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Studies on Carotenoprotein in Aquatic Animals

III. The Relationship between Blue and Purple Carotenoproteins in Crayfish (*Cambarus clarkii*) Exoskeleton

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(Figs. 1-6, Table 1)

The authors' works on carotenoprotein in the crayfish have indicated that the color appearance of the exoskeleton depends on the coexistence of the different colored carotenoproteins and the unbound carotenoids[1),2)]. The bluish color of the exoskeleton is due primarily to the existence of bluish carotenoproteins. Many workers have described this coexistence of various colored carotenoproteins in the crustacean exoskeleton[1),3)~9)]. It is also a wellknown fact that α-crustacyanin converts to β-crustacyanin by certain dissociation. From the native lobster shell, JENCKS and BUTTEN[4)], and BUCHWALD and JENCKS[7)] extracted the yellow pigments with a maximum absorption respectively at 410 and 409 mμ. JENCKS and BUTTEN[4)] reported that the proportion of yellow pigment in extracts varies according to the lobster shells obtained in different seasons. It seems probable that the color variation of the crustacean exoskeleton is due to the changing proportion of the variously colored carotenoproteins and the unbound carotenoids which are affected by environmental and hormonal factors[10)].

In the previous paper[2)], the authors described how a red carotenoprotein (*R*)* obtained from the native exoskeleton is a derivative from the blue carotenoprotein (*B*)* or the purple carotenoprotein (*P*)*. The present paper deals with the relationship between *B* and *P* in order to gain a basic information for the color control of the crustacea.

METHODS

Preparation of Carotenoprotein: The extraction and the purification of three kinds of different colored carotenoproteins were carried out by the method described in the previous paper[1)]. *B* and *P* were respectively homogenous in electrophoretic analysis. *R*, however, was contaminated by a few carotenoid-free proteins.

Electrophoresis: Cellulose acetate and disc electrophoreses were performed in

* *B*, *P*, and *R* stand for blue, purple, and red carotenoproteins respectively obtained from the crayfish exoskeleton.

order to analyze the urea denatured protein and the apoprotein. The cellulose acetate electrophoresis in a veronal buffer at pH 8.6 was carried out by the method described in the previous paper [1),2)]. The cellulose acetate sheets used were Separax of Joko Sangyo Ltd. (6×2 cm) and Sartorius-Membranfilter (7×3 cm). The protein on the sheets was stained with ponceau 3R. The disc electrophooresis was carried out by the method of ORNSTEIN[11)] and DAVIS[12)]. The instrument used was a Toyo CD-8 disc electrophoretic apparatus. A current intensity of 3 mA/gel was charged for 60 minutes. Acrylamido gel was stained with Amido black 10B.

Gel Filtration: The apoproteins and the urea denatured proteins were applied for the gel filtration. Sephadex G-200 was packed in a column of length 45 cm and diameter 1.1 cm. The elution was carried out by a 0.2 *M* sodium acetate solution, and the absorption was measured at various wavelengths.

Molecular Size Determination: The molecular size determinations were carried out by the method of gel filtration employing a Sephadex G-200 column. The column of 45 cm in length and 1.1 cm in diameter was equilibrated with a 0.05 *M* phosphate buffer and caribrated with a series of the proteins which had been purchased as a kit of the molecular weight markers (Mann Res. Lab.). The proteins and their average molecular weight were: myoglobin (M. W. 17,800) from sperm whale, ovalbumin (M. W. 45,000), bovine albumin (M. W. 67,000), γ-globulin (M. W. 160.000) from human, and apo-ferritin (M. W. 480,000) from horse. The void volume of the column was established by dextran blue (M. W. 2,000,000).

Urea Treatment of Carotenoprotein: The carotenoprotein solutions containing a phosphate buffer at pH 7.3 and various concentrated urea were kept for 3 hours at room temperature without illumination. The solutions were then applied for the spectrophotometric determination, the electrophoresis, and the gel filtration.

Preparation of Apoprotein: The apoproteins were prepared from the carotenoproteins by the procedure of CHEESMAN *et al.*[5)] using cold 0.05 *M* potassium thiocyanate and acetone. The apoprotein composition was determined by gel filtration and electrophoresis.

