Properties of the Crustacyanins and the Yellow Lobster Shell Pigment*

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ABSTRACT: The minimum molecular weight of the astaxanthin–protein pigment β-crustacyanin has been found to be 21,800 ± 1000; similar values are reported for α-crustacyanin and a new pigment, γ-crustacyanin. The minimum molecular weight of the yellow lobster shell pigment is 4400. The molecular weights of β-crustacyanin, α-crustacyanin, and γ-crustacyanin are 48,000 ± 5000, 380,000 ± 40,000, and 400,000 ± 40,000, respectively, while that of the yellow pigment is 90,000 ± 10,000. This indicates that β-crustacyanin contains two molecules of astaxanthin and suggests that α-crustacyanin is composed of eight subunits equivalent to β-crustacyanin. The extinction coefficient, oscillator strength, and shape of the absorption bands of the crustacyanins and of astaxanthin are almost identical, suggesting that the energy but not the nature of the electronic transition of astaxanthin is altered by combination with protein; these parameters and also the amino acid composition are different in the yellow pigment. Denaturation of crustacyanin by guanidine hydrochloride or alkali gives separable yellow and colorless fractions which recombine stoichiometrically to the blue pigment. The accessibility of the prosthetic group of the pigments is demonstrated by the rapid reduction of the astaxanthin carbonyl groups with sodium borohydride; this reaction does not result in the formation of a covalent bond between the protein and carotenoid. The bound carotenoid molecules are optically active with maximal molar rotations of 10^5–10^6. The optical rotatory dispersion and circular dichroism spectra in the visible region exhibit evidence for a splitting of the excitation similar to that predicted by exciton theory. Possible mechanisms of the perturbed optical properties of astaxanthin in crustacyanin are discussed and it is suggested that intermolecular interactions of the π-electron systems and/or distortion induced by the protein may account for the altered spectral and rotatory properties.

In this paper we report some of the physical, chemical, and optical properties of the carotenoid–protein lobster shell pigments which, in their denatured state, are responsible for the red color of the boiled lobster: the crustacyanins and the as yet unchristened yellow shell pigment (Wald *et al.*, 1948; Zagalsky and Cheesman, 1963; Jencks and Buten, 1964; Ceccaldi and Allemand, 1964, 1965). The shifts of the absorption maximum of astaxanthin at about 490 to 587–632 mμ in the crustacyanins and to *ca.* 400 mμ in the yellow pigment correspond to changes in excitation energy covering a range of 25 kcal/mole which are brought about by interactions between the carotenoid prosthetic group and the macromolecule. The mechanisms of these interactions are of interest in their own right, but are also of special interest because of the occurrence of a similar shift of between 45 and 245 mμ which is brought about by combination of retinal with opsin in the visual pigments and cannot be accounted for by the imine bond to this protein (Morton and Pitt, 1955; Wald, 1960; Bridges, 1962). Furthermore, an understanding of the mechanism of such interactions may contribute to our understanding of the mechanism of enzyme–substrate interactions.

Zagalsky and Cheesman (1963) and Zagalsky (1964) crystallized and resolved α-crustacyanin and showed that α-crustacyanin (λ_{max} 632 mμ) is a polymer which can be irreversibly converted into a subunit, β-crustacyanin (λ_{max} 587 mμ). Studies of the physical properties of these pigments, which were carried out independently of the work reported here, have confirmed this finding, but have yielded conflicting results as to the molecular weights and minimum molecular weights of the pigments (Cheesman *et al.*, 1966; Kuhn and Kühn, 1966).

Experimental Procedure

Materials and methods were as described in the preceding paper (Buchwald and Jencks, 1968). The pigments were purified by a modification of earlier procedures (Jencks and Buten, 1964; Zagalsky, 1964). All steps were carried out in the cold, avoiding excessive exposure to light. Carefully cleaned and ground lobster shells were extracted for several days with three changes of 0.5 M sodium EDTA (pH 7.5) with constant stirring. The extract was filtered and brought to pH 7.5 with hydrochloric acid. The pigments were precipitated by the addition of 273 g of ammonium

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TABLE I: Spectral Properties of the Purified Pigments.

Pigment	λ_{max} (mμ)	$\nu^{1}/_{2}$[a] (cm^{-1})	ϵ_{max}[b]	f[c]	Other Max (mμ)	Ratio $A_{visible}/A_{ultraviolet}$
α-Crustacyanin	632	4200	1.25×10^5	2.60	370, 320, 278	3.3[d]
β-Crustacyanin	587	4300	1.16×10^5	2.54	360, 315,[e] 278	3.3
γ-Crustacyanin	625	4200	1.19×10^5	2.52	366, 318, 278	3.3
Yellow	409 ±2	3200	1.00×10^5	1.91	300,[f] 255[f]	5.7[g]
Astaxanthin						
In pyridine	492	4200	1.12×10^5	2.35		
In hexane	472	4200	1.24×10^5	2.60		

[a] Half-band width. [b] Molar extinction coefficient, based on astaxanthin content. [c] Oscillator strength. [d] Obtained in only one preparation; other preparations showed a ratio of 2.9–3.1. [e] Shoulder. [f] Broad peak. [g] Obtained in only one preparation.

sulfate/l., collected by centrifugation, and dissolved in 0.05 M potassium phosphate buffer (pH 7). After dialysis against four changes of the same buffer, for a total of 30 hr, the pigment (2 g of protein) was chromatographed on a 600-ml column of DEAE-cellulose (4.5 × 38 cm) which had been prepared according to the method of Peterson and Sober (1962) and thoroughly washed with concentrated and then 0.05 M phosphate buffer (pH 7.0). After the pigments were applied to the column, it was washed with buffer until 10–15% of the absorbance at 620 mμ (β-crustacyanin) had run through. The pigments were then eluted with a linear gradient from 0 to 1.0 M potassium chloride in the same buffer, of 5-l. total volume. When the yellow pigment began to come off the column, the gradient was discontinued and the remainder of the yellow pigment was eluted with 1.0 M potassium chloride in the same buffer.

The major fraction of blue pigments (α- and γ-crustacyanins) was precipitated by 50% saturation with ammonium sulfate, collected by centrifugation, resuspended in 0.05 M potassium phosphate buffer (pH 7), and dialyzed against four changes of the same buffer for a total of 24 hr. This fraction was rechromatographed on DEAE-cellulose in the same manner as described above, except that the gradient was from 0 to 0.5 M potassium chloride in the same buffer. The pigment separated into two peaks, the first of which is γ-crustacyanin.[1]

The pigment from the second peak was precipitated by 50% ammonium sulfate, collected by centrifugation, dissolved in 0.05 M potassium phosphate buffer (pH 7), and dialyzed against four changes of the same buffer for a total of 18 hr. This material was applied to a 400-ml (3.2 × 40 cm) column of DEAE-cellulose equilibrated with the same buffer and washed with buffer until the purple pigment had run through the column. The major fraction was then eluted with 0.25 M potassium phosphate buffer (pH 7). The central fractions with the highest ratio of visible to ultraviolet absorption were pooled, collected by precipitation with 50% saturated ammonium sulfate, redissolved in 0.05 M potassium phosphate buffer (pH 7), and dialyzed against the same buffer. This purified α-crustacyanin was stored in the refrigerator in the dark. After prolonged standing this fraction could be again purified by repetition of the final DEAE chromatography. γ-Crustacyanin which had stood for several months was repurified by the same procedure, except that 0.15 M buffer was used for elution.

Pure β-crustacyanin was obtained as the rapidly eluted fraction in the above steps. It was concentrated by precipitation with ammonium sulfate at 60% saturation, resuspended in 0.05 M potassium phosphate buffer (pH 7), and dialyzed against the same buffer.

The yellow pigment from the first DEAE-cellulose step was chromatographed on a 200-ml (3 × 30 cm) DEAE-cellulose column equilibrated with 0.10 M potassium phosphate buffer (pH 7), with a linear gradient from 0.1 to 0.2 M buffer containing 1.0 M potassium chloride and a total volume of 600 ml. The eluted pigment fractions were concentrated by precipitation with 60% ammonium sulfate, redissolved in 0.05 M potassium phosphate buffer (pH 7), and dialyzed against the same buffer overnight. The purification of the unstable yellow pigment was difficult to reproduce and gave low yields.

About 40–45% of the original absorbance at 620 mμ is recovered as purified α- and γ-crustacyanins and about 10–15% as pure β-crustacyanin. A summary of the spectral properties of the purified pigments is given in Table I.

Cellulose acetate electrophoresis was carried out

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[1] This pigment is probably a component of a blue fraction (λ_{max} 616 mμ) eluted from DEAE-cellulose by washing with 0.15 M phosphate buffer (Cheesman *et al.*, 1966).

