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Studies on Carotenoprotein and Carotenoids of some Micronektonic Crustaceans caught in Sagami and Suruga Bays

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(Figs. 1–4, Tables 1–6)

In general red-colored creatures are found more commonly in waters below 500 m. Among the crustaceans scarlet or blood-red shrimps are characteristic[1]. The biological significance of color appearance of integument in various species has been much discussed. In the twilight zone red coloring is also considered to have a concealing effect. The red rays from the sun are absorbed in the surface waters so a red creature moving below this area will appear not red but black. The dominancy of astaxanthin and its esters in almost all species of deep-sea crustaceans has been reported by HERRING[2].

The contribution of carotenoprotein as well as of carotenoids for the integument color might be of interest. Bluish carotenoproteins are rare in these species[2] The wide distribution of carotenoprotein or carotenoid-containing lipoprotein in crustaceans has been summerized by CHEESMAN *et al*[3]. Astaxanthin preferencially conjugates with protein as carotenoprotein[3～9]. Moreover, besides the color appearance, the possible function of the carotenoprotein or carotenoid-containing lipoprotein is supposed to be a carrier of carotenoid by conjugation with protein or by dissolution in the lipid moiety. Therefore, it is likely that the carotenoid-protein complexes of deep-sea crustaceans have some biochemical significance.

In a previous paper[9], the color variation of crayfish exoskeleton was reported to be due to the coexistence of carotenoproteins and unbound-carotenoids. In this paper the presence of carotenoprotein, carotenoid distribution, and the color appearance in the exoskeleton of deep-sea crustaceans have been investigated. The lipids of some micronectonic shrimps were reported in a separate paper[10].

MATERIALS AND METHODS

Animals

Four species belonging to the order of decapoda, *Acanthephyra quadrispinosa, Sergestes prehensilis, Sergestes lucens, Lucifer sp.*, and a species belonging to the order of euphausiacea, *Euphausia similis,* were collected at night from a surface *circa* 2000 m deep in Suruga and Sagami Bays by means of a large plankton net and Isaacs-Kidd midwater frawl on the cruise of R/V Tansei Maru of the Ocean Research Institute, Tokyo University.

Preparation of pigmented protein

The exoskeletons of *A. quadrispinosa* and *S. prehensilis* were analyzed for carotenoprotein. They were homogenized by a Potter-Elvehem homogenizer with 0.6 *M* ammonium sulfate solution, according to the method used for the extraction of carotenoprotein by CECCARDI & ALLEMAND[11]. The extract by 0.6 *M* ammonium sulfate solution was obtained by centrifuging at 10,000 rpm for 10 minutes. The pigmented protein was salted-out by addition of ammonium sulfate, collected by centrifugation, and redissolved in Tris-glycine buffer at pH 8.6. The thus obtained crude pigmented protein was used for further analysis.

Identification of carotenoid

Carotenoid was repeatedly extracted with acetone. Adding water to the acetone solution, carotenoids were transfered to a petroleum ether layer, then repeatedly washed with water to remove acetone. The petroleum ether extract was dried over anhydrous sodium sulfate and concentrated under nitrogen stream. Thus obtained carotenoids were separated by the thin-layer chromatography using a silicagel G (E. Merck, Darmstadt) plate of thickness 0.25 mm. The solvent systems used are as follows; *n*-hexane, ethylacetate (85:15 v/v) ; benzene, methanol (98:2 v/v). The R*f* values were compared with authentic standards on a plate. Alpha- and β-carotenes, and canthaxanthin were purchased. Echinenone was isolated from the gonad of sea urchin. Cryptoxanthin and zeaxanthin were isolated from maize meal. The pigmented bands on a plate were scratched and re-extracted with acetone. Carotenoids were identified by the following methods ; partition test between 90 % methanol and petroleum ether, saponification with 10 % alcoholic potassium hydroxide, concentrated HCl test, and reduction of keto group with sodium borohydride. The absorption spectra of carotenoids were recorded in a solution of petroleum ether, chloroform, carbon dioxide, and ethanol.

