solvent used from the shortest in *n*-hexane to the longest in carbon disulfide. On the contrary, the introduction of hydroxyl groups into the molecule has little effect on the band shift as mentioned. The introduction of oxo groups has also similar effect provided that its position is not conjugated to the polyene chain[5].

These variations on the characteristic absorption spectra were confirmed by using automatic spectrophotometer (Shimazu UV-200S) and utilized for identifying carotenoids in this study.

c. Special chemical tests

c-1. Reduction

Keto groups of carotenoids can be reduced by adding sodium borohydride to its 95% ethanol solution and standing overnight in a refrigerator. After transferring the reduced carotenoids into petroleum ether from ethanol solution by adding water, their natures can be confirmed. In this way, various types of carotenoids were identified in this study.

c-2. Methylation

Methylation of carotenoids can be accomplished by adding 5 drops of 2N HCl to 5 to 10 ml methanol solution and leaving it at room temperature for 3 hours. After transferring methylated carotenoids into petroleum ether from its methanol solution by adding water, their natures can be confirmed. In this study, various carotenoids were identified after methylation in such a way.

c-3. Epoxide tests

This test is useful to identify various carotenoids. The reason is that if a few drops of 0.1N HCl are added to the ethanol solution of carotenoids and allowed to stand for 3 minutes, such spectral shift as 18–25 nm or 40 nm to shorter wave length from the original wave length was provided with a 5, 6-epoxide or 5, 6 and 5′, 6′-epoxide as exemplified by KRINSKY[6].

In this study, various types of carotenoids were identified through this test.

d. Partition coefficients between solvents

Partition test between *n*-hexane and aqueous methanol proposed by Petrack and Zechmeister[7] can be applicable to identifying carotenoids. In this test, each solvent layer is saturated at first with the other and an isolated carotenoid is dissolved in the layer with high solubility, then its optical density at λ_{max} is measured. This solution is shaken with an equal volume of another solvent in a separating funnel and the optical density of the first solution is measured. By subtracting the optical density of the latter from that of the former, the concentration of this carotenoid in the latter solvent can be obtained. The partition coefficient of this carotenoid between *n*-hexane and aqueous methanol can be thus calculated.

The partition coefficients of carotenoids are very useful to know the numbers of hydroxy group and keto group in carotenoid molecules. In this study, the partition coefficients were obtained for identifying carotenoids.

e. Co-chromatography

Co-chromatography is one of procedures for identifying an unknown substance by sub-

jecting it to chromatography with an authentic compound which should be presumed to be identical with that substance. If this co-chromatography is used, and both compounds give one band on the column or one spot on the sheet of thin layer, it can be confirmed that both are identical.

In this study, the co-chromatography was applied to the thin layer chromatography for identifying carotenes such as α-carotene, β-carotene and γ-carotene using silica gel sheets (Eastman chromatogram sheets) with petroleum ether as a developing solvent. The same sheets and the developer of benzene containing methanol in 3% were also satisfactory used for identifying such xanthophylls as lutein, zeaxanthin, astaxanthin and so on. The R_f values of these carotenoids in thin layer chromatography are shown in Table 4 and the origins of the authentic carotenoids are listed in Table 5.

Table 4. R_f values of carotenoids.
Mobile phase: benzene containing methanol in 3%
Stationary phase: Eastman Chromatogram Sheets

Carotenoids	R_f value
α-Carotene	1.00
β-Carotene	0.97
4-Keto-α-carotene	0.73
Echinenone	0.70
4-Hydroxy-α-carotene	0.67
Isocryptoxanthin	0.54
Canthaxanthin	0.44
Lutein	0.26
Isozeaxanthin	0.24
Zeaxanthin	0.18
α-Doradecin	0.28
Astacene	0.27

Table 5. Authentic carotenoids.

α-carotene	Synthetic compound (F. Hoffmann La Roche Co and Ltd. Basle Switzerland)
Echinenone	〃 〃
Canthaxanthin	〃 〃
Isocryptoxanthin	Reduced echinenone
Isozeaxanthin	Reduced canthaxanthin
Lutein	Isolated from spinach, *Spinacia oleracea*
Zeaxanthin	Isolated from chinese lantern plant, *Physalis alkekengi*
Tunaxanthin	Isolated from tuna fish, *Neothunnus albacora*
α-Doradecin	Isolated from goldfish, *Carassius auratus*
Astaxanthin and astacene	Isolated from prawn, *Penaeus japonicus*

II–5. Quantitative determination

In this study, quantitative determination was made with automatic spectrophotometer and the relative amount of each carotenoid was given as percentage of the total based on its molecular extinction coefficient at λ_{max}. However, the molecular extinction coefficient are not necessarily determined for all carotenoids. In this study some of them had to be estimated in approximation for knowing their concentrations from optical densities. Thus, the molecular extinction coefficient of such carotenoids were presumed from those of carotenoids with similar structures.

III. Carotenoid distribution in aquatic animals

KARRER and JUCKER[9] in 1948, GOODWIN[10] in 1952 and FOX[11] in 1953 have reviewed the literatures on the carotenoids in aquatic animals up to the respective dates. More recent information was given by WEEDON[12] in 1971. This list is mostly concerned for their chemical structures of carotenoids and main resources based on more recent informations.

More specialized reviews have also been published: GOODWIN, Carotenoids in Fish[13], 1951: FOX, Pigments of Fish[14], 1957: GOODWIN, Pigments of Crustacea[15], 1960: GOODWIN, Algal Carotenoids[16], 1971.

No attempt was made by those authors to clarify the biochemical correlation between internal organ carotenoids and integument or carapace carotenoids in aquatic animals. For this object, the present author separated carotenoids from their internal organs and integuments or carapaces and made clear their distributions.

Even though the carotenoid distribution may be clarified in these tissues, there must remain some ambiguities that may confuse us to confirm whether these carotenoids found in these tissues have been originated from their feed or their own capabilities of converting carotenoids toward astaxanthin.

However, a red carotenoid, astaxanthin is seldom detected from both terrestrial plants and algae. Echinenone and canthaxanthin are pink carotenoids found in both plants and animals. Phoenicoxanthin is a red carotenoid proper to animals as well as astaxanthin. Therefore, phoenicoxanthin and astaxanthin must be derived from these carotenoids in algae. Small shrimps are really herbivora but they contain astaxanthin. This tells us the fact that shrimp can convert algal carotenoids to astaxanthin.

The present author was able to clarify the distribution of carotenoids including astaxanthin and phoenicoxanthin in aquatic animals, and the possible metabolic pathways could be presumed through these carotenoids in making use of their structural formulae and their abundance. More accurate consideration of the metabolic pathway among these carotenoids could be performed later through feeding tests of carotenoids employing aquatic animals.

The present author separated carotenoids from a species of starfish, Onihitode, *Acanthaster planci* and isolated individual carotenoids through a column chromatography. The absorption spectrum of astaxanthin isolated from this starfish, gave somewhat different pattern from that of authentic astaxanthin: the spectrum was similar to that of astaxanthin, but changed after

its purification to have two shoulders at 442 nm and at 512 nm differing from no such shoulders of authentic astaxanthin. This stimulated the study to pursue much more in detail. Then one new ketocarotenoid was isolated and confirmed to be 7, 8-didehydroastaxanthin.

During investigating the species of aquatic organisms possessing astaxanthin, the author believed that astaxanthin was separated from orange sea sponge, *Clathria frondifera* (BOWERBANK) because this carotenoid gave λ_{max} at 471 nm close to λ_{max} at 473 nm of astaxanthin. However, the author became soon aware of the difference between both carotenoids because of the existence of the shoulder at 493 nm when the carotenoid from sea sponge was purified. When astaxanthin is reduced, its λ_{max} shift to 450 nm from 473 nm. Therefore, if the carotenoid from sea sponge is identical with astaxanthin, its λ_{max} shift to 450 nm from 471 nm by reduction. The author thus tried to reduced this sponge carotenoid, confirmed the new λ_{max} to be 458 nm and concluded both not to be identical. After trials of various procedures of identification, this carotenoid was confirmed to be 2, 3-didehydro-β, χ-carotene-4-one (tedanin) which was recently discovered by OKUKADO[67].

On the way of studying the distributions of carotenoids in sea sponges, two new carotenoids were found and confirmed their structures to be 3-hydroxy-7, 8-didehydro-β, χ-carotene and 3, 4-didehydro-β, χ-carotene.

III–1. Crustacea

DAVIES et al.[17], KRINSKY[18], CZYGAN[19] and HATA and HATA[20] investigated the carotenoids in *Artemia salina* and have shown that *Artemia* is capable of converting β-carotene into canthaxanthin. The carotenoids in Decapoda were studied by CZECZUGA and CZERPAK[21], GILCHRIST and LEE[22]. The carotenoids in Copepoda were studied by CZECZUGA and CZERPAK[23], and and carotenoids in *Daphnia* were reported by THOMMEN and WECKERNAGEL[24] and HERRING[25]. The carotenoids in Amphipoda were also reported by CZERPAK[26] and CZECZUGA[27]. The distribution and relative abundance of the carotenoids in Branchipoda were investigated by GILCHRIST[28]. WOLF and CORNWELL[29] isolated β-carotene, lutein and astaxanthin in cavernicolous cray fish. BODEA et al.[30,31] reported the isolation of astaxanthin, β-carotene, cryptoxanthin and three unidentified pigments from Copepoda and they observed a close biochemical correlation between astaxanthin and three minor carotenoids.

The pigments of three variant of the marine isopoda, *Idotea montereyensis* were investigated by LEE[32] who isolated β-carotene, echinenone, canthaxanthin, 4-hydroxy-4′-keto-β-carotene, lutein and lutein ester, but no astaxanthin. He also isolated β-carotene, canthaxanthin, isozeaxanthin, echinenone, isocryptoxanthin, 4-hydroxy-4′-keto-β-carotene and lutein, but no astaxanthin in another isopoda species, *Idotea granulosa*.

GILCHRIST and LEE[22] isolated β-carotene, δ-carotene, echinenone, isocryptoxanthin, canthaxanthin, lutein, lutein-5, 8-epoxide, astaxanthin and 4-hydroxy-4′-keto-β-carotene in *Carcinus maenas*.

HERRING[33] studied the carotenoids in *Daphnia magna* and isolated β-carotene, echinenone, canthaxanthin[34], a ketocarotenoid (probably 3-hydroxy-4-keto-β-carotene) and astaxanthin. Alloxanthin has been isolated from the commensal crab, *Pinnotheres pisum*[35] and the sand crab, *Emerita analoga* and its origin can be traced to the algae consumed by these crabs.

Similarly, a common algal pigment, fucoxanthin, was isolated from the barnacle, *Lepas fascicularis* and *Lepas anacles* and from their faecal pellets (HERRING[37]) and peridinin, a characteristic dinoflagellate carotenoid, has been isolated from *Lepas fascicularis*[38].

The carotenoids in spiny lobster, *Panulirus japonicus*[39], tiger prawn, *Penaeus japonicus*[40,41], a swimming crab, *Portunus trituberculatus*[42] were identified. In these Crustacea, ingested labelled β-carotene was converted into astaxanthin through the steps of echinenone canthaxanthin and 3-hydroxy-canthaxanthin[43-46].

The present author attempts to describe about the types and location of the carotenoids in various Crustacea native to southern Japan for aiding the establishment of their metabolic pathways.

Materials and Methods

Fresh sample of *Neomysis intermedia, Ibacus cilatus, Metapenaeopsis* sp., *Penaeopsis* sp., *Penaeus orientalis* and *Squilla oratoria* were purchased at a local fish market. The internal organs and the carapaces were separately collected and the carotenoids were repeatedly extracted with acetone, using the same method as already mentioned.

The pigments were dissolved in about 10 ml of petroleum ether for chromatography using MgO, silical gel and sucrose columns. The carotenoids were separated by the column chromatography and identified by the absorption spectra, the nature on elution from column and the characteristic colors, the special chemical tests and the co-chromatography with authentic carotenoids as already mentioned.

Results and Discussion

The carotenoid pigments in the carapaces and in the internal organs are listed in the order of elution from the columns and the relative amounts of carotenoids are given as percentage (Table 6). Astaxanthin (recovered as astacene) is a by far important pigment than the other, because its abundant occurrences in carapaces give nice market prices. Their concentration are especially high in the carapaces.

Beta-carotene, lutein and zeaxanthin are yellow or orange carotenoids usually found in algae as in the other plants. Therefore, it is natural that these carotenoids are detected from tissues of Crustacea from marine environment.

From carotenoids detected in tissues of these Crustacea, three possible metabolic pathways to astaxanthin are considered as follow: β-carotene→astaxanthin, zeaxanthin→astaxanthin, lutein→astaxanthin (Fig. 3).

One kind of yellow carotenoid, 3, 3′-dihydroxy-ε-carotene (tunaxanthin) are proper to animals. It is quite difficult to put it in these metabolic pathways, because the structure of this carotenoid is remarkably different from those of other carotenoids.

KATAYAMA[43] certified the pathway from β-carotene to astaxanthin through echinenone, canthaxanthin and phoenicoxanthin using tiger prawn, *Penaeus japonicus*.

In the present study, phoenicoxanthin was found in all species tested, β-carotene and cantha-

Table 6. Relative abundances of the carotenoids in Crustacea.

	Carotenoids	Relative abundances (%)				
		Penaeus japonicus	*Panulirus japonicus*	*Portunus trituberculatus*	*Ibacus cilatus*	*Squilla oratoria*
Carapaces	β-Carotene	trace	—	trace	—	—
	Tunaxanthin	3	—	—	—	—
	Echinenone	trace	—	trace	6	1
	Canthaxanthin	trace	12	14	2	1
	Lutein	trace	—	—	—	—
	Zeaxanthin	trace	—	—	—	—
	Phoenicoxanthin	5	2	2	1	trace
	Astaxanthin	90	84	84	90	95
Internal organs	β-Carotene	10	2	2	2	30
	Tunaxanthin	2	—	—	—	—
	Echinenone	3	8	8	trace	—
	Canthaxanthin	6	—	—	—	—
	Lutein	trace	—	—	1	—
	Zeaxanthin	trace	—	—	1	—
	Phoenicoxanthin	3	—	—	—	—
	Astaxanthin	70	88	88	92	70

	Carotenoids	Relative abundances (%)			
		Metapenaeopsis sp.	*Penaeopsis* sp.	*Neomysis intermedia*	*Penaeus orientalis*
Carapaces	β-Carotene	—	5	3	—
	Tunaxanthin	—	—	—	3
	Echinenone	—	2	7	—
	Canthaxanthin	—	4	5	—
	Lutein	—	—	—	—
	Zeaxanthin	—	—	—	—
	Phoenicoxanthin	—	3	3	—
	Astaxanthin	99	83	81	97
Internal organs	β-Carotene	—			—
	Tunaxanthin	—			—
	Echinenone	—			—
	Canthaxanthin	—	Whole	Whole	—
	Lutein	—	bodies	bodies	—
	Zeaxanthin	—			trace
	Phoenicoxanthin	8			4
	Astaxanthin	92			96

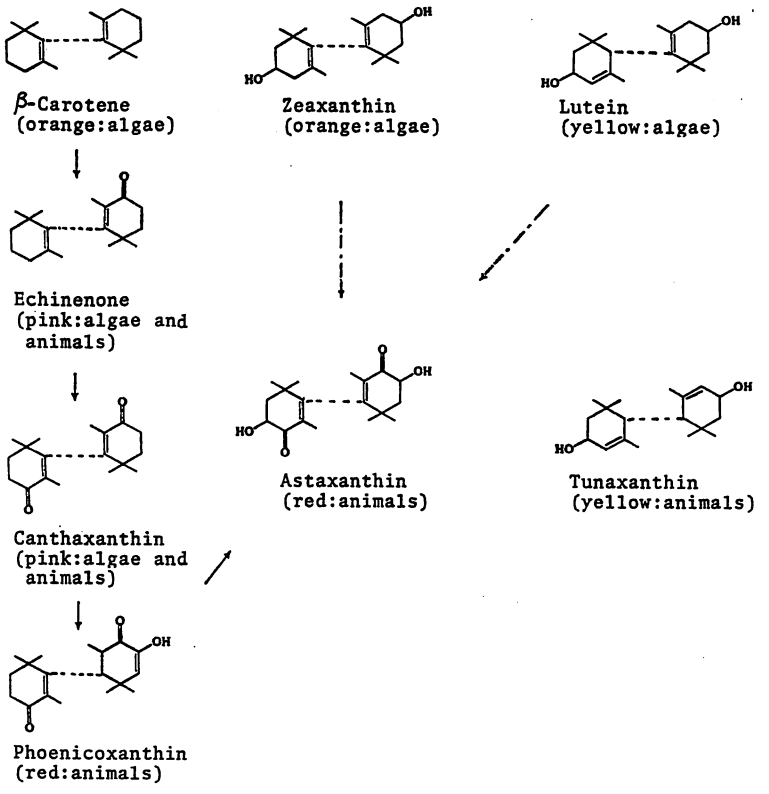


Fig. 3. Possible metabolic pathways considered from the distribution of carotenoids in Crustacea.

——: already confirmed ———: not yet confirmed.
in parentheses, the color and the origins are indicated.

xanthin in 7 species out of 9 species and zeaxanthin in 3 species out of 9 species. Therefore, the precursor of astaxanthin must be phoenicoxanthin. Probably the main pathway of carotenoids is expressed as β-carotene→echinenone→canthaxanthin→phoenicoxanthin→ astaxanthin. This is in accord with the pathway proposed by KATAYAMA. However, another metabolic pathway toward astaxanthin in Crustacea seemed to start from zeaxanthin or lutein.

III–2. Fresh-water red fish

KATAYAMA[47] found α-doradexanthin from skin of goldfish and proposed the metabolic pathway, lutein→α-doradexanthin→β-doradexanthin→astaxanthin. HSU[48] also proposed the same metabolic pathway through feeding tests of goldfish with ^{14}C-lutein. However, HATA and HATA[49] denied the pathway from lutein to astaxanthin and emphasized that lutein can be converted to α-doradexanthin, but the further oxidation of α-doradexanthin can not occur. Instead, they reported that astaxanthin abundantly found in skin of goldfish can be

attributable to the bio-oxidation of zeaxanthin through β-doradexanthin.

The object of this study is to judge which metabolic pathway is correct between those proposed by KATAYAMA[47], HSU[48] and HATA et al.[49] through the distribution of carotenoids in fresh water red fish.