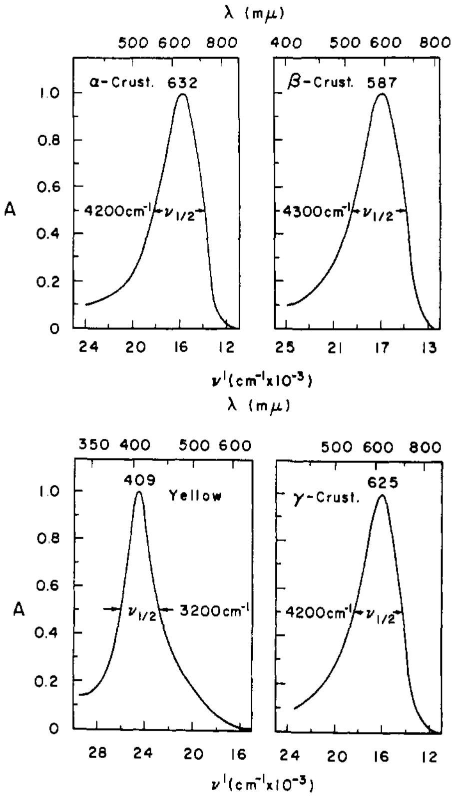


FIGURE 1: Normalized absorption spectra of α-crustacyanin, β-crustacyanin, γ-crustacyanin, and the yellow lobster shell pigment plotted on the frequency scale.

according to the procedure of Kohn (1960) at 4°. The colored zones were marked and the strips were stained for 12–18 hr with 1% Amido Black in a solution containing water–methanol–glacial acetic acid (50:50:10). They were then washed with the same solvent used to dissolve the dye.

Amino acid analyses were carried out by the method of Spackman *et al.* (1958) with a Beckman amino acid analyzer. Hydrolysis of 0.3-mg samples of pigment was carried out in constant-boiling hydrochloric acid; a small amount of brown residue was adsorbed onto the filter paper covering the columns in the analyzer. The analyzer was standardized with an amino acid mixture (Beckman–Spinco) and with known concentrations of L-norleucine and L-2-amino-3-guanidopropionic acid in the buffer used to dissolve the hydrolysate. The concentrations were calculated from the height and half-band width of the peaks. Cysteine and cystine were determined as cysteic acid (Hirs, 1956). The tryptophan content of pigment solutions was determined by the method of Spies and Chambers (1949), with appropriate blank measurements to correct for the small amount of color caused by the carotenoid. Phosphate was determined by the method of Chen *et al.* (1956).

Minimum molecular weights were determined from the carotenoid and nitrogen content of the pigments. The carotenoid concentration was determined spectrophotometrically at 492 mμ after addition of 5–50-μl aliquots of pigment to 1.0 ml of pyridine (Cheesman, 1958). Nitrogen was determined by Kjeldahl digestion and Nessler determination of ammonia (Lang, 1958). The protein concentration was calculated based on a nitrogen content of 15.7%, which was determined from the amino acid composition and which is in agreement with the results of Cheesman *et al.* (1966) and Kuhn and Kühn (1966). All analyses were carried out in duplicate. Extinction coefficients of the pigments in 0.05 M potassium phosphate buffer (pH 7) were determined with the same samples used for minimum molecular weight determinations.

Oscillator strengths were calculated from the relationship $f = 4.3 \times 10^9 \int \epsilon d\nu'$ (Murrell, 1963). The areas under the peaks were measured with a planimeter.

Sedimentation coefficients were determined in a Spinco Model E ultracentrifuge at 59,780 and 52,640 rpm in 0.05 or 0.10 M potassium phosphate buffer (pH 7). The green filter was omitted from the optical system to permit photography of the colored solutions. The plates were read on a Gaertner microcomparator. The sedimentation coefficients were calculated as described by Schachman (1962). Diffusion coefficients were determined in the ultracentrifuge by the method of Ehrenberg (1957) with synthetic boundary cells and corrected to $D_{20,w}$ (Schachman, 1962). α-Crustacyanin was centrifuged at 9945 rpm, γ-crustacyanin at 4908 rpm, and β-crustacyanin at 20,410 rpm for the diffusion measurements. Kodak type II-G plates were exposed for 1 sec in the absence of the green filter. The diffusion coefficient of the yellow pigment was estimated by chromatography on a Sephadex G-200 column according to the method of Ackers (1964).

Molecular weight determination by equilibrium sedimentation was carried out by the method of Yphantis (1964). The yellow pigment was centrifuged at 24,630 and 23,150 rpm and β-crustacyanin at 47,660, 33,450, and 37,020 rpm for 24 hr. Equilibrium was reached in 18 hr. Centrifugation was carried out in double-sector cells with a column size of 2 mm and protein concentrations of 0.10–0.15 mg/ml. Photographs were taken with Kodak type I-N red sensitive plates and a Kodak red filter CS-2-61. Molecular weights were also calculated from the Svedberg relationship (Svedberg and Pedersen, 1940). Molecular weight = $(s/D)[RT/(1 - \bar{v}\rho)]$, in which s is the sedimentation coefficient, D is the diffusion coefficient, $\bar{v}$ is the partial specific volume, and ρ is density.

The partial specific volume of a 0.8% solution of β-crustacyanin which had been dialyzed for 5 days against six changes of 1 l. of water was determined in a 1-ml pycnometer. The protein concentration was determined by drying to constant weight at 105°. The partial specific volumes of α- and γ-crustacyanins were assumed to be the same as that of β-crustacyanin.

Molecular weights were also estimated by molecular exclusion chromatography on Sephadex gel columns (Andrews, 1964). The columns were calibrated with malic dehydrogenase, ovalbumin, cytochrome *c*, dogfish muscle lactic dehydrogenase, and serum albumin.

TABLE II: Amino Acid Composition of the Protein Pigments.[a]

Pigment Prepn:	α-Crustacyanin		β-Crustacyanin	γ-Crustacyanin	Yellow
Acid	1965	1966	1965	1966	1965
Lys	6.90 ± 0.22	5.86 ± 0.04	6.62 ± 0.33	5.97 ± 0.09	4.76 ± 0.13
His	1.62 ± 0.04	2.04 ± 0.03	1.52 ± 0.02	1.52 ± 0.03	2.40 ± 0.05
Arg	4.86 ± 0.11	4.43 ± 0.11	4.3 ± 0.20	4.36 ± 0.02	5.99 ± 0.50
Asp	13.12 ± 0.27	13.20 ± 0.40	13.04 ± 0.40	13.26 ± 0.21	13.60 ± 0.81
Thr[b]	5.61	5.42	5.66	5.75	4.85
Ser[b]	4.79	4.59	4.87	4.69	3.87
Glu	9.73 ± 0.07	10.34 ± 0.8	9.89 ± 0.14	9.88 ± 0.07	14.45 ± 0.47
Pro	5.33 ± 0.03	4.76 ± 0.06	4.03 ± 0.14	4.94 ± 0.10	5.15 ± 0.23
Gly	2.35 ± 0.01	2.78 ± 0.01	2.56 ± 0.04	2.52 ± 0.01	4.02 ± 0.14
Ala	4.91 ± 0.01	4.90 ± 0.01	5.42 ± 0.03	5.01 ± 0.02	5.17 ± 0.20
Cys ($^1/_2$)	2.72 ± 0.01	3.02 ± 0.02	3.03 ± 0.04	3.19 ± 0.03	2.50 ± 0.07
Val[c]	5.59	5.54	6.22	6.15	6.06
Met	0.43 ± 0.06	0.58 ± 0.02	0.50 ± 0.04	0.41 ± 0.01	0.94 ± 0.20
Ile[c]	4.20	4.30	4.22	4.26	6.12
Leu	4.65 ± 0.02	4.82 ± 0.05	4.23 ± 0.04	4.43 ± 0.03	7.60 ± 0.40
Tyr	10.56 ± 0.02	11.11 ± 0.13	11.28 ± 0.32	11.26 ± 0.02	4.39 ± 0.33
Phe	9.54 ± 0.02	9.88 ± 0.04	9.76 ± 0.11	10.13 ± 0.08	5.67 ± 0.27
Trp	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	2.65 ± 0.1

[a] Per cent residue weight (g of anhydro amino acid/100 g of protein). [b] 24-hr value. [c] Maximum value.

Optical rotatory dispersion measurements were carried out with a Cary Model 60 recording spectropolarimeter at 22° with 1-cm path-length cells. Measurements performed at different concentrations of pigment showed adherence to Biot's law. Below 600 mμ the runs were done with a 15-Å slit program and between 650 and 550 mμ with a 100-Å program; in the region of overlap the two scans gave identical results. Circular dichroism spectra were obtained with a Jasco–Durrum recording spectropolarimeter at the Retina Foundation, which was kindly made available by Dr. Béla Nagy. The measurements were taken with Jasco B0045 1-cm cells at room temperature. Each spectrum was preceded and followed by blank measurements on the solvent which showed that the base line was steady. Measurements at different concentrations of the pigments were found to agree within 5%. The molecular ellipticity $[\theta]$ was calculated from the relationship $[\theta] = 3300\Delta\epsilon$ in which $\Delta\epsilon$ is the difference in the absorption of the two components of the circularly polarized light and has the units of degrees cm^2/mole (Crabbé, 1965; Beychok, 1966).