Quantitative determination of carotenoids

For the measurement of carotenoid composition, the carotenoids were separated on the silicagel plate, and the color density of each band was measured on an Ozumor Densitometer 82 with a filter No. 52 (520 nm). The relative amount of carotenoid was given as percentage of the total. The carotenoid content was based on the specific extinction coefficient at the wavelength of maximal absorption in a petroleum ether,

2,000 as the $E^{1\%}_{1\,cm}$.

Disc electrophoresis

The disc electrophoresis of pigmented protein was carried out by the method of ORNSTEIN & DAVIS[12,13]. The current intensity of 3 mA per gel of 6 cm was charged for 60–90 minutes. After migration the protein fractions were stained with amidoblack 10B.

RESULTS

Carotenoid pigments in all species were extracted from a whole animal with acetone. The carotenoid content (mg%) in the whole animal weight are shown in Table 1. The schematic chromatograms of carotenoid composition on a silicagel plate are shown in

Table 1. Carotenoid contents of various crustaceans.

	Acanthephyra quadrispinosa	*Sergestes prehensilis*	*Sergestes lucens*	*Euphausia similis*	*Lucifer sp.*
Content (mg%)	26.60	23.42	11.24	11.80	1.56
Muscle (mg%)	6.64	4.43			
Exoskeleton (mg%)	7.49	8.27			

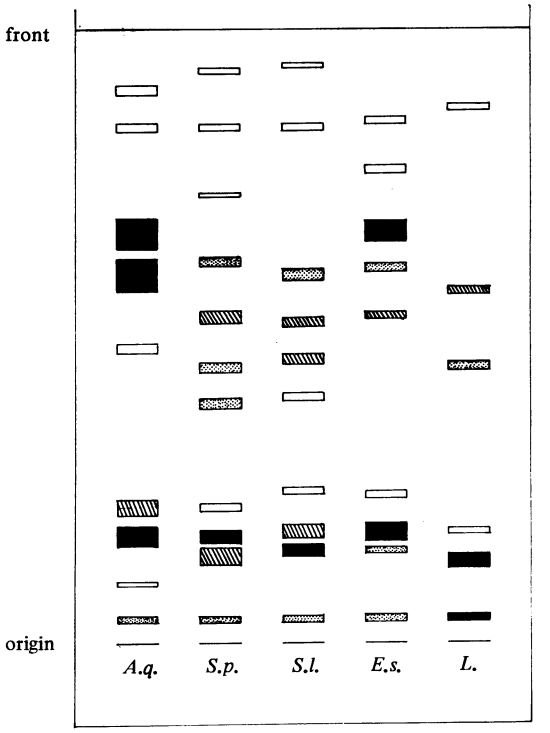

Fig. 1. Thin-layer chromatograms of carotenoids obtained from various crustaceans.
n-hexane and ethylacetate (85:15 v/v).
A.q. *Acanthephyra quadrispinosa* *S.p.* *Sergestes prehensilis*
S.l. *Sergestes lucens* *E.s.* *Euphausia similis*
L. *Lucifer sp.*

Fig. 1. Striking differences in carotenoid composition appeared among the species.