Materials and Methods

Goldfish, Hibuna, fancy red carp and golden yellow carp were purchased at a fish hatchery and killed. The carotenoids in their internal organs and integuments were separately extracted with acetone in a working Waring blender as already stated in II–1. The crude carotenoids thus obtained were dissolved in 10 ml of petroleum ether and subjected to chromatography on a MgO column (magnesium oxide-Hyflosupercel=1: 2). The carotenoids of each band were eluted from the column with acetone and saponified, using the same method as already mentioned in II–2. The saponified pigments were purified on the same MgO column and a sucrose column. Each purified carotenoid was identified by their absorption spectra, the nature on elution from these columns and the characteristic colors, the special chemical tests and the co-chromatography with authentic carotenoids as already mentioned in II–4.

Results and Discussion

The carotenoid pigments in the integuments and the internal organs of fresh water red fish are listed in the order of the elution from the columns and the relative amounts of carotenoids recovered are given as percentage in Table 7.

Table 7. Spectral characteristics and relative abundances of the carotenoids in goldfish, Hibuna, fancy red carp and golden yellow carp.

	Pigments	Spectral characteristics			Relative abundances (%)			
		λ_{max} (nm) in petroleum ether	λ_{max} (nm) in ethanol after reduction	λ_{max} (nm) in chloroform	Gold-fish	Hibuna	Fancy red carp	Golden yellow carp
Integuments	Lutein	422, 446, 475	—	432, 459, 487	14	11	15	45
	Zeaxanthin	421, 451, 481	—	433, 461, 490	5	4	5	5
	α-Doradexanthin	455, 471	422, 446, 476	—	25	24	31	20
	Astaxanthin	473	427, 451, 480	—	55	60	50	30
Internal organs	Canthaxanthin	465	425, 451, 476	—	20	25	25	23
	Lutein	425, 446, 476	—	433, 458, 487	63	61	60	61
	Zeaxanthin	429, 451, 480	—	434, 461, 490	17	14	15	14

HSU and HATA utilized silica gel or MgO for fractionating β-doradexanthin from carotenoids in goldfish. The present author, thus, endeavored to separate β-doradexanthin using both column packed with the same adsorbents. In spite of such endeavor as mentioned, β-doradexanthin was not detected, but lutein, zeaxanthin, α-doradexanthin and astaxanthin

in integuments, lutein, zeaxanthin and canthaxanthin in internal organs of these freshwater red fish. The major carotenoids in their integuments were found to be astaxanthin (50–60%) except golden yellow carp (30%) and lutein (60–63%) in internal organs, respectively. Lutein and zeaxanthin were common carotenoids in internal organs and integuments. The carotenoids proper to their integuments were α-doradexanthin (20–31%) and astaxanthin. The characteristic carotenoid of their internal organs was canthaxanthin (20–25%).

Thus, the present author proposed the possible metabolic pathways of canthaxanthin, lutein and zeaxanthin to astaxanthin as shown in Fig. 4.

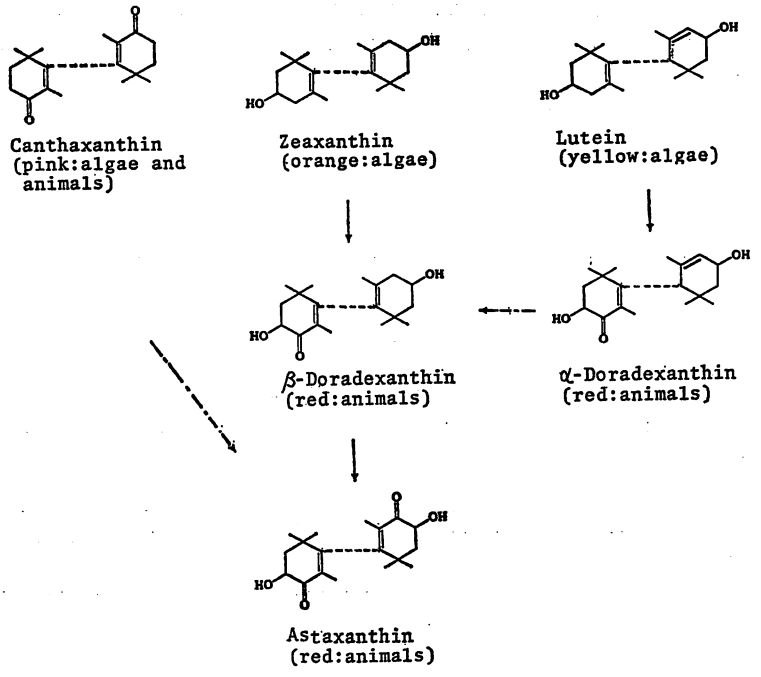


Fig. 4. Possible metabolic pathways considered from the distribution of carotenoids in fresh-water red fish.
——: already confirmed ——: not yet confirmed.
in parentheses, the color and the origins are indicated.

III–3. Marine red fish

THOMMEN and GLOOR[50] reported the presence of canthaxanthin in addition to astaxanthin and β-carotene in sea trout, *Salmo trutta*. Tunaxanthin was first isolated from the Pacific blue fin tuna by HIRAO[51], and its structure was confirmed to be 3, 3′-dihydroxy-ε-carotene by CROZIER[52]. TSUKUDA et al.[53] studied the contents of the carotenoids in marine red fish from the point of view of their coloration. The present author deals with the carotenoids in several marine red fish through their quantitative analyses for contributing to the confirmation of their metabolic pathways.

Materials and Methods

Red sea bream, *Pagrus major* TEMMINCK and SCHLEGEL and crimson sea bream, *Evynnis japonica* TANAKA were purchased at a local fish hatchery and Akaamadai, *Branchiostegus japonicus japonicus,* Kinmedia, *Beryx splendens* LOWE, Sasanohabera, *Pseudolabrus japonicus,* Hachibiki, *Erythrocles schlegeli* (RICHARDSON) and Barahata, *Variola louti* (FORSKAL) were obtained at a local fish market. The internal organs, dorsal and ventral sections and integuments were collected separately and extracted with acetone as already mentioned in II–1. The crude pigments thus obtained were subjected to chromatography using columns of MgO, Micro-Cel C, silica gel and sucrose. The carotenoids were identified through their absorption spectra, nature on elution from columns, colors, special chemical tests and co-chromatography with authentic carotenoids as already mentioned in II–4.

Results and Discussion

The carotenoid pigments in the internal organs and the integuments of marine red fish are listed in the order of elution from columns, and relative amounts of each pigment are given as percentage in Table 8.

From Table 8, it is clearly shown that astaxanthin is the most abundant carotenoid in marine red fish such as red sea bream, crimson sea bream, Kinmedai, Akaamadai,

Table 8. Relative abundances of the carotenoids in marine red fish.

	Caroteno,s	Relative abundances (%)						
		Pagrus major	*Evynnis japonica*	*Beryx splendens*	*Branchiostegus japonicus*	*Pseudolabrus japonicus*	*Erythrocles schlegeli*	*Valiola louti*
Integuments	α-Carotene	2	—	—	—	1	—	—
	α-Cryptoxanthin	—	1	—	—	—	—	—
	Tunaxanthin	17	12	18	9	9	trace	15
	Canthaxanthin	2	1	3	—	—	—	1
	Lutein	12	trace	2	—	—	—	—
	Zeaxanthin	4	1	—	1	2	—	—
	Phoenicoxanthin	—	2	—	—	—	—	—
	Astaxanthin	63	82	77	90	88	99	84
Internal organs	β-Carotene	8	—	2	trace		—	
	Echinenone	5	—	—	—		—	
	Canthaxanthin	7	6	—	—		—	
	Tunaxanthin	8	—	—	—		—	
	Lutein	6	2	—	—		—	
	Zeaxanthin	2	1	—	—		—	
	Astaxanthin	52	91	98	97		99	

Sasanohabera, Hachibiki and Brahata. Secondary abundant carotenoid in the integuments of those fish was tunaxanthin, 3, 3′-didehydroxy-ε-carotene. The red sea bream sample contained α-carotene, β-carotene, lutein and zeaxanthin in either internal organs or integuments. Canthaxanthin was isolated from red sea bream, crimson sea bream, Kinmedai and Barahata though phoenicoxanthin was only isolated from crimson sea bream.

As shown in Table 8, red sea bream and crimson sea bream, which are taxonomically close each other and inhabit in similar waters, contain various kinds of carotenoids, but they always contain tunaxanthin, canthaxanthin, lutein, zeaxanthin and astaxanthin. Katayama found that β-carotene was not converted to astaxanthin in red sea bream through β-carotene feeding test. If 3, 3′-dihydroxy-ε-carotene can be a precursor of astaxanthin, 3, 3′-dihydroxy-ε-carotene must change into lutein or zeaxanthin through isomerization reaction prior to the oxidation reaction. However, lutein could not be converted to astaxanthin, because no α-doradexanthin was found in these carotenoids separated. Therefore, 3, 3′-dihydroxy-ε-carotene seems not to be converted to astaxanthin. There are some possibilities that

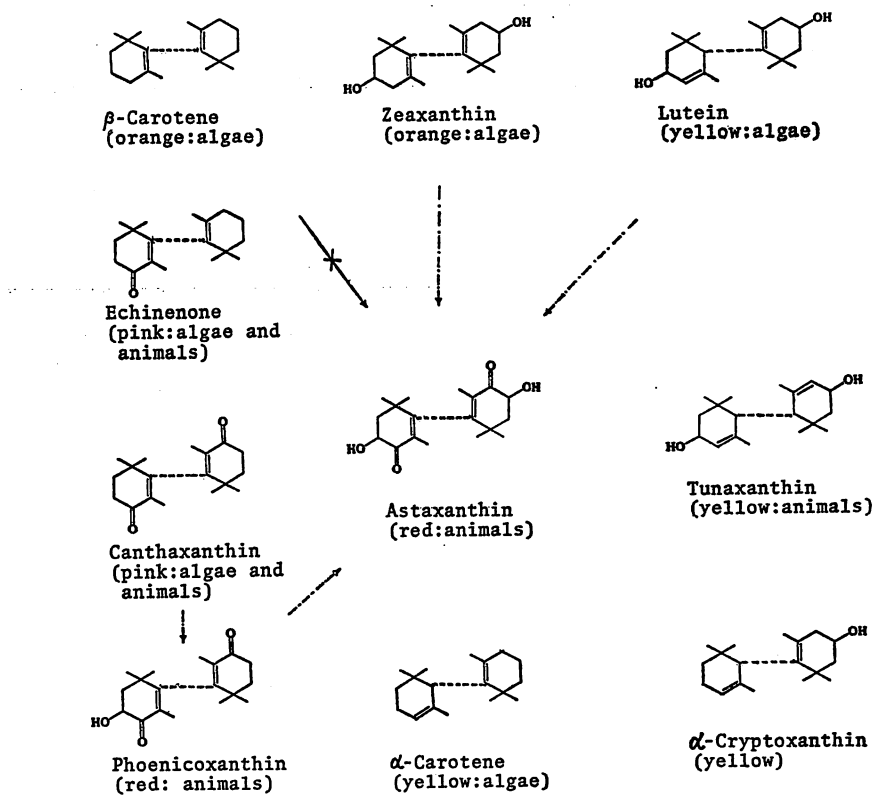


Fig. 5. Possible metabolic pathways considered from the distribution of carotenoids in marine red fish.

—— : already confirmed ——— : not yet confirmed.

in parentheses, the color and the origins are indicated.

canthaxanthin and zeaxanthin convert to astaxanthin in these marine red fish according to the pathways as shown in Fig. 5.

Since only 3, 3′-dihydroxy-ε-carotene and astaxanthin are common carotenoids in these marine red fish, it seems that these fish have no ability to synthesize astaxanthin from plant carotenoids.

Those marine red fish are carnivorous and eat such as crustacean which contain abundantly astaxanthin. Therefore, astaxanthin could be the major carotenoid in their internal organs and integuments of marine red fish.

III–4. Porifera

KARRER et al.[54] recognized the existence of astaxanthin in red sea sponge, *Axinella cristagilli*. CZECZUGA[55] also isolated astaxanthin from *Verongia aerophoba*. DRUMM[56] reported the existence of echinenone, γ-carotene in *Hymeniacidon sanguineum*. In 1957, YAMAGUCHI[57–62] first isolated the aromatic carotenoids, renieratene, isorenieratene and reniera-purpurin from *Reniera japonica*.

After that, the existence of some aromatic carotenoids in such sea sponges as *Cyamon neon* and *Reniera japonica*[63–65] was reported.

In 1974, AGUILLAR-MARTINEZ et al.[66] found a new keto-carotenoid from a sea sponge, *Trikentrion helium* and decided the structural formula to be 3-hydroxy-7, 8-dihydro-κ, χ-carotene-6, 8-dione which was named trikentriorhodin by them. The present author endeavored to know carotenoids in sea sponges, *Clathria frondifera* and *Tedania digitata* and found an abundant unknown carotenoid, then investigated its structural formula through the mass, the NMR and the IR spectrographs and was able to know this carotenoid to agree with trikentriorhodin later, but when this studies had finished in 1975, the AGUILLAR-MARTINEZ's paper was not available. However, no investigator studied the abundance of this carotenoid in porifera, because of no availability of the information concerning about trikentriorhodin. The author was able to clarify its abundance in both sea sponges, *Clathria frondifera* and *Tedania digitata* in making use of the new knowledges which were obtained directly by the author.

In 1975, OKUKADO[67] isolated a new keto-carotenoid from *Tedania digitata* SCHMIDT, decided its structural formula to be 3-hydroxy-2, 3-didehydro-β, χ-carotene-4-one and named tedanin. However, he saponified the carotenoids from this sea sponge for removing fatty materials. Since the saponification of astaxanthin (3-hydroxy-β-carotene-4-one) tends to lose hydrogens from the 2- and 3-positions of this carotenoid, it is ambigious whether tedanin is an artifact or a natural product. The present author, therefore, purified this carotenoid from *Clathria frondifera* (BOWERBANK) omitting the saponification and was able to confirm its structural formula to be really 3-hydroxy-2, 3-didehydro-β, χ-carotene-4-one.

The present author confirmed the existence of two new carotenoids, tedaniaxanthin and tethyatene, and proposed the structure of each carotenoid by measuring its mass, IR or NMR spectra. Also, the author elucidated the various types of the carotenoids in *Clathria frondifera* (BOWERBANK), *Tedania digitata* SCHMIDT, *Haliclona permollis* (BOWERBANK) and *Tethya amamensis* FHIELE, and discussed their metabolic pathways.

Materials and Methods

Four kinds of sea sponges were collected at Kinko bay, Kagoshima. The carotenoids were extracted with acetone by using Waring blender as already mentioned in II–1. The crude carotenoids thus obtained were initially separated through a MgO column (magnesium oxide: Hyflosupercel=1: 2) and then purified according to the procedure shown in Fig. 6, 7, 8. The carotenoids were identified by the absorption spectra, the nature on elution from column and the characteristic colors, the special chemical tests and the co-chromatography with authentic carotenoids as already stated in II–4.

Isolation of new carotenoids and unidentified carotenoids

i) Tedanin and Trikentriorhodin

The procedure of separation of tedanin and trikentiorhodin in *Clathria frondifera* (BOWERBANK) was shown in Fig. 6. A main reddish band (Fr–6) was eluted with ethyl ether-acetic

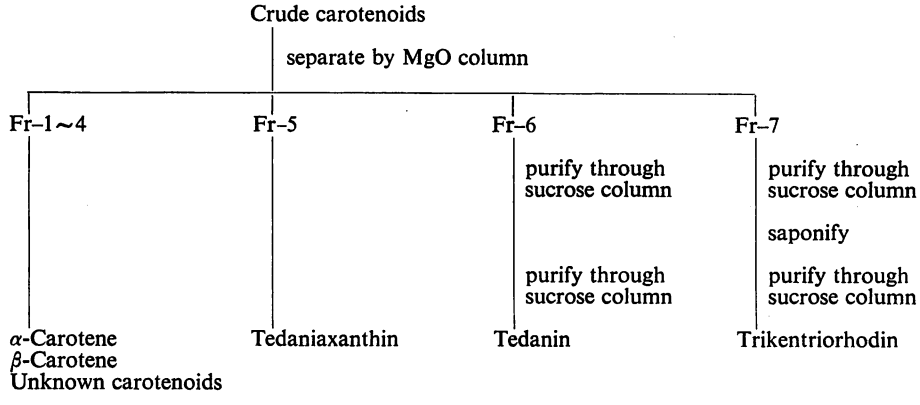


Fig. 6. Isolation of tedanin and trikentriorhodin from the crude carotenoids of *Clathria frondifera* (BOWERBANK).

acid (9: 1) from the MgO column. Carotenoids from the band were transferred from this eluant to petroleum ether by adding water. The petroleum ether solution of the carotenoids was repeatedly washed with water to remove trace of acetic acid, concentrated under reduced pressure, and dehydrated with anhydrous sodium sulphate. The concentrated carotenoids were purified on a dried powered sucrose column, using petroleum ether as a developing solvent. The main pinkish band was eluted with acetone. The carotenoids were transferred from acetone to petroleum ether by adding water. The aqueous layer was discarded. The petroleum ether extract was dehydrated with anhydrous sodium sulphate and evaporated under reduced pressure. The isolated pigment was crystallized from *n*-hexane-ethyl ether in refrigerator.

By using the same procedure, the pigment of Fr–7 was also purified and crystallized from *n*-hexane-ethyl ether.

The structural formula of individual pigments were confirmed by mass, IR and NMR spectra.

The NMR spectrum of each pigment was measured in $CDCl_3$ applying internal TMS at 100 MHz (Nihondenshi MH-100). Each crystalline pigment was ground with potassium bromide, pressed to make a disc and its IR spectrum was measured by using an IR-Spectrophotometer (Shimazu, Type IR-270).

ii) Tedaniaxanthin

The isolation procedure of tedaniaxanthin from *Tedania digitata* SCHMITD is shown in Fig. 7. Tedaniaxanthin was crystallized from *n*-hexane-ethyl ether and its structural formula was proposed from not only the mass, the NMR and the IR spectrographs but the methylation and epoxide tests of this carotenoid.

iii) Tethyatene

The isolation procedure of tethyatene from *Tethya amamensis* FHIELE is shown in Fig. 8.