Results

Spectra and Composition. The spectral properties of the purified pigments are summarized in Table I. The ratio of the maximum absorbance of the principal visible band to that of the ultraviolet band is 3.3 for the best preparations of the crustacyanins, similar to the values reported by others (Cheesman *et al.*, 1966; Kuhn and Kühn, 1966), but most of our preparations of α-crustacyanin showed a ratio of 2.9–3.1, indicating the presence of approximately 10% impurity in these preparations. The minor peaks near 370 and 320 mμ have a ratio of absorbance of 0.15 compared to the major peak and of about 1.0 to each other. Displacement of the major peak to shorter wavelengths in β- and γ-crustacyanins compared to α-crustacyanin is accompanied by smaller displacements of these peaks to shorter wavelengths.

The principal absorption bands of the three crustacyanins are normalized and plotted on a frequency scale in Figure 1. The shape of the three bands is identical and is also the same as that of astaxanthin itself; the widths of the peaks at half-maximal absorbance are all 4200 ± 100 cm^{-1} (Table I). The extinction coefficients, based on the astaxanthin content, are very similar to the extinction coefficient of astaxanthin in hexane. In contrast, the absorption band of the yellow pigment has an entirely different shape, a smaller half-width of 3200 cm^{-1} and a significantly smaller extinction coefficient and oscillator strength.

No fluorescence of β-crustacyanin could be detected, with the exception of an emission maximum near 340 mμ upon excitation at 280 and 290 mμ which is attributed to tryptophan residues in the protein.

The amino acid composition of the pigments is shown in Table II. The composition of the three crustacyanins is very similar but not identical. The composition of the yellow pigment is clearly different from that of the crustacyanins. The higher content of glutamic acid in this protein contributes to its high affinity for DEAE-cellulose. The total phosphorus content

TABLE III: Minimum Molecular Weights of the Lobster Shell Pigments.

Pigment	Year	Min Mol Wt	$E_{1\,\text{cm}}^{1\%}$ $\lambda_{\max}$ (mμ)
α-Crustacyanin	1964	27,800 ± 800	42 ± 2
	1965	28,500 ± 1,000	45 ± 2
	1965	26,600 ± 1,000	47 ± 2
	1966	26,000 ± 1,000	48 ± 2
		Av 27,200 ± 1,200	46 ± 3
β-Crustacyanin	1964	23,400 ± 800	49 ± 2
	1965	21,300 ± 800	56 ± 2
	1966	20,800 ± 1,000	56 ± 2
		Av 21,800 ± 1,000	54 ± 2
γ-Crustacyanin	1966	23,800 ± 800	47 ± 2
Yellow	1965	4,400 ± 300	225 ± 10

of the pigments after precipitation with perchloric acid was found to be less than 0.2 mole of phosphate/mole of astaxanthin in α-crustacyanin and less than 0.3 mole of phosphate/mole of astaxanthin in the yellow pigment.

Analysis for metals carried out by flame photometry by Dr. Bert Vallee on the yellow pigment and on α-crustacyanin showed less than 0.01 mole/mole of astaxanthin of barium, iron, chromium, cobalt, molybdenum, magnesium, strontium, nickel, zinc, manganese, lead, and cadmium. Traces of aluminum and calcium (0.05 and 0.15 mole per mole of astaxanthin, respectively) were detected but probably represent contamination. Thus, there appears to be no significant content of heavy metals in these pigments.

The minimum molecular weights and extinction coefficients of the pigments, based on spectrophotometric determinations of astaxanthin and nitrogen analyses for protein, are summarized in Table III. Based on the accuracy of the astaxanthin and protein analyses, these values are estimated to be accurate within ±5%; the minimum molecular weight of α-crustacyanin is probably too high by an additional 10% because of the presence of impurities in this pigment. The value of 21,800 for the minimum molecular weight of β-crustacyanin is 15% larger than the value of 19,000 reported by Kuhn and Kühn (1966), but is smaller than the value of 36,000 reported by Cheesman *et al.* (1966). The yellow pigment has a much smaller minimum molecular weight of 4400.

Immunological analysis of α- and β-crustacyanins and of the yellow pigment, carried out in collaboration with Drs. Lawrence Levine and Edna Seaman by complement fixation and double diffusion in agar (Wasserman and Levine, 1961; Ouchterlony, 1949), revealed cross-reactions indicative of common antigenic determinants in these three pigments.

Physical Properties. The preparations of β- and γ-crustacyanins sedimented as a single peak in the ultracentrifuge, while α-crustacyanin was found to contain about 5% of a more rapidly sedimenting impurity. The sedimentation coefficients for all of the pigments were found to be independent of concentration between 1 and 5 mg/ml. Electrophoresis of the pigments was carried out on cellulose acetate strips with 0.05 or 0.10 M potassium phosphate buffer (pH 7). The β- and γ-crustacyanins showed a single band; the yellow pigment showed a very faint colorless contaminant in addition to the major band and α-crustacyanin showed about 5% of a colored contaminant which migrates slower than the main band.

The physical constants of the pigments are summarized in Table IV. The molecular weight of α-crustacyanin, determined from the sedimentation and diffusion constants, is 380,000. The molecular weight of β-crustacyanin is 50,000 ± 3000, as determined by sedimentation equilibrium; this value was confirmed by sedimentation and diffusion. It is larger than a value of 37,000, estimated by a comparison of the elution position of this protein from a column of Sephadex G-100 gel with that of calibrating proteins, presumably because of differences in the shapes of these proteins. The molecular weight of γ-crustacyanin is 400,000 ± 40,000. The sedimentation coefficient and diffusion coefficient of this protein are nearly twice those of α-crustacyanin, indicating a more nearly spherical shape of the former protein. The molecular weight of the yellow pigment was found to be 97,000 by sedimentation equilibrium, but the plots for this pigment showed a small amount of curvature and the molecular weight was estimated from the lower portion of the plots. A value of 90,000 was estimated by comparison of the elution position from Sephadex G-200 gel of this protein compared to calibrating proteins and a value of 80,000 was obtained from the sedimentation constant and a diffusion coefficient estimated from Sephadex chromatography (Ackers, 1964). From these results the molecular weight is estimated to be 90,000 ± 10,000.

The ultracentrifuge measurements are based upon a partial specific volume of 0.76, which was measured

TABLE IV: Physical Constants of the Lobster Pigments.[a]

Pigment	$s_{20,w}$	$D_{20,w} \times 10^7$ cm²/sec	D (Sephadex) $\times 10^7$ cm²/sec	$\bar{v}$	Mol Wt[b] $\times 10^{-4}$ g/mole	Mol Wt[c] $\times 10^{-4}$ g/mole	Mol Wt[d] $\times 10^{-4}$ g/mole	Mol Wt[e] $\times 10^{-4}$ g/mole
α-Crustacyanin	12.4 ± 0.3	3.3 ± 0.3		0.76 ± 0.01				38 ± 2.5
β-Crustacyanin	2.8 ± 0.1	(5.8)[f]		0.76 ± 0.01	3.7 ± 0.2	(4.7, 5.0, 5.3) 5.0 ± 0.3		(4.9)[f]
γ-Crustacyanin	24 ± 1	6.05 ± 0.25		0.76 ± 0.01				40 ± 4
Yellow	4.2 ± 0.1		5.2 ± 0.3	0.76[g]	9.0 ± 0.3	(8.6, 9.7, 10.7) 9.7 ± 1.0	8.0 ± 0.8	

[a] All the measurements were carried out with solutions in 0.05 or 0.10 M potassium phosphate buffer (pH 7). Details of the measurements are given in the text. [b] Molecular weight estimated from Sephadex chromatography. [c] Molecular weight determined from sedimentation equilibrium. [d] Molecular weight determined from the Svedberg equation using a diffusion coefficient estimated with data from Sephadex chromatography. [e] Molecular weight determined as in c but with a diffusion coefficient measured in the ultracentrifuge. [f] Value determined only once. [g] Partial specific volume calculated from the amino acid composition.

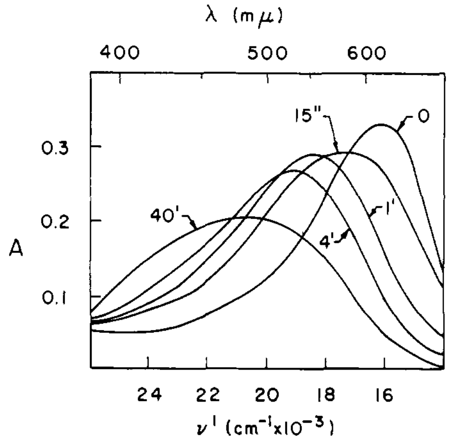


FIGURE 2: Denaturation of α-crustacyanin in 0.2 M sodium carbonate buffer (pH 10.5). The zero time spectrum was obtained with the same concentration of pigment diluted into phosphate buffer (pH 7).

directly for β-crustacyanin and was assumed to be the same for the other crustacyanins. A value of 0.73 was calculated for the partial specific volume from the amino acid analysis (Cohn and Edsall, 1943) and a partial specific volume of 1.0 for astaxanthin (based on the value for β-carotene in the Handbook of Chemistry and Physics, 1965), which would give a molecular weight of β-crustacyanin of 44,000. The partial specific volume of the yellow pigment was calculated from the amino acid and astaxanthin content and introduces a small further uncertainty into the molecular weight of this pigment.

