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Studies on Carotenoprotein in Aquatic Animals

II Reddening of Carotenoprotein obtained from Crayfish (*Cambarus clarkii*)

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(Figs. 1-5, Table 1)

Carotenoids are found in crustacea under various physical and chemical forms, which provide a far wider range of color. The color appearance of the crustacean exoskeleton has been discussed from the view points of the physical structure of the integument, from that of the chemical structure of the carotenoprotein, and of the manner of binding with the prosthetic group[1)~6)]. It is well known that crustaceas can change their color in response to certain stimuli. On the other hand, the "true crustacyanin" converts to a red color by heat, acid or alkali, organic solvents, and other denature agents[7)~10)]. CHEESMANN *et al.*[6)], ZAGALSKY and CHEESMANN[11)], and KUHN and KÜHN[12)13)] showed that α-crustacyanin is a polymer which can be irreversibly converted into a subunit (β-crustacyanin) under certain conditions. Although every colored "true crustacyanin" obtained from many invertebrates has ketocarotenoids as prosthetic group, its absorption maximum has been discussed on the mode of combination between protein and carotenoids[14)].

A considerable amount of information in bluish carotenoproteins has been published by many workers, yet the mechanism of the reddish carotenoprotein is still unopen field. In this paper, we briefly want to describe the reddening of bluish carotenoproteins *in vitro*, and red colored carotenoproteins of crayfish.

MATERIALS AND METHODS

Preparation of Carotenoprotein : The carotenoprotein of the crayfish (*Cambarus clarkii*) exoskeleton was extracted with 0.6 *M* ammonium sulfate solution and precipitated by the addition of ammonium sulfate to half saturation, according to the method described by CECCARDI and ALLEMAND[19)], and CHEESMANN *et al.*[6)] In order to remove the colorless protein, the carotenoprotein was absorbed on a calcium phosphate gel at pH 7.0, then eluted with 0.1 *M* sodium chloride solution. The eluent from the gel consisted of three different colored components, blue, purple, and red carotenoproteins with absorption maximum at 655 mμ, 615 mμ, and a shoulder at 475 mμ, respectively. The separation and the purification of these carotenoproteins were carried out by the method described in the previous

paper[15]. The authors abbreviate blue, purple, and red carotenoproteins as ***B***, ***P***, and ***R***, respectively. As the purification proceeds, ***B*** and ***P*** became respectively homogenous in disc and cellulose acetate electrophoretic determinations, while ***R*** was still contaminated. The carotenoprotein mixture eluted from the calcium phosphate gel was applied for the determination on the influences of temperature and metallic ions.

Electrophoresis : The cellulose acetate electrophoresis was carried out by the method described previously[15]. After migration the sheets of cellulose acetate (1.5 × 6 cm) were stained with ponceau 3R for protein bands. The densitometry of the patterns were obtained with Ozumor Densitometer 82 using a filter No. 50.

Spectrophotometric Determination : The absorption spectra of the carotenoprotein solution were obtained with a Shimadzu IV-50A Automatic Spectrophotometer.

RESULTS

The exoskeleton of the crayfish contains three kinds of different colored carotenoproteins[15]. When ***B*** and ***P*** were exposed to air or strong illumination for long periods at room temperature, they gradually became reddish, and finally turned to a red or pale yellow insoluble substance with lower carotenoid content. Desaltation from the bluish carotenoprotein solution by prolonged dialysis also seemed to accelerate the reddening. It was found that by such reddening the absorption maximum disappeared in the visible region, and a shoulder appeared at 475 mμ which almost corresponded to that of the red carotenoprotein, ***R***. The reddish resultant from ***B*** and ***P*** coincided approximately with ***R*** in the spectrophotometric and the electrophoretic behavior.

The reddish resultant from ***B*** and ***P*** by prolonged preservation are abbreviated as ***Rd***. After the electrophoretic migration, a reddish band was observed on the anode side, this was found to be identical with ***R*** in the electrophoretic mobility as shown in Figure 2. Although the solution of ***R*** was stored for long periods, there was no noticeable change except formation of a pale yellow precipitate. While in ***B*** and ***P*** solution many different bands were observed after prolonged preservation.