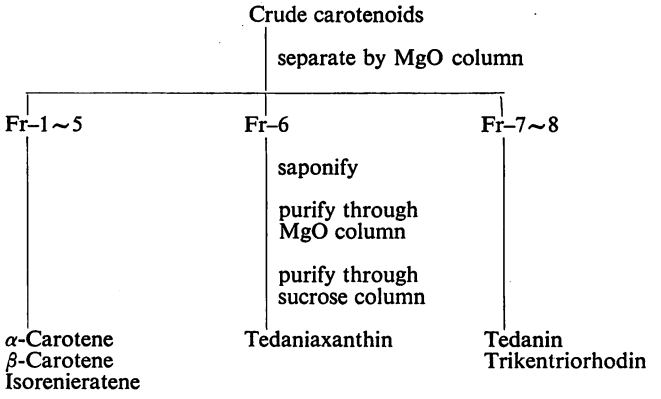


Fig. 7. Isolation of tedaniaxanthin from the crude carotenoids of *Tedania digitata* SCHMIDT.

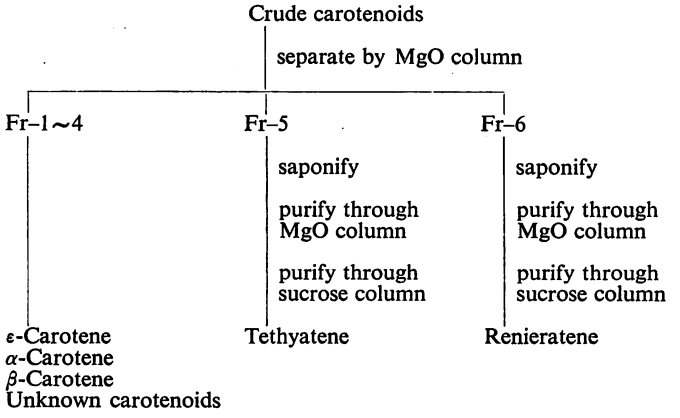


Fig. 8. Isolation of tethyatene and renieratene from the crude carotenoids of *Tethya amamensis* FHIELE.

Also tethyatene was crystallized from *n*-hexane-ethyl ether and its structural formula was proposed by visible absorption spectrum, mass and IR spectra.

Results and Discussion

i) Tedanin

The purified pigment has following characteristics: m.p. 172–173°C, the absorption spectra and the wavelength at λ_{max} in several solvents are shown in Fig. 9 and Table 9.

The pigment was reduced by adding sodium borohydride as already mentioned in II–4–c–1. The absorption spectra of the reduced pigment was also shown in Fig. 9.

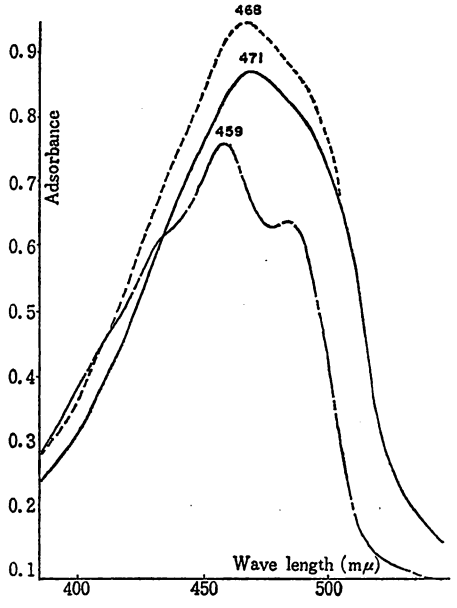


Fig. 9. Characteristic absorption spectra of tedanin.
——, in petroleum ether; ----, in ethanol; ——, after reduction.

Table 9. Wave length at the maximum absorption of tedanin in several solvents.

Solvents	λ_{max} (nm)
Petroleum ether	471
Benzene	486
Carbon disulfide	506
Pyridine	489
Toluene	485
Chloroform	486
Ethanol	468

The high resolution mass spectrographs of this pigment indicated such an empirical formula as $C_{40}H_{48}O_2$=560.36543 (sample: 560.36183). Mass spectrograph shows the peaks at m/e values of M–15 (–CH_3), M–79, M–92, M–106, M–133, M–152, M–158, M–165, M–186, M–198, M–205, M–217, M–231, at high mass region and 43, 69, 91, 133, 157, 173 at low mass region (Fig. 10, 11).

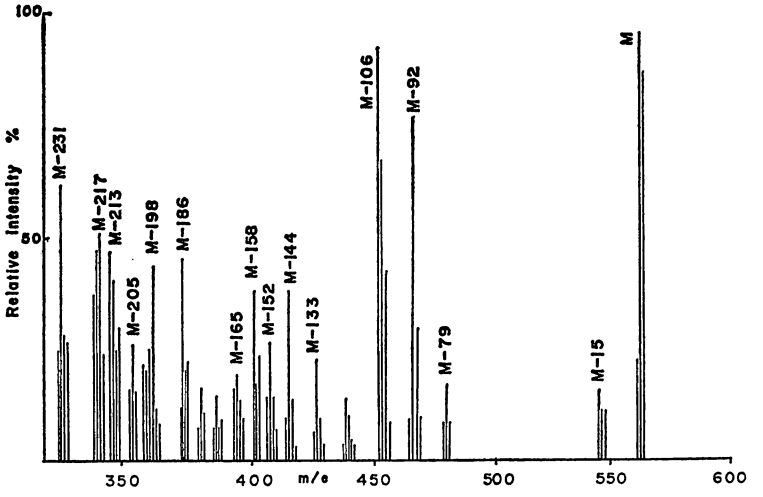


Fig. 10. Mass spectrum of tedanin.

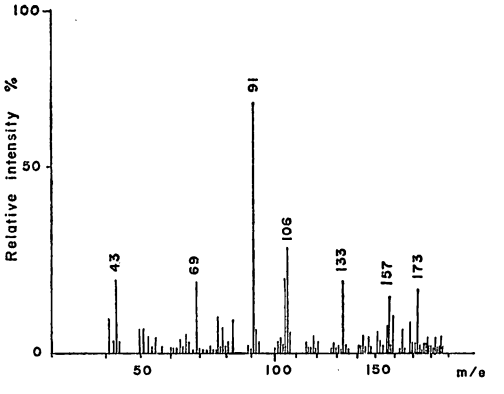


Fig. 11. Mass spectrum of tedanin in low mass region.

M–15 (–CH_3) shows the elimination of a CH_3 fragment. There is a significant M–79 peak which is evidently due to a fragment formed by elimination of a C_6H_7, possibly a methyl cyclopentadienyl radical from the polyene chain[68]. The peaks at M–92 and M–106 are typical ones of carotenoids: M–92 represents the loss of toluene, M–106 the loss of xylene and M–158 the loss of $C_{12}H_{20}$ (presumably the precursor of 2, 6-dimethylnaphthalene) (Fig. 12).

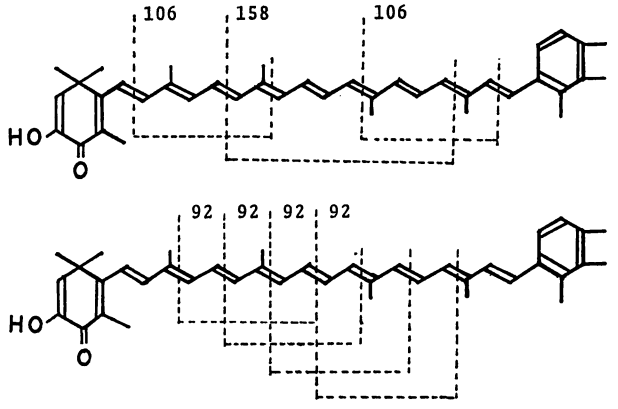


Fig. 12. Schematic representation of the possible ways of formation of M–92, M–106 and M–158 from tedanin.

The fragments of toluene (91), xylene (106) and dimethylnaphthalene (157) can also be detected in the low mass region. As a rule the peaks at M–92, M–106 and M–158 are mainly due to fragments of thermal decomposition products[70].

The compounds possessing the aromatic end groups such as chlorobactene and okenone all exhibit the m/e 133 as an intense peak in the low mass region and the M–133 in the high mass region, while this is not observed in any of the other spectra[69,70]. The peaks at M–173 and M–186 also show the cleavage of 9′–10′ and 11′–12′ bonds (Fig. 13).

The peaks at M–152, M–165, M–205, M–217 and M–231 show the presence of astacene type end group[68,70] (Fig. 13).

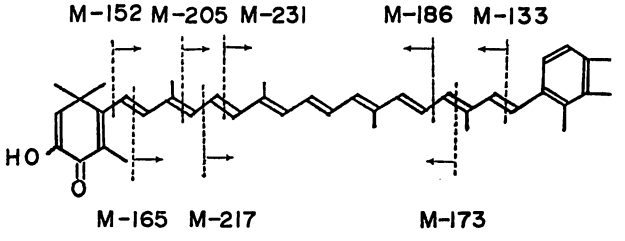


Fig. 13. Schematic representation of the possible ways form ation of M–133, M–173, M–186, M–152, M–165, M–205, M–217 and M–231 from tedanin.

The NMR spectrum of this pigment was measured in $CDCl_3$ using internal TMS at 100 MHz (Fig. 14). There are signals at τ 8.74 (6H), τ 8.05 (6H), τ 8.02 (3H), τ 7.98 (3H), τ 7.95 (3H), τ 7.84 (3H), τ 7.75 (6H) and τ 4.00–2.70 (ca. 18H). Signals in spectral range between τ 4.00–2.70 were not clear, so these signals were not discussed.

There was only one signal as a geminal methyl at τ 8.75 and it shows that one end group has a geminal methyl, but another has no geminal methyl. The signal ranges between τ 8.05

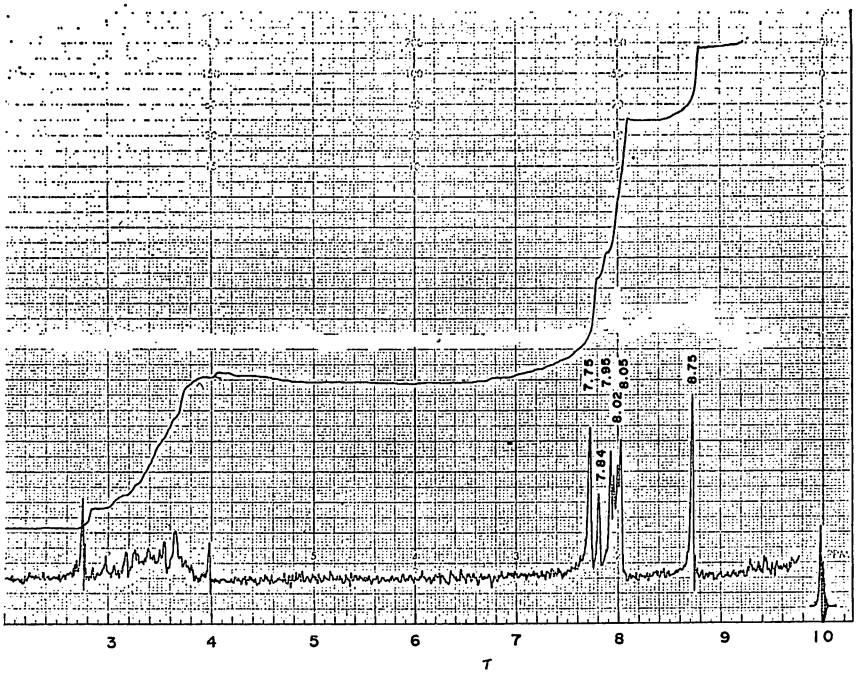


Fig. 14. NMR spectrum of tedanin.

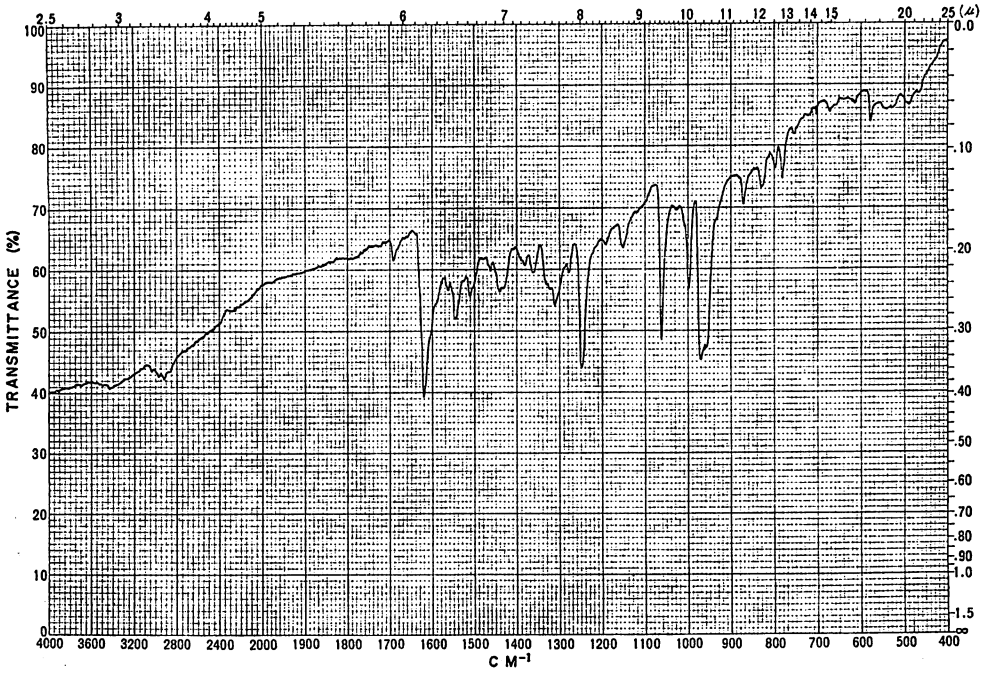


Fig. 15. Infrared spectrum of tedanin.

to τ 7.98 are attributable to the methyl groups on the 9-, 13-, 13′- and 9′-positions of this carotenoid. The signals at τ 7.78 and τ 7.84 are peculiarly unique to the carotenoids possessing the aromatic end group and indicate the existence of χ-carotene type end group such as renierapurpurin[68,71,72,73].

From these results, this pigment can be concluded to be 3-hydroxy-2, 3-didehydro-β, χ-carotene-4-one (tedanin) as shown in Fig. 13. The IR spectrum was also identical with that of tedanin (Fig. 15).

ii) Trikentriorhodin

The purified pigment has such characteristics as m.p. 154–155°C λ_{max} in petroleum ether 480 nm (Fig. 16).

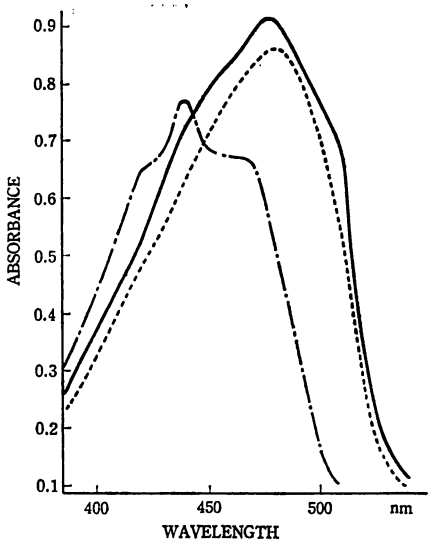


Fig. 16. Characteristic absorption spectra of trikentriorhodin.
——, in petroleum ether; ----, in ethanol; ———, after reduction.

The pigment was reduced by adding sodium borohydride as already mentioned in II–4–c–1. The absorption spectrum of the reduced pigment was shown in Fig. 16.

The high resolution mass spectrograph of this pigment indicated such an empirical formula as $C_{40}H_{52}O_3$=580.39165 (sample: 580.38809). Mass spectrum shows the presence of peaks at M–18, M–79, M–92, M–106, M–124, M–133, M–155, M–186, M–197, M–198 and M–216 in high mass region and 43, 55, 69, 109, 127, 133 and 173 in low mass region (Fig. 17, 18). It was already stated in (i) with respect to the peaks at M–79, M–92, M–106 and M–198 in high mass region and 43, 69 and 91 in low mass region. M–18, M–124 and M–216 show that this pigment has at least one OH group. Among these peaks, M–133, M–186, M–155 and M–187 in high mass region and 109, 127 and 133 in low mass region are characteristic.

The peaks at M–133, M–186 and 133 indicate the existence of aromatic end group as already explained in (i) (Fig. 19).

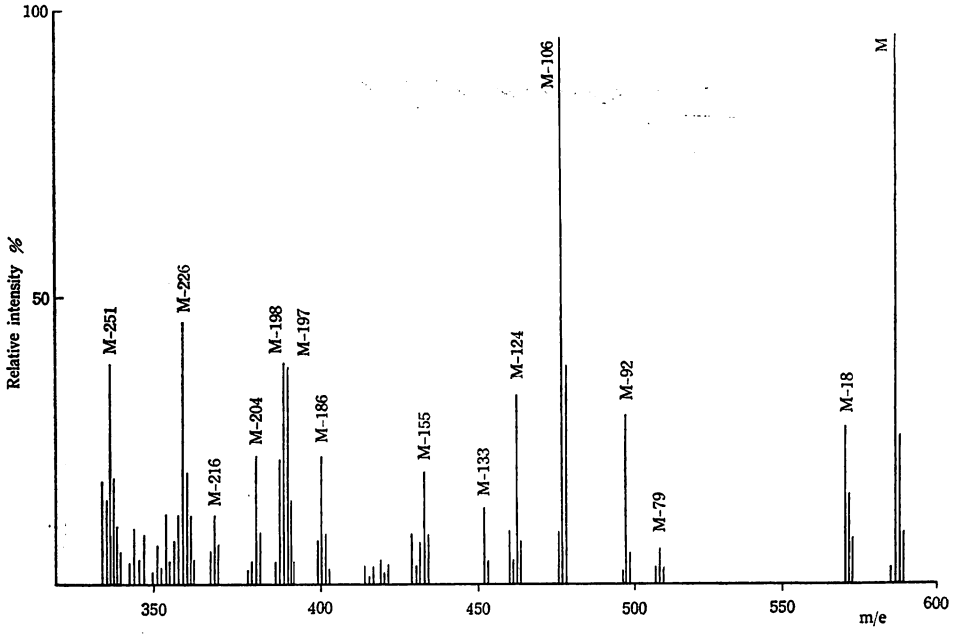


Fig. 17. Mass spectrum of trikentriorhodin.