Denaturation. In the presence of nonpolar denaturing agents the lobster pigments are denatured to red products with spectra similar to that of astaxanthin, while in the presence of denaturing agents of the urea–guanidinium class which contain free hydrogen atoms they are denatured to yellow pigments with absorption maxima near 400 mμ; in the presence of acid and base the crustacyanins are denatured to yellow products with absorption maxima near 440 and 400 mμ, respectively (Jencks and Buten, 1964). The denaturation of α-crustacyanin as a function of time at pH 10.5 is shown in Figure 2. There is a gradual shift of the absorption maximum to lower wavelength with a broadening of the peak and no indication of an isosbestic point; at pH 11.5 the absorption maximum is at 397 mμ and in still stronger base it shifts back to 413 mμ. This gradual shift through a large number of intermediate absorption bands has been observed with a number of other denaturing agents and requires either that denaturation is a continuous process or that it proceed through a large number of intermediate species.

Upon ultracentrifugation, the yellow product of denaturation at pH 12 separates into a more rapidly sedimenting yellow component with an $s_{20,w}$ of 5.6 S and a slower colorless fraction with an $s_{20,w}$ of 2.1 S. Chromatography on Sephadex gel gave a rough estimate of the molecular weight of the colorless fraction of 20,000–

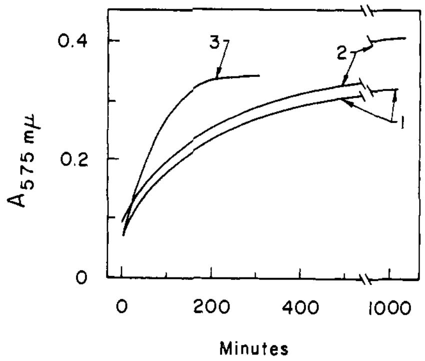


FIGURE 3: Renaturation of denatured α-crustacyanin reconstituted from the separated yellow and colorless fractions at 42.4° in 0.1 M potassium phosphate buffer (pH 7). The samples contained 0.05 mg of the yellow fraction in tubes 1 and 2 and 0.1 mg in tube 3, which was added to 0.12 mg of the colorless fraction in tubes 1 and 3 and 0.24 mg in tube 2.

24,000. Kuhn and Kühn (1966) have reported a molecular weight of less than 10,000 for this fraction.

The yellow product of denaturation in 3 M guanidine hydrochloride also separates into a rapidly sedimenting yellow fraction and a slower colorless fraction upon ultracentrifugation; the values of $s_{20,w}$ for these fractions are 18 and 2.1 S, respectively, after correction for the density and viscosity of the solution according to Kawahara *et al.* (1965). After removal of the guanidine by dialysis in the cold, the yellow fraction sediments with a value of $s_{20,w}$ of 5.5 S at 2° while at room temperature it sediments very rapidly and sticks to the bottom of the cell. At both temperatures the colorless material sediments with a value of $s_{20,w}$ of 2.0 ± 0.1 S. Upon Sephadex chromatography the yellow fraction migrates with the excluded volume through G-200 gel, while the colorless material is a broad, asymmetric peak corresponding to a molecular weight of approximately 20,000–24,000. These results are in agreement with those of Cheesman *et al.* (1966) for the products of denaturation in 6 M urea and in 0.01 M sodium hydroxide. It is apparent that the yellow product of these denaturation reactions undergoes aggregation to a high molecular weight material. Treatment of the native yellow lobster pigment (mol wt 90,000) with 3 M guanidine hydrochloride was also found to give a large aggregate which is completely excluded from Sephadex G-200 gel.

The stoichiometry of the denaturation of α-crustacyanin in the presence of 3 M guanidine hydrochloride was determined by measuring the astaxanthin and protein contents of the yellow and colorless fractions, which were separated by chromatography on Sephadex G-100 gel with a quantitative recovery of total protein. The minimum molecular weight of the yellow product is 6700 ± 900, 50% higher than that of the native yellow lobster pigment. The protein contents of the colorless and yellow proteins were found to correspond to 77 and 23% of the total protein, respectively, corresponding to a ratio of 3.4 for the protein content of the two samples. Renaturation of α-crustacyanin which had been denatured with 3 M guanidine hydrochloride and from which the denaturing agent had been removed by dialysis in the cold, was found to follow first-order kinetics after an initial faster reaction and gives a product with an absorption maximum between 570 and 600 mμ. The rates were found to be independent of pH between pH 7.6 and 8.1 and to increase with decreasing ionic strength and increasing temperature. The first-order rate constants at 39, 42, and 46° give a linear Arrhenius plot with a large activation energy, on the order of 55 kcal/mole.

The kinetics of the renaturation reaction studied with the separated colorless and yellow fractions are shown in Figure 3. The rate of the reaction is independent of the concentration of the colorless fraction and is increased approximately twofold by doubling the concentration of the yellow fraction. However, under the conditions of this experiment the *extent* of renaturation is dependent upon the concentration of colorless pigment and is independent of the concentration of yellow pigment. No renaturation was observed with either the yellow or colorless fractions alone. From the ratio of 3.4 for the protein contents of the colorless and yellow products of the denaturation reaction it is predicted that the amount of the colorless fraction is limiting in the renaturation reaction of runs 1 and 3 and the yellow fraction is limiting in run 2. The extent of the renaturation reaction in these fractions is as expected from this stoichiometry and the amount of blue pigment recovered in run 2 is within 10% of that predicted for a stoichiometric recombination of the fractions according to this ratio, indicating that both fractions must be present in the correct stoichiometric relationship to each other in order that the maximum amount of renaturation may take place.

It was shown that the yellow material produced by guanidine denaturation of α-crustacyanin undergoes renaturation in the presence of the colorless protein obtained from β-crustacyanin and *vice versa.* On prolonged storage, γ-crustacyanin as well as α-crustacyanin were found to be partially converted into β-crustacyanin, which could be separated by chromatography on DEAE-cellulose.

Chemical Reactivity of the Carotenoid. In order to determine the accessibility of astaxanthin and the nature of its binding to the protein in the crustacyanins, the rate of reduction of astaxanthin in the native pigments by sodium borohydride was measured. Attempts to measure the rate of reduction of pure astaxanthin by sodium borohydride in aqueous pyridine and aqueous isopropyl alcohol did not give results which were useful for comparison with the rate of reduction of the bound pigment due to complications introduced by the presence of these mixed solvents. In the presence of pyridine the rate of astaxanthin reduction shows a roller coaster dependence upon the pyridine concentration, which is apparently caused by mixed micelle formation at low concentrations and a pronounced solvent effect at higher concentrations of pyridine. In aqueous isopro-

TABLE V: Reaction of Sodium Borohydride with α- and β-Crustacyanins at pH 10 and 25°.[a]

Crustacyanin				
Pigment	α	α	β	β
[KCl] (M)	0.4	0	0.4	0
$[BH_4^-]$ (M)	0.02	0.02	0.02	0.02
k_{obsd} (min^{-1} × 10^2)	3.8 ± 0.2	5.8 ± 0.2	5.65 ± 0.2	5.9 ± 0.3
Number of runs	5	3	3	3

[a] The solutions contained 0.10 M sodium borate buffer and the reactions were followed by the decrease in absorbance at 650 mμ. The pigments were incubated in the reaction mixture for 10 min and the reactions were started by the addition of a small volume of sodium borohydride in 0.001 M sodium hydroxide. At the end of the reaction the pH of each reaction mixture was found to be 9.96 ± 0.06 in all of the runs.

pyl alcohol the reaction at low borohydride concentration shows a pronounced lag which is abolished by prior incubation of borohydride in this solvent and an increasing rate with increasing time of prior incubation, suggesting that borohydride reacts with isopropyl alcohol to form a more reactive reducing species.

The second-order rate constant for the reduction of astaxanthin by excess sodium borohydride in 0.1 M sodium borate buffer (pH 10) in water in the presence of 1% Brij 35 detergent was found to be 11 ± 2 M^{-1} min^{-1} over a range of borohydride concentration from 5×10^{-3} to 10^{-2} M. The individual runs are pseudo-first order, the rate is not significantly affected by changing the concentration of detergent from 1 to 5%, and the rate constant is similar to that in 30% pyridine, in which the astaxanthin is in solution. These facts suggest that the polar carbonyl groups which undergo reduction are at the surface of the detergent micelle and are exposed to the aqueous solvent for the reaction with borohydride. Nuclear magnetic resonance measurements have shown that the polar molecules nitrobenzene and *N,N*-dimethylaniline are adsorbed at the surface of micelles of cetyltrimethylammonium bromide, whereas the less polar molecules isopropylbenzene and cyclohexane are incorporated into the nonpolar interior of the micelle (Eriksson and Gillberg, 1966).