The carotenoprotein mixture eluted from the calcium phosphate gel was dissolved into a 0.05 *M* phosphate buffer at pH 7.3, and heated at various temperature in a water bath. When the heating exceeded 60°C, ***B*** and ***P*** turned red with the shifted absorption maximum, somewhat lowered absorbance, and the changed electrophoretic mobilities as shown in Figure 1 and 3. When the temperature was raised until below 60°C and then cooled off again, the solution exhibited a certain type of heat denaturation with color change. The reddish solution, however, immediately reversed to the original color and to the original electrophoretic mobility. The colorless proteins which had appeared during the heating did not disappear in the restoration of the carotenoprotein. After boiling,

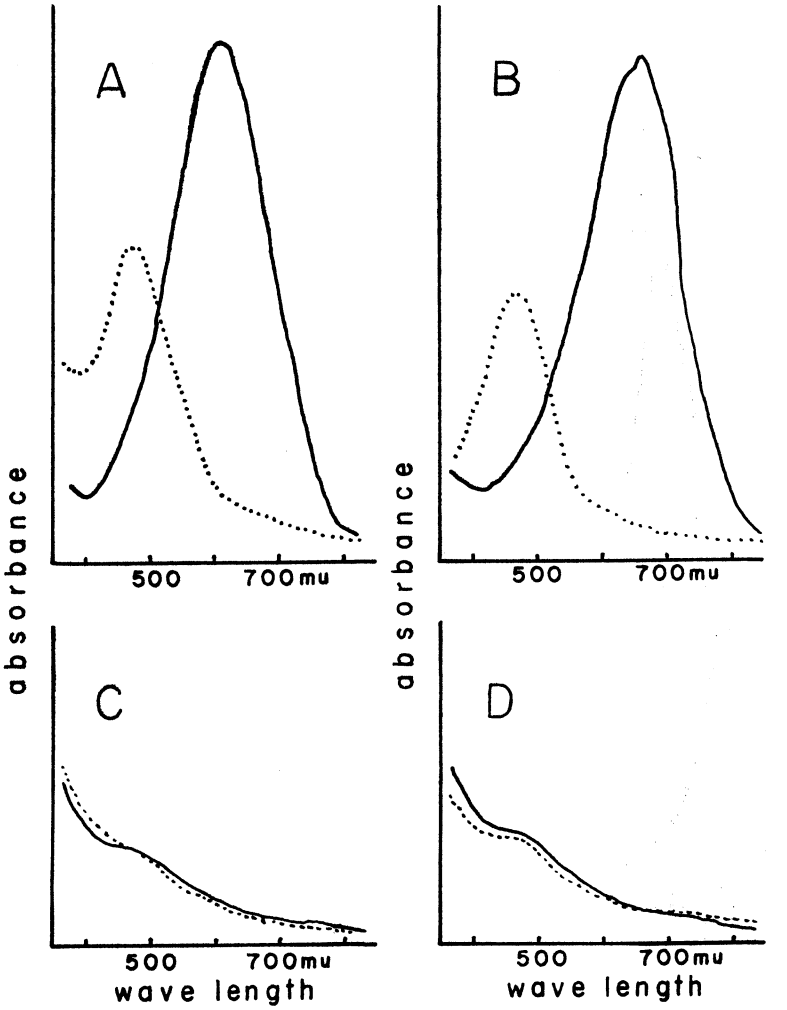


Fig. 1 Absorption spectra of carotenoproteins before (——) and after (·····) heating at 100°C for 5 minutes, in 0.05 *M* pH 7.3 phosphate buffer.

A, blue carotenoprotein (*B*)

B, purple carotenoprotein (*P*)

C, red carotenoprotein (*R*)

D, resultant formed by prolonged preservation of *B* and *P* (*Rd*)

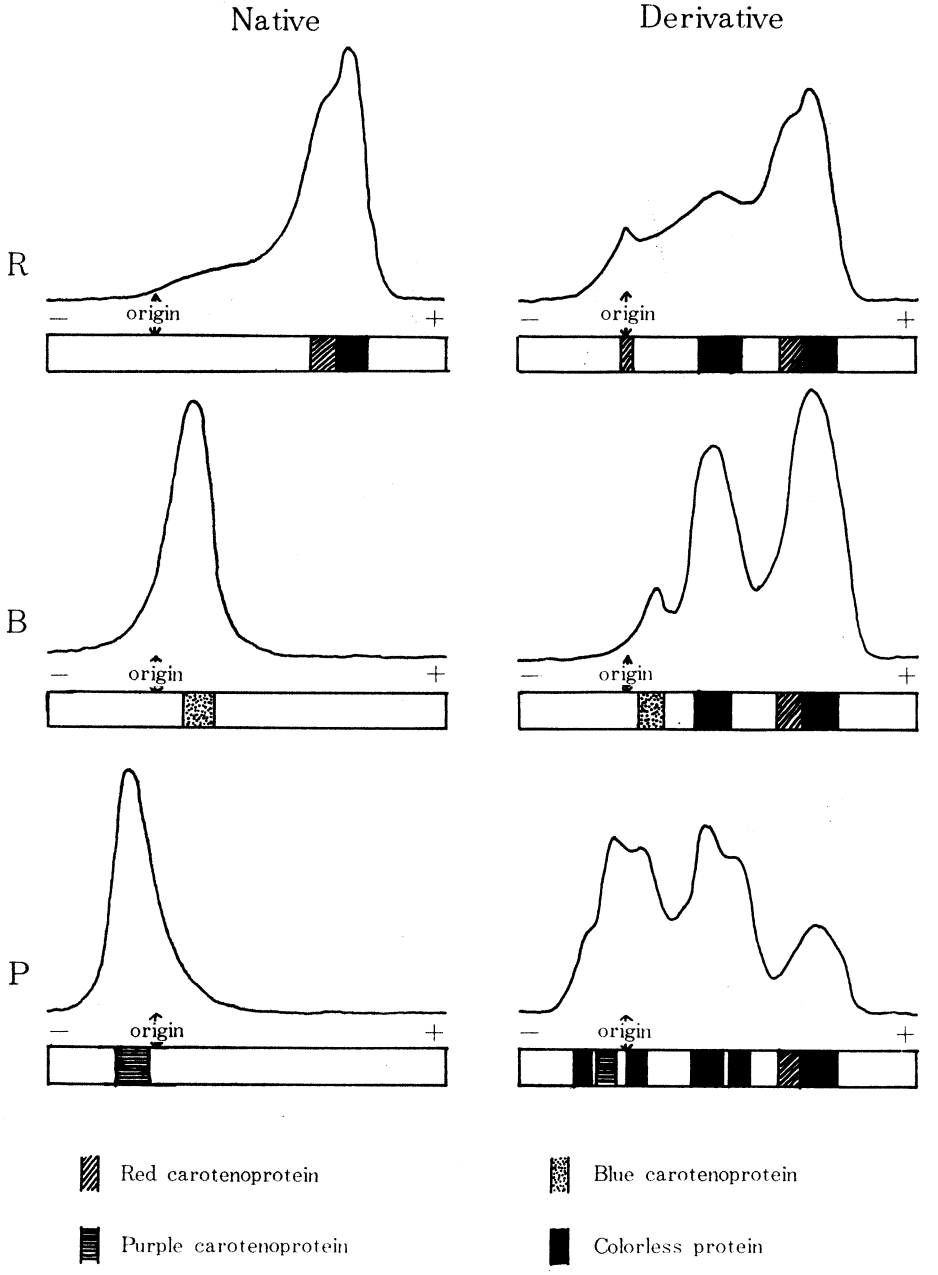


Fig. 2 Electrophoretic diagrams on cellulose acetate of isolated carotenoproteins, *R*, *B*, and *P* (left), and their derivatives (right) by prolonged preservation. Diagrams were obtained after staining with ponceau 3R.

the resultants from *B* and *P* were indistinguishable in the gel filtration, spectrophotometry and electrophoresis, however, *R* showed no spectral change at visual region but lowered electrophoretic mobility. After boiling the exoskeleton, it became impossible to extract the carotenoprotein (*Rh*) with a 0.6 *M* ammonium sulfate.