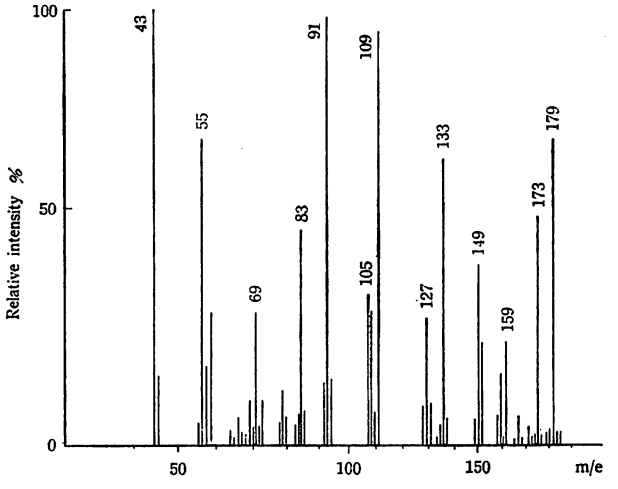


Fig. 18. Mass spectrum of trikentriorhodin in low mass region.

The peaks at M–155, 109 and 127 show the existence of 3-hydroxy-κ-carotene-6-one which was found in capsorubin or capsanthin[74-78]. The peak at M–197 indicates the presence of oxo-group at the 8-position[76,77].

The NMR spectrum of this pigment shows signals at τ 9.16, τ 8.82, τ 8.68, τ 8.03, τ 7.96, τ 7.82, τ 7.72 and τ 4.40–2.80 (Fig. 20). There were three signals at τ 9.16, τ 8.82 and

Fig. 19. Schematic representation of the possible ways of formation of M–18, M–133, M–155, M–186 and M–197 from trikentriorhodin.

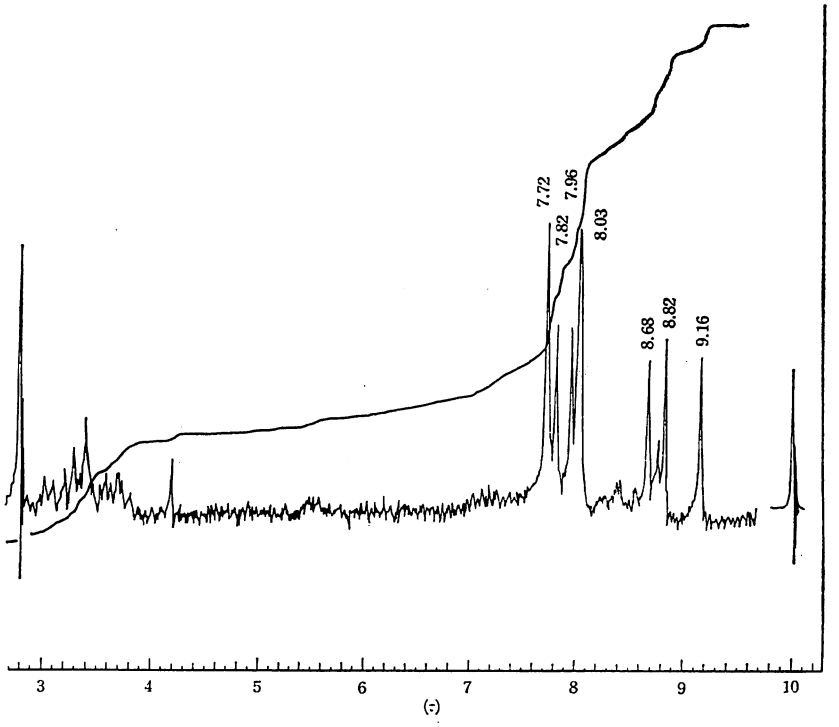


Fig. 20. NMR spectrum of trikentriorhodin.

τ 8.68 as methyl group bound to the saturated carbon and they show one end group to be residual structure of 3-hydroxy-κ-carotene-6-one[74,79–82]. Signals between τ 8.03 to 7.96 are attributable to methyl groups at the 9-, 13-, 13′- and 9′-positions of polyene chain. Signals at τ 7.82 and τ 7.72 are unique to the carotenoids possessing χ-carotene end group[68,71,72,73].

The IR spectrum of this pigment was shown in Fig. 21. The characteristic absorption bands were obtained at 3450 cm^{-1} (–OH), 2950–2860 cm^{-1} ($-CH_3$, $=CH_2$), 1600 cm^{-1} (=CO, β-diketone) and 965 cm^{-1} (trans –CH=CH–).

From the mass spectrum and NMR spectrum, it is clear that this pigment possesses 3-

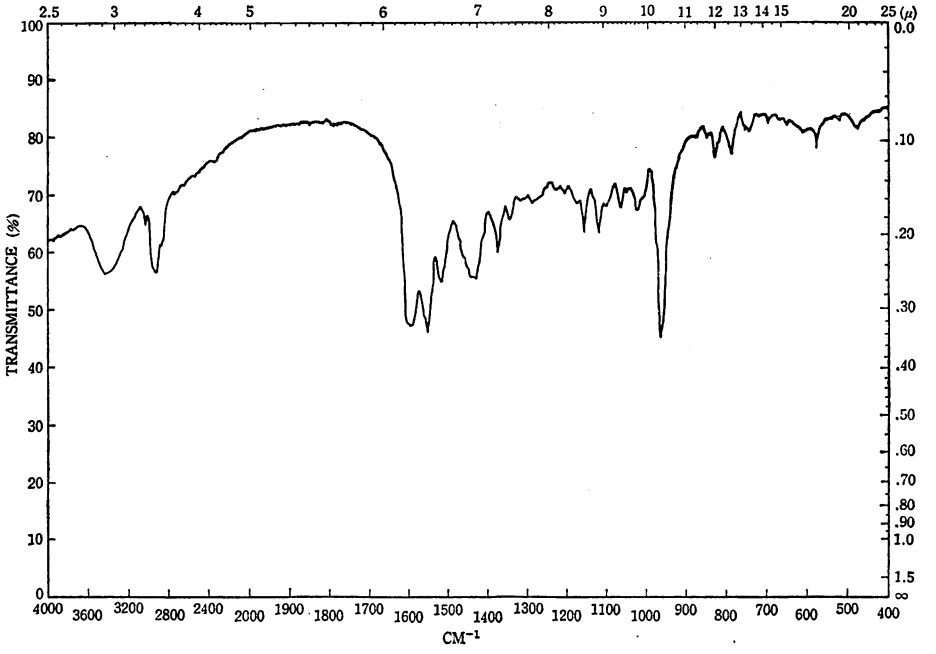
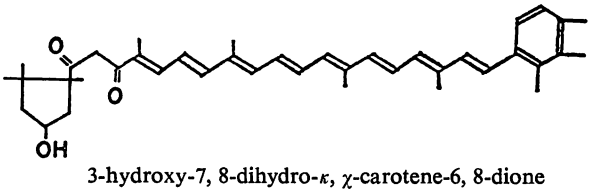
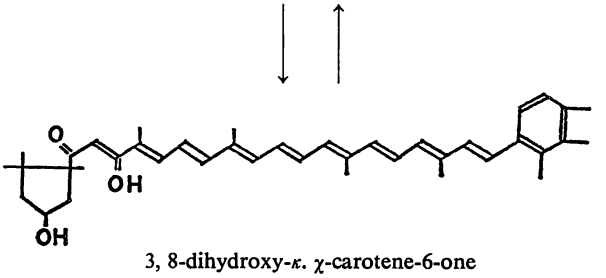


Fig. 21. Infrared spectrum of trikentriorhodin.

hydroxy-κ-carotene-6-one at one end and χ-carotene at opposite end of the molecule. The characteristic IR absorption due to carbonyl group was observed at 1600 cm^{-1}, though IR absorption of carbonyl group of 3-hydroxy-κ-carotene-6-one should be observed at 1650 cm^{-1} [79,81]. This shows that this carotenoid has two oxo groups at the 6-position and 8- or 4-position, giving β-diketone[83,84]. If there are two oxo groups at the 6- and β-position



3-hydroxy-7, 8-dihydro-κ, χ-carotene-6, 8-dione



3, 8-dihydroxy-κ. χ-carotene-6-one

Fig. 22. Structure of trikentriorhodin.

of the 6-position without changing the structure of 3-hydroxy-κ-carotene-6-one at one end of the molecule, this β-position should be the 8-position. The peak at M–197 can support this configuration of β-diketone (Fig. 19).

The structure of this carotenoid was confirmed to be 3-hydroxy-7, 8-dihydro-κ, χ-carotene-6, 8-dione on the basis of these mass, NMR and IR spectra. A compound with β-diketone has been well known to be tautomeric.[83,85] Thus, the tautomer of carotenoid must be 3, 8-dihydroxy-κ, χ-carotene-6-one as shown in Fig. 22.

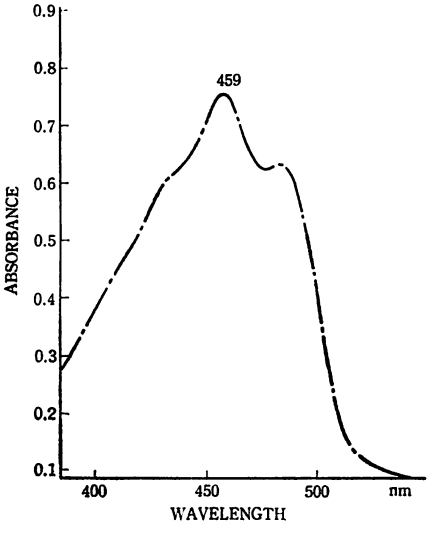


Fig. 23. Characteristic absorption spectrum of tedaniaxanthin.

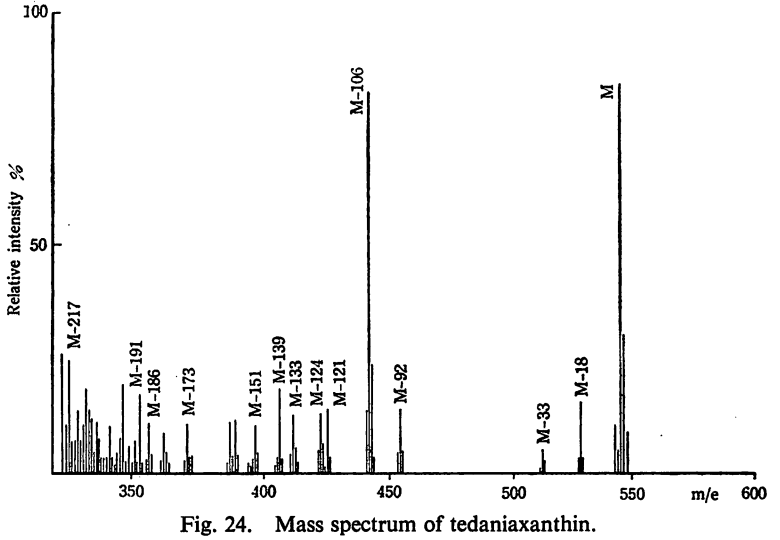


Fig. 24. Mass spectrum of tedaniaxanthin.

iii) Tedaniaxanthin

The isolated pigment has such characteristics as m.p. 164–165°C, λ_{max} in petroleum ether 433, 459, 486 nm (Fig. 23).

The high resolution mass spectrograph of this pigment indicated such an empirical formula as $C_{40}H_{50}O$=546.38617 (sample: 546.386044). The mass spectrograph shows the presence of peaks at M–92, M–106, M–133 and M–151 (Fig. 24). The peak at M–133 shows the existence of such carotenoids as renieratene, renierapurpurin, chlorobactene and okenone having an aromatic end group at least[69,70,77]. The peaks at M–92 and M–106 are typical ones common to carotenoids. The peaks at M–151, M–191 and M–217 show the presence of mono-hydroxy-7, 8-didehydro-β-carotene end group (Fig. 25).

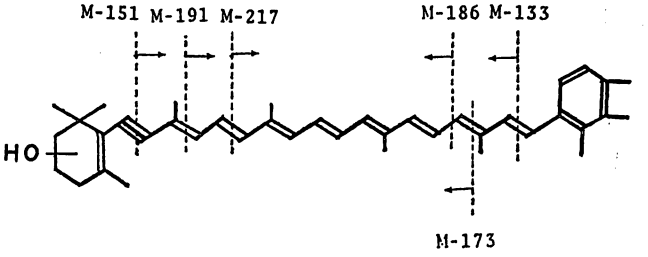


Fig. 25. Schematic representation of the possible ways of formation of M–133, M–173, M–186, M–151, M–191 and M–217 from tedaniaxanthin.

The NMR spectrum revealed C-methyl resonance at τ 7.72 and τ 7.81, indicating the existence of χ-carotene end group as renierapurpurin[68,73]. These signals at τ 8.87, τ 8.81 and τ 8.08 suggested the existence of 3-hydroxy-7, 8-didehydro-β-carotene (Fig. 26).

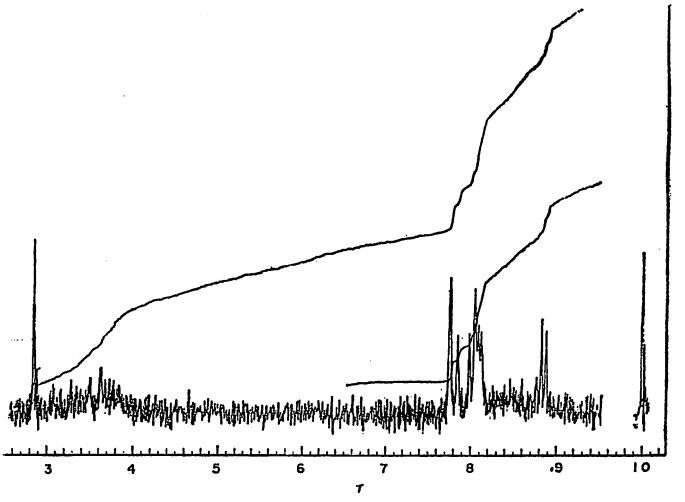


Fig. 26. NMR spectrum of tedaniaxanthin.

The IR spectrum shows characteristic absorption bands at 3440 cm^{-1} (hydroxy) and 975 cm^{-1} (conjugated double bonds) (Fig. 27).

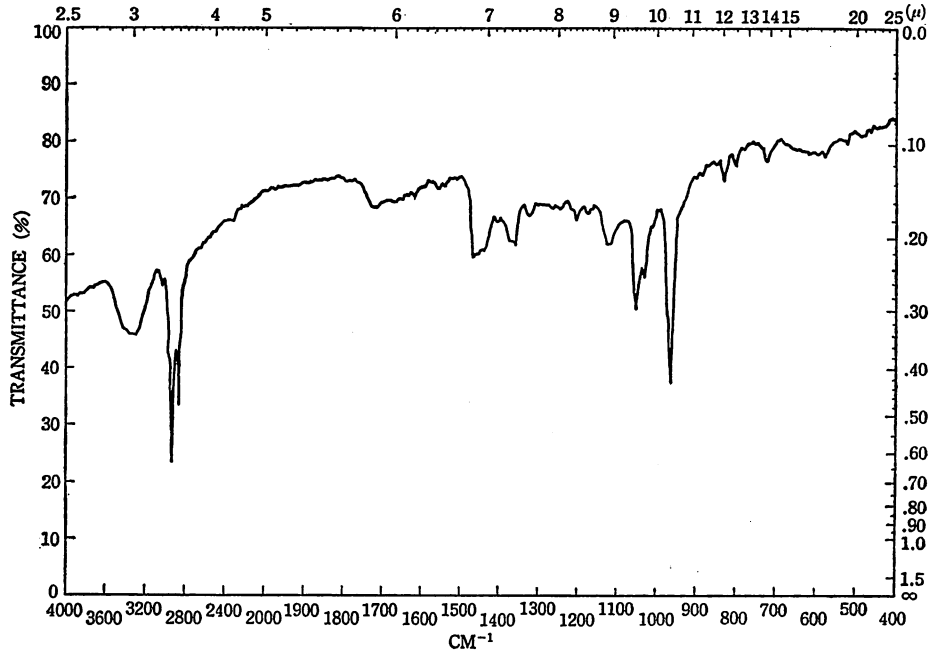


Fig. 27. Infrared spectrum of tedaniaxanthin.

From these results, the position of hydroxy group linking to 7, 8-didehydro-β-carotene is still ambiguous. In the case of hydroxylated carotenoids, the position of carbon linking to hydroxy group is generally the 2-, 3- or 4-position. Therefore, the author performed such methylation test and epoxide test as already mentioned in II–4–c for determining this position. The methylation test can be positive when hydroxy group links to allylic position in β-ionone ring, but negative if it does not link to the allylic position. Since the methylation test was negative, the position of hydroxy group was regarded to be the 2- or 3-position in β-ionone ring. And the negativity of epoxide test of this carotenoid suggested the possibility of 3-position.

The proposed structure of this carotenoid based on these visible absorption, IR, NMR,

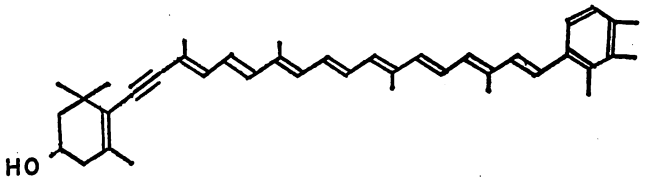


Fig. 28. Proposed structure of tedaniaxanthin.

mass spectra, and the results of methylation and epoxide tests is shown in Fig. 28.

iv) Tethyatene

The isolated pigment has such characteristics as m.p. 139–141°C, λ_{max} in petroleum ether 468 nm (Fig. 29). This pigment was reduced with sodium borohydride. The absorption maximum of this pigment did not shift by the reduction as demonstrated in Fig. 29.

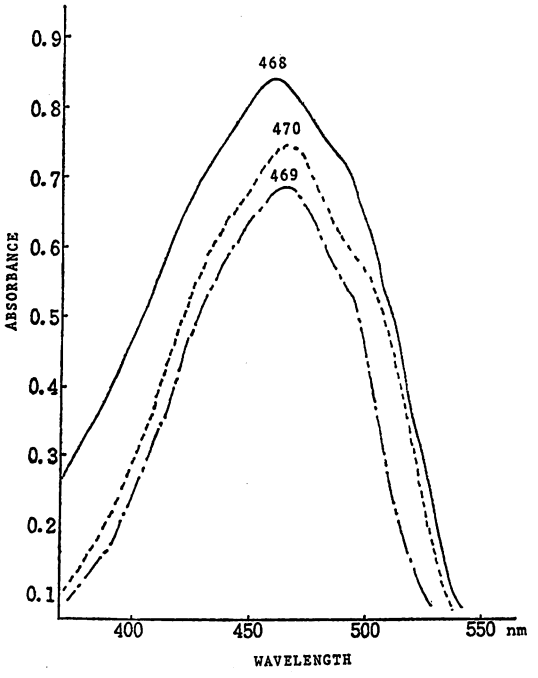


Fig. 29. Characteristic absorption spectrum of tethyatene.
——, in petroleum ether; ----, in ethanol; ———, after reduction.