The pseudo-first-order rate constants for the reduction of the astaxanthin prosthetic group of α- and β-crystacyanins in 0.1 M sodium borate buffer (pH 10) in the presence and absence of 0.4 M potassium chloride are shown in Table V. The pseudo-first-order rate constants were found to be not strictly linear with respect to borohydride concentration, but the rate constants shown in Table V correspond to second-order rate constants of 1.9-3 M^{-1} min^{-1}, which are not very much smaller than that for the reaction of borohydride with astaxanthin in water in the presence of detergent and are similar to the value of 1.5 M^{-1} min^{-1} for the reaction of borohydride with acetone in water (Davis and Carter, 1966). The pseudo-first-order rate constant for α-crustacyanin of 3.8×10^{-2} min^{-1} is increased to 5.8×10^{-2} min^{-1} in the absence of potassium chloride. The latter value is similar to that for β-crustacyanin and suggests that dissociation of α-crustacyanin in the absence of salt to a subunit, similar to β-crustacyanin (Cheesman *et al.*, 1966), causes a small increase in rate.

The reduction of crustacyanin causes a loss of the characteristic blue color caused by interaction of astaxanthin with the apoprotein and gives a product with absorption maxima at 520, 475, and 445 mμ, similar to those of crustaxanthin, the expected reduction product of astaxanthin (Figure 4). Extraction with ether gives a product with the same spectrum as crustaxanthin in ether. Spectra at intermediate times during the reduction show the presence of an intermediate with a larger absorption at 520 mμ than either the starting material or the product (Figure 4). In a parallel experiment the carotenoid was extracted with ether several times during the course of the reduction and was subjected to thin-

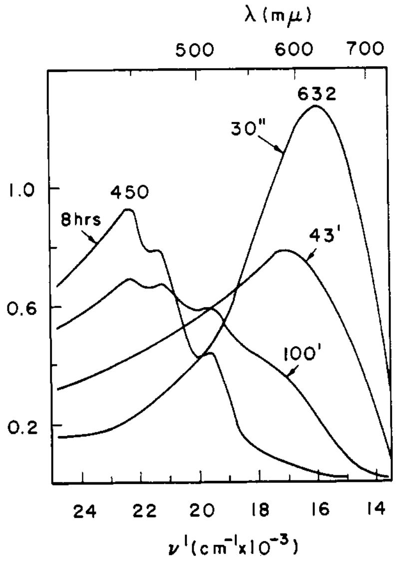


FIGURE 4: The reaction of α-crustacyanin with 5×10^{-3} M sodium borohydride in 0.1 M sodium borate buffer (pH 10) containing 0.4 M potassium chloride at 25°.

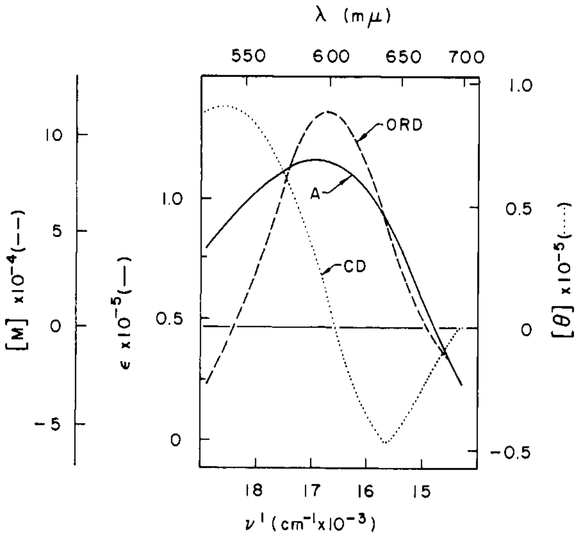


FIGURE 5: Optical rotatory dispersion, circular dichroism, and absorption spectra of β-crustacyanin in 0.05 M potassium phosphate buffer (pH 7). The optical rotatory dispersion spectra were obtained with solutions containing 0.5–1.3 $\times$ 10^{-5} M and the circular dichroism spectra with 1.7 and 2.2 $\times$ 10^{-5} M astaxanthin in the pigment solution.

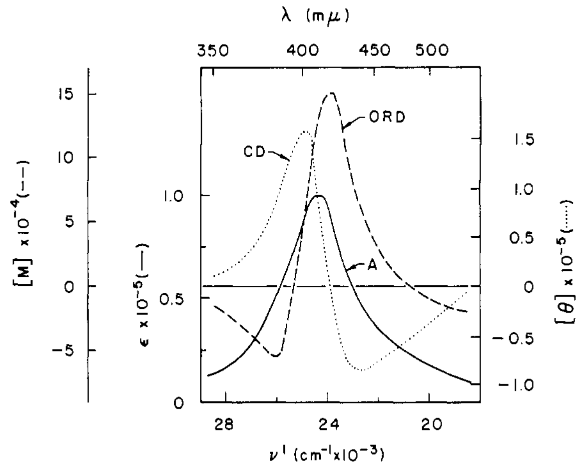


FIGURE 6: Optical rotatory dispersion, circular dichroism, and absorption spectra of the yellow pigment in 0.05 M potassium phosphate buffer (pH 7). The optical rotatory dispersion spectra were obtained with three solutions containing 0.67–1.2 $\times$ 10^{-5} M and the circular dichroism spectra with three solutions containing 1.17–3.34 $\times$ 10^{-5} M astaxanthin in the pigment solutions.

layer chromatography on silica gel K (Eastman Kodak Co.) with a solvent of equal volumes of acetone and petroleum ether (bp 30–60°) (Schimmer, 1967). The intermediate is apparent as a spot which migrates with an R_F values intermediate between that of astaxanthin and crustaxanthin and is present at a maximum concentration of approximately one-third of the total carotenoid during the reaction. These results suggest that the reduction proceeds in two steps, corresponding to the reduction of the two carbonyl groups of astaxanthin with the intermediate formation of a pigment containing a single carbonyl group.

Reduction of the yellow lobster pigment with 0.1 M sodium borohydride in 0.01 M sodium borate buffer (pH 9) was complete in 10 min and caused a shift in the absorption maximum from 415 to 405 mμ. A similar experiment with astaxanthin dispersed from pyridine into 1 M sodium perchlorate gave a shift in the absorption maximum from 410 to 392 mμ in less than 10 min. These reactions could not be followed kinetically because of the small spectral shift, but the rapid rates of reduction indicate that the carbonyl groups are not protected from the aqueous solvent in the yellow pigments and aggregates. The fact that the product of the reaction is also yellow indicates that the carbonyl groups are not required in order to obtain the yellow color. On prolonged standing the carotenoid product precipitates and upon redissolving in organic solvents it was found to have the characteristic spectrum of crustaxanthin.

Optical Rotatory Dispersion and Circular Dichroism. The optical rotation of β-crustacyanin shows a positive maximum with a molar rotation of 110,000 at 600 mμ, near the absorption maximum at 587 mμ, and a Cotton effect at 545 mμ, well below the absorption maximum (Figure 5). The molar rotations of the pigments are based on their astaxanthin content. The circular dichroism is maximal at 540 mμ and zero at 600 mμ. The optical rotation also exhibits a maximum of negative rotation with a molar rotation of 150,000 at 370 mμ associated with the absorption maximum at 360 mμ and a negative rotation maximum at 233 mμ corresponding to the peptide backbone.

The optical rotatory dispersion spectrum of α-crustacyanin is very similar to that of β-crustacyanin except that the rotational maxima associated with the visible peaks are displaced toward longer wavelength, corresponding to the shifts of the absorption maxima toward longer wavelength. The rotatory strengths are approximately the same as those of β-crustacyanin. The exact position of the maximal rotation at over 600 mμ could not be resolved because of instrumental limitations. The absorption maximum at 370 mμ is associated with a maximum of negative rotation at 377 mμ.

The optical rotation of the yellow pigment exhibits a positive maximum with a rotatory strength of 150,000 at 415 mμ, corresponding to the absorption maximum at 409 mμ, a negative peak at 383 mμ, and a trough at 233 mμ corresponding to the peptide backbone (Figure 6). The magnitude of the rotation maximum of this pigment in the visible region is *larger* than that of the peptide backbone at 233 mμ. The circular dichroism spectrum shows positive and negative maxima below and above the absorption maximum and crosses the base line at 415 mμ, near the absorption maximum. Esti-

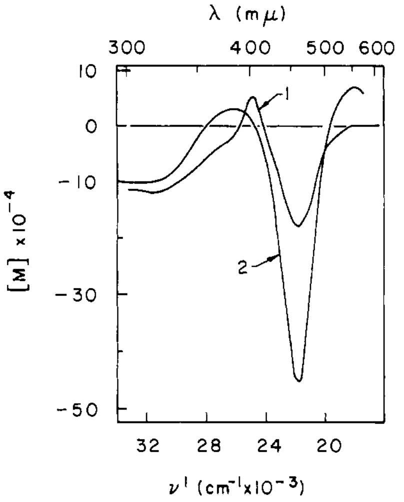


FIGURE 7: Optical rotatory dispersion of α-crustacyanin denatured in 3 M guanidine hydrochloride in 0.05 M potassium phosphate buffer (pH 7). The absorption spectra of the denatured pigment showed an absorption maximum at 408 mμ and a broad shoulder at 460 mμ with a ratio of A_{408}/A_{460} = 1.75–2.0.