In order to observe the reversibility of carotenoprotein after boiling at 100°C, the reddened substance, which is abbreviated as *Rh*, was sealed in a cellophane tube and preserved with changing the outer solution (phosphate buffer) at a low temperature for 4 months. The reversibility was determined by the cellulose acetate electrophoresis and the gel filtration on Sephadex G-200 of length 45 cm and diameter 1.1 cm. Colorless protein, *B*, *P*, and *R* were found in the electrophoretic analysis. The partial reversibility to the original carotenoprotein was proven by keeping it at a low temperature (Figure 3). *Rh*, which is the abbreviation for the reddened substance resulted by boiling, showed different electrophoretic patterns and absorption spectra from *Rd*. However, when the reddened products, *Rd* and *Rh*, and *R* were passed through the column of Sephadex G-200, their eluted positions were almost identical.

The stability of the carotenoprotein for the presence of metallic ion was determined by spectrophotometry and cellulose acetate electrophoresis at pH 8.6. As the source of metallic ions, the following salts were used: sodium chloride (Na^+), potassium chloride (K^+), calcium chloride (Ca^{++}), magnesium chloride (Mg^{++}), copper chloride (Cu^{++}), ferric sulfate (Fe^{+++}), ferrous chloride (Fe^{++}), zinc chloride (Zn^{++}), mercuric chloride (Hg^{++}), barium chloride (Ba^{++}), lead chloride

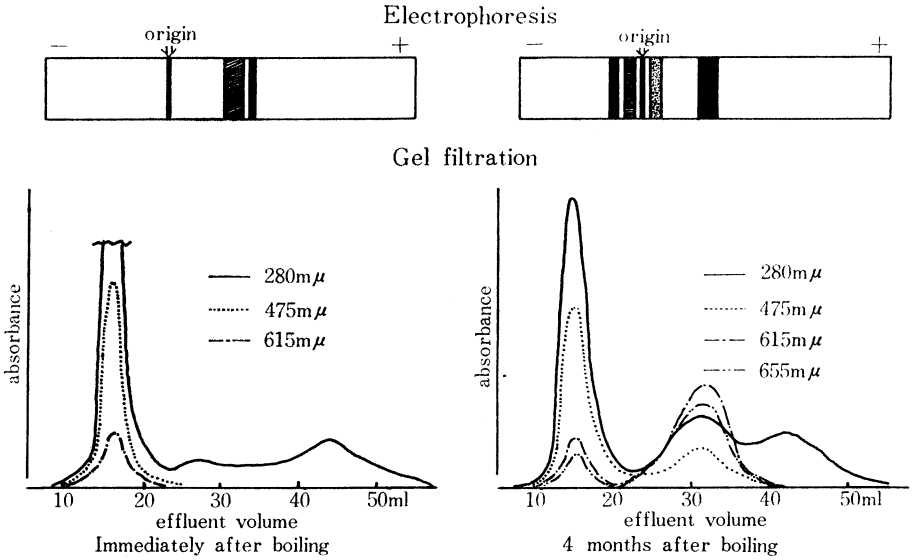


Fig. 3 Electrophoresis on cellulose acetate and gel filtration of boiled carotenoprotein. Footnote of the indivisual band in the electrophoretic diagram is indicated in Figure 2.

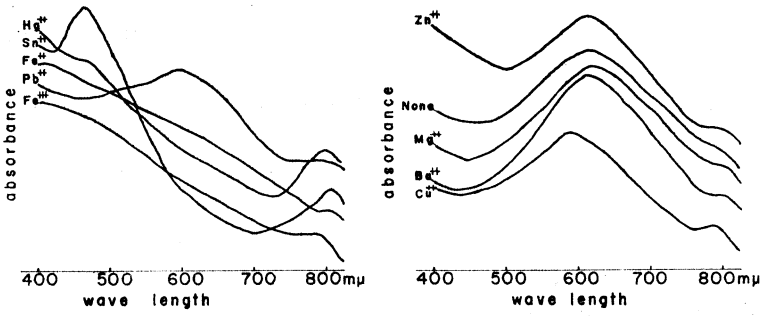


Fig. 4 Absorption spectra of the carotenoprotein mixture after addition of various metallic ions. The spectra in Na^+, K^+, and Ca^{++} are similar with that of Mg^{++}, and are omitted.

(Pb^{++}), and stannic chloride (Sn^{++}). Each salt solution was added to the salt-free carotenoprotein solution so as to be a 0.005 *M* metallic ion concentration. The absorption spectra were measured in relation to the blank after standing for 24 hours (Figure 4). And they were applied to the cellulose acetate electrophoresis after removing of the ions by dialysis. In the presence of certain metallic ions, ***B*** and ***P*** transformed into yellow or orange products. The reddish products with an absorption maximum at 470 mμ and 462 mμ showed in the presence of Hg^{++} and Sn^{++}, respectively. Moreover, Fe^{++} and Fe^{+++} were effective in decomposing ***B*** and ***P***. And the removal of their metallic ions by dialysis did not give a reversal of

Table 1 Spectrophotometric behavior of carotenoprotein mixture in the presence of various metallic ions.

Metal	In metal solution			After removal of the metal		
	Emax.	C. I.*	Color	Emax.	C. I.*	Color
None	620mμ	2.01	blue	605mμ	2.29	blue
Na^+	625mμ	1.77	blue	615mμ	1.91	blue
K^+	620mμ	1.75	blue	605mμ	2.11	blue
Ca^{++}	620mμ	1.87	blue	605mμ	1.90	blue
Mg^{++}	620mμ	2.05	blue	605mμ	1.96	blue
Ba^{++}	618mμ	1.73	blue	605mμ	1.56	blue
Pb^{++}	595mμ	2.64	blue	610mμ	1.44	blue
Hg^{++}	470mμ**	3.41	brown	575mμ	2.43	brown
Cu^{++}	590mμ	1.64	purple	595mμ	2.26	purple
Sn^{++}	467mμ	4.21	red	475mμ	8.31	red
Zn^{++}	615mμ	1.96	blue	615mμ	1.13	blue
Fe^{++}	—	—	green	480mμ**	—	green
Fe^{+++}	—	—	yellow	480mμ**	—	yellow

* Color intensity: calculated from the absorbance ratio of 280 mμ to the maximum absorption at visual region.

** The absorption maxima were not clear.

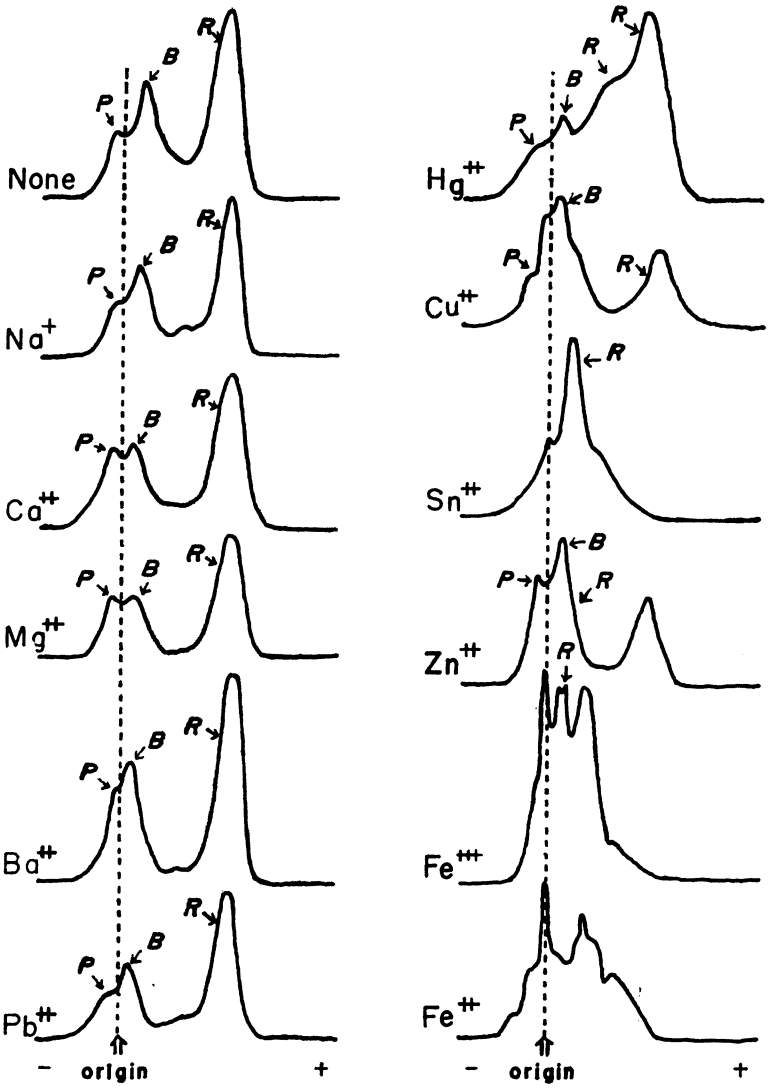


Fig. 5 Densitometry of cellulose acetate electrophoresis of carotenoprotein mixture which treated with various metallic ions.

P purple band, *B* blue band, *R* red band

changed color to the original color.

The color intensity was determined from the absorbance ratio of 280 mμ to the maximum absorption at visual region. It was found that the addition of certain metallic ions to the carotenoproteins gave somewhat intence color than metal-free solution. Figure 5 shows the electrophoretic diagrams of carotenoproteins treated with metallic ions, from which the ions were removed by dialysis before application on the cellulose acetate film. Na^+, K^+, Ca^{++}, Mg^{++}, and Ba^{++} had no influence upon the absorption maxima and the electrophoretic behavior of the carotenoprotein. However, Cu^{++}, Hg^{++}, Sn^{++}, Fe^{++}, and Fe^{+++} were effective in decomposing to yellowish or orangish substances with shifted absorption maximum and lowered electrophoretic mobility. Furthermore, these metallic ions gave colorless proteins. While Pb^{++} shifted absorption maximum to 595 mμ, the removal of the ion turned the original color without inhibition of the electrophoretic behavior. Spectrophotometric behaviors of the carotenoprotein mixture in the presence of metallic ions and in the removal of them are summerized in Table 1.

DISCUSSION

It has been demonstrated by many workers that the color changes of carotenoproteins are brought about by heat, oxidation, dehydrogenation, organic solvents, or other denaturation reagents which give a red product as principle with the absorption maximum of the prosthetic group[7)~10),14)16)17)]. The reversible or irreversible change in physical properties were also found in *B* and *P* obtained here. If *B* and *P* were let leave for a long period, they turned reddish first, then became a pale insoluble substance. The amount of carotenoid in *Rd* seemed less than that of *B* or *P*. The most probable explanation seems to be that preservation under strong illumination or air conditions brought autoxidation to *Rd* about. However, the protein-bound carotenoid is more stable than the form of free carotenoid[10)].

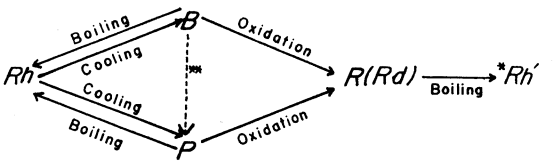
It has been reported that β-crustacyanin appears by denaturation of α-crustacyanin, and that the molecular weight becomes lower by dissociation[6)11)13)17)]. But in our results the reddish carotenoproteins, *R*, *Rd*, and *Rh*, were found to be of higher molecular weight than *B* and *P*. It was suggested that the reddish carotenoproteins with a higher molecular weight were the polymerized units of *B* or *P*. The difference of physical properties between them probably depended on a change in the protein structure or in the difference of stoichiometric amount of the prosthetic group.

Rh derived from *B* and *P* showed identical properties with *R*, while some differences were observed between *R* and *Rh*. Therefore *R* was suggested to be a derivative from *B* and *P*. Thus reddening of the bluish carotenoproteins seems to result from a metabolic action even in the pigmented layer of the exoskeleton. It was suggested that *R*, as reported in the previous paper[15)], is not an artifact resulted from the bluish carotenoprotein during the extraction, because the bluish

carotenoproteins were relatively stable under the nitrogen phase at low temperature.

It is interesting to discuss the metabolism of the red carotenoprotein in the native exoskeleton. LEE[3)4)] has isolated three pigments from marine isopods and discussed the mechanism of the various color appearances. Red cuticles are primarily found to contain unbound carotenoids, while green cuticles have a bluish carotenoid-protein complex. In *Daphnia* GREEN[2)] found that light promotes the deposition of carotenoids in the fat cells. In the crayfish having a thick calcified exoskeleton, the color appearance might contribute to the coexistence of unbound carotenoids and various colored carotenoproteins.

The carotenoprotein described by WALD et al.[16)] denatures by heating at 100°C. And they stated that if heated to 60°C, the color change is reversible after cooling off. When the bluish carotenoproteins obtained from the crayfish were heated above 60°C, they became red. ***B*** and ***P*** shifted their electrophoretic mobility and absorption maxima to 475 mμ. However, after keeping of ***Rh*** at a low temperature, a part of it gradually reversed to its original bluish color. A scheme of relationship between the carotenoproteins of crayfish is tentatively summerized as follows.



* *Rh'* is derived from *Rd* or *R* by boiling. It differes from *Rh* in spectrophotometric behavior.

** This relation will be reported in the next paper of this series.[20)]

Therefore the restoration was possible in the reddened carotenoprotein resulted from ***B*** and ***P*** by heating at a temperature under than 100°C. In general, the color of a boiled lobster is believed to be that of astacin, which is the oxidation product of astaxanthin derived from the cleavage of carotenoproteins by heating. Further investigation is necessary to explain the relationship between these different colored carotenoproteins.

It has been reported that the absorption maximum of the crustacyanin has been shifted to short wave length by desaltation[6)11)13)17)]. Addition of considerable concentration of salts restores the spectrum almost completely into the original. The bluish carotenoproteins might be stabilized by the addition of Na^+, K^+, Ca^{++}, Mg^{++}, Ba^{++}, Zn^{++}, and Pb^{++}. The presence of Fe^{++}, Fe^{+++}, Sn^{++}, Hg^{++}, and Cu^{++}, however, caused irreversible color change to the red or yellow products. GOTO *et al.*[18)] have determined the decomposing effect of some metallic ions on carotene in carrot. And they report that Fe^{+++}, Pb^{++}, Na^+, and K^+ are effective in the

decomposing of carotene when the sample is boiled in the solution. Therefore the stability of carotenoids for metallic ions seems to differ in mode of combination to protein.

SUMMARY

1. The red carotenoprotein present in the native exoskeleton of the crayfish (***Cambarus clarkii***) seemed to be a derivative resulted from the oxidation of blue or purple carotenoproteins.

2. The red carotenoprotein extracted from the exoskeleton was different in physical properties from the reddened one resulted by boiling.

3. The red carotenoproteins resulted from boiling and autoxidation had a lesser carotenoid content and showed a higher molecular weight than the bluish carotenoproteins.

4. It was found that the carotenoprotein reddened by heating at 100°C was partially reconverted to the original bluish carotenoproteins.

5. The presence of 0.005 *M* Fe^{+}, Fe^{++}, Sn^{++}, Hg^{++}, and Cu^{++} caused irreversible denaturation of the carotenoproteins.

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水産動物のカロチノプロテインに関する研究

Ⅱ アメリカザリガニのカロチノプロテインの赤変について

中川　平介・鹿山　光・浅川　末三

1. 天然の甲殻に存在する赤いカロチノプロテイン（R）は青又は紫カロチノプロテイン（B, P）の自動酸化により生じたものと考える.
2. R は B 又は P を 100°C に加熱して生じた赤いカロチノプロテイン（Rh）とは二・三性質を異にする.
3. B 及び P の自動酸化，加熱により生ずる R, Rh は B, P よりカロチノイド含量が少なく，分子量は大きい.
4. B 及び P を 100°C 加熱して生じた Rh を長期間保存すると 部分的ではあるが元の B, P に回復することを認めた.
5. 0.005 M の Fe^{++}, Fe^{+++}, Sn^{++}, Hg^{++}, Cu^{++} の存在によりカロチノプロテインは不可逆的に変性する.