The high resolution mass spectrograph of this pigment indicated such an empirical formula as $C_{40}H_{50}$=530.39120 (sample: 530.38459). The mass spectrum shows the presence of peaks at M–2, M–15, M–92, M–106, M–133, M–158, M–175, M–188, M–198 and M–201 (Fig. 30).

The visible absorption spectrum suggested this carotenoid to be a keto-carotenoid, but both high resolution mass spectrum and IR spectrum, and the result of reduction test indicated that this carotenoid is a hydrocarbon, but not a keto-carotenoid. The mass spectrum giving these peaks of M–135, M–175, M–188 and M–201 shows the presence of a didehydro-β-carotene end group[68]. Among these carotenoids possessing a didehydro-β-carotene end group, only a 3, 4-didehydro-β-carotene gives such an absorption spectrum as that of keto-carotenoid.[86,87] These peaks in mass spectrum and visible absorption spectrum strongly support that this pigment has a 3, 4-didehydro-β-carotene end group at least.

Another end group of this pigment is regarded to be an aromatic ring because of the presence

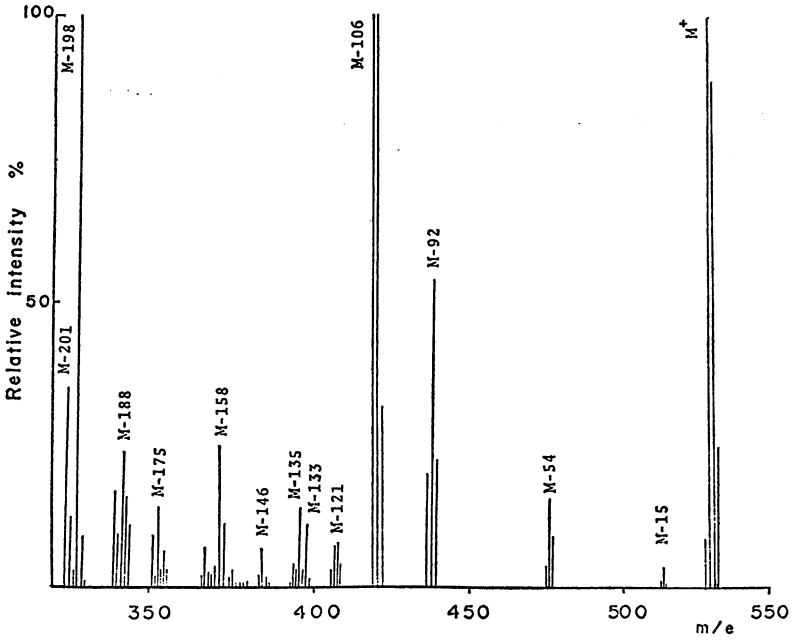


Fig. 30. Mass spectrum of tethyatene.

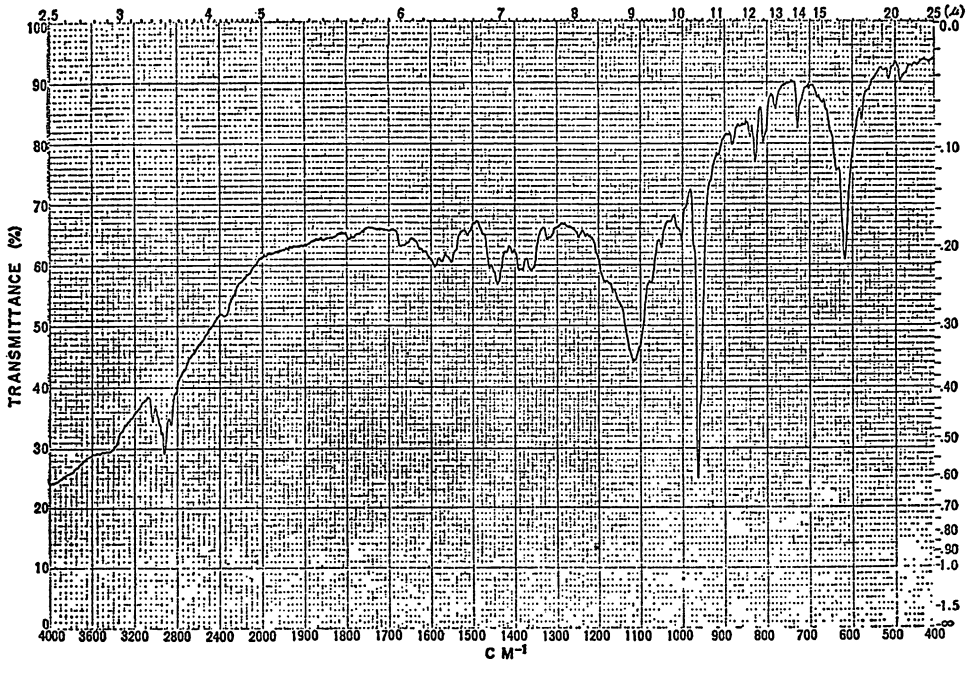
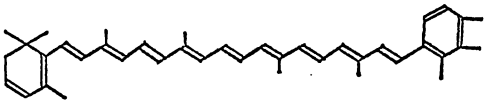


Fig. 31. Infrared spectrum of tethyatene.

of peak at M–133 as exemplified in mass spectra of chlorobactene and okenone[68,72]. The visible absorption maximum of this pigment shows that the aromatic end group is a χ-carotene residue.

The structural formula of this carotenoid is proposed as shown in Fig. 32. This is a new aromatic carotenoid which was identified as 3, 4-didehydro-β, χ-carotene. Since this aromatic carotenoid was separated from *Tethya amamensis* FHIELE, it was named tethyatene by the author.



3, 4-didehydro-β, χ-carotene

Fig. 32. Proposed structure of tethyatene.

Carotenoids from four species of sea sponges are listed in the order of elution from columns together with their relative abundances in Table 10.

Table 10. Relative abundances of the carotenoids in sea sponges.

Carotenoids	Relative abundances (%)			
Names	*Clathria frondifera*	*Tedania digitata*	*Haliclona permollis*	*Tethya amamensis*
ε-Carotene	—	—	—	7
α-Carotene	4	2	—	9
β-Carotene	7	7	27	11
Tethyatene	—	—	—	16
Isorenieratene	—	5	—	—
Renieratene	—	—	—	39
Lutein	—	—	9	—
Zeaxanthin	—	—	10	—
Tedaniaxanthin	5	16	—	—
Tedanin	55	37	—	—
β-Doradexanthin	—	—	10	—
Astaxanthin	—	—	31	—
Trikentriorhodin	14	24	—	—
Unknown	17	9	13	18

It is very difficult to find out the possible precursor of tedanin among α-carotene, β-carotene, isorenieratene, tedaniaxanthin and trikentriorhodin in *Clathria frondifera* and *Tedania digitata*.

The possible metabolic pathway of astaxanthin in *Haliclona permollis* can be proposed as follow: Zeaxanthin→β-Doradexanthin→Astaxanthin.

It has been reported by GOODWIN[88] that in sponges, hydrocarbon carotenoids preponderate over the oxygen-containing xanthophylls which are absent frequently. However, their distributions of carotenoids in three species of *Clathria frondifera* (BOWERBANK), *Tedania digitata*

SCHMIDT and *Haliclona permollis* (BOWERBANK) show that the main carotenoid is such keto-carotenoids as tedanin or astaxanthin, but not hydrocarbon carotenoids. Therefore, the emphasis of GOODWIN seems to be of limited value.

Seemingly, these sea sponges can be divided into two groups according to their main carotenoid: a) Sponges containing hydrocarbon carotenoids, b) Sponges containing keto-carotenoids.

It is very interesting to note that only sea sponges have many aromatic carotenoids, excepting bacteria and algae[89-94]. This suggests that microorganisms which are commensal with or ingested by sea sponges, may synthesize these aromatic carotenoids, otherwise, these sea sponges should have the ability of converting β-ionone ring into aromatic ring.

The existence of tethyatene suggests that there can be such a conversion that β-ionone ring changes into aromatic ring as φ-carotene ring in sea sponges, because 3, 4-didehydro-β-carotene ring can be regarded as an intermediate from β-ionone ring to φ-carotene ring[95]. The same reaction might be occurred in sea sponges, but the possible precursor of χ-carotene ring has not been elucidated as yet.

III–5. Echinodermata

The carotenoids in starfish were first studied by EULER[96] who isolated "Asterinsäure" from *Asteria rubens*. SØRENSEN et al.[97] confirmed that "Asterinsäure" were identical with astaxanthin, and main carotenoid was considered to be astaxanthin in starfish. DE NICOLA[98] reported the existence of β-carotene, echinenone, hydroxy-ketocarotenoids and astaxanthin in *Ophidiaster ophidianus*. He also reported the existence of β-carotene, echinenone, hydroxy-ketocarotenoids and astaxanthin in *Asteria panceri*[99].

FRANCIS et al.[100] recently showed that "Asterinsäure" was a mixture of 7, 8, 7′, 8′-tetradehydroastaxanthin and 7, 8-didehydroastaxanthin, and they isolated 7, 8, 7′, 8′-tetradehydroastaxanthin in crystalline state, but not 7, 8-didehydroastaxanthin as pure crystals.

The author isolated an astaxanthin like pigment from Onihitode *Acanthaster planci* and confirmed it to be 7, 8-didehydroastaxanthin. The existence of β-carotene, echinenone, lutein, zeaxanthin and 7, 8-didehydroastaxanthin was also clarified by the author in these starfish such as Yatsudehitode, *Coscinasterias acutispina*, Ooakahitode, *Leiaster leachi*, and Hitode, *Asteria amurensis*. Besides these carotenoids, α-cryptoxanthin, canthaxanthin, tunaxanthin diatoxanthin and 7, 8, 7′, 8′-tetradehydroastaxanthin were found in Onihitode.

Materials and Methods

1. Method of determining the structure of an astaxanthin like pigment from Onihitode:

Onihitode were collected from the coastal waters of Okinawa. The pigments were extracted from the shell of the animals with acetone as already mentioned in II–1. The pigments were dissolved in about 10 ml of petroleum ether and subjected to the column chromatography using magnesium oxide — Hyflosupercel (1 : 2) and petroleum ether. The extract from the main band was mixed with petroleum ether, washed well with water and evaporated under

vacuum. The pigments were dissolved in about 20 ml of petroleum ether and subjected again to the chromatography using a sucrose column and petroleum ether. The isolated pigment from the main band was crystallized from *n*-hexane.

This pigment was reduced with sodium borohydride and the absorption spectrum of the reduced pigment was measured. The high resolution mass spectrum of this pigment was also measured. The crystalline pigment was ground with potassium bromide, pressed to make a disc and the infrared spectrum was measured.

2. Separation and identification of the carotenoids in starfish:

Ooakahitode, Yatsudehitode and Hitode were collected in the bay of Kinko, Kagoshima. The carotenoids were extracted with acetone, using the same method as already mentioned. The carotenoids were purified through the same column chromatography and the purified carotenoids were identified by their absorption spectra, nature on elution from column, colors and co-chromatography with authentic carotenoids.

Results and Discussion

The isolated astaxanthin like pigment has such characteristics as m.p. 187–189°C, λ_{max}, in *n*-hexane 473 nm (Fig. 33). The λ_{max} of this pigment shifted from 475 nm to 449 nm in ethanol by reduction (Fig. 34). The characteristic absorption spectrum of this pigment resembles those of astaxanthin and 7, 8, 7′, 8′-tetradehydroastaxanthin in various solvents

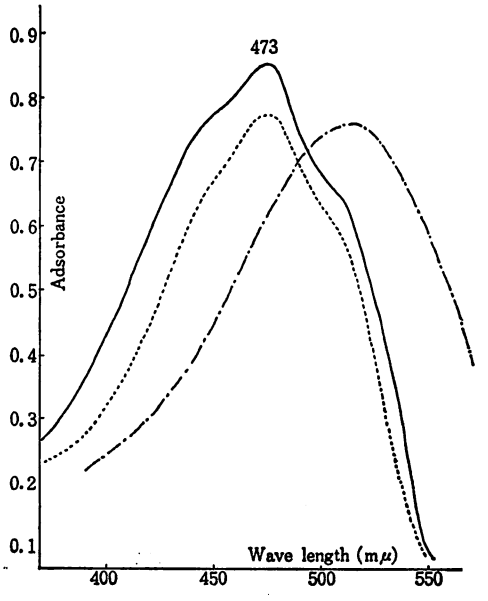


Fig. 33. Characteristic absorption spectrum of 7, 8-didehydroastaxanthin.

——, in hexane; — — —, lin pyridine; - - - -, in benzene.

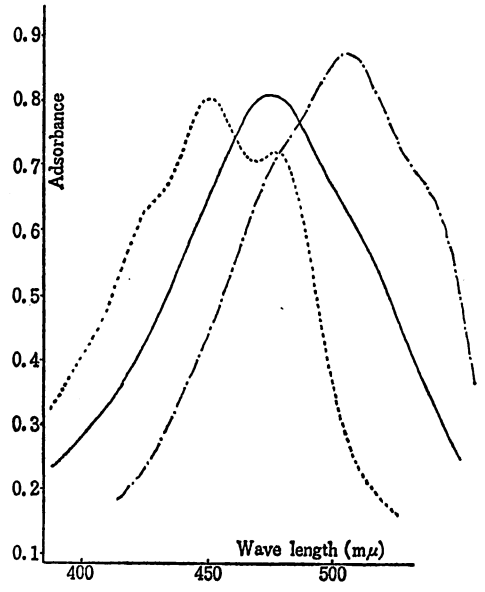


Fig. 34. Characteristic absorption spectrum of 7, 8-didehydroastaxanthin.
——, in EtOH; ———, in CS_2; ----, after reduction.

(Figs. 33, 34).

The high resolution mass spectrum of this pigment indicated that this pigment has an emperical formula $C_{40}H_{50}O_4$=594.37091 (sample: 594.36826). In the mass spectrum of this pigment, the peaks at M–92 and M–106 are due to the typical fragments formed from carotenoids as already mentioned in III–4. The peaks at M–16, M–32, M–153, M–167, M–207, M–219, M–231, M–233, M–245 are also observed (Figs. 35, 36).

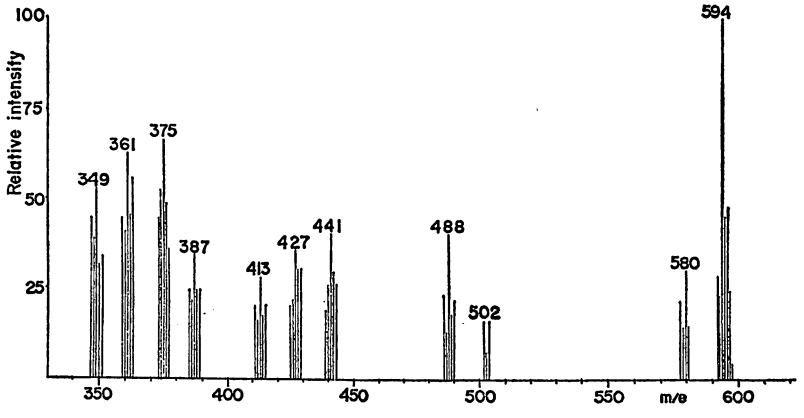


Fig. 35. Mass spectrum of 7, 8-didehydroastaxanthin.

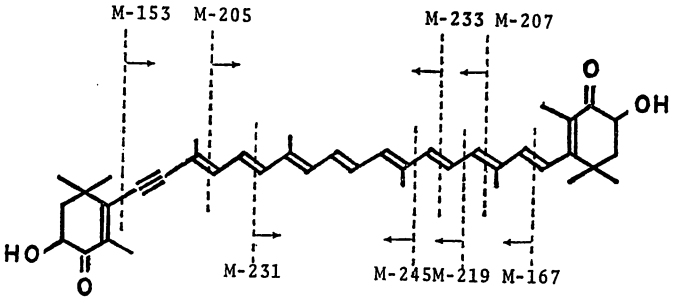


Fig. 36. Schematic representation of the possible ways of formation of M–153, M–205, M–231, M–167, M–207, M–219, M–233 and M–245 from 7, 8-didehydroastaxanthin.

The occurrence of peaks at M–16 and M–32 indicates the presence of two hydroxy groups, the main peaks except M–153 and M–231 can be seen in the mass spectrum of astaxanthin (Table 11). The peak at M–153 indicates the presence of the triple bond at 7, 8-position and also can be found in the mass spectrum of 7, 8, 7′, 8′-tetradehydroastaxanthin, but not in that of astaxanthin (Fig. 36) (Table 11).

Table 11. Characteristic fragments of astaxanthin like pigment, astaxanthin and 7, 8, 7′, 8′-tetradehydroastaxanthin.

Fragments	Astaxanthin like pigment	Astaxanthin	7, 8, 7′, 8′-Tetradehydroastaxanthin
M	594	596	592
M–16	578	580	*
M–32	562	564	*
M–92	502	504	500
M–106	488	490	486
M–153	441	—	439
M–154	—	442	—
M–167	427	429	—
M–205	*	—	387
M–207	387	389	—
M–219	375	377	—
M–231	363	—	361
M–233	361	363	—
M–245	349	351	—

*: not clear peak

The characteristic infrared absorption bands were obtained at 3400 cm^{-1} (–OH), 2940 and 2850 cm^{-1} ($-CH_3$, $=CH_2$), 2150 cm^{-1} (–C≡C–), 1600 cm^{-1} (=C=O) as indicated in Fig. 37.

From these results, the astaxanthin like pigment was confirmed to be 7, 8-didehydroastaxanthin with the structure indicated in Fig. 36.