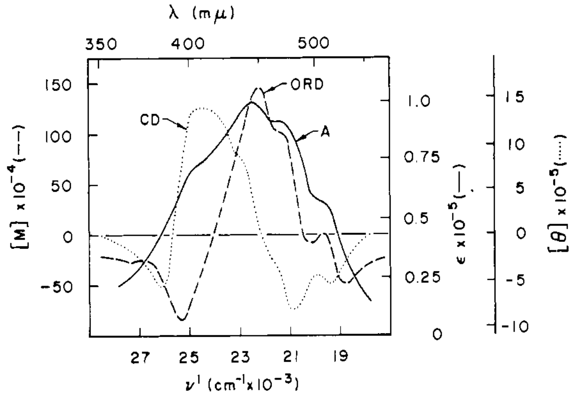


FIGURE 8: Optical rotatory dispersion (- - -), circular dichroism (· · ·), and absorption spectra (—) of α-crustacyanin after reduction with 0.02 M sodium borohydride in 0.1 M sodium borate buffer (pH 10). The concentrations of astaxanthin in the pigment before reduction were 1.0 and 0.24 × 10^{-5} M in the two solutions.

mates based on the molar rotation at 233 mμ and assuming that all of the rotation at this wavelength is caused by the peptide backbone suggest that the content of α helix in β-crustacyanin and the yellow pigment is low, approximately 6% (Fasman, 1963).

Denaturation of α-crustacyanin with 3 M guanidine hydrochloride gives a product with an absorption maximum at 408 mμ and a broad shoulder at 460 mμ, which exhibits a sharp maximum of negative rotation at 460 mμ and a smaller maximum of positive rotation near 400 mμ (Figure 7). The relative amplitudes of these two rotational maxima vary from one preparation to another, as shown in the figure. The molar rotation of the negative maximum can be as large as 450,000. Similar results, but with a smaller negative rotation maximum, were obtained with α- and β-crustacyanins denatured with 0.1 M sodium hydroxide.

Denaturation of β-crustacyanin with 90% dimethyl sulfoxide or 8 M tetramethylurea to a product with maximal absorption at 490 mμ was found to lead to a complete loss of optical rotation in the visible region which could be detected at concentrations of pigment similar to those used in the above experiments.

The absorption, optical rotatory dispersion, and circular dichroism spectra of α-crustacyanin which had been reduced with sodium borohydride are shown in Figure 8. Identical results were obtained with γ-crustacyanin. The absorption spectrum is similar to that of crustaxanthin dispersed in water or salt suspensions, with four absorption maxima. The optical rotatory and circular dichroism spectra are complex and suggest that most or all of these electronic transitions contribute to the rotational spectra. The maximal molar rotation of this product is 1,500,000.

Discussion

Composition and Stoichiometry. Before considering possible mechanisms for the changes in the optical properties of astaxanthin upon incorporation into crustacyanin, it is important to know whether the smallest blue pigment molecule contains one or several carotenoid prosthetic groups; *i.e.*, whether mechanisms which involve intermolecular electronic interaction should be considered. Our determinations of the molecular weight of β-crustacyanin by sedimentation equilibrium and sedimentation and diffusion measurements in the ultracentrifuge give a value of 50,000 ± 3000, based upon a measured partial specific volume of 0.76; a partial specific volume of 0.73 would give a molecular weight of 44,000. In either case the minimum molecular weight per astaxanthin molecule of 21,800, based upon a measured molar extinction coefficient of 1.12 × 10^{-5} for astaxanthin in pyridine (Buchwald and Jencks, 1968), requires that each molecule of β-crustacyanin contain two molecules of astaxanthin. A molecular weight of 37,000 estimated from the elution position of β-crustacyanin from Sephadex gel is in agreement with a value of 35,000 reported by Cheesman *et al.* (1966), but this method is not reliable for the estimation of the molecular weight of proteins of varying shape and composition (Ackers, 1964). Kuhn and Kühn have reported in a preliminary communication (1966) values of 19,000 for the minimum molecular weight and 36,000–39,500 for the molecular weight of β-crustacyanin, which would also give a content of two astaxanthin molecules in each molecule of crustacyanin.

The molecular weight of 380,000 for α-crustacyanin is also larger than the values of 307,000–320,000 reported by Kuhn and Kühn (1966). Cheesman *et al.* (1966) have estimated a molecular weight on the order of 650,000 for α-crustacyanin by Sephadex chromatography, but ultracentrifugal measurements by Gammack and Raper (quoted by Cheesman *et al.*, 1966) suggest a considerably lower value. The molecular weight of β-crustacyanin and the minimum molecular weight of α-crustacyanin correspond to a composition of 16 molecules of astaxanthin and 8 molecules equivalent to β-crustacyanin for each molecule of α-crustacyanin. This is in agreement with Kuhn and Kühn (1966) who report lower values of both the minimum molecular weight and the molecular weight.

γ-Crustacyanin is a previously unreported pigment which is similar to α-crustacyanin in its spectrum and its molecular weight of 400,000, but which has a diffusion coefficient and sedimentation coefficient nearly twice as large as α-crustacyanin, indicating that although its composition is similar, its shape is more nearly spherical than that of α-crustacyanin.

The different amino acid composition is one of several properties which distinguish the yellow lobster shell pigment from the crustacyanins. The molecular weight of approximately 90,000 and minimum molecular weight of 4400 correspond to a content of 20 molecules of astaxanthin in each molecule of pigment; however, the possibility that the content is 16, corresponding to that of α- and γ-crustacyanins, is not definitely ruled out.

The yellow pigment formed from the crustacyanins by denaturation in the presence of guanidine hydrochloride or base is spectrally similar to the native yellow pigment, but has a higher minimum molecular weight and exists as a large aggregate. The denaturation process is accompanied by the release of a smaller protein(s) of mol wt 20,000–24,000, similar to the apoprotein described by Cheesman *et al.* (1966). The maximum degree of renaturation of crustacyanin from the yellow and colorless pigments requires a stoichiometric ratio of these two proteins which corresponds closely to that in the native protein. This is further evidence for a definite stoichiometric composition of the crustacyanins and suggests that the reversible denaturation reaction may be formulated according to eq 1. The observation that the *rate* of the renaturation reaction is dependent only on the concentration of the yellow pigment suggests that either this pigment undergoes a rate-determining structural change which makes the recombination reaction possible or that the surface of this pigment contains a limited number of sites to which molecules of the colorless protein are bound and that these sites are saturated with colorless protein.

$$\underset{\alpha\text{-crustacyanin}}{n\left(\frac{2\text{ astaxanthins}}{48{,}000\text{ mol wt}}\right)_8} \rightleftharpoons \underset{\text{yellow}}{\left(\frac{16\text{ astaxanthins}}{110{,}000}\right)_n} + \underset{\text{colorless}}{12n(20{,}000\text{–}24{,}000)} \qquad (1)$$

Mechanism of the Spectral Shift. The following mechanisms may be considered as possible explanations for the perturbations of the energy of the electronic excitation and the optical rotatory dispersion spectrum of astaxanthin which are brought about by incorporation into crustacyanin. An important experimental fact which is pertinent to all of these mechanisms is that the large shift of the position of the absorption maximum of astaxanthin upon incorporation into crustacyanin is not accompanied by significant changes in the extinction coefficient, the shape of the absorption band, or the oscillator strength, compared to astaxanthin in hexane or methanol. This almost certainly means that there is no large change in the *nature* of the electronic transition and the relative importance of excitations to different vibrational levels in the free carotenoid and in the crustacyanins; it is the over-all excitation *energy* that is altered. On the other hand, the large difference in the optical rotatory properties of free and bound astaxanthin is a further indication that some important change in the properties of astaxanthin must occur upon incorporation into crustacyanin.

CHEMICAL ALTERATION. The presence of carbonyl groups in astaxanthin and the known formation of an imine between retinal and opsin in rhodopsin (Morton and Pitt, 1955; Wald, 1960; Bownds and Wald, 1965) suggest the possibility of a chemical reaction between astaxanthin and the apoprotein in crustacyanin. The strongest evidence against such a reaction is the fact that crustacyanin is readily reduced by sodium borohydride, to give a product which can be easily separated from the protein by extraction with organic solvents. Reduction of an imine by borohydride would give a stable secondary amine which could not be separated from the protein by mild procedures. It has previously been shown that crustacyanin can be reversibly denatured by alkali in the presence of 2 M hydroxylamine, which might be expected to interfere with an imine structure by reacting with either the imine or the free carbonyl groups of astaxanthin (Jencks and Buten, 1964).