Acanthephyra quadrispinosa The exoskeleton color of this species was uniform-brilliant-red. The soft exoskeleton was separated from the hypodermis as much as possible. The absorption spectrum of the fragment of carapace obtained by using an opal glass method showed its maximum at 463 nm (Fig. 2). Any remarkable shift of the absorption spectrum did not occur after removal of the pigmented protein nor after heating the carapace at 100°C. The acetone extract from whole animal was separated into 9 fractions by a thin-layer chromatography on a silicagel plate developed by *n*-hexane and ethylacetate as solvent system, as shown in Fig. 1. Fraction 1 from the solvent front had its absorption maxima at 451 and 479 nm and a shoulder at 425 nm in petroleum ether, and 463 and 492 nm in chloroform. The concentrated HCl test was negative. The absorption spectra, partition test, and R*f* value before and after saponification also showed the characteristics of β-carotene. Fraction 2 with R*f* 0.84, which was commonly found in other species too, was not identified. Fraction 3 showed its absorption maxima at 455, 476, and 504 mm in petroleum ether, and epiphasic in partition test, before and after saponification. The absorption spectrum was probably in agreement with those of isocarotene (retro-dehydrocarotene) obtained from plant by ASCHMAWI *et al*[14]. The fraction was suggested to be a derivative of isocarotene. Fraction 4 showed its absorption maxima at 472 nm in petroleum ether. The absorption property, R*f* value, and partition test of the fraction before and after saponification were indistinguishable from those of astaxanthin-ester obtained from crayfish exoskeleton[9]. After saponification the resulted carotenoid was inseparable with astacin. The reduced product of the unsaponifiable matter showed a β-carotene structure. Therefore fraction 4 was identified as astaxanthin-ether. Fraction 5 with an R*f* value of 0.45 was not identified. Fraction 6 revealed its absorption maxima at 453, 475, and 506 nm in petroleum ether before and after saponification. The absorption spectrum of fraction 6 resembled to that of fraction 3. After saponification, the partition behavior changed to hypophasic, and the R*f* value decreased. These phenomena indicated the presence of di-hydroxyl groups. Therefore, the fraction was suggested as esterified eschltxanthin, which has

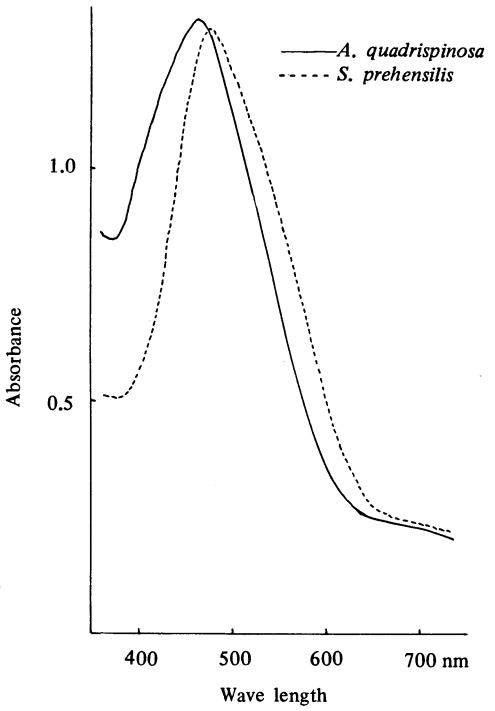

Fig. 2. Absorption spectra of the carapace.

been reported by KARRER & LEUMAN[15]. Fraction 7 was the most abundant carotenoid in this species. Its absorption maximum was at 472 nm in petroleum ether, and did not change after saponification. After following reduction of saponification product, the abosrption spectrum showed β-carotene-like spectrum. Its R*f* value was identical with those of the authentic canthaxanthin and astacin. When the fraction was co-chromatographed on a silicagel plate by using the solvent system, benzene and methanol, no traces of astaxanthin were found in it. The fraction was identified as canthaxanthin. Fraction 8 showed similar R*f* with phoenicoxanthin. Fraction 9 was the lowest in R*f*, showing its single absorption maximum at 473 nm in petroleum ether. The partition test was hypophasic before and after saponification. Its R*f* value increased and became indistinguishable from that of astacin after saponification. These results indicated the presence of di-hydroxyl groups which might change to carbonyl groups by saponification. Therefore, the fraction was identified as astaxanthin.

The exoskeleton was homogenized with 0.6 *M* ammonium sulfate, then centrifuged. The oil globule of the top layer was removed so as to make dissolution into petroleum ether possible. The absorption maximum at 470–472 nm was observed in the petroleum ether solution. The oil globule contained 0.8 per cent carotenoid in total carotenoid. The extract with 0.6 *M* ammonium sulfate gave brown color and the absorption maximum was at 475 nm (Fig. 3). The absorption spectrum of the pigmented protein extracted from the egg of this species was measured in the same way. The absorption

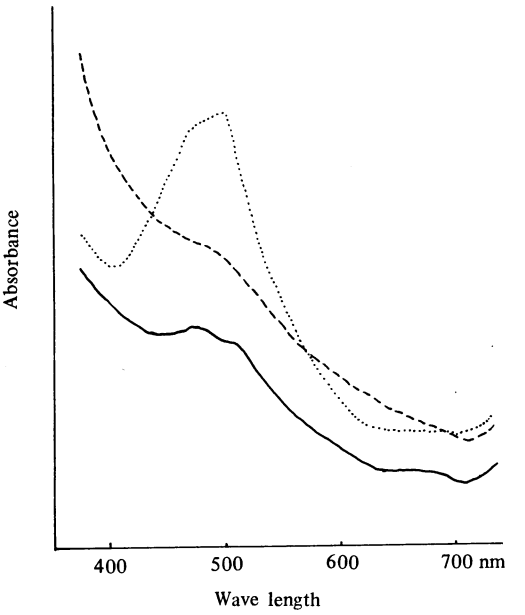

Fig. 3. Absorption spectra of extracts with 0.6 *M* ammonium sulfate from the exoskeleton and egg of deep-sea decapods.
—— *Acanthephyra quadrispinosa* exoskeleton
- - - - - *Sergestes prehensilis* exoskeleton
..... *A. quadrispinosa* egg

spectrum showed a clear single peak at 495 nm in ammonium sulfate solution. By adding solid ammonium sulfate to the pigmented extract from the exoskeleton, the resulted brown protein was applied for disc electrophoresis at pH 8.6. After migration one brown protein and four colorless proteins were detected. The carotenoids of the prosthetic group of the pigmented protein were extracted by a mixture of methanol and chloroform. Applyed on silicagel thin-layer chromatography, the extract showed one spot corresponding to astaxanthin.

Table 2. Absorption maxima and percentage composition of the carotenoid from *Acanthephyra quadrispinosa.*

No.	Rf	Pet. ether	Ethanol	Chloroform	Identification	%
1	0.90	428*–451–479	428*–454–481	438*–463–492	β-carotene	2.3
2	0.84					2.0
3	0.67	455–476–504				20.7
4	0.60	472	480	491	Astaxanthin ester	21.8
5	0.48					2.0
6	0.22	453–475–506				10.5
7	0.18	466	476–9	485–7	Canthaxanthin	31.1
8	0.10				Phoenicoxanthin	trace
9	0.04	473	476	489	Astaxanthin	9.5

* Indicates an obtuse peak.

Sergestes prehensilis The exoskeleton of this species was uniformly red, tingen with violet. The absorption maximum of the carapace was at 475 nm, somewhat similar to that of *Acanthephyra,* as shown in Fig. 2. No shift of the absorption spectrum was observed even after treatment with 0.6 *M* ammonium sulfate or heating. In carotenoid composition of whole animal, eleven pigmented bands were observed on a silicagel plate. Fraction 1 gave a β-carotene-like R*f* value, but authentic α- and β-carotenes could not separate from each other in this analytical condition. Therefore, the identification of the fraction requires further experiment. Fractions 2 and 3 were not identified. Fractions 4 and 5 showed the typical properties of astaxanthin ester. They gave a symmetrical single peak in petroleum ether, indicating the presence of di-keto groups. Both fractions were suggested to be astaxanthin esters, because the two separated spots were probably due to the structural difference of the alkyl groups. Fractions 6 and 7 both gave a similar absorption spectrum. They were equal to those of isocryptoxanthin and cryptoxanthin, respectively, and unchangeable by saponification. Reducation changed the absorption maxima to be 405, 427, and 452 nm in petroleum ether and was epiphasic in the partition test. The fraction was probably astaxanthin monoester, showing the same characteristics as astacin after saponification. Distribution of astaxanthin monoester in crustacea was reported by MATSUNO *et al.*[16] Fraction 9 with relatively high concentration showed the absorption maxima at 460 and 474 nm in petroleum ether. The absorption spectrum was somewhat resembling to that of

α-doradexanthin ester as reported by KATAYAMA *et al.*[17,18] The identity was not checked. Fraction 10 was characteristic in this species, and was not detected in the other species analyzed. Although the partition test was epiphasic, it became hypophasic after saponification. The absorption spectrum and R*f* value indicated the presence of di-hydroxyl groups. Fraction 11 was identified as astaxanthin from the chromatographic and spectrophotometric behaviors before and after saponification.

The pigmented protein extracted from the exoskeleton was centrifuged, then three distinct layers appeared : a pigmented oil globule, an aqueous layer, and the bottom residue. A fairly high amount of red oil globules, of which the absorption maxima lied at 465 nm in petroleum ether, was found. The carotenoid content in the oil globules corresponded to about 25 per cent of total carotenoid in the exoskeleton. The absorption spectrum of the middle layer which included the pigmented protein of the exoskeleton was characterized by a minor peak at about 485 nm (Fig. 3). The carotenoid of the prosthetic group extracted from the pigmented protein showed 6 components on a silicagel plate under the use of benzene and methanol as developing solvent, although only 2 components showed on a plate developed with a mixture of *n*-hexane and ethylacetate. In 6 components, astaxanthin and one unknown pigment were predominant, while two other components with higher R*f* value than astaxanthin were secondary, and two other components with lower R*f* value were subsidiary. The chromatogram and its densitometry of the carotenoid are illustrated in Fig. 4.

Table 3. Absorption maxima and percentage composition of the carotenoids from *Sergestes prehensilis*

No.	R*f*	Pet. ether	Chloroform	CS_2	Identification	%
1	0.93					0.7
2	0.84					0.8
3	0.73					trace
4	0.62	473	486	499	Astaxanthin ester	8.1
5	0.53	465	479		Astaxanthin ester	15.5
6	0.45	435*–459–487		457*–480–510*		11.7
7	0.39	433*–457–488	453*–475–502			9.1
8	0.22	472			Astaxanthin monoester	3.6
9	0.17	460–474				21.8
10	0.14	435*–462–480				16.7
11	0.04	465	483	495	Astaxanthin	11.9

* indicates an obtuse peak.

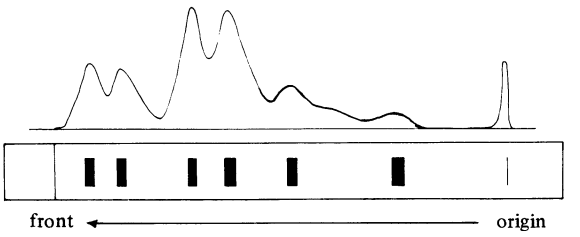

Fig. 4. Thin-layer chromatogram and its densitometry of the prosthetic group carotenoid of the pigmented protein obtained from *Sergestes prehensilis* exoskeleton. Carotenoids were developed with the mixture of benzene and methanol (98:2 v/v).

Sergestes lucens The carotenoid content of this species was lower than that of *S. prehensilis*. Only 11.24 mg% occured in whole animal of this species, while there was 23.42 mg% in *S. prehensilis*. Ten components were separated as carotenoids on a silicagel plate. Between two *Sergestes* species, the carotenoid composition was hardly similar, especially in some components with lower R*f* values. Fraction 1 was recognized as carotene from its high R*f* value. Fraction 2 was not identified. Fractions 3 and 4 were confirmed to be astaxanthin ester from the results of R*f* values and absorption properties. Fraction 5 showed an asymmetrical single absorption peak in petroleum ether, and proved to contain mono-keto group. After saponification the absorption spectrum changed to a symmetrical peak at 482 nm. The spectral shift suggested that the formation of a carbonyl group from a hydroxyl group had occured by saponification. Furthermore, saponification converted its partition behavior from epiphasic to hypophase. At least one of the hydroxyl groups was probably esterified with the alkyl group. Fractions 6 and 7 were not identified. Fraction 8 was identified as canthaxanthin which had not been detected in *S. prehensilis*, showing properties identical with those of authentic canthaxanthin in R*f* value, and the absorption spectrum before and after reduction. Fraction 7 resembled somewhat to fractions 6 and 7 of *S. prehensilis* in

Table 4. Absorption maxima and percentage composition of the carotenoid from *Sergestes lucens*

No.	R*f*	Petroleum ether	Identification	%
1	0.94			0.8
2	0.84			0.7
3	0.60	473	Astaxanthin ester	11.0
4	0.52	470	Astaxanthin ester	18.6
5	0.46	466	Ester of monohydroxy monoketo β-carotene	12.8
6	0.40			2.6
7	0.25	434*–458–486		4.7
8	0.18	473	Canthaxanthin	18.3
9	0.15	473	β-doradexanthin monoester	22.3
10	0.04	475	Astaxanthin	8.3

* indicates an obtuse peak.

the maximal absorption, but differed in the chromatographic behavior. Dominant component fraction 9 showed a symmetrical absorption peak at 473 nm in petroleum ether. The partition behavior and absorption spectrum suggested the presence of di-keto groups. Therefore the fraction might be β-doradexanthin monoester. Fraction 10 was identified as astaxanthin.

Euphausia similis The whole extract was applied for the thin-layer chromatography. In total 9 components of carotenoid separated on a plate. Fractions 1 and 2 were not identified. Fraction 2 of this species was of a similar absorption maximum with fractions 3 and 6 of *A. quadrispinosa.* This fraction was likely to be a derivative from isocarotene. Fraction 3 appeared in large amounts, and did not differ from authentic echinenone on a thin-layer plate nor in absorption property. Therefore, it was identified as echinenone. Fractions 4 and 5 were identified as astaxanthin ester by their R*f* values and absorption spectra compared with a well-defined one. Fraction 6 with absorption maximum at 465 nm in petroleum ether was considered to be astaxanthin monoester from the results of the partition test and chromatography before and after saponification. Dominant carotenoid in the species, fraction 7, showed the characteristic absorption maximum and R*f* value of canthaxanthin. Fraction 8 revealed the characteristic of β-doradexanthin in its absorption spectrum and R*f* value. Fraction 9 was confirmed as astaxanthin.

Table 5. Absorption spectra and percentage composition of the carotenoids from *Euphausia similis*

No.	R*f*	Petroleum ether	Identification	%
1	0.85			0.5
2	0.77	454–473–503		2.3
3	0.67	473	Echinenone	24.7
4	0.61	475	Astaxanthin ester	10.5
5	0.53		Astaxanthin ester	3.4
6	0.24	465	Astaxanthin monoester	6.1
7	0.18	472	Canthaxanthin	37.2
8	0.15	470	β-doradexanthin monoester	8.1
9	0.04	472	Astaxanthin	7.4

Table 6. Percentage composition of the carotenoids from *Lucifer sp.*

No.	R*f*	Identification	%
1	0.87		trace
2	0.57	Astaxanthin ester	10.7
3	0.45		9.5
4	0.18	Canthaxanthin	6.6
5	0.13		41.5
6	0.04	Astaxanthin	32.4

Lucifer sp. The exoskeleton had a pink color resulting from small red pigmented chromatophores. It should be noted that the concentration of carotenoid in this species was pretty low. The carotenoids were devided into 6 fractions on a silicagel plate. They revealed the R*f* values corresponding to the following components; astaxanthin esters, canthaxanthin, astaxanthin, and 3 unidentified components. The polar unidentified xanthophyll differed from phoenicoxanthin or astaxanthin as dominant carotenoid.

DISCUSSION

The recent development of thin-layer chromatographic technique for the examination of carotenoid made possible rapid analysis even for the small amounts of pigment. The carotenoids of 5 species belonging to crustaceans were analyzed on a rather smaller scale. All species examined contained about 10 carotenoid components. Mono- and di-esterified astaxanthin would be distinguished on the thin-layer plate. HERRING[2] found the presence of astaxanthin ester as the major pigments in decapods. A fairly high amount of carotenoids existed in the form of ester. Percentage composition of astaxanthin and its esters ranged from 20–40 per cent in all the species analyzed.

Some unidentified carotenoids found in *A. quadrispinosa* and *E. similis* were probably isocarotene derivatives, which had never been reported in crustacea. It is noteworthy that the spectrophotometric determination showed no α-carotene derivatives in these species analyzed, although it is well known that carotenoids such as lutein are distributed widely in crustacean.

The taxonomic proportion of carotenoid distribution have been reported for the phytoplanktonic species[19]. The specific pigments are frequently associated with particular phylum or even with a class or order. However, the significance of the specific colors of the exoskeleton and carotenoid composition of various species is not yet clearly accounted for in crustaceans. Between *S. prehensilis* and *S. lucens,* the pigment arrays were different. The former showed the carotenoid with α-doradexanthin ester-like spectrum as major carotenoid, while the latter contained a yet unidentified xanthophyll ester as dominant.

The exoskeleton's carotenoproteins of two deep-sea decapods were analyzed. The brown pigmented protein could be extracted by 0.6 *M* ammonium sulfate. However, considering that the carotenoid content and color intensity of the pigmented protein were minute, the contribution for color appearance might be negligible. It is well known that blue or green carotenoproteins are rare in deep-sea decapods[2]. A bluish protein did not occur in the exoskeleton of two species analyzed. In *S. prehensilis,* the oil globule which could be readily separated from the homogenate of exoskeleton contained large amounts of carotenoid, corresponding at least to 25 per cent of the exoskeleton carotenoid. Only one brown protein was detected in the exoskeleton

of *S. prehensilis* or *A. quadrispinosa.* Therefore, the exoskeleton was suggested to be pigmented by receive its pigmentation from the oil globule. The carapaces of *A. quadrispinosa* and *S. prehensilis* showed an absorption maxima at 465 and 475 nm, which is somewhat resembling to that of astaxanthin in water in the presence of detergent[4]. The possibility seemed to exist that a small amount of astaxanthin or other xanthophylls were connected with the large molecular protein in some manners; and almost all carotenoids existed in free form in the exoskeleton. Therefore, the red exoskeelton was due to the presence of large amounts of unbound xanthophylls more than to protein-bound xanthophylls.

The presence of carotenoid-containing protein was confirmed in the exoskeleton of two species, whereas it was not clear whether the pigmented protein was either carotenoprotein containing a stoichiometrical amount of carotenoids as prosthetic group, or carotenoid-containing lipoprotein. It was suggested that the pigmented protein functioned not for the color appearance, but for the carotenoid-carrier protein. The presence of carotenoprotein could increase the absorption area of light by hypso- or bathochromic shift. Thus color of the animal living in feeble light environment was probable corelated to camouflage or uptake of light.

SUMMARY

Thin-layer chromatographic procedure using silicagel was employed for the examination of the carotenoids of five species of crustacean. The carotenoids were separated in about 10 components in all species analyzed on a thin-layer plate. Besides astaxanthin and its esters, several xanthophylls were also detected. The small amounts of carotenoid-containing protein were obtained from the exoskeleton of *Acanthephyra quadrispinosa* and *Sergestes prehensilis.* It seems likely that the pigmented protein has more biochemical than pigmentary significance.

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相模湾，駿河湾産甲殻類のカロチノプロテイン及びカロチノイド

中川 平介・鹿山 光

相模湾，駿河湾産の5種類の甲殻類についてカロチノイド，及びそのうちの2種類について甲殻の色素蛋白質の分析を行った。全ての甲殻類のカロチノイドは薄層クロマトグラフィーで約10成分に分離し，そのうちアスタキサンチン，アスタキサンチンエステル，及び未同定のキサントフィルが主成分であった。

Acanthephyra quadrispinosa 及び *Sergestes prehensilis* の甲殻からカロチノイドを含む色素蛋白質を分離したが，この蛋白質は量的にみて，色素としての作用よりもカロチノイド運搬のような生化学的な機能を果すと考えられる。