Amino Acid Composition: The apoproteins were subjected to HCl hydrolysis for 24 hours, and the amino acid analysis was made by a Liquid Chromatography of the Hitachi 034 Type. The amino acid composition was expressed in per cent of residue weight. Tryptophane and amide contents have not been determined in this study.

Spectrophotometric Determination: Absorption curves were obtained with a Shimadzu IV-50 Automatic Spectrophotometer.

RESULTS

On the absorption spectra (Fig. 1) of three kinds of carotenoproteins, *B* and *P* underwent a small hypsochromic shift by the addition of urea (1 *M*). A gradual spectral shift was also observed by the addition of urea, in the course of time. In

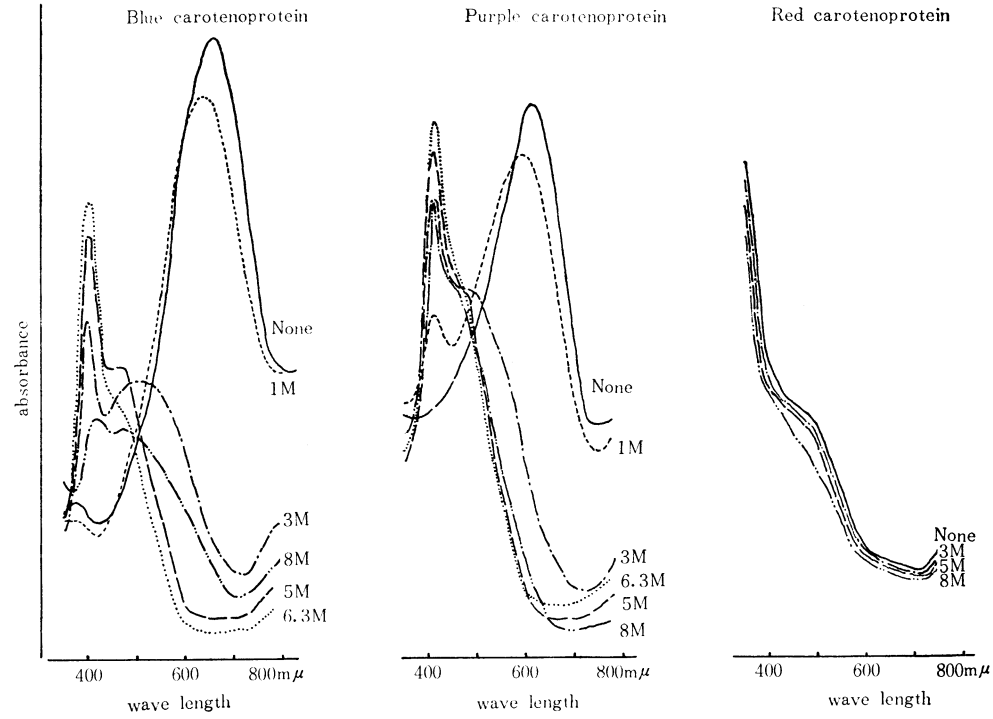

Fig. 1. Spectral changes of three carotenoproteins in the presence of various concentration of urea.

the presence of 1 *M* urea, *B* with blue color turned to purple with an absorption maximum at 620 mμ. The blue and purple bands in the electrophoretic patterns were also observed, and finally faded in the presence of concentrated urea. Then *B* and *P* gradually turned to a yellowish color with an absorption maximum at 400–500 mμ, and then lost their color. The spectral shift of *B* seemed to be somewhat reversible in the lower concentration of urea, the restoration did not occur more than 3 *M*. *R*, however, it showed great stability spectrophotometrically even under the existence in the concentrated urea. Figure 2 and 3 are the electrophoretic patterns of the urea-treated carotenoproteins after the elimination of urea by dialysis. *B* and *P* dissociated into several components in the presence of 1 *M* urea concentration. An increment of colorless bands was temporarily observed in the disc electrophoresis after staining with Amido black 10B. When *B* was placed in a 1 *M* urea solution, it dissociated to a dominated purple component with several other colorless components. The purple component showed good agreement with *P* in electrophoretic mobility and color appearance. The colorless components temporarily increased and then progressively disappeared with a denser urea concentration. At 5 *M* urea the degradation became more pronounced, as *B* and *P* yielded colorless components. It was found electrophoretically that *R* also was partially degradated into some components over 5 *M* urea.