Kuhn and Sorensen (1938a,b) showed that astaxanthin is converted into a blue product in strong alkali in the absence of oxygen and suggested that the product of this reaction is an enolate tetraanion and that the same type of ionization could account for the blue color of crustacyanin. The shift of the absorption maximum of astaxanthin to longer wavelengths in the presence of strong acids could equally well be proposed as a model for crustacyanin or the visual pigments (Blatz, 1964). Both the alkaline and the acid products undergo rapid decomposition in the presence of oxygen, in contrast to crustacyanin which is stable for months, and both would be converted into a nonionized form upon exposure to neutral pH; if any such mechanism accounted for the color of crustacyanin the astaxanthin prosthetic group would have to be deeply buried within the protein so that no contact with solvent or oxygen would be possible. The fact that the rate of reduction by sodium borohydride of astaxanthin in crustacyanin is only slightly reduced compared to astaxanthin at the surface of micelles in aqueous solution shows that the carotenoid is readily accessible to the solvent and effectively rules out these mechanisms. Furthermore, the chromophore of anionic or protonated astaxanthin is quite different and would be expected to give an absorption band with a different shape compared to that of astaxanthin itself, as is observed in the case of protonated astaxanthin. The similarity of the absorption bands of astaxanthin and crustacyanin is therefore further evidence against these mechanisms. The finding that a blue pigment can be formed from crustacyanin apoprotein and astacene (3,3′,4,4′-oxo-β-carotene) shows that ionization in the 3 position is not required for formation of the blue color, but does not rule out the formation of an enolate ion at the 2 position (Lee and Zagalsky, 1966).

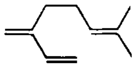
The absence of significant amounts of metal ions in crustacyanin and the yellow pigment and the fact that the pigments are stable to prolonged exposure to 0.5 M EDTA during the extraction procedure are evidence against any role of a metal ion in determining the spectral properties of crustacyanin or the yellow pigment.

CHARGE-TRANSFER ABSORPTION. The spectrum of crustacyanin is not that of a classical charge-transfer complex, because a charge-transfer absorption is a *new* band which appears upon interaction of a donor and acceptor molecule and which is accompanied by only minor modifications of the original absorption bands of the reactants (Mulliken, 1952). The absorption of crustacyanin is not a new band, but represents a *shift* of the position of the absorption band of astaxanthin without a change in shape. No significant change in the absorption spectrum of astaxanthin was observed in the presence of durene, chloranil, or indole (Buchwald and Jencks, 1968).

MEDIUM EFFECTS. The difference in the environments of a chromophore in solution and bound to a protein could give rise to a spectral shift from a simple medium or solvent effect. The absorption maxima of dyes which have a large difference in the polarity of their ground and excited states, such as the merocyanins, undergo large shifts in different solvents, depending on the charge solvating ability of the medium (Brooker *et al.*, 1951, 1965) and the absorption maximum of 2-(4′-hydroxyphenylazo)benzoic acid undergoes a shift from 348 to 480 mμ upon binding to serum albumin, which is similar to that induced by transfer from water to organic solvents or micellar detergents (Baxter, 1964). However, the absorption band of astaxanthin shows only a small dependence on the nature of the solvent; the shifts which are observed are a function only of the refractive index and bear no relation to the charge solvating ability or dielectric constant of the solvent (Buchwald and Jencks, 1968). An ordinary medium effect, therefore, cannot account for the spectral shifts of astaxanthin in crustacyanin or the yellow pigment; the refractive index of proteins of approximately 1.6 would give an absorption maximum at only 500 mμ.

POLARIZATION. The anomalously short wavelength of maximum absorption of polyenes with nonequivalent alternating single and double bonds, compared to compounds such as the cyanines in which the bonds are equivalent, has been explained by the free electron and linear combination of atomic orbital–molecular orbital theories (Kühn, 1949; Dewar, 1952; Ooshika, 1957; Longuet-Higgins and Salem, 1959; Platt, 1961). Platt (1959) has suggested that polarization of an alternating polyene system could make the bonds more nearly equivalent and shift the absorption maximum to a longer wavelength. Somewhat similar mechanisms have been proposed to account for the shifts of the carotenoid absorption maxima in crustacyanin by Cheesman *et al.* (1967) and in the visual pigments by Kropf and Hubbard (1958) and Dartnall and Lythgoe (1965). It is predicted by theory and observed in the merocyanins, the spectral shifts of which can be described in terms of a polarization effect, that such polarization will result in a narrowing and change in shape of the absorption band (Brooker *et al.*, 1947; McRae, 1958). The spectral shifts which occur upon protonation of astaxanthin may be explained in terms of a polarization effect, because protonation will make the single and double bonds more nearly equivalent (Buchwald and Jencks, 1968), and these spectra also exhibit a narrowing and change in shape compared to that of astaxanthin. The close similarity of the shapes of the absorption bands of astaxanthin and of crustacyanin, therefore, is difficult to explain in terms of a polarization mechanism; the same difficulty exists for the visual pigments, which exhibit a very similar shape of the absorption band in pigments with absorption maxima ranging from 440 to 620 mμ (Dartnall, 1962).

ELECTRONIC INTERACTIONS. The presence of two carotenoid molecules in the smallest blue crustacyanin subunit demands that mechanisms based on intermolecular electronic interactions be considered as possible explanations for the spectral shift and optical rotatory properties of these pigments. It was concluded in the previous paper that the yellow color and optical rotatory dispersion properties of the yellow pigment and of astaxanthin aggregates in the presence of certain salts result from interaction of the π-electron systems of the aggregated molecules; according to exciton theory, a spectral shift in this direction would involve a parallel alignment of the transition dipoles (Buchwald and Jencks, 1968). A similar interaction in which the transition dipoles are oriented head to tail could cause a shift in the absorption maximum to longer wavelengths (Levinson *et al.*, 1957; Kasha, 1963). The increase in the absorption maximum of myrcene (I) from 225 (characteristic of a diene) to 278 mμ upon adsorption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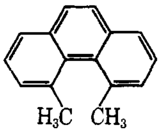
onto silica gel has been attributed to an overlap of the π electrons of the isolated double bond with the orbitals of the diene system which is enforced by the steric constraints of the adsorbed state (Leermakers *et al.*, 1966). However, a simple increase in the length of the conjugated system which might be brought about by an interaction of this kind between the carotenoid molecules in crustacyanin would not be expected to cause a large shift in the absorption maximum because of the already mentioned insensitivity of the absorption maximum of long-chain alternating polyenes to chain length. More important, as far as the authors are aware there are no known examples of large shifts in the absorption maximum of aggregated molecules which are not accompanied by large changes in the shape of the absorption bands and theoretical treatments according to both exciton and classical theory predict that the strong coupling which gives rise to such large shifts will also give rise to changes in band shape and, in the limit, to sharp, narrow bands such as are observed in the *J* bands of large dye aggregates (West and Carroll, 1966; Simpson and Peterson, 1957; Kasha, 1963; DeVoe, 1964; Fulton and Gouterman, 1964). The large shift in the absorption maximum of benzene in *p*-cyclophanes, from 38,000 to 30,000 cm^{-1}, has been attributed to intermolecular electronic interaction and explained by a modified exciton treatment which takes into account the possibility of electron transfer upon excitation; however, these shifts are also accompanied by changes in the shape and intensity of the absorption spectra (Cram *et al.*, 1954; Vala *et al.*, 1966). Thus, in the case of the crustacyanins and also in the case of rhodopsin, which contains only a single molecule of retinene (Hubbard, 1954), there does not exist adequate experimental or theoretical precedent at the present time to support an explanation based on intermolecular electronic interactions for the large spectral shifts of the chromophore.

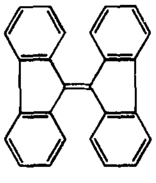
The strong optical rotation which is induced in dyes and coenzymes upon combination with optically active macromolecules can be caused either by the induction of asymmetry into the monomeric chromophore by the polymer, as in the case of 1,10-phenanthroline bound to alcohol dehydrogenase and, probably, dyes bound to polynucleotides at very low dye:polynucleotide ratios, or by the induced formation of dimers or polymers in which the chromophores are arranged in a stereospecific, asymmetric structure, such as a helix (Stryer and Blout, 1961; Ulmer *et al.*, 1961; Mason, 1964; Yamaoka and Resnik, 1966). The presence of two or more chromophore molecules in each molecule of lobster pigment and the splitting of the absorption band which gives rise to the rotation suggest that interaction between the chromophores affects the rotatory properties.

The optical rotatory dispersion spectrum of the yellow pigment (Figure 6) is similar to that of the yellow astaxanthin aggregate (Buchwald and Jencks, 1968) with a strong maximum positive rotation near the position of the absorption maximum and Cotton effects above and below the absorption maximum. The circular dichroism spectrum of this pigment crosses the base line near the absorption maximum and exhibits positive and negative maxima below and above the absorption maximum, respectively, in accord with the optical rotatory dispersion spectrum. This evidence for splitting of the absorption band into two neighboring bands of opposite rotational direction is similar to that observed in polyadenylic acid and the dinucleotide ApA and may be explained by exciton theory (Tinoco *et al.*, 1963; Warshaw *et al.*, 1965; Van Holde *et al.*, 1965). A similar splitting of the absorption band of the two carotenoid molecules in crustacyanin into two adjacent bands of opposite rotatory direction provides a reasonable explanation for the similar relationships of the optical rotatory dispersion and the circular dichroism spectra to the absorption band of β-crustacyanin.