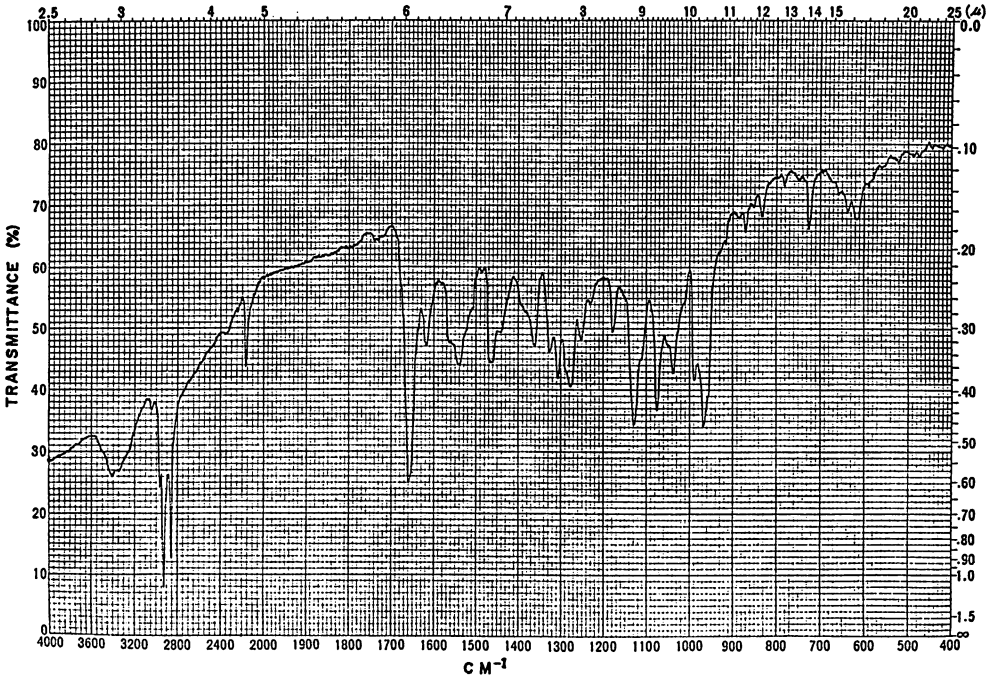


Fig. 37. Infrared spectrum of 7, 8-didehydroastaxanthin.

Table 12. Spectral characteristics and relative abundances of the carotenoids in starfish.

Pigments	Spectral characteristics		Relative abundance (%)			
	λ_{max} (nm) in petroleum ether	λ_{max} (nm) after reduction	*Acanthaster planci*	*Leiaster leachi*	*Asterias amurensis*	*Coscinasterias acutispina*
α-carotene	432, 444, 466					trace
β-carotene	430, 451, 477		1.5	12.3	5.6	9.0
Echinenone	455	428, 449, 477	3.3	0.4	0.6	trace
α-cryptoxanthin	423, 444, 472		2.4			
Canthaxanthin	460	—, 448, 476	2.0			
Lutein	424, 444, 473		5.3	3.6	2.1	3.6
Zeaxanthin	—, 448, 475		4.7	10.3	11.1	12.0
Tunaxanthin	418, 441, 471		1.1			
Diatoxanthin	—, 449, 476		5.1			
Astaxanthin	472	—, 451, 477	2.6			
7, 8-Didehydroastaxanthin	473	—, 451, 478	63.2	64.3	68.3	50.9
7, 8, 7′, 8′-Tetradehydroastaxanthin	472	—, 450, 477	3.0			15.7
Unknown			5.7	9.0	12.3	8.6

The carotenoid pigments in starfish: Onihitode, *Acanthaster planci*, Ooakahitode, *Leiaster leachi*, Hitode, *Asteria amurensis*, and Yatsudehitode, *Conscinasterias acutispina* are listed in Table 12.

The major carotenoid in starfish was considered to be astaxanthin. TSUMAKI et al.[101] also reported that the major carotenoid in Itomakihitode, *Asteria pectinifera* was astaxanthin. However, the evidence presented here shows that the main carotenoid in four kinds of starfish is 7, 8-didehydroastaxanthin, and it is the first time to prove the occurrence of 7, 8-didehydroastaxanthin with the isolation in crystalline state in combination with physico-chemical characteristics.

The chromatographic separation of the carotenoids from Ooakahitode, Yatsudehitode and Hitode revealed the existence of β-carotene, echinenone, lutein, zeaxanthin and 7, 8-didehydroastaxanthin in these starfish. Besides these carotenoids, α-cryptoxanthin, cantha-xanthin, tunaxanthin, astaxanthin, diatoxanthin and 7, 8, 7′, 8′-tetradehydroastaxanthin were found in Onihitode.

IV. Metabolic pathways of carotenoids in specialized aquatic animals

By separating and identifying the carotenoids in various aquatic animals, the metabolic pathways were discussed in the previous chapter.

The present author tried to delineate more in detail the metabolic pathways from plant carotenoids to astaxanthin by measuring the assimilation of astaxanthin in their flesh after having fed tiger prawn, goldfish and red sea bream on a certain diet added with various carotenoids for finding out the precursors of astaxanthin.

HSU, HATA and HATA, and KATAYAMA already confirmed metabolic pathways by feeding aquatic animals with labelled carotenoids, but those results were not always consistent with one another.

The attention should be focused to the following two points brought about during the investigation of bioconversion of labelled carotenoids to astaxanthin in aquatic animals.

1. In various aquatic animals, astaxanthin occurs as ester forms rather than free one. It is very difficult to isolate astaxanthin ester from other carotenoids, since there exists astaxanthin in the forms of monoester or diester. So it is the most convenient method to purify them as astacene after having saponified.

When astacene was purified on such strong adsorbent columns as silica gel, MgO, alumina or Micro-Cel C columns, astacene was adsorbed on the top of column and hardly eluted from the column by using polar solvent. As shown in Table 1, β-carotene tends to decompose when purified on the columns packed with strong adsorbents and the decomposed product remains on the top of column. When astacene is separated from the other carotenoids by using columns packed with adsorbents, an astacene fraction is usually contaminated with these decomposed products.

For clarifying the metabolic pathways of carotenoids, ^{14}C- or ^{3}H-labelled carotenoids are often utilized. When the astacene fraction is separated from the labelled carotenoids through these column chromatography after feeding tests, this fraction tends to be contaminated with

the decomposed labelled carotenoids, even though test animals actually could not convert the administrated carotenoids into astaxanthin. This often lead us to erroneous conclusions.

In order to exclude these failures, the separated astacene fraction should be purified through such a weak adsorbent column as a sucrose column after having separated this fraction using a certain strong adsorbent column.

2. Another point is that colored samples must be bleached with U.V.-light and then left overnight in the dark place to allow the U.V.-light induced chemical luminescence to decay before counting the radioactivities of the labelled carotenoids using a scintillation counter. If we omit these operations, the astaxanthin fraction gives usually an extraordinary high radioactivity.

From these reasons as medtioned above, there is some doubt on the results of feeding tests using radioactive carotenoids.

In order to make clear the metabolic pathways from plant carotenoids to astaxanthin, the author fed aquatic animals on the artificial diet added with pure carotenoids and compared the relative contents of astaxanthin in their tissues of test animals with those in the control group. The most conclusive and practical evidence could be obtained by this method.

The author is now intending to elucidate systematically the oxidation position of β-ionone ring through these feeding tests of tiger prawn, goldfish and red sea bream with zeaxanthin and canthaxanthin.

IV-1. Crustacea

The pigments of three variants of the marine isopoda, *Idotea montereyensis* and *Idotea granulosa* (RATHKE) were investigated by LEE[102,103] 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 suggested as follows:

β-carotene⟶echinenone⟶4-hydroxy-4′-keto-β-carotene⟶canthaxanthin

LEE[104,105] also investigated pigmentation of isopod, *Idotea resecata*.

GILCHRIST and LEE[22] isolated α-carotene, β-carotene, echinenone, isocryptoxanthin, canthaxanthin, lutein, zeaxanthin lutein-5, 8-epoxide, astaxanthin and 4-hydroxy-4′-keto-β-carotene from *Carcinus maeans*, a certain Crustacea, Decopoda. A metabolic pathway in this animal was also proposed.

DAVIES et al.[17] found that California strains of *Artemia salina* converted β-carotene to echinenone and echinenone to canthaxanthin without any apparent intermediate.

HERRING[33] studied the carotenoids in *Daphnia magna* fed with *Chlorella pyrenoidosa* and isolated β-carotene, echinenone, canthaxanthin, a detocarotenoid (probably 3-hydroxy-4-keto-β-carotene) and astaxanthin. He suggested that the animals can form echinenone, canthaxanthin and astaxanthin from β-carotene. Alloxanthin has been isolated from the commensal crab, *Pinnotheres pisum*, during the study of the carotenoid metabolism by CAMBELL[35] and from the sand crab, *Emerita analoga*, during the reproducible study of possible role of carotenoids by GILCHRIST and LEE[36]. The origin of this pigment appears to be algal carotenoids consumed by these crabs. Similarly, a common algal pigment, fucoxanthin, was isolated

from barnacles, *Lepas fascicularis* and *Lepas anacles* and from their faecal pellets[37].

KATAYAMA et al.[43-45] confirmed that a prawn and a lobster can convert β-carotene to astaxanthin when fed these crustaceans with $^{3}H_2$-labelled β-carotene, and a metabolic pathway in these animals was proposed as follows: β-carotene→echinenone→canthaxanthin→phoenicoxanthin→astaxanthin.

Tritium tends to move from originally labelled compounds to the other compounds while purified, so the author fed tiger prawns on ^{14}C-labelled β-carotene and canthaxanthin respectively in order to confirm the metabolic pathway proposed by KATAYAMA et al.[43].

As already mentioned in chapter II–2, besides β-carotene it is possible that zeaxanthin and lutein may be the precursors of astaxanthin in these animals. Recently the existence of β-doradexanthin in some crustacean was reported by SIMPSON[106] and MATSUNO et al.[107] and suggested that zeaxanthin might be converted to astaxanthin. The author confirmed that there is another metabolic pathway from zeaxanthin to astaxanthin, but not from lutein to astaxanthin in tiger prawn thorugh feeding tests of zeaxanthin and lutein.

It was confirmed that tiger prawns can convert β-carotene, canthaxanthin and zeaxanthin to astaxanthin, and also transfer dietary astaxanthin to tissues.

At the same time, the pigmentation of tiger prawns was confirmed to be improved by feeding these carotenoids.

Spirulina, a blue green algae rich in β-carotene and canthaxanthin were found to imporve their coloration of tiger prawns by feeding them.

Materials and Methods

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

a) Preparation of ^{14}C-labelled β-carotene.

Phycomyces blakeleeanus was grown aerobically in 1 liter Erlenmeyer flask. To each flask containing 300 ml of a certain cultural medium, which CHICHESTER et al.[108] used for incorporating labelled leucine into carotene of *Phycomyces*, the spore suspension of this organism was added. Mevalonic acid-2-^{14}C was added to this medium so as to become the radioactivity of 0.2 mCi/l. The cultures were kept on a shaker under scattered light for a week at 25±2°C. The mycelium mats were harvested at the point when mycelium began to turn yellow. The mats were disrupted in acetone by using Waring blender. The homogenate was filtered and treated by the way as already stated in II–1 and purified through a MgO column (magnesium oxide — Hyflosupercel=1: 2). The β-carotene fraction was purified again through an alumina column. Beta-carotene was eluted from a specified band on the alumina column with acetone and crystallized from mixed solvents of petroleum ether and ethanol, then dried in a vacuum desicator.

b) Radioactive β-carotene feeding experiment.

Sea sand was placed in a tank (60×30×30 cm) at the thickness of 5 cm. The sea water was kept at 25°C and aerated at a rate of 400 ml per minute. Thirty tiger prawns (ca. 0.8 g) were acclimatized for five days with the artificial diet (Table 13). Carbon fourteen labelled

Table 13. Composition of the artificial diet for feeding tests.[43]

Constituent	Amount	Constituent	Amount
Defatted soybean powder	200.0 g.	Inositol	100.0 mg.
Dry pulp yeasts	40.0 g.	*p*-Aminobenzoic acid	50.0 mg.
Mineral mixture*	15.0 g.		
DL-Methionine	1.0 g.		
Green algae powder	4.0 g.	Salt 1	
Unpurified soybean oil	24.0 g.	K_2HPO_4	2.31 g.
Cholesterol	0.2 g.	KCl	0.724 g.
Vitamin A (natural)	4000 I.U.	$MgSO_4$	1.14 g.
Vitamin B_1 (thiamin-HCl)	2.5 mg.	$FeSO_4 \cdot 7H_2O$	0.108 g.
Vitamin B_2	25.0 mg.	$Ca_3(PO_4)_2$	2.11 g.
Vitamin B_6 (pyridoxine)	15.0 mg.	$MnSO_4 \cdot 7H_2O$	0.0154 g.
Vitamin C	1000.0 mg.	$CaCO_3$	1.29 g.
Vitamin D	800.0 I.U.	$ZnSO_4$	0.0154 g.
Vitamin E (natural tocopherol)	16.0 mg.	$CuSO_4$	0.0154 g.
Vitamin K_3	250.0 mg.	Salt 2	
Nicotinic acid	150.0 mg.	$CaCO_3$	1.0 g.
Calcium pantothenate	250.0 mg.	$Ca_3(PO_4)_2$	2.0 g.
Choline-HCl	1000.0 mg.	*Mineral mixture: —	
Biotin	0.4 mg.	(Salts 1 and 2 were mixed as	
Folic acid	2.0 mg.	follows: Salt 1: Salt 2=2: 1)	

For the feeding test of red sea bream with this artificial diet, peptone of bonito viscera was mixed in 5% by weight as a good appetizer.

β-carotene was dissolved in soybean oil and added to the artificial diet. Those tiger prawns were fed daily enough with this artificial diet containing ^{14}C-β-carotene for 21 days. The pigments were then extracted and purified as stated in II–1 and II–2.

The purified pigments were transferred to counting vials, dried, dissolved in toluene (5 ml) and bleached with UV-light and left overnight in the dark place for removing the substances emitting the UV-induced luminescence, then the ^{14}C-activity was assayed after adding 5 ml of the double strength scintillation solution (0.1 g POPOP and 1.2 g PPO in 100 ml of toluene) using a Beckman Scintillation System Model LS–230.

2. Feeding tests with zeaxanthin and β-carotene.

Tiger prawns (ca. 10 g) were obtained from a local hatchery and separated into three groups. Each group has ten tiger prawns. The first control group was fed for 21 days with the artificial diet containing pure zeaxanthin for same period. The last third group was fed on the same diet but containing pure β-carotene for the same period. The tiger prawns were collected and the pigments were extracted with acetone and purified as already mentioned in II–3. The pigments were identified through various ways as stated in II–4.

3. Feeding test with lutein.

Prawns (ca. 1 g) were separated into two groups. Each group had fifty tiger prawns. The first control group was fed for 21 days with the artificial diet and the second group on the artificial diet containing 40 mg% lutein for the same period.

The tiger prawns were collected, and the pigments were extracted with acetone and purified as already mentioned in II–3. The amounts of astaxanthin were determined, respectively.

4. Feeding test with astaxanthin.

Fourty tiger prawns (ca. 1 g) separated into two groups and each had twenty tiger prawns. The first control group was fed on the artificial diet and the second group was fed on the artificial diet containing 20 mg% pure astaxanthin for the same period. The purification and identification of the pigments in these tiger prawn were performed in accordance with the procedures as stated in II–3 and II–4.

5. Feeding tests with alfalfa, corn gluten, *Spirulina* and canthaxanthin.

Thirty-five tiger prawns (ca. 1 g) were placed in each tank. Feeding experiments were performed with five kinds of artificial diets, with no special additive (control, Tank No. 1), enriched with 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 tiger prawns were killed and the pigments were extracted and purified on the column by using the same method as already mentioned in II–3.

Results and Discussion

1. Possibilities of conversion from dietary β-carotene and canthaxanthin to astaxanthin.

Table 14 shows the results of feeding tests of tiger prawns with ^{14}C-β-carotene. This tells us the fact that β-carotene converts to astaxanthin in these tiger prawns. The results of the feeding test on the *Spirulina* diet rich in β-carotene are in accord with the test of β-carotene labelled with ^{14}C (Table 18).

Table 14. Conversion of ^{14}C-β-carotene into astaxanthin.
β-Carotene in the food
(Total count: 1,230,000 cpm)
(Specific activity: 820,000 cpm/mg)

Carotenoids in the body	Total counts recovered	Distribution of radioactivity (%)
β-Carotene	712	18.7
Lutein	20	0.5
Zeaxanthin	0	—
Astaxanthin	2988	78.6
Unidentified carotenoids	82	2.1

It was clarified that astaxanthin in tiger prawns[43], a swimming crab[45] and spiny lobster[44] was labelled when they were fed on the artificial diet containing $^{3}H_2$-β-carotene, and it was presumed that β-carotene can be converted to astaxanthin through echinenone, canthaxanthin

and 3-hydroxy-canthaxanthin.

From Table 18, it was confirmed that canthaxanthin supplied with food was converted clearly to astaxanthin in tiger prawn. This result is consistent with the possible pathway proposed by KATAYAMA[43].

2. Possibility of conversion from dietary zeaxanthin to astaxanthin.

Recently the existence of β-doradexanthin[47] in same crustaceans was reported by MATSUNO[107] and SIMPSON[106], these authors suggested that zeaxanthin can be converted to astaxanthin via β-doradexanthin.

Table 15. Possibilities of conversion from dietary β-carotene and zeaxanthin to astaxanthin in tiger prawn.

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

Table 15 showing the results of feeding tests of tiger prawns with pure zeaxanthin tells us that zeaxanthin yields the high concentration of astaxanthin.

In this study, zeaxanthin was fed to tiger prawn and the conversion of this carotenoid to astaxanthin was assured. Therefore, it is clear that there is another metabolic pathway from zeaxanthin to astaxanthin than that from canthaxanthin to astaxanthin in tiger prawn. It is apparent that zeaxanthin represents to make a more efficient pathway to astaxanthin through β-doradexanthin than that from β-carotene to astaxanthin. (Table 15).

3. Possibility of conversion from dietary lutein to astaxanthin.

Table 16 showing the results of feeding tests of tiger prawns with pure lutein indicates that lutein cannot be converted to astaxanthin.

Table 16. Possibility of conversion from dietary lutein to astaxanthin in tiger prawn.

Diet / Pigment	Artificial diet (control)	Artificial diet containing 40 mg% lutein
Astaxanthin found (μg/g body wt.)	4.98	5.09

4. Possibility of transfer from dietary astaxanthin to tissues in tiger prawn.

Table 17 shows us the fact that astaxanthin can be transferred directly to their bodies, probably into the carapaces of tiger prawn.

Tiger prawn can convert β-carotene and canthaxanthin to astaxanthin and also can convert zeaxanthin to astaxanthin via β-doradexanthin.

Table 17. Possibility of transfer from dietary astaxanthin to tissues in tiger prawn.

	Astaxanthin found (μg/g body wt.)
None (control)	1.01
Astaxanthin (20 mg% in the diet)	1.81

5. Pigmentation of tiger prawn by adding several carotenoid sources.

Table 18 lists the results of tiger prawn feeding tests on the artificial diet and those added with 10% corn gluten, 10% alfalfa, 3% canthaxanthin and 10% *Spirulina*.

Spirulina, a blue green algae rich in β-carotene (Table 19), and the synthetic canthaxanthin were found to yield the higher concentration of astaxanthin than either corn gluten or alfalfa. The tiger prawns fed on *Spirulina* showed superlative red and those fed on canthaxanthin had comparable red (Table 18), but the others showed to differ outstandingly in the pigmentation.

Table 18. Contents of astaxanthin in the tiger prawn fed on diets containing corn gulten, alfalfa, canthaxanthin and *Spirulina*.

Diet / Pigment	Artificial diet (control)	Artificial diet containing			
		10% Corn gluten	10% Alfalfa	3% Canthaxanthin	10% Spirulina
Astaxanthin found (μg/g body wt.)	3.4	5.4	5.5	10.4	18.4
Main carotenoid and contents in diet		Zeaxanthin (2.5 mg%)	Lutein (8 mg%) Zeaxanthin (2 mg%)	Canthaxanthin (3%)	β-Carotene (30 mg%) Zeaxanthin (2.5 mg%)

Table 19. Spectral characteristics and relative abundance of carotenoids in *Spirulina plantensis*.

Compound	%	Spectral characteristics
α-carotene	7.0	418, 443, 472
β-carotene	67.4	425, 447, 473
echinenone	6.8	453
cryptoxanthin	6.0	422, 446, 472
pigment 425	2.5	405, 426, 452
hydroxy-echinenone	1.4	460
lutein	2.7	(420), 445, 468
zeaxanthin	5.8	425, 448, 473
euglenanone	trace	452

The major carotenoid in corn gluten is zeaxanthin, but its contents are lower than those of β-carotene in *Spirulina*. In alfalfa, the most abundant carotenoid is lutein and also contains small amounts of zeaxanthin. Therefore, it is possible that astaxanthin is biosynthesized from zeaxanthin and β-carotene. However, contents of zeaxanthin in corn gluten and alfalfa are too low to improve color of prawns.

From the results of these feeding tests and the distribution of carotenoids in tiger prawn, the metabolic pathways to astaxanthin in tiger prawn can be proposed as shown in Fig. 38.

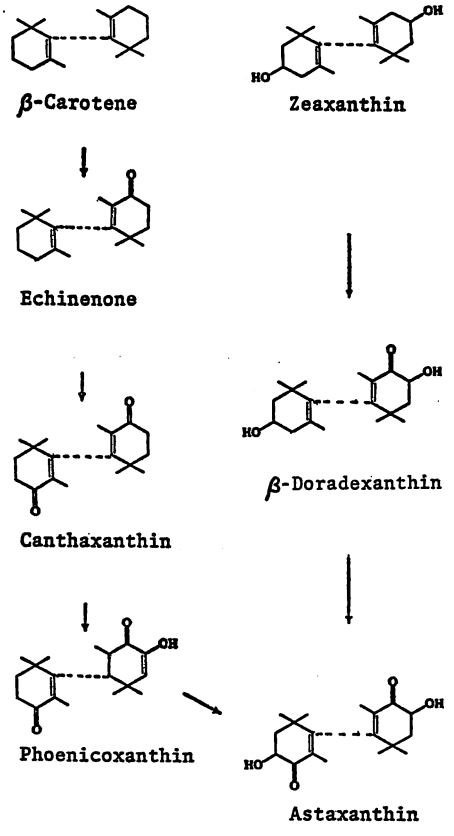


Fig. 38. Metabolic pathways to astaxanthin in tiger prawn.

Canthaxanthin with oxo groups at the 4- and 4′-positions was confirmed to be converted to astaxanthin, therefore, tiger prawns can have the capability of oxidizing the 3- and 3′-positions of β-ionone rings of carotenoids. Zeaxanthin with hydroxy groups at the 3 and 3′-positions was also confirmed to be converted to astaxanthin, so tiger prawns possess the power of oxidizing the 4- and 4′-positions of β-ionone rings of carotenoids.

Beta-carotene can be converted to astaxanthin in tiger prawn tissues through coupling oxidation at the 3- and 3′-, and the 4- and 4′-positions of β-ionone rings. Thus, its metabolic pathway from β-carotene to astaxanthin was presented as follows:

$$\beta\text{-carotene} \xrightarrow[4\text{ position}]{\text{oxidation at}} \text{echinenone} \xrightarrow[4'\text{ position}]{\text{oxidation at}} \text{canthaxanthin}$$

$$\xrightarrow[3\text{ position}]{\text{oxidation at}} \text{phoenicoxanthin} \xrightarrow[3'\text{ position}]{\text{oxidation at}} \text{astaxanthin.}$$

From these results and distributions of the carotenoids in various species of crustacean, it may be said that Crustacea have the capabilities of oxidizing the 3- and 3′-positions, and the 4- and 4′-positions of β-ionone rings of carotenoids (Fig. 39).

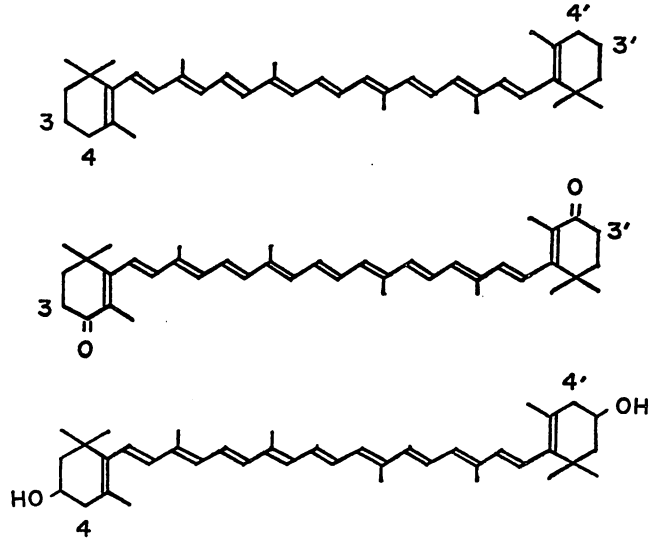


Fig. 39. Oxidation position of β-carotene, canthaxanthin and zeaxanthin through the metabolic pathway of Crustacea.

Therefore, when tiger prawn or other crustaceans are cultured in fish farms, β-carotene, canthaxanthin, zeaxanthin or astaxanthin should be supplemented to their diet for maintaining their natural color, or improving their faded color during the culture with compounded feed. *Spirulina* and crab waste which contain those carotenoids are also efficient for the maintenance or the improvement of color in Crustacea.

IV–2. Fresh-water red fish

As stated in chapter II–2, zeaxanthin, lutein and canthaxanthin might be precursors of astaxanthin in fresh-water red fish.

The possible metabolic pathway from lutein to astaxanthin through α-doradexanthin and β-doradexanthin in goldfish was first proposed by KATAYAMA et al.[47]. Later HSU et al.[48] confirmed that dietary ^{14}C-lutein contributes to the radioactivities of α-doradexanthin, β-doradexanthin and astaxanthin in goldfish tissues. However, HATA and HATA[49] denied the

pathway from lutein to astaxanthin and emphasized that lutein can be converted to α-doradexanthin, but the further oxidation of α-doradexanthin can not proceed. Instead, they stated that zeaxanthin is converted to astaxanthin.

Hsu[48] reported that β-carotene could be converted to astaxanthin by feeding goldfish on ^{14}C-β-carotene and proposed the following pathway: β-carotene→ketocarotenoids→ astaxanthin.

Therefore, the author fed goldfish on β-carotene, canthaxanthin, lutein and zeaxanthin for clarifying the metabolic pathway from these carotenoids to astaxanthin.

The amounts of astaxanthin were increased by feeding goldfish on lutein and zeaxanthin, but not increased by feeding with β-carotene and canthaxanthin. From these results, goldfish has not the capabilities of converting β-carotene and canthaxanthin to astaxanthin, but zeaxanthin and lutein to astaxanthin.

Materials and Methods

1. Feeding tests with β-carotene and canthaxanthin.

Healthy goldfish (about 10 cm in length) were purchased at a local fish hatchery. Three tanks (60×30×30 cm) were prepared. Eighteen goldfish were placed in each tank and acclimatized for a month to the artificial diet mentioned in Table 13. The first control group of goldfish was fed on the artificial diet. The second group of goldfish was fed with the artificial diet containing pure β-carotene at 200 mg%, and the third group of goldfish was fed on the artificial diet containing pure canthaxanthin at 200 mg%. All three groups were cultured for 31 days. These goldfish were separately collected and the pigments were extracted with acetone as already mentioned in II–1, 2. The pigments were purified and identified as stated in II–3, 4.

2. Feeding tests with lutein and zeaxanthin.

Goldfish (about 10 cm in length) were obtained from a local fish hatchery and were separated into three groups. The first control group was fed with the artificial diet. The second group was fed with the artificial diet containing pure lutein at 20 mg% and the third group was also fed with the artificial diet containing pure zeaxanthin at 200 mg%. All three groups were raised for 31 days. The carotenoid pigments in these goldfish were extracted with acetone and purified through the columns as already mentioned in II–1, 2, 3. The pigments were identified by the same procedures as already stated in II–4.

3. Feeding test with astaxanthin.

Goldfish were separated into two groups. The first control group was fed with the artificial diet and the second group was fed with the artificial diet containing astaxanthin at 20 mg%. Both groups were cultured for 31 days. The pigments were extracted and purified through the column as already mentioned in II–1, 2, 3, and identified as mentioned in II–4.

Results and Discussion

1. Possibilities of conversion from dietary β-carotene and canthaxanthin to astaxanthin.

It is clearly shown in Table 20 that β-carotene could not be converted to astaxanthin in the goldfish by feeding with the diet containing pure β-carotene. This result agrees with that

Table 20. Possibilities of conversion from dietary β-carotene and canthaxanthin to astaxanthin in goldfish.

Diet / Pigment	Artificial diet (control)	Artificial diet containing	
		200 mg% Pure β-carotene	200 mg% Pure canthaxanthin
Astaxanthin found (μg/g body wt.)	31.3	30.2	32.3

obtained through the feeding tests of goldfish, red carp and fancy red carp with the same diet containing ^{3}H-labelled β-carotene[43]. It was also shown in Table 20 that canthaxanthin could not be converted to astaxanthin by feeding with the diet containing canthaxanthin.

2. Possibilities of conversion from dietary lutein and zeaxanthin to astaxanthin.

It has been clarified that α-doradexanthin is an intermediate on the biosynthetic pathway from lutein to astaxanthin. This was confirmed by feeding goldfish with the diet containing ^{14}C-lutein by HSU et al.[48]. Table 21 lists the results of feeding experiments of pure lutein and zeaxanthin to goldfish. It was confirmed that lutein could be converted to astaxanthin by feeding. These results are consistent with those obtained by HSU et al.[48]. It was also found that zeaxanthin could be converted to astaxanthin by feeding. These results are consistent with the results by feeding goldfish with the diet containing labelled zeaxanthin[109,110].

Table 21. Possibilities of conversion from dietary lutein and zeaxanthin to astaxanthin in goldfish.

Diet / Pigment	Artificial diet (control)	Artificial diet containing	
		20 mg% Pure lutein	200 mg% Pure zeaxanthin
Astaxanthin found (μg/g body wt.)	31.4	52.1	51.1

3. Possibility of transfer from dietary astaxanthin to tissues of goldfish.

It was already confirmed that astaxanthin could be accumulated in the tissues of goldfish, red carp and fancy red carp by feeding with the diet containing ^{3}H-labelled astaxanthin[111].

Goldfish were fed with artificial diet containing pure astaxanthin at 20 mg% and it was confirmed that astaxanthin in the feed could be transferred to their tissues (Table 22). The colors of goldfish were improved with plentiful red color.

Table 22. Transfer of astaxanthin from diet to the body of goldfish.

Carotenoids in body / Carotenoids in diet	Carotenoids found (μg/g body wt.)	Astaxanthin found (μg/g body wt.)	Relative abundance of astaxanthin
None	23.2	12.3	1.00
Astaxanthin	31.7	20.7	1.67

From the results of these feeding tests and the distributions of carotenoids in fresh water red fish, the metabolic pathways to astaxanthin in goldfish can be proposed as shown in Fig. 40.

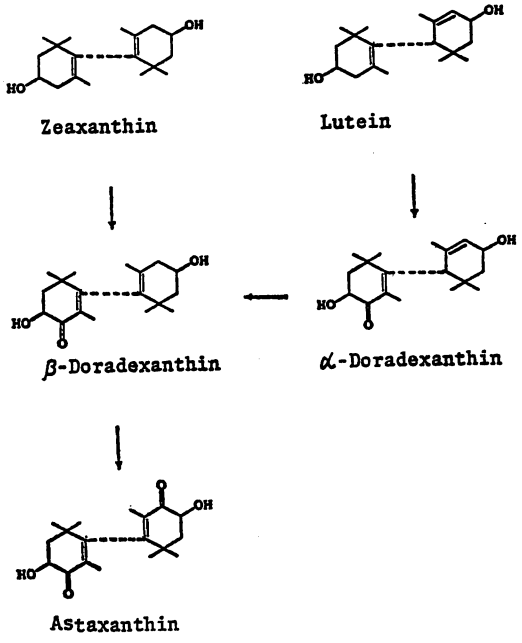


Fig. 40. Metabolic pathways to astaxanthin in goldfish.

Goldfish cannot convert canthaxanthin to astaxanthin. This tells us that they have not any ability to oxidize the 3- and 3′-positions of β-ionone rings of canthaxanthin.

Since goldfish can convert zeaxanthin to astaxanthin, this shows us that they can oxidize the 4- and 4′-positions of β-ionone rings of zeaxanthin with hydroxy groups at the 3- and 3′-positions.

Since fresh-water red fish such as Hibuna, *Carassium auratus* v., fancy red carp, *Cyprinus carpio* LINNE and golden yellow carp, *Cyprinus carpio* LINNE v. revealed that the constituents

of their carotenoids are analogous with those of goldfish, the pattern of the biochemical oxidation of carotenoids was presumed to be identical with that of goldfish. Therefore, it can be summerized that these fresh water red fish can oxidize only the 4- and 4′-positions of β-ionone rings of such carotenoids with hydroxy groups at the 3- and 3′-positions as zeaxanthin and lutein (Fig. 41).

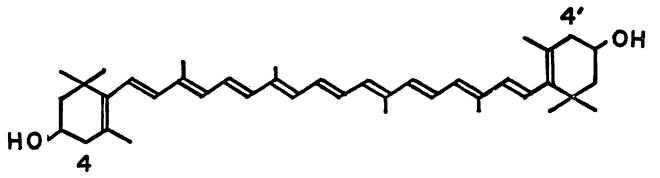


Fig. 41. Oxidation positions of zeaxanthin through the metabolic pathway of fresh-water red fish.

From these results, zeaxanthin or lutein must be included in their feed for maintaining their bright red color or improving once faced color during their culture with artificial diets.

IV–3. Marine red fish

KATAYAMA et al.[112] found that the faded color of cultured red sea bream, *Pagorus major* TEMMINCK and SCHLEGEL was caused by the lack of astaxanthin in their skins. These authors examined the stomach contents of wild sea bream and found partly digested *Squilla oratoria* and other Crustacea which contain astaxanthin and canthaxanthin. KATAYAMA et al.[44] fed red sea bream and crimson sea bream, *Evynis japonica* TANAKA, with the diet containing β-carotene and found that these fish could not convert β-carotene to astaxanthin. On feeding red sea bream and crimson sea bream with ^{14}C-labelled astaxanthin, it was confirmed that these fish was able to transfer the pigment only from the food into their integuments[113,114].

The present author undertook to reaffirm the results obtained by KATAYAMA and also to confirm their pigmentation by feeding red sea bream on β-carotene and astaxanthin.

Obviously, it was elucidated that red sea bream cannot convert β-carotene to astaxanthin, since the amounts of astaxanthin in their tissues were not increased by feeding on β-carotene. However, they were able to transfer astaxanthin in the diet to their integuments which showed to increase in the amounts of astaxanthin.

Zeaxanthin and canthaxanthin, had been regarded as the precursors of astaxanthin in marine red fish, were separately supplied to red sea bream. The results showed this fish can convert neither zeaxanthin nor canthaxanthin to astaxanthin, because no increases in the content of astaxanthin in their tissues were observed through these feeding tests.

A similar feeding test was performed using red sea bream for determining the possibility of the conversion from lutein to astaxanthin, but the result showed that red sea bream were not able to convert lutein to astaxanthin because of no increase in the amount of astaxanthin in their tissues.

Materials and Methods

1. Feeding experiments of β-carotene and astaxanthin.

Forty red sea breams (8–10 g) were placed in each tand (1×1×1 m) and acclimatized for 1 month to the artificial diet (Table 13).

The fish in tank 1 were fed every day with 20 g artificial diet for 31 days, the fish in tank 2 with the artificial diet+200 mg% β-carotene, and the fish in tank 3 with the artificial diet +20 mg% astaxanthin for same periods, respectively.

2. Feeding experiments of canthaxanthin and zeaxanthin.

Three aquaria were prepared. Ten red sea breams (40–45 g) were placed in each tank and acclimatized for 34 days to the artificial diet. The fish in tank 1 were then fed every day with 15 g artificial diet for 31 days as control, the fish in tank 2 with the artificial diet+200 mg% canthaxanthin and fish in tank 3 with the artificial diet+200 mg% zeaxanthin for the same periods.

3. Feeding experiments of lutein.

Fifteen red sea breams (30–35 g) were placed in two tanks and acclimatized for 20 days to the artificial diet. The fish in tank 1 were then fed every day with 25 g artificial diet for 31 days, the fish in tank 2 with the artificial diet+20 mg% lutein for the same periods, respectively.

The fish tested were killed and the pigments were extracted and purified using the same method as already mentioned in II–1, 2, 3. The carotenoids were identified by the same method as already stated in II–4 and the amounts of astaxanthin were determined.

Results and Discussion

1. Possibilities of conversion from dietary β-carotene to astaxanthin and transfer of dietary astaxanthin to tissues.

Table 23 shows the results of β-carotene and astaxanthin feeding experiments. From these results, it was confirmed that red sea bream cannot convert β-carotene to astaxanthin but they can only transfer astaxanthin from diets to their tissues.

Observing the pigmentation through the carotenoid feeding experiments, red sea bream fed on astaxanthin diet are clearly demonstrated to be more red than those fed on control and β-carotene diet.

The β-carotene diet group revealed a high mortality and a low growth rate when compared with those of the astaxanthin diet group and the control diet group. Thus, it was concluded that the feeding red sea bream with a large amount of β-carotene (200 mg%) in harmful.

Table 23. Possibilities of conversion from dietary β-carotene to astaxanthin and transfer of dietary astaxanthin to tissues in red sea bream.

Pigments \ Diet	Control diet	Diet containing	
		200 mg% β-carotene	20 mg% astaxanthin
Astaxanthin found (μg/g body wt.)	—	—	0.414
β-carotene found (βg/g body wt.)	—	0.040	—
Tunaxanthin found (μg/g body wt.)	0.088	0.076	0.079

2. Possibilities of conversion from dietary canthaxanthin and zeaxanthin to astaxanthin.

The feeding tests of red sea bream on canthaxanthin and zeaxanthin proved that this fish cannot convert them to astaxanthin in their bodies, but deposit them in integuments, because of the rather decrease in the contents of astaxanthin and the clear increase in the contents of canthaxanthin or zeaxanthin in their integuments as shown in Table 24.

Table 24. Possibilities of conversion from dietary canthaxanthin and zeaxanthin to astaxanthin in red sea bream.

Pigments \ Diet	Control diet	Diet containing	
		200 mg% canthaxanthin	200 mg% zeaxanthin
Astaxanthin found (μg/g body wt.)	0.104	0.096	0.095
Zeaxanthin found (μg/g body wt.)	trace	trace	0.319
Canthaxanthin found (μg/g body wt.)	—	1.250	—

3. Possibility of conversion from dietary lutein to astaxanthin.

Table 25 shows the results of lutein feeding experiments. From these results, it was confirmed that red sea bream cannot convert lutein to astaxanthin.

Table 25. Possibility of conversion from dietary lutein to astaxanthin in red sea bream.

Pigments \ Diet	Control diet	Diet containing 20 mg% lutein
Astaxanthin found (μg/g body wt.)	—	—
Lutein found (μg/g body wt.)	0.042	0.100

From the feeding tests of red sea bream on canthaxanthin and zeaxanthin, it is evident that they have not any ability to oxidize the 3- and 3′-positions and the 4- and 4′-positions of β-ionone rings of carotenoids.

Seemingly, marine red fish cannot convert any kinds of carotenoids to astaxanthin, but only transfer carotenoids from feed to their tissues. Therefore, such materials as mysis and crab waste which contains astaxanthin must be included in their feed for maintenance of their natural bright color, if these marine red fish are cultured for commercial objects.

IV–4. Porifera

Several studies on the distribution of carotenoids in sea sponges have been performed. The metabolism of the carotenoids in porifera has not been studied as yet.

The present author has succeeded to isolate two kinds of new carotenoids from two species of sea sponge, *Tedania digitata* SCHMIDT and *Tethya amamensis* FHIELE and proposed their structural formulae.

The major carotenoids in four species of sea sponge were determined: tedanin in *Clathria frondifera* (BOWERBANK) and *Tedania digitata* SCHMIDT, astaxanthin in *Haliclona permollis* (BOWERBANK) and renieratene in *Tethya amamensis* FHIELE.

From the distribution of carotenoids in these sponges, the metabolic pathways to their main carotenoids are proposed.

The precursor of tedanin in *Clathria frondifera* (BOWERBANK) and *Tedania digitata* SCHMIDT can not be presumed. The precursor of astaxanthin in *Haliclona permollis* (BOWERBANK) was presumed to be zeaxanthin because of the coexistence of β-doradexanthin as the necessary intermediate. The precursor of renieratene was presumed to be tethyatene found by the present author.

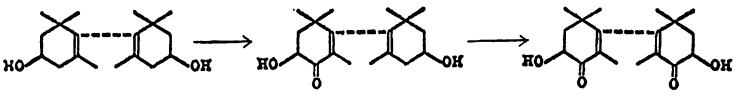
Results and Discussion

The author isolated new carotenoids, tedaniaxanthin and tethyatene, and their structures have been proposed to be 3-hydroxy-7, 8-didehydro-β, -χcarotene and 3, 4-didehydro-β, χ-carotene. The main carotenoids were astaxanthin in *Haliclona permollis* (BOWERBANK), tedanin in *Clathria frondifera* (BOWERBANK) and *Tedania digitata* SCHMIDT and renieratene in *Tethya amamensis* FHIELE, respectively, as shown in Table 10.

From the distribution of carotenoids in these four species of sea sponge, sea sponges seemed to be divided into two groups depending on their main carotenoids: one group (a) containing hydrocarbon carotenoid and another group (b) ketocarotenoids. The former such as *Tethya amamensis* FHIELE is devoid of the ability to oxidize carotenoids, but latter such as *Clathria frondifera* (BOWERBANK), *Tedania digitata* SCHMIDT and *Haliclona permollis* (BOWERBANK) has the ability to oxidize carotenoids.

In *Haliclona permollis* (BOWERBANK), the metabolic pathway to the main carotenoid, astaxanthin, can be suggested as follows from distribution of zeaxanthin (10%), β-doradexanthin (10%) and astaxanthin (31%).

The main carotenoid in *Clathria frondifera* (BOWERBANK) and *Tedania digitata* SCHMIDT is



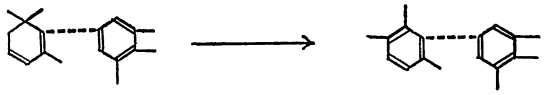
Zeaxanthin β-Doradexanthin Astaxanthin

not astaxanthin but tedanin. The precursor of tedanin cannot be presumed.

It is still ambiguous whether the formation of astaxanthin from zeaxanthin in *Haliclona permollis* (BOWERBANK), can be attributable to its own ability or bacteria commensal with this sponge. However, it has been well known that bacteria and algae seldom produce astaxanthin. This suggests that this metabolic pathway can be proceeded by Haliclona own ability. If so, this sea sponge can have the ability to oxidize the 4-position of β-ionone ring with hydroxy group at the 3-position.

It can say that some sea sponge seem to have the ability to oxidize zeaxanthin to astaxanthin like fresh-water red fish, but some have not any ability to oxidize them, though the numbers of test animals are limited at the present time.

HERZBERG and JENSEN[95] synthesized very easily 3′, 4′-didehydro-chlorobactene with φ-carotene ring (1, 2, 5-trimethyl benzene ring) from myxoxanthophyll with β-ionone ring (3-hydroxy-β-carotene ring) through 3, 4-didehydro-β-carotene ring which were postulated to exist during the reaction using catalysts of dry hydrochloric acid and chloroform. However, this reaction suggested the possibility of the aromatization of β-ionone ring through the 3, 4-didehydro-β-carotene ring. If such a metabolic pathway exists elsewhere in biota, the carotenoids with the 3, 4-didehydro-β-carotene ring must be discovered from biota. The present author first found out tethyatene which possesses the 3, 4-didehydro-β-carotene ring. Thus, it was concluded that the precursor of renieratene in *Tethya amamensis* FHIELE is tethyatene.



Tethyatene Renieratene

IV–5. Echinodermata

DE NICOLA[98] reported the metabolic pathway from β-carotene to astaxanthin in *Ophidiaster ophidianus* as follows:

β-carotene⟶cryproxanthin⟶3′-hydrosy-echinenone⟶β-doradexanthin⟶ astaxanthin.

He also proposed the following biosynthetic route from β-carotene to astaxanthin in *Asterias panceri*[99].

β-carotene⟶echinenone⟶3′-hydroxy-echinenone⟶β-doradexanthin⟶ astaxanthin.

The present author isolated an astaxanthin like pigment from Onihitode and made clear its structure to be 7, 8-didehydroastaxanthin. The author also proposed the possible metabolic pathways from diatoxanthin to 7, 8-didehydroastaxanthin, from alloxanthin to 7, 8, 7′, 8′-tetradehydroastaxanthin and from zeaxanthin to astaxanthin, and from β-carotene to astaxanthin (Prawn-Type) in Onihitode.

Results and Discussion

DE NICOLA emphasized that β-carotene converts to cryproxanthin as the first step of oxidation, then cryptoxanthin to 3′-hydroxy-echinenone in *Asterias panceri*[99]. This shows us the fact that the biochemical oxidation must occur at the 3 position of ionone ring for producing cryproxanthin from β-carotene. And, next oxidation occurs at the 4-position of another ionone ring, when cryptoxanthin is converted to 3′-hydroxy-echinenone in accordance with the Nicola's proposal.

The structure of β-carotene is symmetric and two ionone rings occupy the opposite positions in the molecule. Therefore, if one ionone ring can be oxidized through an enzymatic reaction, the oxidation of another ionone ring should occur at the same position prior to starting the oxidation of the other positions of ionone ring. If this conception is correct, there should be synthesized zeaxanthin, instead of 3′-hydroxy-echinenone from cryproxanthin. However, there was not detected cryptoxanthin as seen in his data. The present author was not able to detect cryptoxanthin, but echinenone in Onihitode as well as in *Asterina panceri* studied by NICOLA. Therefore, it is difficult to propose the metabolic pathway from β-carotene to astaxanthin including cryptoxanthin as a necessary intermediate.

The present author found β-carotene, echinenone and canthaxanthin, but not 3′-hydroxy-echinenone in Onihitode. The metabolic pathway such as β-carotene→echinenone→canthaxanthin conflict with the conception of the enzymatic oxidation of β-carotene as mentioned above.

Furthermore, NICOLA showed that 3′-hydroxy-echinenone is converted to β-doradexanthin and β-doradexanthin to astaxanthin. These pathway is also incompatible with the enzymatic oxidation, because of the same reason. NICOLA and the present author found astaxanthin in starfish, although their genera were different. However, astaxanthin suggests the existence of either β-doradexanthin or phoenicoxanthin. NICOLA took the choice of β-doradexanthin. If β-doradexanthin is an intermediate in this pathway, the carotenoid expected as the precursor should be zeaxanthin in accordance with the enzymatic oxidation. Obviously, he could not find out zeaxanthin in the starfish used. With this reason, he had to insert 3′-hydroxy-echinenone as the precursor of β-doradexanthin, although there was out of accord with the specificity of the oxidizable position of carotenoids due to enzymatic actions.

The present author took the choice of phoenicoxanthin as the precursor of astaxanthin, because canthaxanthin was found as the precursor of phoenicoxanthin. In this study, phoenicoxanthin was not detected, probably because of the fast velocity of reaction between phoenicoxanthin and astaxanthin.

The present author isolated a new ketocarotenoid, 7, 8-didehydroastaxanthin as a main

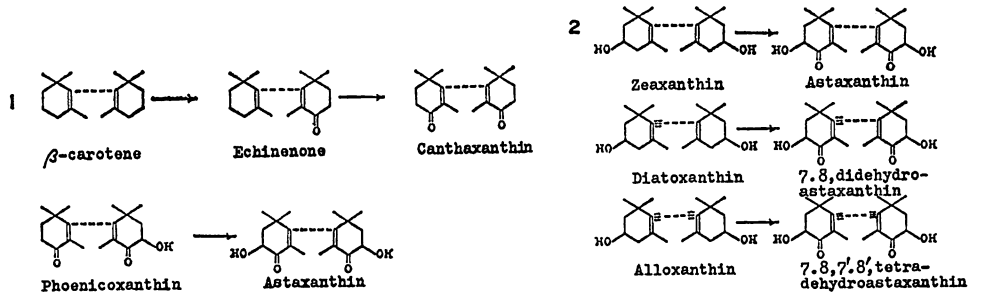


Fig. 42. Possible metabolic pathways in Onihitode, *Acanthaster planci*.

carotenoid in Onihitode, although astaxanthin, zeaxanthin, diatoxanthin and 7, 8, 7′, 8′-tetradehydroastaxanthin were also found simultaneously. No paper has reported that the aquatic animals can dehydrogenize carotenoids at 7, 8-positions forming a triple bond in their metabolism. Therefore, the precursor of 7, 8-didehydroastaxanthin should be diatoxanthin rather than astaxanthin.

Through the same pattern of the carotenoid oxidation, astaxanthin can be produced from zeaxanthin and 7, 8, 7′, 8′-tetradehydroastaxanthin from alloxanthin.

V. Conclusion

It has been well known that aquatic animals can not synthesize carotenoids, but alter only special carotenoids through certain metabolic pathways.

In order to elucidate some of these metabolic pathways of carotenoids in tissues of aquatic animals, carotenoids were extracted from their bodies, purified through column chromatography and identified through their characteristic absorption spectra, nature on elution from columns and characteristic colors, special chemical tests and co-chromatography with authentic carotenoids.

The author discovered three new carotenoids from aquatic animals and proposed their structural formulae. Among three new carotenoids, two were isolated from *Tedania digitata* SCHMIDT and *Tethya amamensis* FHIELE, and their structures were proposed to be 3-hydroxy-7, 8-didehydro-β, χ-carotene and 3, 4-didehydro-β, χ-carotene. The names of tedaniaxanthin and tethyatene were proposed for these two aromatic carotenoids, respectively. The last new carotenoid, an astaxanthin like pigment was isolated from starfish, Onihitode (*Acanthaster planci*) and its structure was established to be 7, 8-didehydroastaxanthin.

In Crustacea, it was presumed by KATAYAMA[43] that β-carotene can be converted to astaxanthin through echinenone, canthaxanthin and phoenicoxanthin during feeding test of ^{3}H-β-carotene.

The present author confirmed by feeding test with canthaxanthin that tiger prawn can convert canthaxanthin to astaxanthin. In this case, the oxidation could occur at the 3- and 3′-positions of canthaxanthin. The author also confirmed by feeding test with zeaxanthin that they can convert zeaxanthin to astaxanthin. Obviously, the oxidation could occur at the

4- and 4′-positions of zeaxanthin. Therefore, the present author proposed a new metabolic pathway from zeaxanthin to astaxanthin through β-doradexanthin.

In fresh-water red fish, KATAYAMA et al.[47] and HSU[48] proposed that goldfish can convert lutein to astaxanthin through α-doradexanthin. HATA and HATA[49] confirmed that goldfish can convert zeaxanthin to astaxanthin. The present author fed goldfish with zeaxanthin and knew that the oxidation occurs at the 4- and 4′-positions of zeaxanthin forming astaxanthin. A similar test was performed by feeding goldfish with lutein and it was concluded that goldfish can convert lutein to astaxanthin. The author also fed goldfish with canthaxanthin for elucidating whether they can oxidize at the 3- and 3′-position of canthaxanthin or not, and know the fact that this oxidation does not take place.

KATAYAMA et al.[44] performed feeding tests with ^{3}H-β-carotene and ^{3}H-astaxanthin using crimson sea bream and red sea bream, and presumed from the distribution of radioactivities of carotenoids that they cannot convert β-carotene to astaxanthin, but transfer astaxanthin from feed to their own tissues. The author tried to clarify by feeding tests whether red sea bream can convert canthaxanthin and zeaxanthin to astaxanthin or not and confirmed that these reactions do not take place in their body. Therefore, it is clear that red sea bream can neither oxidize carotenoids at the 3- and 3′-positions of β-ionone rings with oxo groups at the 4- and 4′-positions, nor those at the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions and only the capability is to transfer astaxanthin from diet to their tissues.

These results allow us to consider that there are three types of carotenoid oxidation as represented by three types of aquatic animals such as tiger prawn, goldfish and red sea bream.

1. Prawn-Type carotenoid oxidation: Crustacean can oxidize carotenoids at the 3- and 3′-positions of β-ionone rings with oxo groups at the 4- and 4′-positions and also at the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions. These tell us that they can convert β-carotene, canthaxanthin and zeaxanthin to astaxanthin. Therefore, crustacean should be fed on the feed supplemented with β-carotene, canthaxanthin or zeaxanthin for preventing their body color to fade.

2. Goldfish-Type carotenoid oxidation: Most of fresh-water red fish in Japan belong to Cyprinidae. They can only oxidize carotenoids at the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions, but not oxidize carotenoids at the 3- and 3′-positions of β-ionone rings with oxo groups at the 4- and 4′-positions. In other words, they can convert zeaxanthin and lutein to astaxanthin, but not convert canthaxanthin and β-carotene to astaxanthin. Therefore, these fresh-water red fish should be fed on the diet mixed with either zeaxanthin or lutein for maintaining their bright red color or improving their faded color to bright red color.

3. Sea bream-Type carotenoid oxidation: Marine red fish can not oxidize carotenoids at the 3- and 3′-positions of β-ionone rings with oxo groups at the 4- and 4′-positions and also at the 4- and 4′-positions of β-ionone rings with hydroxy groups at the 3- and 3′-positions. In other words, they can not convert β-carotene, canthaxanthin and zeaxanthin to astaxanthin. They can only transfer zeaxanthin, lutein, canthaxanthin and astaxanthin from feed to their own tissues. Therefore, astaxanthin should be supplemented to their diet for the maintenance

of their color or the improvement of their color faded during cultures.

In the case of porifera, sea sponges could be divided into two groups: one group has not any ability to oxidize carotenoids as exemplified in *Tethya amamensis* FHIELE, but another has the ability to oxidize them. In latter group, *Haliclona permollis* (BOWERBANK) can be included, because the existence of zeaxanthin, β-doradexanthin and astaxanthin suggested that this sponge has the distribution and metabolic pathways similar to those of goldfish. Therefore, this sponge was presumed to have the ability to oxidize the 4-position of β-ionone ring with hydroxy group at the 3-position. Sea sponges, *Clathria frondifera* (BOWERBANK) and *Tedania digitata* SCHMIDT do not contain any appreciable amount of astaxanthin but tedanin as a major carotenoid.

One of echinoderms, Onihitode seems to belong to organisms with the capability of Prawn-Type carotenoid oxidation, because its distribution of carotenoids was similar to that of tiger prawn.

In this study, it was found that Micro-Cel C has a certain catalytic hydroxylation of β-carotene and can produce isocryptoxanthin with slight amounts of isozeaxanthin and echinenone. This warned investigators engaging in the studies of carotenoid chemistry.

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