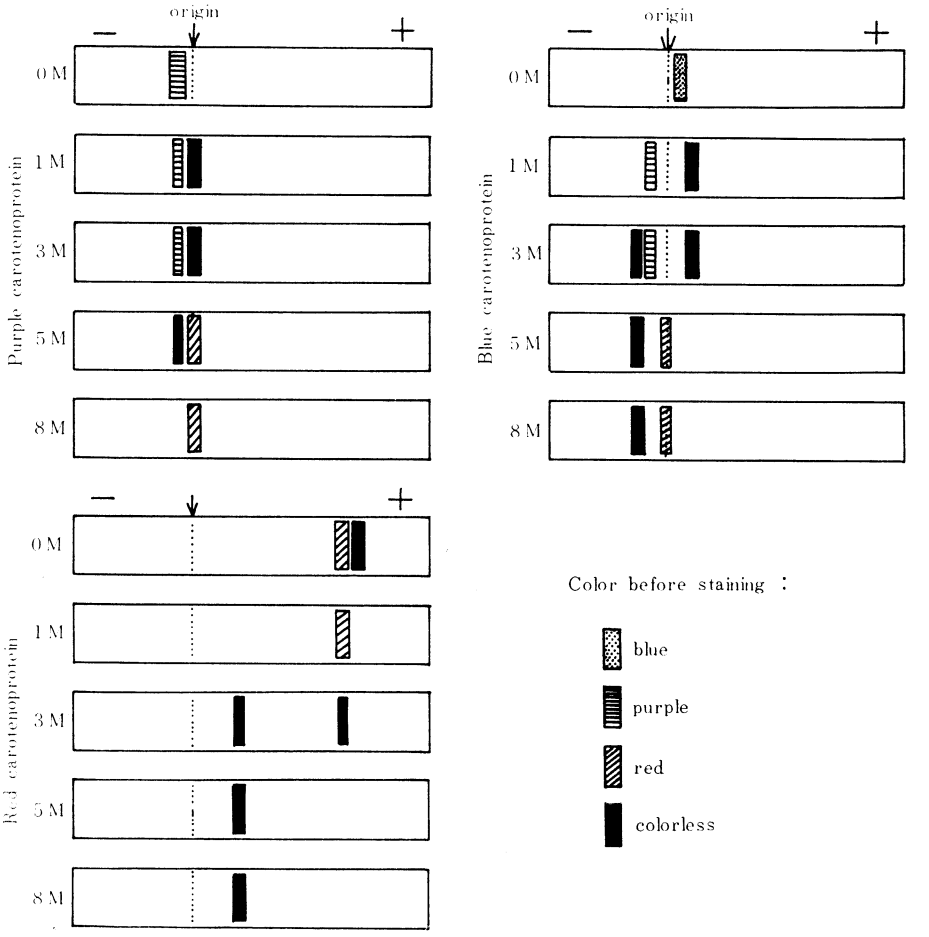

Fig. 2. Changes of cellulose acetate (Separax) electrophoretic patterns of the carotenoproteins in the presence of various concentration of urea. Veronal buffer pH 8.6, a current intensity 1 mA/cm 30 minutes.

B was degradated by mixing with 3 *M* urea for 3 days. The product was placed on a column of Sephadex gel, which previously equilibrated with a 0.2 *M* sodium acetate solution. And some fractions with different molecular size and color were obtained. The elution patterns were obtained by measuring the absorption at different wavelengths (Fig. 4). The eluents showed at least 3 components with different absorption maxima. The salting-out and dialyzed eluents were applied to disc electrophoresis. The eluents of the fraction number 6–8 had an absorption maximum at 402 mμ. In the disc electrophoresis of the eluents, the reddish substance remained in the origin without migration. The eluents of the fraction number 9–12 of strong bluish color, and showing an absorption maximum at 650 mμ, were separated into two colored components and several colorless components in the disc electrophoresis. Two colored components were identified from the electrophoretic

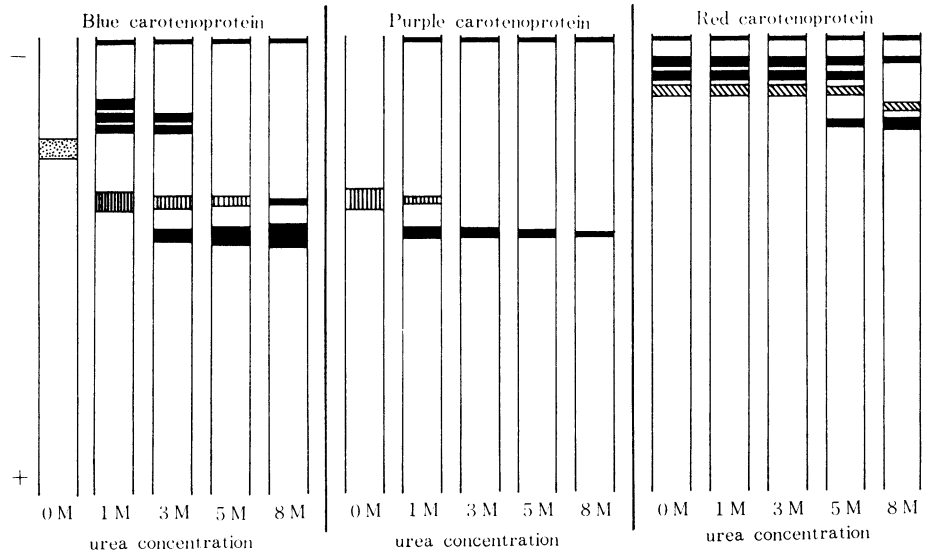

Fig. 3. Changes of disc electrophoretic pattern of the carotenoproteins in the presence of various concentration of urea. The explanation of bands were shown in Fig. 2.

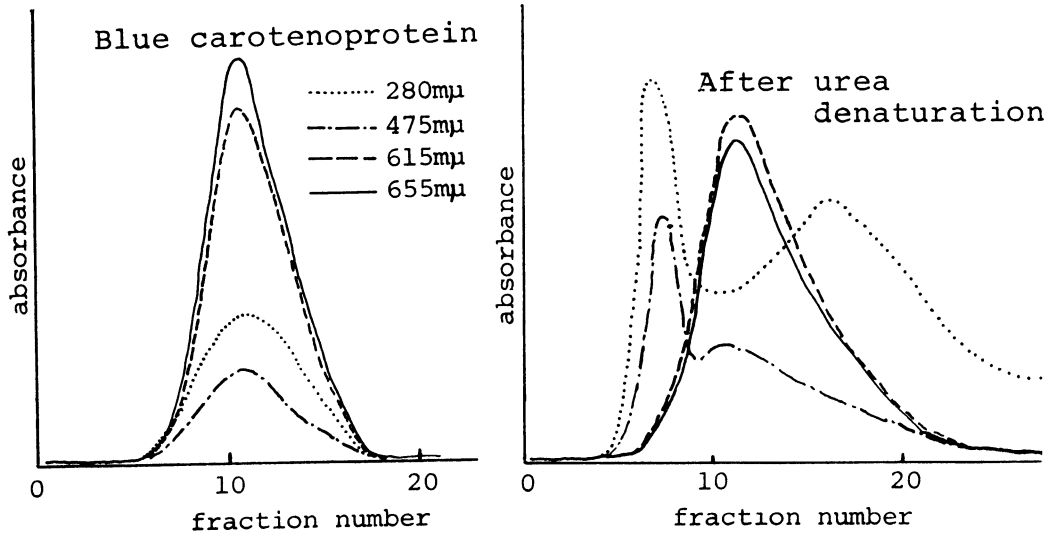

Fig. 4. Elution patterns on gel filtration in the blue carotenoprotein and its derivatives after the denaturation with 3 M urea.

mobility, the elution position on gel filtration, and the absorption spectrum: the one was *B* which remained without denaturation, and the other was *P* which was derived from *B*. The eluents of the fraction number 13–17 with their absorption maximum at 615–620 mμ were the mixture of either *P* or colorless low molecular components,

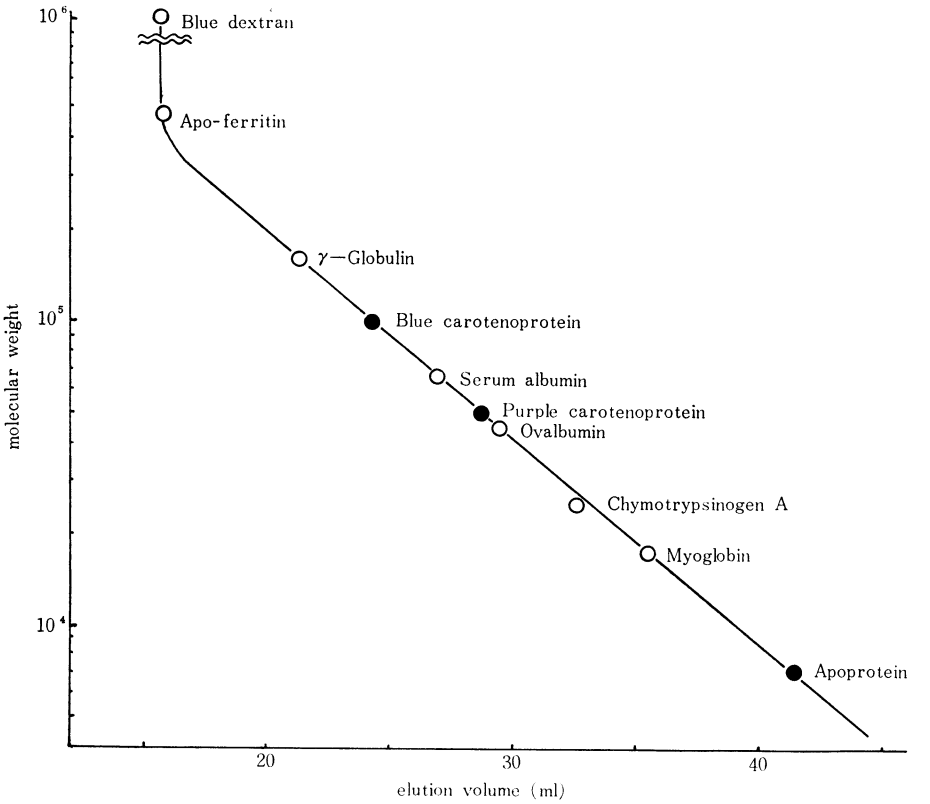

Fig. 5. Molecular weight determination of the carotenoproteins and the apoprotein of crayfish exoskeleton by gel filtration.

When a series of globular proteins were applied to a column of Sephadex gel, an almost linear relationship was obtained in the total effluent volumes corresponding to the maximum concentrations of the different proteins in the effluent against the logarithms of the known molecular weight.

The carotenoproteins and their derivatives were passed through the column, and their effluent volumes were applied to the figure obtained by the method mentioned above. Figure 5 shows the result obtained. It became evident that the molecular size of *B* was between that of a bovine serum albumin (M. W. 67,000) and that of a γ-globulin (M. W. 160,000). Therefore the molecular weight of *B* was of the order of about 10^5. *P*, whose elution rate was slower than *B*, was found to be of the order about 5×10^4. The elution patterns of the apoproteins prepared from *B* and *P* were similar in the gel filtration. The dominant apoprotein components of them came to elution at the position of the order of about 8×10^3. The reddish carotenoprotein derivative (*Rh*), prepared from *B* or *P* by heating at 100°C for 5 minutes, and *R* were also passed through the gel. Their elution positions were faster than that of γ-globulin and near to that of blue dextran.

The electrophoretic patterns of the apoprotein prepared from *B* and *P* were

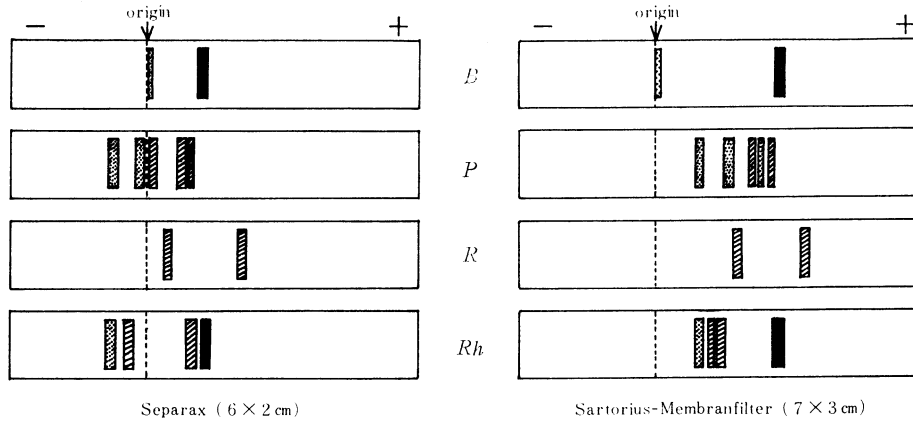

Fig. 6. Apoprotein constituents of the carotenoproteins obtained from the crayfish exoskeleton on cellulose acetate electrophoresis. Veronal buffer pH 8.6, a current intensity 1 mA/cm 30 minutes. The solid bands represent major fractions, the diagonal lines represent bands of medium intensity, and dots of faint intensity.

quite different each other. The apoprotein of ***B*** showed two bands in different domonancies on the cellulose acetate electrophoresis at pH 8.6. Five bands were observed in the apoprotein of ***P*** (Fig. 6). The apoprotein of ***Rh*** indicated four bands which quite differed from that of ***R*** in electrophoretic pattern.

Table 1. Amino acid composition* of the carotenoproteins

Amino acid	*B*	*P*
Asp	14.75	12.35
Thr	7.10	4.68
Ser	6.08	4.21
Glu	12.92	22.79
Pro	5.47	1.92
Gly	3.82	2.38
Ala	5.72	5.90
Val	6.71	4.43
Met	0.28	0.47
Leu	4.13	3.60
Ileu	6.16	9.31
Tyr	8.34	3.12
Phe	9.55	3.81
Lys	4.47	9.00
His	1.21	2.32
Arg	2.34	8.34
NH_3	0.96	1.35

* Per cent of residue weight (g of anhydro amino acid per 100 g of protein)

The amino acid compositions of *B* and *P* are given in Table 1. The high amount of aspartic acid and glutamic acid, and absence of cystin of cysteine were observed in the carotenoproteins obtained from the crayfish exoskeleton.

DISCUSSION

It has already been described that *B* and *P* irreversibly converted to *R* upon the autoxidation. The authors have observed that *B* turned to purple with an absorption maximum at 615 mμ by certain treatments. Several reports[1),3)~9)] described the coexistence of different colored carotenoproteins as chromophore in a crustacean exoskeleton. These carotenoproteins undergo the spectral shifts which relate to the change in bounding properties with the prosthetic group or in the complex quaternary structure of the proteins[14)]. Removal of salt by dialysis, in dilute solutions of the protein, yields a product, α′-crustacyanin, which readily aggregates to α-crustacyanin on addition of salt, but is converted by prolonged dialysis into a form, β-crustacyanin[5)]. By prolonged dialysis to desaltate, the solution of *B* turned to *P*, *R*, and carotenoid-free proteins.

In general, concentrated urea was often used to dissociate the protein into the subunits. The presence of the concentrated urea in a crustacyanin readily dissociates into several subunits[4),5),6),9)]. It was also found that *B* irreversibly dissociated to *P* and colorless components in the presence of concentrated urea. Therefore, it is quite apparent that *B* and *P* had distinctly different composition each other, but it is probable to exchange each other in the exoskeleton. *R* was composed by aggregation of the several subunits differing from that of *Rh*.

B had characteristic properties distinguished from other crustacyanins, the absorption maximum leaning toward the longer side of the wavelength and having a lower molecular size. Heterogeneity of *B* and *P* could be apparently explained, not only in amino acid composition, but in the apoprotein constituent. α- and β-crustacyanins seem to be composed of two different apoproteins, of which subunits are linked together by the carotenoids. While the apoprotein of *B* seemed to consist of two subunits, that of *P* was fractionated into further subunits by electrophoresis and gel filtration. The apoprotein obtained from *Rh* with higher molecular size than *B* showed to be composed of subunits different from those of the other carotenoproteins in the crayfish exoskeleton.

The amino acid composition of *B* was found to be similar though not identical with the carotenoproteins of other species[8)], however, clearly different from that of *P*. Cystin or cysteine content of the carotenoproteins in this study differed from those presented by BUCHWALD and JENCKS[7)], and KUHN and KÜHN[6),9)], but were similar to those of the carapace carotenoprotein of *Eriphia* as presented by ZAGALSKY *et al*[8)].

The molecular size determination of *B* and *P* by the gel filtration measurement gave the value of 10^5 and 5×10^4, respectively. Judging from the results of carotenoid analysis, it might be considered that *B* molecules contained more astaxanthin

molecules than *P*. *B*, which seems to be stabilized on the tertiary and quaternary structures by the prosthetic group carotenoid according to the explanation of CHEESMAN and PREBBLE[15], probably dissociated to *P* and several carotenoid-free proteins, when the prosthetic group carotenoid was eliminated under certain conditions; then finally resulted in *R* and carotenoidf-ree proteins. In this study, the large hypsochromic shift seemed to occur when the carotenoid was eliminated from the carotenoprotein. It was interesting to observe that the thus resulted carotenoid-free proteins revealed an electrophoretic mobility identical to that of the apoproteins. GREEN[16] stated that light promotes the deposition of carotenoids in the fat body of *Daphnia*. Therefore the appearance of *P* and *R*, or the deposition of unbound carotenoids in the exoskeleton is suggestive in this respect. Since α-crustacyanin is converted into β-crustacyanin with lower molecular weight and shifted absorption maximum, *R* was considered to be derived from *B* or *P* by the decrement of carotenoid content and by the aggregation of protein moiety.

The earlier observations noted the role of the blood lipoprotein as a carrier of carotenoid by dissolving in the lipid component, and protein-carotenoid complexes of different types could occur in a wide variety of organisms[17]. CECCALDI[18] reported electrophoretically two kinds of carotenoproteins, corresponding to α- and β-crustacyanins in the blood of *Homarus*. The role of various colored carotenoproteins in exoskeleton are of interest in their own right. It is also of interest that the possibility of the occurrence of the derived carotenoproteins, *i. e.* β-crustacyanin, yellow pigment, *P* and *R* in native circular organ as chromophore of the exoskeleton.

SUMMARY

From the results of the electrophoretic and the amino acid determinations, the apoproteins prepared from blue carotenoprotein (*B*) and purple carotenoprotein (*P*) of the crayfish (*Cambarus clarkii*) exoskeleton were found to consist of different subunit.

P, molecular size about 5×10^4, was derived from *B* with the molecular size about 10^5 by urea treatment. *B* was able to dissociate to *P* and carotenoid-free proteins due to the decrement of the prosthetic group carotenoid content under the certain conditions, these thereafter resulted by aggregation in a red carotenoprotein (*R*) with lower carotenoid content.

The possibility of conversion among three carotenoproteins in the exoskeleton under certain conditions was discussed.

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水産動物のカロチノプロテインに関する研究

Ⅲ アメリカザリガニ甲殻の青色カロチノプロテインと紫色カロチノプロテインの関係

中川 平介・鹿山 光・浅川 末三

アメリカザリガニ甲殻から得た青色カロチノプロテイン（以下Bと略記）と紫色カロチノプロテイン（以下Pと略記）のアポ蛋白質の分析結果より，それぞれのアポ蛋白質は異ったサブユニットで構成されていることを明らかにした．

約10万の分子量をもつBは尿素の存在により分子量約5万のPに変化する。Bは，ある条件下で，補欠分子族のカロチノイド含量が減少することにより，Pおよびカロチノイドを含まない蛋白質に解裂し，最終的には縮合して赤色カロチノプロテインを生ずる．甲殻内においてもBとPは相互変換が起りうると考えた．