DISTORTION. If ethylene or a polyene is twisted about the double bond the energy of the ground state is increased and the energy of the excited state is lowered, with a consequent decrease in the excitation energy and increase in the wavelength of the absorption band (Mulliken and Roothaan, 1947; Platt, 1961). In the case of the polyene, the rotation will decrease the bond order of the double bond and thereby make the single and double bonds of the conjugated chain more nearly equivalent. There are experimental precedents for spectral shifts of this kind. The strain on the partial double bond (or, more precisely, on a bond with no node in the ground state) of 4,5-dimethylphenanthrene (II) induced by the methyl groups results in a shift of the absorption maximum by -2300 cm^{-1}, corresponding to a decrease in the excitation energy of 6.6 kcal, without a change in the extinction coefficient; this shift is in accord with molecular orbital calculations of the expected changes in excitation energy (Cromartie and Murrell, 1961). The absorption maximum of dibiphenyleneethylene (III), which is forced to undergo partial rotation about the double bond between the two aromatic systems by steric interference of the benzene rings, is at 458 mμ. This is at a *higher* wavelength than the maximum at 444 mμ of dibiphenylenebutadiene, which contains a longer conjugated system but is not subject to strain about the double bond, and is also considerably higher than the value of 380 mμ which has been estimated for the dibiphenylene system in the absence of strain by analogy with other unstrained systems (Bergmann and Hirshberg, 1950; Bergmann *et al.*, 1950). Rotation by 180° about the single carbon–carbon bond of butadiene or other polyenes is known, on both theoretical and experimental grounds, to cause a shift of the absorption maximum to longer wavelengths (Allinger and Miller, 1964). A shift of this kind to the *s-cis* configuration has been proposed to account for the red shift of the chromophore in the acyl group of certain acyl-chymotrypsins (Charney and Bernhard, 1967). Hammond and coworkers have shown that electron transfer can take place from an excited sensitizer molecule to an acceptor, such as stilbene, in which the energy of the lowest available orbital is as much as 10 kcal higher than the energy of the excited sensitizer; the fact that transfer is observed in such cases is evidence that the acceptor molecule is twisted in such a



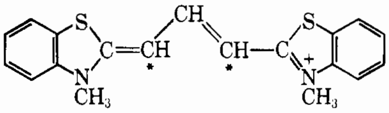
II



III

way as to decrease the energy of its lowest vacant orbital and decrease the energy gap between the ground and lowest excited states (Herkstroeter and Hammond, 1966, and references therein).

The changes in absorption maximum of cyanine dyes which are caused by distortion of the conjugated system are usually accompanied by decreases in extinction coefficient (Brooker *et al.*, 1947), but this is not always the case. The absorption maximum of the cyanine dye IV at 554 mμ undergoes a small shift to 567 mμ if the starred carbon atoms are connected by methylene groups to give a six-membered ring, but shifts to 594 mμ with no change in the extinction coefficient or band shape if one methylene group is omitted to give a five-membered ring; there are other, similar examples of the effect of the geometry of the unsaturated system on the position of the absorption maximum (Riester, 1966).



IV

If a polyene system is twisted through a *small* angle, rotation will occur about the single bonds in preference to the double bonds, conjugation will be decreased, and the absorption maximum will shift to shorter rather than to longer wavelengths. However, examination of molecular models of astaxanthin reveals that if the methyl groups are immobilized and the β-ionine ring is bound to the protein so as to be twisted out of the plane of the conjugated polyene, it is possible to localize the strain in double rather than single bonds. In this connection it should be noted that development of the blue color of crustacyanin requires the presence of a carbonyl group at the 4 and 4′ positions (Lee and Zagalsky, 1966) and that reduction of crustacyanin with borohydride, which will change the shape of the β-ionine ring of astaxanthin by converting the carbon atoms at the 4 and 4′ positions from planar sp^2 into tetrahedral sp^3 hybridization, causes immediate loss of the blue color. One of the most attractive features of the strain hypothesis is that it provides a simple explanation for the fact that partial denaturation of crustacyanin gives products with absorption maxima which gradually shift from that of the starting material to that of astaxanthin. This demands either the existence of a large number of intermediates or a continuous denaturation process, which can be easily visualized according to the strain hypothesis. As the tertiary and quaternary structure of the protein undergoes progressive loosening there is a gradual relaxation of the distortion of the polyene.

The strain mechanism also provides a reasonable explanation for the spectral shifts of retinal bound to opsin in the visual pigments, which occur over a large range of wavelengths without a significant change in the shape of the absorption band. Yoshizawa and Wald (1963) have suggested a strain mechanism to account for the absorption band of prelumirhodopsin, an intermediate in the bleaching of the visual pigments. The utilization of strain induced by interaction with a protein to reduce the energy gap between the ground and excited states of a pigment is analogous to its possible utilization in enzyme–substrate complexes to reduce the energy gap between the ground state and an activated complex (Lumry, 1959; Jencks, 1966, and references therein). It has recently been proposed that distortion increases the energy of the ground state and the rate of *cis–trans* isomerization of azo dyes in the presence of proteins (Lovrien and Linn, 1967) and of *trans*-retinal to 11-*cis*-retinal at low temperature in the site on iodopsin which fits the latter isomer (Yoshizawa and Wald, 1967).

Stereospecific distortion which introduces asymmetry into a chromophore leads to the development of optical activity with a much larger rotational strength than is found in molecules in which the asymmetry is in an atom adjacent to the chromophore; the maximum specific rotation of hexahelicene is 230,000 (Moscowitz, 1961; Stryer and Blout, 1961; Charney, 1965). The large molar rotations of 110,000 for β-crustacyanin and of 1,500,000 for crustacyanin which has been reduced with borohydride may be caused by such stereospecific distortion as well as by interaction between the chromophores. The circular dichroism of the single retinal chromophore of rhodopsin in the visible region is similar in magnitude to that of crustacyanin and must result from this type of binding (Crescitelli *et al.*, 1966).

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Added in Proof

The complete report of the work of the Kuhn and Kühn (1967) indicates that the most important differences from the results reported here arise from the smaller partial specific volume of crustacyanin which was obtained by these workers. The experimental results and conclusions regarding the stoichiometry and properties of crustacyanin are in general agreement with those reported here; further experiments will be required to determine whether differences in the properties of the pigments from different species account for other relatively small differences in experimental results.

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On the Kinetics of Metal-Catalyzed Adenosine Triphosphate Dephosphorylation*

Thomas G. Spiro, William A. Kjellstrom, Marielouise Zeydel, and Ronald A. Butow

ABSTRACT: Analysis of Tetas and Lowenstein's rate data (*Biochemistry 2*, 351 (1963)) on metal ion catalysts of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) dephosphorylation reveals that the role of the metal is not limited to the acceleration of water attack on phosphate linkages or of elimination of metaphosphate. Such mechanisms are enhanced by chelation of manganese(II) to ATP, but are actually inhibited for the manganese ADP chelate, although hydroxyl attack is accelerated in the latter case. For the copper(II)–ATP systems a completely new mechanism dominates the kinetics, one involving proton attack on the dimeric hydroxy chelate, $[\mathrm{CuATP(OH)}]_2^{6-}$. Involvement of this dimer is confirmed by a study of the concentration dependence of the rate. On dilution, the pseudo-first-order rate constant decreases in parallel with the dimer concentration. A similar mechanism is probably operative in the Zn(II)–ATP system.

Divalent metal ions are required in most enzymatic reactions of ATP.[1] The interaction of metal ions with ATP in nonenzymatic systems has been studied in considerable detail. Stability constants have been measured (Sillén and Martell, 1964) and the sites of attachment of some metal ions to ATP have been determined (Cohn and Hughes, 1962; Brintzinger, 1963; Schneider *et al.*, 1964).

The influence of metal ions on the kinetics of nonenzymatic reactions of ATP, and also ADP, have been investigated by Lowenstein and coworkers (1958a,b, 1961, 1963). Of particular interest is their study on the hydrolysis (dephosphorylation) reaction (Tetas and Lowenstein, 1963). The results obtained are complex and were not interpreted quantitatively. The authors did, however, offer some suggestions on mechanisms. They proposed that chelation of ATP and ADP by metal ions serves to render the terminal phosphate susceptible to nucleophilic attack by water, or to elimination, and that specific differences among metal ions might result from changes in chelate structure. Schneider and Brintzinger (1964) made a more detailed study of copper(II) catalysis of ATP dephosphorylation, and proposed a specific chelate structure to account for the exceptional activity of Cu(II). The differences ob-

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[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate.