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STUDIES ON THE BLUE ASTAXANTHIN-PROTEINS OF *VELELLA VELELLA* (COELENTERATA: CHONDROPHORA)

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Velella velella is a surface living oceanic chondrophore exhibiting the typical blue pigmentation of the neuston. In the present study the properties of the blue astaxanthin-proteins of the mantle tissue are reported.

The pigments have been separated into four main fractions by gel filtration (V620, V600, V570 and V545) with λ_{max} 620 nm, 600 nm, 570 nm and 545 nm, respectively, and their molecular sizes were estimated. Pigment V620 is a polymeric form, possibly a tetramer, of pigment V600. Analysis of the gradient elution profile of V600 in gel filtration at low chloride ion concentration provides evidence that the pigment is an octamer of V570-size units. Pigment V570 separates in preparative acrylamide electrophoresis into six components. Gel filtration, SDS-electrophoresis and phase-partition experiments show that these are dimers of apoprotein (V545-size) units of molecular mass *ca.* 2.3×10^4. Estimates of minimum molecular mass based on astaxanthin content reveal that V620 and V600 have two astaxanthin prosthetic groups per apoprotein subunit. The pigments are simple proteins with free sulphydryl groups and negligible tyrosine contents.

Pigments V620 and V600 possess specific anion-binding sites which have an influence on the visible and circular dichroism spectra, and on the quaternary structure of the pigments. In the absence of halide ions the pigments dissociate reversibly forming pigments with λ_{max} 583 nm (V600-size) and λ_{max} 565 nm (V570-size). The anion-dependence of the spectra of the native pigments and of the components, V583 and V565, separated in halide-free gel filtration, have been investigated. Equations are derived linking the spectral changes and anion-dependent association processes to the anion concentration. The order of effectiveness of anions for the changes ($Br^- > I^- > Cl^- > NO_3^-$, CNS^-) does not follow the Hofmeister series. The changes take place at low anion concentrations (10^{-5}–10^{-1} M) with a negative temperature dependence and are partially abolished by blocking lysine residues; higher anion concentrations are required to bring about alterations in the spectra of the separated components V583 and V565. The halide-dependent quaternary structural changes of the pigments have been followed in gel filtration on Bio-Gel columns equilibrated with phosphate buffer containing low concentrations of KCl.

In order to explain the high affinity of the pigments for anions and the order of anion effectiveness, it is proposed that the anion-binding sites consist of clusters of basic residues which impose stereospecific restrictions on the anion binding. These sites are progressively lost in the absence of halide ions and following gel filtration of halide-free solutions of the pigments. ^{36}Chloride-binding measurements, using a gel filtration equilibrium technique, indicate that V600 may have three such sites per apoprotein subunit. The temperature dependence of chloride binding and of the spectral alterations of the pigment show that occupation of the sites causes relatively minor changes in the polypeptide conformations of the native pigment; surface spreading of the pigment revealed only a small increase in the stability of the tertiary and quaternary structure of the pigment in the presence of chloride ions. Pigment V570 and the apoprotein do not possess halide-ion binding sites of high affinity.

The astaxanthin prosthetic groups of pigments V620 and V600 are optically active with large molar ellipticity values in the visible region; pigment V570 shows a low molar ellipticity value at the visible absorption maximum. The origin of the optical activity of the carotenoid in the complexes is discussed. Exciton splitting, attributed to carotenoid-carotenoid interaction, is observed in the circular dichroism (c.d.) spectrum of V620 but only in the presence of halide ions. It is suggested that occupation of the anion-binding sites of the pigment affects the relative orientation or distance apart of the chromophores. A comparison of the c.d. spectrum of the pigments in the far ultraviolet did not reveal any major alteration in protein conformation resulting from anion binding. The c.d. spectrum of V600 in the far ultraviolet and the amino acid composition of the pigment are consistent with a high contribution of β-structure to the configuration of the protein.

Investigations into the specificity of carotenoid attachment have shown that although astaxanthin combines most efficiently with the apoprotein, other carotenoids, including acetylenic and cyclopentene derivatives, are able to effect quaternary structural changes

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of the protein. The carotenoid effects dimerization of the apoprotein in the presence of a 4-keto group in one ring and the presence in either ring of an additional hydrophilic substituent; dimerization of the apoprotein is also obtained with the half-carotenoid, 15-15′-dehydro-8′-apo-β-carotene-8′-al-3,4-dione. Both 4- and 4′-keto groups and additional hydrophilic substituents in the carotenoid structure are prerequisites for the further polymerization of the protein. Carotenoproteins showing chloride-dependent spectral shifts are obtained only with carotenoids containing a keto substituent in both rings.

Introduction

It was reported by one of us in an earlier publication (Herring 1971) that the spectra of the blue astaxanthin-proteins of *Velella* and *Porpita* were sensitive to conditions of temperature and ionic strength. A further study of the *Velella* pigment, reported here, has revealed that the degree of saturation of specific halide-ion binding sites in the proteins is responsible for the variations in the absorption spectra of the proteins in the visible region. It is probable that retention of halide-ions by the proteins during the purification procedures (cf. Silhavy, Szmelcman, Boos & Schwartz 1975) was responsible for some of the previous observations.

Materials

The following materials were employed in the protein studies: DEAE-cellulose (Whatman DE32); calcium phosphate gel, prepared by the method of Keilin & Hartree (1937); Munktell cellulose powder (L.K.B., Sweden); Celite hyflo supercel (Koch-Light, England); Polyethylene glycol 6000 (B.D.H., Poole); Dextran T500 (Pharmacia, Uppsala); Agarose (Oxoid, London).

Sephadex G-25 (fine grade), G-75 and G-200 and Agarose 4B and 6B from Pharmacia, Uppsala, and Bio-Gel P-300 from Bio-Rad, California, were used in gel filtration studies.

Protein solutions were concentrated by a Diaflo ultra-filtration (UM10 membranes) apparatus (Amicon, Holland); Sephadex G-25 was used for buffer exchange.

Acrylamide reagents for electrophoresis were purchased from Koch-Light, England, Pyronine G from B.D.H., England, and Coomassie Brilliant Blue G250 from Serva, Germany.

Chemicals were of Analar grade and solvents were redistilled before use. $CHCl_3$ and CH_2Cl_2 were washed with a solution of NaCl to remove any free HCl and dried over Na_2SO_4. Pyruvic aldehyde (40 % in water) and phenylglyoxal were purchased from Koch-Light, England; *N*-ethylmaleimide (NEM), from B.D.H., England.

Methylacetimidate hydrochloride, prepared by the method of Hunter & Ludwig (1962), was converted into the acetate by stirring solutions with excess of the anion-exchanger De-Acidite FF(SRA 66) (Permutit Co., London) in the acetate form; 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) hydrochloride (Sigma chemicals, U.S.A.) was similarly converted into the acetate; 1-cyclohexyl-3(2-morpholinyl-(4)-ethyl) carbodiimide metho-*p*-toluene sulphonate (CMC) was purchased from Aldrich Chemical Co., U.S.A.

Phosphate buffers were prepared by adjusting the pH of solutions of KH_2PO_4 with Aristar KOH (B.D.H., Poole). 0.5 M buffer contained $< 2 \times 10^{-6}$ M Cl^-.

Radioactive $Na^{36}Cl$ (> 3 mCi per g Cl), purchased from the Radiochemical Centre, Amersham, contained 7.72 mg Cl per ml.

Animals were obtained during the course of a cruise of R.R.S. *Discovery* in the eastern North Atlantic in 1972. They were collected in nets towed at the surface, drained of excess seawater

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and deep-frozen whole at −20 °C for the duration of the cruise. In the laboratory the animals were thawed and the tissue residue was filtered from the deep blue pigment solution. The residue was homogenized in an equal volume of 0.1 M phosphate buffer pH 7.2, and centrifuged. The blue supernatant was pooled with the previous extract, and the blue pigment was fractionally precipitated between 20 % and 60 % ammonium sulphate saturation. The precipitated pigment was resuspended in 60 % saturation ammonium sulphate and stored in a deep freeze for 8 months prior to the present investigations. The tentacles tend to be shed during capture, and the pigment is therefore derived primarily from the mantle tissues.

Methods

(*a*) *Purification*

The *Velella* carotenoproteins were purified by a modification of the calcium phosphate gel chromatography procedure used previously (Herring 1971). The protein, dissolved in 0.2 M KCl–0.2 M phosphate buffer, pH 5.9, was mixed with a quantity of calcium phosphate gel suspended in the same solution and the pH was adjusted to 5.9. The gel was washed thoroughly with 0.2 M KCl–0.2 M phosphate buffer, pH 5.9, and the blue carotenoproteins were eluted with 0.2 M KCl–0.1 M phosphate buffer pH 7 containing 30 % saturation ammonium sulphate. The carotenoproteins were precipitated at 50 % ammonium sulphate saturation, dissolved in 3 ml 1 M KCl–0.04 M phosphate buffer, pH 7, and separated into pigments differing in size by gel filtration on a column of Sepharose 6B (70 cm × 2.5 cm) equilibrated with the same solution. The pigments were purified by rechromatography on columns of Agarose 4B, 6B or Sephadex G75, depending on their size, equilibrated with the 1 M KCl–0.04 M phosphate buffer, pH 7.

(*b*) *Molecular size and subunit structure*

Estimates of the sieve coefficients and Stokes radii of the carotenoproteins and their derivatives were obtained in gel filtration studies. Columns of Agarose 4B and 6B, Sephadex G75 and G200, and Bio-Gel P300, were equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7, and standardized as described by Ackers (1967).

Molecular masses were calculated from the Stokes radii using the classical equation of Siegel & Monty, as previously outlined (Zagalsky, Ceccaldi & Daumas 1970), assuming values for the frictional ratios (see Results section) and taking the partial specific volumes calculated from the amino acid compositions. Estimates of molecular mass were also obtained from lg (molecular-mass/effluent volume) plots (Andrews 1964).

The partition behaviour of the proteins in poly(ethylene glycol)-dextran two-phase systems was investigated as described by Sasakawa & Walter (1972). The proteins (0.1 mg/ml, final concentration) were partitioned in phosphate buffers, pH 5–8.5, in the presence of the following salts (final concentration given): 0.2 M KCl and 0.2 M KBr for the *Velella* pigments λ_{max} 600 and 620 nm; 0.1 M NaCl and 0.05 M Na_2SO_4 for the *Velella* pigments λ_{max} 570 and 545 nm. The partitioning was followed at the visible absorption maximum of the pigments. Isoelectric points were obtained from the cross-partitions, and molecular masses derived by using the standard curve given by Sasakawa & Walter.

The minimum molecular masses were calculated from the nitrogen and carotenoid contents of solutions of the carotenoproteins in 0.05 M phosphate buffer, pH 7, as previously described (Zagalsky & Herring 1972). The change in maximal extinction of the carotenoid following

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dissociation from the complexes with pyridine (55 % by vol.) was calculated, measuring the extinction of the native pigments following the addition of equal volumes of 1 M KCl to samples of the protein solutions. Carotenoid was rapidly lost when the dissociation was carried out in the presence of chloride (cf. Buchwald & Jencks 1968*b*). Subunit size was estimated by acrylamide disk electrophoresis (5, 8, 10 and 15 % acrylamide gels), as described by Banker & Cotman (1972) but with the buffer system of Laemmli (1970). The carotenoproteins were dissociated with sodium dodecylsulphate (SDS)-mercaptoethanol (Laemmli 1970).

A study of the reversible dissociation of the *Velella* carotenoprotein (λ_{max} 600 nm) at low chloride ion concentration was made by an analysis of the gel filtration at 5 °C of a solution of the protein (160 ml, 0.16 mg/ml; flow rate 5 ml/h) dissolved in 10^{-3} M KCl–0.1 M phosphate buffer, pH 7, on a column of Agarose 6B (void volume = 82 ml) equilibrated with the same solution. The stoichiometry of the dissociation was obtained by an analysis of the concentration gradient pattern of the trailing edge of the carotenoprotein, followed at 580 nm. The degree of polymerization n was estimated from the relationship,

$$n = \frac{3V'_{min} - \sigma_m - 2\sigma_p}{3V'_{min} - 2\sigma_m - \sigma_p}, \quad \text{(Ackers \& Thompson 1965)}$$

where σ_m and σ_p are the molecular sieve coefficients for the monomer and polymer, respectively, and V' is the normalized elution volume of the minimum in the concentration gradient pattern for the trailing edge of the carotenoprotein zone.

(*c*) *Apoprotein preparation*

2 ml carotenoprotein (0.2 %) in 0.2 M LiCl–0.01 M phosphate buffer, pH 6.7, was stirred with ice-cold 30 ml acetone. The precipitated protein was redissolved in the ice-cold 0.2 M LiCl–0.01 M phosphate buffer and the precipitation process was repeated. The apoprotein was dissolved in 2 ml 0.2 M KCl–0.04 M phosphate buffer, pH 7, and dialysed against the same solution for 3 h in the cold to remove excess acetone. The apoprotein was then transferred to 1 M KCl–0.04 M phosphate buffer, pH 7, by using a column of Sephadex G25, and separated from unchanged carotenoprotein and aggregated apoprotein by gel filtration on a column of Sephadex G75 equilibrated at 5 °C with the same solution, as described by Cheesman, Zagalsky & Ceccaldi (1966).

A crude division of the apoprotein was made into protein not adsorbed on to a DEAE-cellulose column from 0.005 M phosphate buffer, pH 7, and protein adsorbed and then eluted with 0.25 M phosphate buffer, pH 7.

(*d*) *Specificity studies*

Powdered cellulose was washed thoroughly with solutions in the order: acetone; distilled water; 1 M KCl–0.04 M phosphate buffer, pH 7; 2 % EDTA, pH 7.5; water; acetone. The cellulose was air-dried and solutions of the carotenoid (0.1–0.3 mg/ml) in acetone, methylene-dichloride, chloroform or other suitable solvent (2.5 ml) were stirred with the cellulose (3 g) to make a slurry. The solvent was evaporated under a stream of nitrogen and the cellulose was washed with ice-cold deaerated distilled water. A solution (8 ml) of the apoprotein (*ca.* 1 mg/ml) in 1 M KCl–0.04 M phosphate buffer, pH 7 was stirred for 24 h in the dark at room temperature with the carotenoid-adsorbed cellulose. The slurry was filtered through a layer of supercel which had been washed thoroughly with 1 M KCl–0.04 M phosphate buffer, pH 7. The filtrate was concentrated, centrifuged and the nature of the reconstituted carotenoproteins was investigated

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following separation on columns of Sephadex G75 equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7.

Reconstitution with astaxanthin was also carried out in the absence of chloride ions, with the apoprotein dissolved in 0.2 M phosphate buffer, pH 7.

(*e*) *Electrophoresis*

Homogeneity was tested by disk electrophoresis (see, for example, Smith 1968) with 0.5 % agarose–3 % acrylamide for the gel material (Peacock & Dingman 1968); 0.25 M tris-HCl, pH 7.5, and 0.1 M phosphate buffer, pH 7 were used as buffers.

Proteins were dissociated by dissolution in 0.05 M HCOOH–0.01 M NaOH buffer, pH 3.1, or in 6 M urea-tris-glycine buffer, pH 8.6 (Righetti & Secchi 1972) and disk electrophoresis carried out in 15 % acrylamide gels containing the same buffers ($\pm$0.01 M KCl). Gels were fixed with 20 % sulphosalicylic acid, transferred to 12.5 % trichloracetic acid and stained for protein with Coomassie blue G250 (Diezel, Kopperschläger & Hofman 1972), and for carbohydrate as described by Smith (1968).

Preparative electrophoresis was carried out at 15 °C in 7.5 % acrylamide gels with a PAGE-apparatus (Shandon Ltd, Surrey) in the manner suggested by Righetti & Secchi (1972); gels were prerun for 10 h in the 20 mM tris-glycine buffer, pH 8.7 to remove persulphate.

(*f*) *Compositions*

Methods used in the analyses of the compositions of the carotenoproteins have been described in an earlier publication (Zagalsky *et al.* 1970). Comparisons of amino acid compositions were made in the manner proposed by Marchalonis & Weltman (1971). Values for the partial specific volumes of pigments were derived from the amino acid compositions, taking the specific volume of astaxanthin as 1.0 (Buchwald & Jencks 1968*b*).

The *N*-ethylmaleimide (NEM) method of Diez, Osuga & Feeney (1964) was used to determine the sulphydryl content of the proteins: 1 ml of a solution of NEM (0.1 mg/ml) in 0.1 M phosphate buffer, pH 7, was mixed with 2 ml protein (0.5 mg/ml) dissolved in the phosphate buffer with and without 1 M KCl. Corrections for the hydrolysis of NEM and the absorption of the pigments at 300 nm were obtained from controls made without protein and without NEM, respectively. The spectrum of the proteins in the visible region were unaltered following the treatment with NEM.

(*g*) *Modification of amino acid groups*

Amidination was carried out as described by Hunter & Ludwig (1962): protein (*ca.* 0.05 mg/ml), dissolved in 0.2 M phosphate buffer, pH 8.5, was treated with methylacetimidate acetate (0.8 mg/ml) for 3 h at 25 °C.

Modification of arginine residues was performed as follows (Takahashi 1968): protein (*ca.* 0.05 mg/ml), dissolved in 0.2 M phosphate buffer, pH 7.5, was incubated at 25 °C for 1–7 h with phenylglyoxal hydrate (0.4 mg/ml); reaction of the protein (0.05 mg/ml) with pyruvic aldehyde (0.1 ml/ml) was carried out in 0.2 M phosphate buffer, pH 8.5, at 25 °C for 3 h.

Carboxyl groups were amidated following reaction with water-soluble carbodiimides (Armstrong & McKenzie 1967; Wilchek, Frensdorff & Sela 1967): proteins (*ca.* 0.05 mg/ml) dissolved in 1 M ammonium sulphate–0.2 M phosphate buffer, pH 5.8, were treated with CMC (0.25 mg/ml) or EDC-acetate (0.15 mg/ml) for 1–5 h at 25 °C.

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(*h*) *Circular dichroism spectra*

Circular dichroism spectra were kindly measured by Dr P. M. Scopes (Westfield College, London University) between 200 and 750 nm for solutions of the complexes in 0.2 M phosphate buffer, pH 7, both in the presence and absence of 0.5 M KCl. Results were expressed in terms of molar ellipticity for the carotenoid absorption (degrees cm^2/dmol) and mean residue ellipticity for the protein absorption (degrees cm^2/dmol).

(*i*) *Surface spreading*

The effectiveness of chloride ions in preventing spreading of the carotenoproteins at the air/water interface was kindly investigated by Mr C. J. Cookson, Bedford College, using a Langmuir trough of conventional pattern at 25 °C. The proteins (0.4–0.5 mg/ml) dissolved in 0.05 M phosphate buffer, pH 7, were spread (see Cheesman *et al.* 1966) over 0.2 M phosphate buffer, pH 7, containing 10^{-5}–10^{-1} M KCl, and the surface pressure-area were continuously recorded. Results were expressed as area occupied (m^2/mg) at a surface pressure of 10 dyn/cm. The decrease in the area occupied, compared to spreading on 0.2 M phosphate buffer, pH 7, was calculated as a percentage of the total decrease in area achieved with the strongest chloride solution (0.1 M).

(*j*) *Halide-binding sites*

The chloride-ion binding properties of the carotenoproteins were investigated with radioactive chloride ($Na^{36}Cl$) by a gel-filtration equilibrium method (Fairclough & Fruton 1966) on Sephadex G25. The proteins (0.2–0.3 %) were dialysed for 48 h, at the temperature of the gel filtration, against solutions of 0.2 M phosphate buffer, pH 7, containing 10^{-3}–10^{-2} M KCl and 0.1–0.3 ml radioactive NaCl solution per litre. Samples (0.5 ml) were passed through the Sephadex G25 column (36 cm × 2 cm; flow rate 2.5 ml/h) equilibrated with the dialysate and 3.5 ml fractions were collected. The carotenoprotein, distributed in 1–2 fractions, was analysed for ^{36}Cl and carotenoid. Radioactive measurements were made in the Triton X-100 liquid scintillant (system tT21) suggested by Patterson & Greene (1965) with a Packard (Series 3000, model 3380) liquid scintillation apparatus. The vials contained: 0.4 ml protein fraction, 0.1 ml of an identical unreactive phosphate-KCl solution, 0.8 ml 0.1 M KCl and 10 ml liquid scintillant. A quench curve was obtained with vials containing: 0.4 ml radioactive dialysate, 0.1 ml of solutions of the carotenoproteins dialysed against an identical unreactive phosphate-KCl solution, 0.8 ml 0.2 M KCl and 10 ml liquid scintillant. The carotenoproteins retained their characteristic colour and gave clear solutions in the scintillant. Samples of the fractions from gel filtration were diluted 10–15 times with 0.05 M phosphate buffer, pH 7, and the carotenoid content was determined following dissociation with pyridine, as described above.

Results

(*a*) *Purification*

The *Velella* pigment was resolved into several carotenoprotein components, differing in size, by gel filtration on columns of Agarose 6B. The two main proteins represented 75 % and 15 % of the total pigment and had λ_{max} at 600 and 620 nm, respectively, in 1 M KCl–0.04 M phosphate buffer, pH 7. Pigment λ_{max} 620 (V620) was purified on columns of Agarose 4B; the smaller-size pigment, λ_{max} 600 nm (V600) was purified on columns of Agarose 6B and subsequently

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separated from low molecular-size pigments (V570 and V545) on columns of Sephadex G75; V570 was purified on columns of Sephadex G75 and separated from the more strongly retarded V545. Most studies were carried out with V600, as there were insufficient amounts of the other pigments for extensive investigations.

Aggregated *Velella* pigment, eluting in the void volume on Agarose 4B columns, redissolved in 1 M KCl–0.02 M mercaptoethanol–0.04 M phosphate buffer, pH 7; rechromatography of this material gave mainly V600, with some of the pigments V620 and V570. The amount of aggregated material was considerably reduced when 10^{-3} M NEM was included in the column buffers. Pigments V600 and V570 were slowly and irreversibly formed from V620 dissolved in 1 M KCl–0.04 M phosphate buffer, pH 7, and stored at 4 °C. Both pigment V620 and V600 showed a tendency to form insoluble aggregates on storage (soluble on treatment with mercaptoethanol).

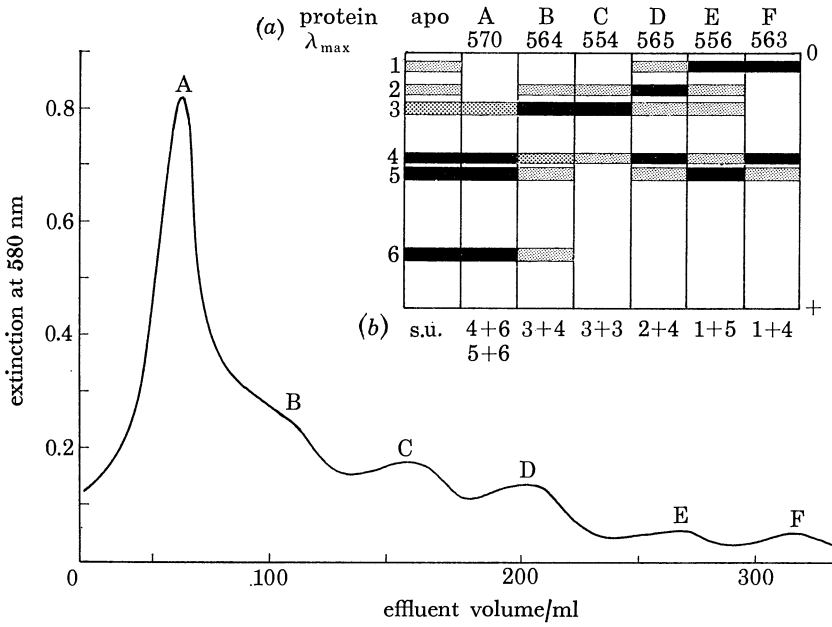

FIGURE 1. Preparative electrophoresis of V570 (*ca.* 5 mg protein in 3 ml 10 mM tris-glycine buffer, pH 8.7) in a 7.5% acrylamide gel (electrophoretic buffer: 20 mM tris-glycine, pH 8.7; potential gradient: 35 V/cm; temperature: 16 °C; fraction volume: 5 ml). Inset: disk electrophoresis of separated pigment fractions and of the apoprotein (prepared by acetone precipitation and purified by Sephadex G75 gel filtration) in 15% acrylamide gels (electrophoretic buffer: 6 M urea-20 mM tris-glycine, pH 8.7); the position (nm) of the absorption maxima (*a*) of the pigment fractions in 1 M KCl–0.04 M phosphate buffer, pH 7, and their probable subunit compositions (*b*) are shown.

(*b*) *Homogeneity*

The *Velella* pigments V600 and V620 gave small amounts of two more mobile purple components, in addition to the blue carotenoprotein band, in electrophoresis in tris-HCl buffer, pH 7.5. An increased amount of the purple pigments was observed when electrophoresis was carried out in phosphate buffer, pH 7. The absence of spreading between the blue and purple bands indicated that there was no rapid dissociation–association between these components.

An identical electrophoretic pattern was given by the apoprotein of the *Velella* pigment V600, prepared by acetone precipitation, by dissolution in formate buffer, pH 3.1, or by heating at 50 °C for 30 min in 6 M urea–tris–glycine buffer, pH 8.7. In electrophoresis at pH 3.1 the

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apoprotein separated into two purple components closely similar in mobility. Resolution into six purple components was achieved when the electrophoresis was carried out with tris-glycine buffer, pH 8.7 (figure 1). The apoprotein of V620 was indistinguishable in electrophoresis from that of V600.

The pigment V570, purified on a column of Sephadex G75, was resolved into six components in preparative acrylamide electrophoresis at pH 8.7 (figure 1). Each partially purified fraction gave a distinctive electrophoretic pattern following dissociation in 6 M urea–tris–glycine buffer, pH 8.7; the probable subunit composition of each fraction, deduced from the electrophoretic studies, and the position of the visible absorption maximum, is given in figure 1.

(*c*) *Halide-dependent spectral and structural alterations*

The spectra of V620 and V600 altered on removal of halide ions from the pigment solutions; the position of the absorption maxima shifted hypsochromically to 570–582 nm and 572–583 nm respectively, in 0.2 M phosphate buffer, pH 7, the precise position depending on the temperature. The E_{660}/E_{560} ratio for solutions of V600 (0.04 mg/ml) in 0.05 M or 0.5 M phosphate buffer pH 7, increased by 10–11 % on lowering the temperature from 25 to 5 °C, and by 6 % at 5 °C and 25 °C on transferring the pigment from 0.05 to 0.5 M phosphate buffer; the ratio increased by 18 % on changing the pigment from solution in 0.05 M phosphate buffer at 25 °C to 0.5 M phosphate buffer at 5 °C. The original spectra were rapidly and completely restored on immediate readdition of KCl to 1 M (90 % recovery within 15 s for 10^{-4} M KCl at 4 °C). Only partial recovery of the spectra was obtained with solutions of the pigments in 0.2 M phosphate buffer pH 7, left for prolonged periods in the absence of halide ions, particularly at room temperature.

The solubility of V620 and V600 decreased in the absence of halide ions, and they had only a low solubility in 0.02 M phosphate buffer pH 7.

The pigments V620 and V600, dissolved in 0.2 M phosphate buffer, pH 7, separated into two pigment fractions following gel filtration on columns of Agarose 6B, Sephadex G200 or Bio-Gel P300 equilibrated with the same buffer. The larger-size blue component V583 (λ_{max} 583 nm at 5 °C, 580 nm at 24 °C) had the same elution volume as V600 filtered on columns equilibrated with the buffer containing 1 M KCl. A considerable quantity of the smaller-size purple component V565 (λ_{max} *ca.* 565 nm at 5 °C, *ca.* 560 at 24 °C) was adsorbed onto the bed material when Agarose 6B and Sephadex G200 columns were used for the separation, particularly with columns used for the first time and with samples of low pigment content. Adsorption was minimal on columns that had been in constant use and were thereby saturated with the pigment. The adsorbed pigment was eluted from the bed materials with 1 M KCl as a blue pigment mixture, λ_{max} 590–610 nm. There was no adsorption of the purple component with Bio-Gel P300 columns (see figures 2*a* and 3*a*). The relative proportion of carotenoid associated with the two pigment fractions separated from V600 on Bio-Gel P300 columns equilibrated with 0.2 M phosphate buffer, pH 7 (figure 3*a*) was *ca.* 65–70 % with V583 and *ca.* 35–30 % with V565. The proportions were not significantly different whether the transference to phosphate buffer and gel filtration was performed at 5 or 25 °C or whether 0.05 or 0.5 M buffers were employed for these purposes. The precise absorption maxima of the fractions were dependent on temperature and on the ionic strength of the phosphate buffers. Pigment V620 gave a greater proportion of the smaller fragment; about 60 % of the total carotenoid was associated with this fraction (figure 2*a*)

There was no evidence in the differentiated gel filtration elution profiles (Winzor & Scheraga

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1964) of an association–dissociation equilibrium between the two components (figures 2*a* and 3*a*) for either V620 or V600.

The position of the absorption maximum of pigment V583 returned to between 590 and 598 nm in the presence of 1 M KCl; on transference to 0.2 M phosphate buffer, pH 7 and rechromatography on Bio-Gel P300, V583 alone was eluted. In the presence of 1 M KCl the low molecular-size V565, isolated from V620 and V600 (figures 2*a* and 3*a*) partially repolymerized. The percentage of the low molecular-size V570 nm in 1 M KCl, irreversibly formed in the chloride-free buffer, varied between 20–60 % for V600 and 5–15 % for V620, depending on the temperature and time taken for the Bio-Gel filtration. The polymerized proteins, separated from unchanged low molecular-size pigments on columns of Sephadex G75 equilibrated with 1 M KCl–0.04 M phosphate

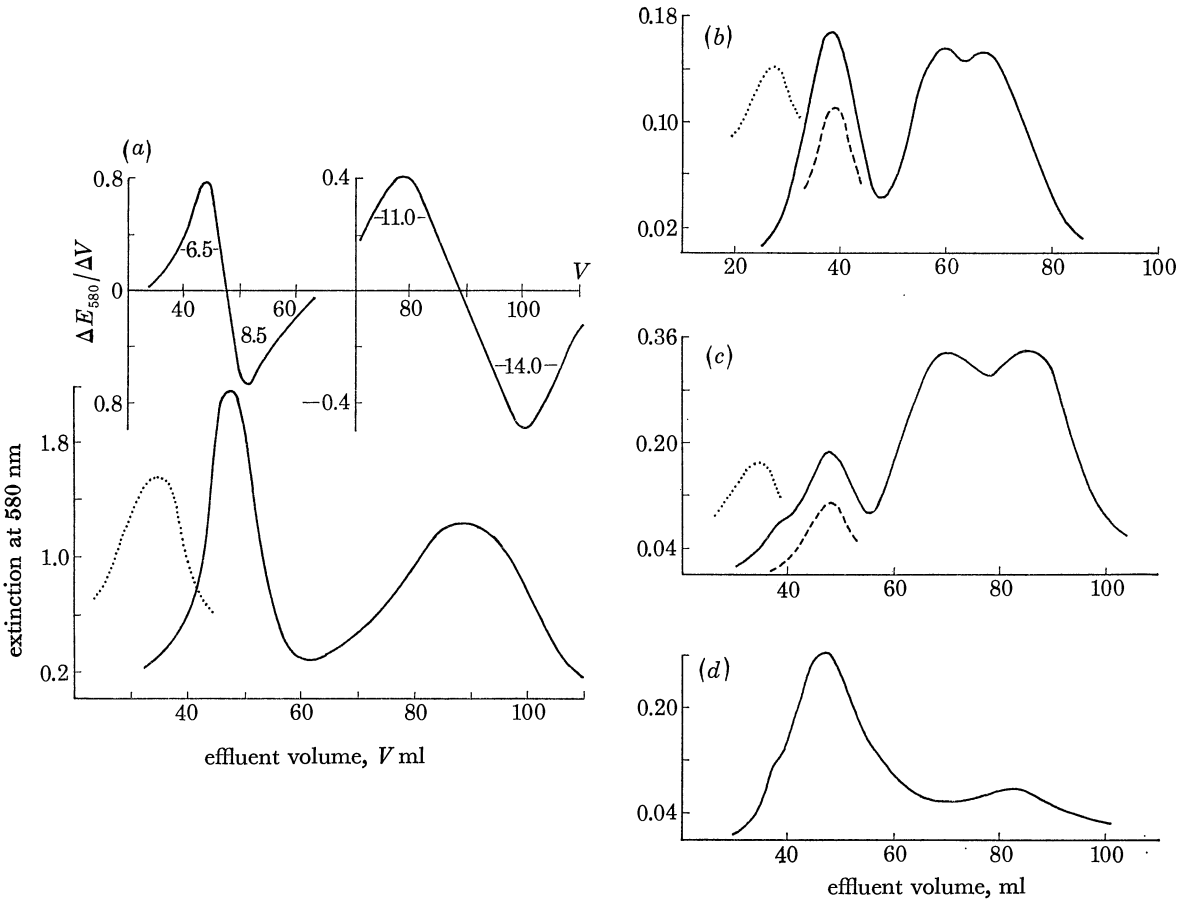

FIGURE 2. Gel filtration elution profiles of V620 on Bio-Gel P300 at 16 °C. (*a*) Column equilibrated with 0.2 M phosphate buffer, pH 7 (3.5 ml sample, 1.9 mg protein/ml; void volume: 34.5 ml; flow rate: 4 ml/h; fraction volume: 2.5 ml). Curve (. . .): elution profile of pigment with the column equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7. The inset shows the first difference plot and band widths at half-maximal values of $\Delta E/\Delta V$ for the filtration in the absence of chloride ions. (*b*) Column equilibrated with 0.2 M phosphate buffer, pH 7, containing 1.0×10^{-4} M KCl (5 ml sample, 0.1 mg protein/ml; void volume: 27.5 ml; flow rate: 4 ml/h; fraction volume: 2.5 ml). Elution profiles of V620 (. . .) and V600 (- - -) with the column equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7, are shown. (*c*) Column equilibrated with 0.2 M phosphate buffer, pH 7, containing 1.0×10^{-3} M KCl (5 ml sample, 0.3 mg protein/ml; void volume: 37.5 ml; flow rate: 4 ml/h; fraction volume: 2.5 ml). Elution profiles of V620 (. . .) and V600 (- - -) with the column equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7, are shown. (*d*) Column equilibrated with 0.2 M phosphate buffer, pH 7, containing 4.5×10^{-3} M KCl (4 ml sample, 0.15 mg protein/ml; void volume: 37.5 ml; flow rate: 4 ml/h; fraction volume: 2.5 ml).

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buffer, pH 7, had absorption maxima at *ca.* 600 nm and 615 nm, depending on whether they originated from V600 or V620 respectively. The pigment λ_{max} *ca.* 600 gave the purple (V565) and blue (V583) components following transference to 0.2 M phosphate buffer pH 7, and re-chromatography on Bio-Gel in this buffer; the purple component was formed in greater relative proportion than for the native V600.

The absorption maximum of V583, derived from V620 and V600, was restored to between 590 and 598 nm by the addition of KCl to 1 M.

The absorption maxima of the large- and small-size fragments of V620 and V600 were

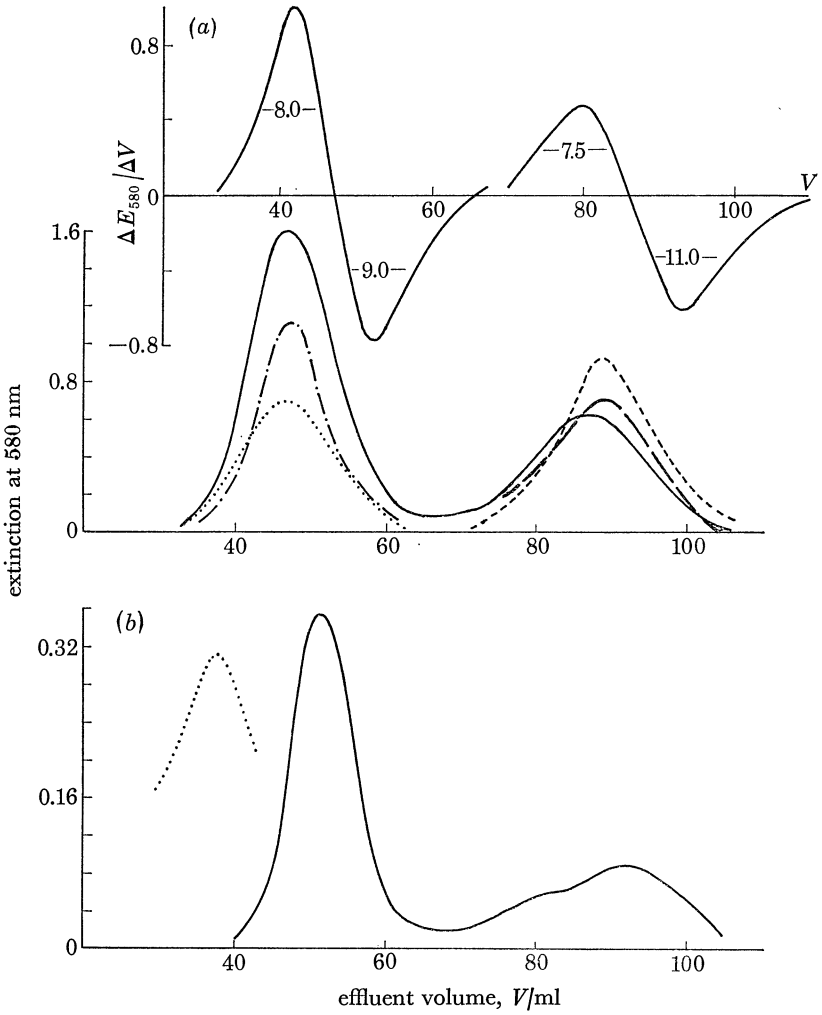

FIGURE 3. Gel filtration elution profiles of V600 and its derivatives on Bio-Gel P300 at 16 °C. (*a*) Column equilibrated with 0.2 M phosphate buffer, pH 7 (void volume: 34.5 ml; flow rate: 4 ml/h; fraction volume: 2.5 ml). Smooth curve, V600: 5 ml sample, 0.6 mg protein/ml; curve (. . .): rechromatography of large-size fragment, V583 (5 ml sample, 0.2 mg protein/ml); curve (- - -): rechromatography of small-size fragment, V565 (5 ml sample, 0.25 mg protein/ml). Curves (-·-·-) and (— — —) are for V600 (2.5 ml sample, 0.6 mg protein/ml) and V570 (2 ml sample, 0.5 mg protein/ml), respectively, with the column equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7. The inset shows the first difference plot and band widths at half-maximal values of $\Delta E/\Delta V$ for the filtration of V600 in the absence of halide ions (smooth curve, above). (*b*) Column equilibrated with 0.2 M phosphate buffer, pH 7, containing 1.0×10^{-2} M KCl (3.5 ml sample, 0.15 mg protein/ml; void volume: 37.5 ml; flow rate: 4 ml/h; fraction volume: 2.5 ml). (. . .) Elution profile for V620 with the column equilibrated with 1 M KCl–0.4 M phosphate buffer, pH 7.

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unaltered on storage (6 weeks) at 4 °C in 0.2 M phosphate buffer pH 7, but the ability to change in colour on addition of KCl was almost completely lost.

Attempts were made to study the changes in the quaternary structures of V620 and V600, accompanying the halide-dependent spectral shifts, by following the elution profiles of the pigments on Bio-Gel columns equilibrated with phosphate buffer containing low concentrations of KCl. The small-size pigment, V565, obtained in halide-free filtration of V620 (figure 2*a*), partially reassociated when KCl was included in the equilibrating buffer (figure 2); it formed a pigment of intermediate size (a dimer, λ_{max} *ca.* 580 nm) at 10^{-4} M KCl and polymerizes further at higher KCl concentrations to give V600 and, in some Bio-Gel separations (figure 2), small amounts of V620. The dimer associated in the presence of 1 M KCl to form V620 nm. A partial reassociation of the small-size fraction from V600 (figure 3*a*) to form a dimer, is evident only at the highest KCl concentration employed (10^{-2} M, figure 3*b*). There is a gradual bathochromic shift in the position of the absorption maximum of the large-size fraction of V600 and of V620 with increasing concentration of KCl in the eluting buffer. The absorption maximum of this fraction in the presence of 1 M KCl was at 590–600 nm for V620 (following separations at the lower chloride concentrations) and for V600; at the higher concentrations of KCl (4×10^{-3} M and 1.0×10^{-2} M) used for the Bio-Gel runs of V620, the absorption maximum of the native pigment was recovered.

The dependence of the absorption spectra on halide ion concentration, and the ability of a range of different anions to restore the spectral characteristics of the pigments were investigated in the following manner: solutions of the pigments in 0.2 M phosphate buffer, pH 7, were incubated for 2 h (overnight in preliminary experiments) with different concentrations of the anion under investigation. The fractional increase in extinction at 660 nm, $\bar{V}$, was then measured (ratio of the change in 660 nm absorption on addition of anion, to the total change in 660 nm absorption for saturation of the halide ion binding sites with 1 M KCl at 4 °C). At this wavelength the chloride-free and chloride-saturated pigments differ most in extinction.

If a simplified view is taken of the structural and spectral changes accompanying the binding of anions, the fractional increase in the extinction at 660 nm, $\bar{V}$, may be related to the number of anion binding sites and their apparent association constant. Thus, if it is supposed that, on binding anions, V565 associates to give V600, with no intervening steps, the equilibrium constant (K_1) for the process

$$x\mathrm{V565} + nxA^- \rightleftharpoons \mathrm{V600},$$

is given by

$$K_1 = \frac{[\mathrm{V600}]}{A^{nx}[\mathrm{V565}]^x}, \tag{1}$$

where x is the degree of association, and n is the number of anions bound per molecule V565. It can be shown, provided the binding of each chloride ion gives rise to an equal increment in the extinction at 660 nm, that

$$\frac{1-\bar{V}}{\bar{V}} = \frac{1}{xK_1A^{nx}[\mathrm{V565}]^{x-1}}. \tag{2}$$

For independent and identical anion-binding sites, characterized by the same apparent binding constant (k), $K_1 = k^{nx}K_p$, where K_p is an association constant for the polymerization of the V565 subunits.

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Similarly for the binding of anions by pigment λ_{max} 583 (V583) with the formation of V600:

$$\mathrm{V583} + nxA^- \rightleftharpoons \mathrm{V600},$$

$$K_2 = \frac{[\mathrm{V600}]}{A^{nx}[\mathrm{V583}]},$$

and thus

$$\frac{1-\bar{V}}{\bar{V}} = \frac{1}{K_2 A^{nx}}, \tag{3}$$

where $K_2 = k'^{nx}$ for independent and identical anion-binding sites of apparent association constant k'.

For the mixture of pigments V583 and V565 obtained on removing bound anions from V600, the following processes may be considered to occur on readdition of halide ions.

$$\begin{array}{ll} \mathrm{V583} + nxA^- & \overset{+\mathrm{Cl}^-}{\underset{-\mathrm{Cl}^-}{\rightleftharpoons}} \\ & \mathrm{V600} \\ x\mathrm{V565} + nxA^- & \overset{+\mathrm{Cl}^-}{\underset{-\mathrm{Cl}^-}{\rightleftharpoons}} \end{array}$$

and

$$\frac{1-\bar{V}}{\bar{V}} = \frac{K_2 + xK_1[\mathrm{V565}]^{x-1}}{xK_1K_2A^{nx}[\mathrm{V565}]^{x-1}}, \tag{4}$$

provided V565 and V583 give an equal contribution to the extinction increment at 660 nm on conversion to V600.

Similarly, for the polymerization

$$mx\mathrm{V565} + nmxA^- \rightleftharpoons \mathrm{V620},$$

and

$$\frac{1-\bar{V}}{\bar{V}} = \frac{1}{mxK_3A^{nmx}[\mathrm{V565}]^{mx-1}}, \tag{5}$$

where

$$K_3 = \frac{[\mathrm{V620}]}{A^{nmx}[\mathrm{V565}]^{mx}} \tag{6}$$

and m is the degree of association of V620 from subunits of the size of pigment V600. For independent and identical anion-binding sites of apparent binding constant k'', $K_3 = k''^{nmx}K_p'$, where K_p' is an association constant for the polymerization of V565 subunits. For the mixture of pigments present in halide-free buffer solutions of V620 the associations that take place on addition of halide ions may be taken as:

$$\begin{array}{ll} mx\ \mathrm{V565} + nmxA^- & \overset{+\mathrm{Cl}^-}{\underset{-\mathrm{Cl}^-}{\rightleftharpoons}} \\ & \mathrm{V620} \\ m\ \mathrm{V583} + nmxA^- & \overset{+\mathrm{Cl}^-}{\underset{-\mathrm{Cl}^-}{\rightleftharpoons}} \end{array}$$

and

$$\frac{1-\bar{V}}{\bar{V}} = \frac{K_4 + x^m\sqrt{(K_3/K_4)}/[\mathrm{V565}]^{x-1}}{mxK_3K_4A^{nmx}[\mathrm{V565}]^{mx-1}}, \tag{7}$$

where

$$K_4 = \frac{[\mathrm{V620}]}{A^{nmx}[\mathrm{V583}]^m}. \tag{8}$$

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Plots of $\bar{V}$ and lg $((1-\bar{V})/\bar{V}$ against $(\gamma[A^-])$ are shown for a number of anions at 4 and 24 °C (figure 4); the activities (γ) were calculated from the equation, $-\lg\gamma = 0.5\sqrt{\mu}/1+2\sqrt{\mu}$ derived for chloride ions (Scatchard, Scheinberg & Armstrong 1950), and assumed, as an approximation, to hold for the other anions tested.

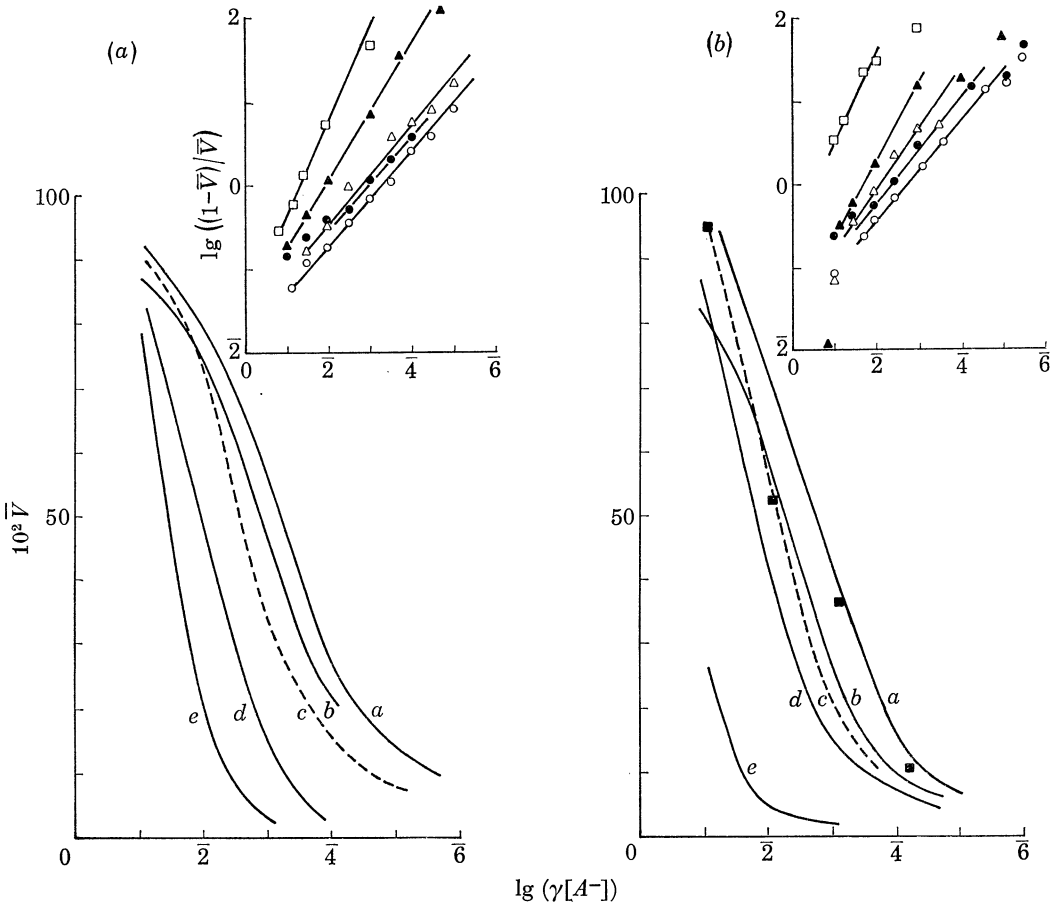

FIGURE 4. Plots of $\bar{V}$ (increase in extinction at 660 nm on addition of anion to a given concentration divided by maximal increase in extinction at 660 nm with the anion under investigation) against log anion activity (lg $(\gamma[A^-])$ at (*a*) 4 °C and (*b*) 24 °C for V600 dissolved in 0.2 M phosphate buffer, pH 7 (0.054 mg protein/ml). Curve *a* for bromide ions (○); curve *b* for iodide ions (●); curve *c* for chloride ions (△); curves *d* and *e* are for the large-size fragment (▲), V583 (0.055 mg protein/ml) and small-size fragment (□), V565 (0.085 mg protein/ml), respectively, separated in halide-free gel filtration of V600 (see figure 2*a*), treated with chloride ions. The change in surface spreading of V600 in 0.05 M phosphate buffer, pH 7, with varying concentrations of KCl in a subphase of 0.2 M phosphate buffer, pH 7, at 25 °C is shown (■): points represent the decrease in area occupied compared to spreading on 0.2 M phosphate buffer, pH 7, calculated as a percentage of the total decrease in area achieved with the subphase containing 0.1 M KCl. The insets show plots of lg $((1-\bar{V})/\bar{V})$ against lg $(\gamma[A^-])$.

The approximate linearity of the plots of lg $((1-\bar{V})/\bar{V})$/lg $(\gamma[A^-])$ for the pigments (table 1) implies that: $|\lg xK_1| \gg |(x-1)\lg[\mathrm{V565}]|$ in equation (2); $|xK_1[\mathrm{V565}]| \gg |K_2|$ and $|\lg xK_1K_2| \gg |(x-1)\lg[\mathrm{V565}]|$ in equation (4); $|\lg mxK_3| \gg |(mx-1)\lg[\mathrm{V565}]|$ in equation (5); $|K_4| \gg |x^m\sqrt{(K_3/K_4)}\,[\mathrm{V565}]^{x-1}|$ and $|\lg mxK_3K_4 \gg |(mx-1)\lg[\mathrm{V565}]|$ in equation (7). Equation (4) may then be written

$$\frac{1-\bar{V}}{\bar{V}} \approx \frac{1}{K_2A^{nx}} \qquad (9)$$

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and equation (7),

$$\frac{1-\bar{V}}{\bar{V}} \approx \frac{1}{mxK_3A^{nmx}[\mathrm{V565}]^{mx-1}}. \qquad (10)$$

The order of effectiveness of the anions for the spectral alterations of the pigments was $Br^- > I^- > Cl^- > NO_3^-, CNS^-$ (figure 4; table 1); the changes were independent of the nature of the cation and showed a negative enthalpy dependence. Complete recovery of the spectra of V620 and V600 was not achieved with CNS^- and NO_3^-. At high concentrations of CNS^- (0.2–1.0 M) the absorption maxima of the pigments shifted hypsochromically from *ca.* 590 nm in 0.1 M KCNS to 540–60 nm at 4 °C in 1 M KCNS. The positions of the absorption maxima of V620 and V600 were at 600 and 595 nm, respectively, following addition (to 1 M) of $NaNO_3$

TABLE 1(A). SLOPES AND INTERCEPTS OF PLOTS OF lg $((1-\bar{V})/\bar{V})$ AGAINST lg $(\gamma[A^-])$ FOR V620 AND ITS DERIVATIVES†

	V620					
	−slope‡		coefficient of correlation‡		$(\gamma[A^-])$ at lg $((1-\bar{V})/\bar{V}) = 0$	
anion	4 °C	24 °C	4 °C	24 °C	4 °C	24 °C
Br^-	0.44	0.52	0.996	0.939	2.5×10^{-4}	1.1×10^{-3}
I^-	0.61	0.67	0.997	0.997	6.6×10^{-4}	2.2×10^{-3}
Cl^-	0.59	0.69	0.995	0.991	9.2×10^{-4}	4.9×10^{-3}
NO_3^-	0.95	1.11	0.950	0.965	1.5×10^{-3}	2.8×10^{-2}
CNS^-	0.70	0.33	0.973	0.944	4.0×10^{-3}	2.6×10^{-2}
Large molec. mass fraction separated in halide-free Bio-Gel filtration (V583)						
Cl^-	0.71	0.79	0.995	0.990	1.1×10^{-2}	1.6×10^{-2}
Small molec. mass fraction separated in halide-free Bio-Gel filtration (V565)						
Cl^-	0.77	0.95	1.00	0.998	9.2×10^{-3}	1.5×10^{-2}

TABLE 1(B) SLOPES AND INTERCEPTS OF PLOTS OF lg $((1-\bar{V})/\bar{V})$ AGAINST lg $(\gamma[A^-])$ (FIGURE 4) FOR V600 AND ITS DERIVATIVES†

	V620					
	−slope‡		coefficient of correlation‡		$(\gamma[A^-])$ at lg $((1-\bar{V})/\bar{V}) = 0$	
anion	4 °C	24 °C	4 °C	24 °C	4 °C	24 °C
Br^-	0.51	0.58	0.993	0.969	5.5×10^{-4}	1.8×10^{-3}
I^-	0.50	0.59	0.992	0.994	0.95×10^{-3}	5.4×10^{-3}
Cl^-	0.59	0.59	0.987	0.980	1.5×10^{-3}	5.8×10^{-3}
NO_3^-	0.94	0.83	0.942	0.996	5.3×10^{-3}	7.9×10^{-2}
CNS^-	0.48	0.40	0.962	0.986	3.8×10^{-3}	1.4×10^{-2}
Large molec. mass fraction separated in halide-free Bio-Gel filtration (V583)						
(*a*) in 0.2 M phosphate buffer pH 7						
Cl^-	0.74	—	0.940	—	2.9×10^{-3}	—
(*b*) left overnight at 4 °C in 1 M KCl–0.2 M phosphate buffer, pH 7, and transferred to 0.2 M phosphate buffer, pH 7						
Cl^-	0.83	0.80	0.999	0.998	9.1×10^{-3}	1.3×10^{-2}
Small molec. mass fraction separated in halide-free Bio-Gel filtration (V565)						
Cl^-	1.20	1.15	0.998	0.980	2.9×10^{-2}	0.21

† Proteins dissolved in 0.2 M phosphate buffer, pH 7.

‡ Slope and coefficient of correlation of least squares straight line. Values given in tables 1(A) and (B) are for $\bar{V} = 0.1$–0.9.

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at 4 °C. The original spectrum of V600 was almost completely restored when KCl was added (to 1 M) to solutions of the pigment containing 1 M $NaNO_3$ or < 0.3 M KCNS. Pigment V600 in 0.1 M KCNS–0.2 M phosphate buffer, pH 7, separated into polymeric pigment, λ_{max} *ca.* 590–95, and low molecular-size purple pigment, λ_{max} 565, on columns of Sephadex G75 equilibrated at 4 °C with the same solution; the absorption maximum of the purple pigment shifted to 590–595 nm on adition of KCl to 1 M. An insignificant amount of small-size purple pigment was separated in gel filtration of V600 dissolved in 0.1 M $NaNO_3$–0.2 M phosphate buffer, pH 7, on columns of Sephadex G75 equilibrated with the same solution. The following anions were ineffective (at 0.1 M and 4 °C) in changing the spectra of halide-free V600: F^-, ClO_4^-, CO_3^{2-}, HSO_3^-, HCO_3^-, SO_3^{2-}, SO_4^-, CCl_3COO^-, IO_4^- and Ac^-.

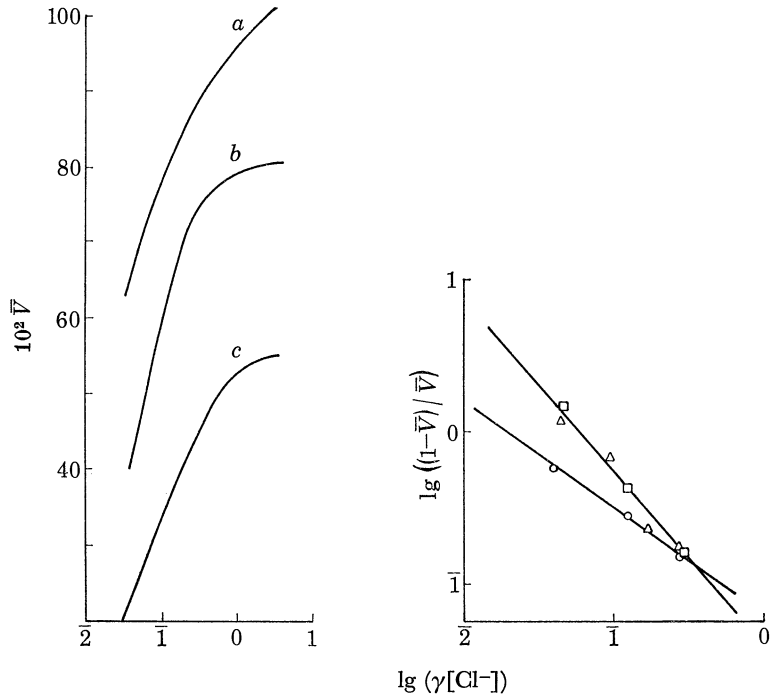

FIGURE 5. Plots of $\bar{V}$ (increase in extinction at 660 nm on addition of chloride ions to a given concentration divided by maximal increase in extinction at 660 nm for control sample with chloride ions at 25 °C) and lg $((1-\bar{V})/\bar{V})$ against log chloride ion activity (lg (γ[Cl$^-$])) at 25 °C for V600 (0.023 mg protein/ml) in 0.2 M phosphate buffer, pH 8.5. Curve *a*, following incubation of the pigment for 3 h at 25 °C (control ○); curve *b* following treatment with methylacetimidate (△); curve *c*, after treatment with pyruvic aldehyde (□).

The spectra of the components separated from V620 and V600 in Bio-Gel filtration with chloride-free buffer show a dependence on chloride ion concentration quite distinct from the unresolved pigments in 0.2 M phosphate buffer (table 1). Reversal of the altered sensitivity to chloride ions of the larger component of V600 (pigment λ_{max} 583) was not obtained by incubation in 1 M KCl for 24 h. This is evident from the lg $((1-\bar{V})/\bar{V})$ lg (γ[Cl$^-$]) plots for the pigment subsequently transferred to 0.2 M phosphate buffer with a Sephadex G 25 column (figure 4; table 1*b*).

The pH dependence of the spectral changes effected by an intermediate concentration of KCl (3×10^{-3} M) for V600 dissolved at fixed concentration in 0.2 M phosphate buffers was investigated over the stability range of the carotenoid-protein linkage (pH 5.5–8.6). The values

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for $\bar{V}$ were of similar magnitude at all pH values; a slight enhancement of the bathochromic shift caused by the chloride occurs at low pH.

Treatment of chloride-free V600 with mercaptoethanol, NEM, carbodiimides or phenylglyoxal did not result in an alteration in the magnitude of the extinction increase at 660 nm obtained with 3×10^{-3} M KCl, and the 600 nm maximum was recovered in 1 M KCl. There was, however, an apparent decrease in the effectiveness of chloride for the spectral modifications following treatment with methylacetimidate and pyruvic aldehyde; the latter treatment partially abolished the chloride-dependent spectral shift (figure 5). The absorption maximum of the methylacetimidate-treated pigment was at 595 nm in the presence of excess KCl.

(*d*) *Surface spreading*

The effect of the addition of chloride ions to the subphase on the ability of V600 (in 0.05 M phosphate buffer, pH 7) to spread at an air–water interface is shown in figure 4*b*; there was an 8 % decrease in the spread area (m^2/mg) over a subphase chloride concentration range of 10^{-5}–10^{-1} M at a surface pressure of 10 dyn/cm. The spread area for pigment λ_{max} 570 and for the small- and large-size fragments separated from V600 in halide-free gel filtration, dissolved in 0.05 M phosphate buffer, pH 7, was unaffected by the inclusion of KCl (10^{-5}–10^{-1} M) in the subphase.

TABLE 2. AMINO ACID COMPOSITION (MOLE %)† OF V600‡

Lys	6.9 (15.9)	Thr	6.1 (14.1)
His	1.8 (4.1)	Ser	10.6 (24.4)
Arg	3.3 (7.6)	Glu	11.2 (25.8)
CySH	3.5 (8.1)	Pro	3.7 (8.5)
Asp	10.1 (23.3)	Gly	12.2 (28.1)
Val	6.6 (15.2)	Ala	8.2 (18.9)
Ileu	4.1 (9.4)	Phe	4.1 (9.4)
Leu	6.2 (14.3)	Tyr	nil
		Met	1.7 (3.9)

† Tryptophan and amide contents were not determined.
‡ Residues/apoprotein of molecular mass 2.35×10^4 in parentheses.

TABLE 3. AVAILABLE SULPHYDRYL GROUPS OF *VELELLA* PIGMENTS PER MINIMUM MOLECULAR MASS (BASED ON ASTAXANTHIN CONTENT), DETERMINED USING NEM IN THE PRESENCE AND ABSENCE OF 1 M KCl (AVERAGE OF TWO DETERMINATIONS)

	+KCl	−KCl
V620	1.2	2.9
V600	3.0	3.0

(*e*) *Compositions*

The amino acid composition of V600 is given in table 2. The pigments V620, V583 and V570 did not differ significantly from V600 in amino acid content; the values of $S\Delta\phi$ (differences in the mole percent values for each amino acid, summed and squared) for comparisons of the compositions of pairs of the pigments were less than 10, a value obtained by us in separate analyses of a given protein. The two apoprotein fractions of V600 separated in DEAE-cellulose chromatography (subunits 1–3 (figure 1, inset) not adsorbed, subunits 4–6 adsorbed and eluted from anion-exchanger) were considered to be identical in composition to each other and to V600 ($S\Delta\phi < 10$).

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The sulphydryl content of V600 and V620 in the presence and absence of 1 M KCl is given in table 3. The absorption at 300 nm by the pigments limits the accuracy of the values. No difference in sulphydryl content was found for V600, however, in the presence or absence of KCl. The pigment V600 was devoid of hexosamine and protein-bound phosphorus; V600 and V620 did not stain for carbohydrate in acrylamide gels following electrophoresis.

(*f*) *Absorption spectra*

The absorption spectra of V620 and V600, dissolved in 0.025 M phosphate buffer containing 0.2–1.0 M KCl, were independent of temperature between 0 and 25 °C, and of pH between 5.5 and 8.6. The spectra were unaltered following treatment of the pigments in the presence of 1 M KCl with methylacetimidate, pyruvic aldehyde, phenylglyoxal or carbodiimides. The absorption spectra of V583, V620 and V600 were unaltered following treatment of the proteins in 0.2 M phosphate buffer pH 7, with 1.0 M mercaptoethanol or reaction of sulphydryl groups with NEM in 1 M KCl–0.04 M phosphate buffer, pH 7. A considerable proportion of pigment λ_{max} 570 precipitated on addition of high concentrations (0.2 M) of mercaptoethanol to solutions of the pigment in 1 M KCl–0.04 M phosphate buffer, pH 7; the carotenoid-protein linkage was partially disrupted by the treatment (λ_{max} 545 nm).

The extinction coefficients $E^{0.1\,\%}_{\text{vis. max.}}$ of V620 and V600 were estimated as 9.8 ± 1.1 and 11.6 ± 2.0, respectively. The dissociation of the carotenoid-protein linkage with pyridine resulted in decreases in the extinction at the visible absorption maximum of the carotenoid of 14.3 % for V620, 13.7 % for V600 and *ca.* 10 % for V570. The pigments had $E_{280}/E_{\text{vis. max.}}$ values of *ca.* 0.2. V583 had an $E^{0.1\,\%}_{583}$ value of 10.9 ± 0.7 in 0.2 M phosphate buffer, pH 7, at 4 °C; there was a 13.7 % decrease in the extinction at the visible absorption maximum of the carotenoid following treatment with pyridine.

The positions of the visible absorption maxima of solutions of V583 and V570 occurred at longer wavelength at low temperature and high ionic strength. In 0.05–0.5 M phosphate buffers, pH 7, and at temperatures between 4 and 25 °C they varied between 580 and 583 nm and between 560 and 570 nm respectively, the precise position depending on the temperature and ionic strength. In 1 M KCl–0.04 M phosphate buffer pH 7, the maxima were at 583 and 570 nm at both 4 and 24 °C.

The half-band widths ($V_{\frac{1}{2}}$) and oscillator strengths (f) of the main absorption band of V620 and V600 in 1 M KCl–0.04 M phosphate buffer, pH 7, were 40.0×10^2 cm^{-1} ($V_{\frac{1}{2}}$) and 2.63 (f), and 39.0×10^2 cm^{-1} ($V_{\frac{1}{2}}$) and 2.38 (f), respectively. V570 had values of $V_{\frac{1}{2}}$ and f of 43.0×10^2 cm^{-1} and 2.61 in 1 M KCl–0.04 M phosphate buffer, pH 7, at 5 °C; V583 showed values of $V_{\frac{1}{2}}$ and f of 37.0×10^2 cm^{-1} and 2.54 respectively in 0.2 M phosphate buffer, pH 7 at 5 °C.

(*g*) *Circular dichroism spectra*

The circular dichroism spectrum of V620 dissolved in 0.5 M KCl–0.2 M phosphate buffer, pH 7, in the visible region (see Zagalsky 1976), shows a positive maximum at 630 nm, a negative maximum at 535 nm and crosses the base line at 578 nm. The positive and negative maxima have molar ellipticity values based on astaxanthin content of 1.3×10^6 and 5.3×10^5 degrees cm^2/dmol, respectively. A negative peak associated with the absorption band centred at 345 nm has a molar ellipticity value of 7.5×10^5 degrees cm^2/dmol. In the absence of chloride ions the dichroism spectrum has a single positive maximum at 570 nm, close to the visible absorption maximum in 0.2 M phosphate buffer, pH 7, with a molar ellipticity value of 6.2×10^5

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degrees cm²/dmol. The value of the negative maximum at 342 nm is lower in the absence of chloride ions, at 3.2×10^5 degrees cm²/dmol.

The circular dichroism of V600 dissolved in 0.5 M KCl–0.2 M phosphate buffer, pH 7, has a positive maximum in the visible region at 595 nm with a molar ellipticity value of 1.8×10^5 degrees cm²/dmol. There is evidence of slight splitting of the band into a double peak, with a small negative maximum of molar ellipticity 2.2×10^4 degrees cm²/dmol at 520 nm. The positive band, which crosses the base line at 544 nm, is asymmetric with considerable trailing of the descending limb towards higher wavelengths (up to 750 nm). In the absence of chloride ions a single positive maximum of molar ellipticity 2.3×10^5 degrees cm²/dmol occurs at 572 nm. The pigment has a negative maximum at 350 nm of molar ellipticity 6.3×10^4 and 7.9×10^4 degrees cm²/dmol in the presence and absence of chloride ions, respectively.

Pigment V570 has a positive maximum at 585 nm of molar ellipticity 6.7×10^4 degrees cm²/dmol in the presence and absence of chloride ions; it shows no dichroism at 340–50 nm, but has a positive peak of molar ellipticity 6.7×10^4 at 310 nm.

The circular dichroism spectra of the three pigments in the far ultraviolet, unaltered in the absence of chloride ions, have negative maxima of large magnitude at 215 nm. A value of 14.3×10^3 was estimated for the mean residue ellipticity of V600 at this wavelength.

The pigments showed weak dichroism in the near ultraviolet (250–320 nm) with positive absorption peaks at *ca.* 280 nm. A value of 500 degrees cm²/dmol was obtained for the mean residue ellipticity at 280 nm for V600 in the presence and absence of chloride ions. The dichroism spectrum of V620 in the 250–300 nm region was distorted by the more intense neighbouring bands; the effects of altered ionic environment on the absorption in this region alone could not, therefore, be distinguished. The dichroism spectrum of V570 between 250 and 320 nm was identical in the presence and absence of chloride ions.

(*h*) *Molecular size*

The Stokes radii of the carotenoproteins and derivatives, obtained in gel filtration studies, are shown in table 4, and estimates of their molecular mass, for different values of frictional coefficient (f/f_0), presented in table 5. Both the mean frictional coefficient (1.25) of the proteins utilized for standardization of gel filtration columns and values of f/f_0 of analogous crustacean carotenoproteins were taken for the calculations of molecular masses of pigments. The values of f/f_0 given in table 5 for V583 and V600, V570 and the apoprotein are those of α-, β- and apocrustacyanin (Gammack, Raper, Zagalsky & Quarmby 1971), respectively; the value of f/f_0 for V620 is that of the mandible carotenoprotein of *Aristeus antennatus*, which shows a similar large size in gel filtration. A partial specific volume of 0.72, calculated from the amino acid composition, was used in the molecular mass calculations.

The elution volumes of the *Velella* pigments in gel filtration experiments were unaltered when 10^{-3} M NEM or 0.02 M mercaptoethanol were included in the column equilibrating buffer.

The pigments V570 and V545 had partition coefficients (K) of 0.49 and 1.05, respectively, in the dextran-poly(ethyleneglycol) two-phase systems; there is reasonable agreement between the values of molecular mass obtained for the pigments in gel filtration and from the values of K (table 5). Pigment V600 had a K value of 0.02; a considerable amount of the protein (*ca.* 30 %) was present at the interface. The larger molecular size pigment V620 partitioned solely between the interface and lower phase at all pH. The isolectric points derived from the partition experiments for V545, V570 and V600 were *ca.* pH 4.8, 6.0 and 6.3.

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An estimate of the stoichiometry (n) of the halide-dependent dissociation–association of V600 was obtained from analysis of the gradient elution profile shown by the pigment in gel filtration at low chloride concentration. It was assumed that the equilibrium between the undissociated V583 and V600 did not influence the position of the minimum in the solute gradient, and that the association was not perturbed by solute-gel matrix interactions at the temperature (4 °C) of the experiment. Pigment V600 was eluted in gel filtration on columns of Agarose 6B equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7, with a molecular sieve coefficient (σ_p) of 0.331. The value taken for σ_m (0.619) was that of the irreversibly formed V570 on columns of Agarose 6B equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7. The normalized, reduced coordinates of the minimum (V') in the gradient elution experiment was 0.536. A stoichiometry $n \gg 8$ was derived with these values in equation (1).

TABLE 4. STOKES RADII (Å) OF *VELELLA* CAROTENOPROTEINS AND DERIVATIVES, DERIVED FROM GEL FILTRATION†

	Sephadex		Agarose		Bio-Gel	
gel filtration material *Velella* proteins	G75	G200	4B	6B	P300	mean value
V620	—	—	128.4	105.5	—	117.4
V600† and V583‡	—	67.4	70.2	70.2	71.6	69.8
V570	29.5	30.3	—	33.7	26.3	29.9
V545	22.3	—	—	—	—	22.3
apoprotein	22.3	—	—	—	—	22.3

† Columns equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7 at 4 °C.
‡ Columns equilibrated with 0.2 M phosphate buffer, pH 7 at 4 °C.

TABLE 5. MOLECULAR MASSES OF *VELELLA* CAROTENOPROTEINS AND DERIVATIVES

method ...	1	2	3	4	5
Velella proteins					
V620	—	2.1×10^6 (1.25) 8.0×10^5 (1.92)	—	2.35×10^4	$13.4 \pm 2.0 \times 10^3$
V600 and V583	5.2×10^5 (i)	6.1×10^5 (1.25) 3.4×10^5 (1.52)	—	2.35×10^4	$12.0 \pm 1.0 \times 10^3$ (*a*) $11.7 \pm 2.0 \times 10^3$ (*b*)
V570	4.6×10^4 (ii)	4.8×10^4 (1.25) 5.7×10^4 (1.18)	*ca.* 5.0×10^4	—	
apoprotein and V545	2.30×10^4 (ii)	2.0×10^4 (1.25) 2.5×10^4 (1.16)	—	2.35×10^4	

1. Gel filtration; values derived from elution volume against lg (molec. mass) plots.
2. Gel filtration; values calculated from Stokes radius (table 1), f/f_0 given in parentheses.
3. Partition; two-phase system, dextran-poly(ethyleneglycol).
4. SDS-acrylamide electrophoresis.
5. Minimum molecular mass based on astaxanthin content.

Gel filtration bed material: (i) Sephadex G200, (ii) Sephadex G75. (*a*) pigment V583, (*b*) pigment V600

The dissociated V600 and V620 gave identical patterns in SDS-acrylamide electrophoresis. Anomalous behaviour was not observed in gels of different acrylamide concentration. A Ferguson plot, for the main protein band, of mobility relative to pyronine G against acrylamide concentration was linear, with free electrophoretic mobility (intercept, M_0) of 0.98 and retardation coefficient (slope, K_R) of 15×10^{-3}. The subunit size, given in table 5, was estimated from a K_R/lg mol. mass plot for standard proteins of known subunit size; the value is close to that obtained for the apoprotein in gel filtration. Traces of three additional smaller-size components

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($8–13 \times 10^3$ mol. mass in 15 % acrylamide gels), more intensely staining in samples left for prolonged periods (24 h, 37 °C) in dissociating buffer, were probably formed by covalent bond cleavage of the 2.35×10^3 mol. mass subunit.

The presence of two astaxanthin prosthetic groups per apoprotein subunit for the *Velella* pigments is established from the measurements of minimum molecular weight based on astaxanthin content (table 5).

(*i*) *Chloride ion binding measurements*

A limited number of $^{36}Cl^-$ binding measurements are presented in table 6 for the *Velella* pigments. Availability of protein was a restriction on the number of binding experiments made; the pigments were not recoverable from the liquid scintillant. The reliability of the results is highly dependent on the accuracy of fit of the quench points to a quench curve; the variability of the results, reflected in the high values for the standard deviations in the determinations, is a consequence of the approximate nature of this curve. The limit of detection of bound chloride for the protein concentrations used in the experiments was estimated as *ca.* 0.2 mol Cl per minimum molecular mass (based on astaxanthin content).

TABLE 6. NUMBER OF CHLORIDE ION BINDING SITES OF THE *VELELLA* PIGMENTS OCCUPIED PER MINIMUM MOLECULAR MASS (BASED ON ASTAXANTHIN CONTENT) AT DIFFERENT CONCENTRATIONS OF CHLORIDE, DETERMINED BY MEASUREMENT OF $^{36}Cl^-$ BINDING IN SEPHADEX G25 GEL FILTRATION EQUILIBRIUM STUDIES

		Cl^- bound per minimum molec. mass		
carotenoprotein†	Cl^- activity/M	2 ± 2 °C	12 ± 2 °C	22 ± 2 °C
V620	8.16×10^{-4}	1.37 ± 0.20§	—	—
V600	8.16×10^{-4}	0.77 ± 0.12‡	0.73 ± 0.24‡	0.78 ± 0.38§
	3.26×10^{-3}	1.14 ± 0.48§	1.03 ± 0.61§	0.95 ± 0.40§
V573	8.16×10^{-4}	Nil‡ (< 0.2)	—	—

† Single gel filtration run at each KCl concentration.
‡ 5 determinations of bound $^{36}Cl^-$ on a single gel filtration fraction.
§ 5 determinations on each of two gel filtration fractions.

Let it be assumed for V600 dissolved in 0.2 M phosphate buffer that: (1) 30 % of the carotenoid is associated with V565 and 70 % with V583 (see above) and (2) the following reactions take place on addition of chloride ions:

$$V565 + Cl^- \rightleftharpoons VCl \qquad V583 + Cl^- \rightleftharpoons V'Cl$$

$$VCl + Cl^- \rightleftharpoons VCl_2 \qquad V'Cl + Cl^- \rightleftharpoons V'Cl_2$$

$$VCl_{(4n_{Cl}-1)} + Cl^- \rightleftharpoons VCl_{4n_{Cl}} \qquad V'Cl_{(4xn_{Cl}-1)} + Cl^- \rightleftharpoons V600$$

$$xVCl_{4n_{Cl}} \rightleftharpoons V600$$

where n_{Cl} is the number of chloride ions bound per astaxanthin prosthetic group, $4n_{Cl}$ is the number of chloride ions bound by V565, and x, the degree of association of V565 for the formation of V600, is taken as 8 (see above). It is further assumed that the chloride ion binding sites are independent and identical with apparent binding constant k (see Hammes & Wu 1974).

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Then the average number ($\bar{r}$) of chloride ions bound per carotenoid prosthetic group for V600 is given by: $\bar{r} = 0.3\bar{r}_1 + 0.7\bar{r}_2$, where $\bar{r}_1$ is the average number of chloride ions bound per carotenoid prosthetic group for V565 and given by:

$$\bar{r}_1 = \frac{n_c k[\mathrm{Cl}^-] + n_{\mathrm{Cl}}\, x[\mathrm{V600}]/[\mathrm{V565}]}{1 + k[\mathrm{Cl}^-] + x[\mathrm{V600}]/[\mathrm{V565}]}$$

and $\bar{r}_2$ is the average number of chloride ions bound per carotenoid prosthetic group for V583 and given by:

$$\bar{r}_2 = \frac{n_{\mathrm{Cl}} k[\mathrm{Cl}^-]}{1 + k[\mathrm{Cl}^-]}.$$

TABLE 7. THERMODYNAMIC DATA FOR THE CHLORIDE-DEPENDENT FORMATION OF V620 AND V600 AND FOR THE BINDING OF CHLORIDE IONS TO THE HALIDE-FREE PIGMENT DERIVATIVES

carotenoprotein (see figure 6)	lg $K(k)$† 4 °C	lg $K(k)$† 24 °C	$\Delta H_{4\,^\circ\mathrm{C}}$‡ / kJ mol^{-1}	ΔG / kJ mol^{-1} 4° C	ΔG / kJ mol^{-1} 24 °C	$\Delta S_{4\,^\circ\mathrm{C}}$ / J K^{-1} mol^{-1}	$\Delta S_{4\,^\circ\mathrm{C}}/\Delta H_{4\,^\circ\mathrm{C}}$
V620							
(i) halide-free pigment, V$^{1\prime}$565	lg K_3†§ (1)						
	790	650	−11000	−4200	−3700	−24000	2.2
(ii) large-size pigment, V^{3}583, isolated in halide-free gel filtration	lg K_2‡‖ (1)						
	94	86	−620	−800	−490	−420	0.7
	lg k¶						
	2.0	1.8	−13.0	−10.0	−10.0	−8.8	
(iii) small-size pigment, V$^{2\prime}$565, isolated in halide-free gel filtration	lg K_3§ (1)						
	600	560	−3200	−3200	−3200	−250	0.08
V600							
(i) halide-free pigment, V^{2}583	lg k¶ (2)						
	2.9	2.5	−27	−15	−14	−46	1.7
	lg K_2††						
	140	120	−1300	−730	−690	−2200	
	lg K_2†† (1)						
	140	110	−2200	−700	−610	−5400	2.5
	lg k¶						
	2.8	2.2	−46	−15	−13	−113	
(ii) large-size pigment, V^{3}583, isolated in halide-free gel filtration	lg K_2‖ (1)						
	98	91	−580	−520	−540	−220	0.4
	lg k¶						
	2.0	1.9	−12	−11	−11	−5	
(iii) small-size pigment, V$^{4\prime}$565, isolated in halide-free gel filtration	lg K_1¶‡‡ (1)						
	110	74	−3200	−610	−420	−9500	3.0

† Values derived (1) from the plots of lg $((1-\bar{V})/\bar{V})$ against lg $(\gamma[\mathrm{Cl}^-])$, figure 4 and table 1 and (2) estimated from the $^{36}\mathrm{Cl}^-$-binding data, table 6.

‡ Enthalpy change determined from the slope of Arrhenius plots for values of K or k at 4 and 24 °C.

§ Equilibrium constant for the Cl^--dependent formation of V620 from V565.

‖ Equilibrium constant for the Cl^--dependent formation of V590–600 (see text) from V583.

¶ Average value for the apparent chloride association constant.

†† Equilibrium constant for the Cl^--dependent formation of V600 from V583.

‡‡ Equilibrium constant for the Cl^--dependent formation of pigment $\lambda_{\max}$ *ca.* 600 nm (see text) from V565.

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If, as an approximation, the values for [V600]/[V565] at (γ[Cl$^-$]) = 8.16×10^{-4} M and 3.27×10^{-3} M are taken as the value of $\bar{V}/(1-\bar{V})$ at these concentrations (0.49 and 1.17 at 4 °C and 0.23 and 0.60 at 24 °C, respectively, read from figure 4*a*), then substitution of $n_{Cl} = \frac{3}{2}$ and $k = 800$ and 350 in the above equation gives values for $\bar{r}$ of 1.17 and 0.95 (at (γ[Cl$^-$]) = 3.27×10^{-3} M and 0.78 and 0.53 (at (γ[Cl$^-$]) = 8.16×10^{-4} M), in reasonable agreement with the ^{36}Cl binding data at 2 and 22 °C. The average values for the thermodynamic constants of chloride–protein association derived from the apparent chloride-binding constants at 2 and 22 °C are given in table 7.

Estimates for the apparent chloride-binding constants at 4 and 24 °C for V583 before gel filtration (from measurements on V600 in halide-free buffer) and afterwards (separated from V620 or V600 in halide-free buffer) were derived from equations (9) and (3), using values of (γ[Cl$^-$]) at lg $((1-\bar{V})/\bar{V}) = 0$ (table 1) and with $n = 6$ and $x = 8$; the values are given together with the relevant thermodynamic data in table 7. The product formed from the filtered pigment had λ_{max} 590–600 in the presence of excess KCl.

The equilibrium constants (K_1 and K_3) for the halide-dependent polymerizations of the small-size fragments (V565) isolated in the halide-free gel filtration of V620 and V600 were calculated at 4 and 24 °C from equations (5) and (2), respectively, with $x = 8$ and $m = 4$ (see earlier) for values of (γ[Cl$^-$]) and [V565] at lg $((1-\bar{V})/\bar{V}) = 0$. The fraction of V570 (irreversibly-formed derivative) present in each sample was estimated following gel filtration on columns of Sephadex G75 equilibrated with 1 M KCl–0.04 M phosphate buffer, pH 7; the value for [V565] at lg $((1-\bar{V})/\bar{V}) = 0$ in each case was taken as the product of half the initial pigment concentration and (1 – the fraction of V570 present in the sample). The equilibrium and thermodynamic constants for the reactions are given in table 7.

Values of K_3 and the thermodynamic constants for the formation of V620 were obtained from equation (10), in a similar manner; the concentration of V565 (M) at lg $((1-\bar{V})/\bar{V}) = 0$ was taken as half the initial concentration (60 % of the total pigment concentration in halide-free buffer, see earlier).

(*j*) *Specificity of carotenoid attachment*

The results of studies on the specificity of carotenoid combination with the apoprotein of V600 are given in table 8. Only those carotenoids having the ability to alter the quaternary structure of the apoprotein are included. The spectra of the carotenoid–protein complexes in 0.1 M phosphate buffer, pH 7, are recorded at 5 °C in the presence of 2 M KCl and, in some cases, following removal of KCl on columns of Sephadex G25. A more detailed summary, listing those carotenoids unable to bind or to alter the quaternary structure of the apoprotein, is included in a review of the specificity of carotenoid–protein association (Zagalsky 1976).

Reconstitution with astaxanthin, in the absence of chloride ions, gave the dimeric pigment. V565. The protein associated further in the presence of KCl to give a high proportion of V600

Discussion

It seems probable from the present work that some, at least, of the previously recorded discrepancies in the position of the visible absorption maximum of the *Velella* mantle pigment (see Herring 1971) may be attributed to dissociation of the native protein (λ_{max} 620) and loss of specifically-bound anion.

It cannot, however, be assumed that the blue pigmentation of *Velella in vivo* can be ascribed to

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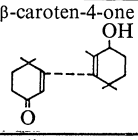
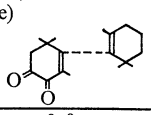
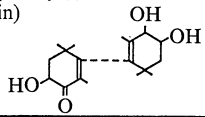
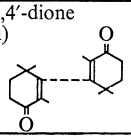
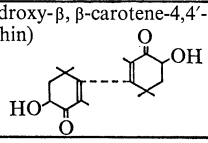
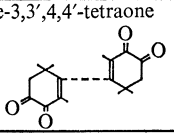
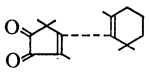
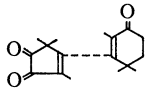
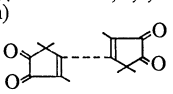
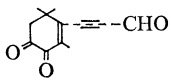
TABLE 8. SPECIFICITY OF COMBINATION OF THE APOPROTEIN OF V600 WITH CAROTENOIDS

carotenoid	% recombination†	absorption maxima‡/nm		
		excluded from Sephadex G75	'β -size'	'apo-size'
4′-hydroxy-β, β-caroten-4-one 	9		5 % (510–515)	95 % 492 (±Cl⁻)
β,β-carotene-3,4-dione (euglenanone) 	11		75 % 517	25 % 495
3,3′,4-trihydroxy-β, β-caroten-4-one (idoxanthin) 	16		90 % 535 (±Cl⁻)	10 % 515 (±Cl⁻)
β, β-carotene-4,4′-dione (canthaxanthin) 	2		15 % 560–570	85 % 510
3,3′-dihydroxy-β, β-carotene-4,4′-dione (astaxanthin) 		90 % 600 (+Cl⁻) 580 (−Cl⁻)	8 % 570	2 % 545
astaxanthin-3,3′-diacetate	6		10 % 560–570	90 % 510 §
β, β-carotene-3,3′,4,4′-tetraone (astacene) 	51	10 % 585 (+Cl⁻) 530 (−Cl⁻)	25 % 535 (+Cl⁻) 520 (−Cl⁻)	65 % 505 (±Cl⁻)
15,15′-dehydroastacene	14	80 % 552 (+Cl⁻) 537 (−Cl⁻)	10 % 537 (+Cl⁻) 515 (−Cl⁻)	10 % 490
2-nor β-carotene, β-carotene-3,4-dione 	6		95 % 625, 405 (±Cl⁻)	
2-nor β-carotene, β-carotene-3,4,4′-trione 	10	98 % 725, (480–490) (+Cl⁻) 695, (480–490) (−Cl⁻)		
15,15′-dehydro-2-nor β-carotene, β-carotene-3,4,4′-trione	32	95 % 660, 455 (+Cl⁻) 630, 440 (−Cl⁻)	5 % 640, 440 (+Cl⁻) 627, 430 (−Cl⁻)	
2,2′-dinor-β, β-carotene-3,3′,4,4′-tetraone (violerythrin) 	14	15 % 670 (+Cl⁻) 650 (−Cl⁻)	75 % 645 (+Cl⁻) 635 (−Cl⁻)	10 % (520)
15,15′-dehydroviolerythrin	41	75 % 670 (+Cl⁻) 645 (−Cl⁻)	23 % 650 (+Cl⁻) 637 (−Cl⁻)	2 %
15,15′-dehydro-8′-apo-β-carotene-8′-al-3,4-dione 	51		95 % 508 (±Cl⁻)	

† % recombinations are derived from the extinction of the complexes at the main absorption maximum, assuming identical extinction coefficients and taking the recombination obtained with astaxanthin as 100 %. Carotenoid-protein complexes are separated in gel filtration using Sephadex G75 into protein excluded from the gel, protein having a similar elution volume to V570 and that showing a similar size to the apoprotein.

‡ Absorption maxima of the complexes dissolved in 0.1 M phosphate buffer, pH 7, are recorded at 5 °C in the presence of 0.2 M KCl and, where stated, in the absence of KCl.

§ 'Apo-size' material contains astaxanthin and astaxanthin diacetate.

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V620 alone. Populations of the animals often contain individuals whose visible colour ranges from clear blue, consistent with a preponderance of V620, to deep royal blue, suggesting the presence of pigments with absorption maxima at shorter wavelengths. Halide-dependent spectral differences are unlikely to be significant. Seawater contains Cl^- at concentrations slightly in excess of 0.5 M, and similar tissue concentrations would saturate the anion binding sites. The other factors determining the relative proportions of different pigments *in vivo* remain unknown.

(*a*) *Molecular size*

The state of aggregation of V620 could not be determined with any certainty, since the frictional coefficients of the pigments were not measured. The stoichiometry of association from units of V600 may be between 2, for an asymmetric protein ($f/f_0 = 1.92$), and 6, for a globular protein ($f/f_0 = 1.25$). A degree of polymerization greater than the dimer is inferred from the partition of the pigment between the lower phase and interface of the dextran-poly(ethyleneglycol) two-phase system (Sasakawa & Walter 1972). The protection of two-thirds of the available sulphydryl groups in the association (table 3) may be reconciled with a tetramer-structure for V620. On this basis, the pigment has a molecular mass of 1.5×10^6 and frictional coefficient of 1.40. The contribution of hydration to the frictional coefficient (f/f_0 hydr.) was calculated as 1.16; the mass of the solvent associated with 1 g anhydrous pigment was obtained from the amino acid composition of the protein (Kuntz 1971), taking half the acidic amino acids as amidated. The frictional coefficient of asymmetry (f/f_0 asym.) of the pigment is then 1.20, corresponding to an axial ratio for a prolate or oblate ellipsoid of 6.0 (Tanford 1967). The agreement in molecular size estimates obtained for the apoprotein and V570 by the various methods (table 5) indicates that the proteins are fairly symmetrical. The results show that V570 is a dimer of the apoprotein (mol. mass *ca.* 2.35×10^3) and has four astaxanthin prosthetic groups. It seems reasonable to conclude that pigment V600 is an octamer (mol. mass *ca.* 380 000) of subunits of the size of V570, both from the molecular size determination in gel filtration (with $f/f_0 = 1.5$) and from the stoichiometry ($n = 8$) obtained by analysis of the trailing edge of the gel filtration elution profile in dissociation–association equilibrium. However, complete reliance cannot be placed on the stoichiometry determination as the estimated value is close to the upper limit of the method (Ackers & Thompson 1965); perturbation of the equilibrium by protein monomer-gel matrix interaction, although minimized by using an extensively employed Agarose 6B column, may also have had an influence on the result.

The apparent heterogeneity of the apoprotein and of V570 may have partly resulted from differences in degree of amidation, either natural or, more likely, caused by the facile deamidation of asparaginyl and glutaminyl residues during storage of the pigment (Scotchler & Robinson 1971; McKerrow & Robinson 1971.) Although two apoprotein components were observed in electrophoresis at pH 3.1, where differences in mobility due to variations in amide content are minimized, there was no significant difference in the amino acid compositions of these components of the apoprotein preparation. The apoproteins, if distinct, are evidently closely related in composition and may be genetic variants of a single protein unit. The protein is related to the crustacean astaxanthin-proteins in amino acid composition; a low content of aromatic amino acids is characteristic of the carotenoproteins of crustaceans living at the sea surface (see Zagalsky 1976). The denaturation of V570 with high concentrations of mercaptoethanol and the reaction with NEM of six, out of the total of eight, cysteine residues per apoprotein, may indicate the presence of a single intramolecular disulphide bond per apoprotein; it

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is likely to be buried in the structures of the polymeric pigments as it is protected in these from reaction with mercaptoethanol. The electrophoretic results show, in agreement with molecular mass estimates, that the pigments λ_{max} 554–570 are composed of pairs of apoprotein units.

It is expected that the association between apoprotein units in the pigments largely involves hydrogen-bond and electrostatic interactions, in view of the low content of hydrophobic amino acids and small value (3.22 MJ; 772 kcal, see Zagalsky 1976) for the average hydrophobicity. The inverse temperature dependence of the halide-sensitive bathochromic shifts in the spectra of V620 and V600 is also indicative of the greater importance of these forces in the accompanying tertiary and quaternary structural changes.

The presence of 4 astaxanthins per subunit of molecular mass 4.7×10^4 suggests that van der Waals–London dispersion interaction between the highly polarizable prosthetic groups may make a significant additional contribution to the stability of the quaternary structures (Zagalsky 1976).

(*b*) *Anion-binding sites*

The order of effectiveness of high concentrations of anions in influencing diverse physical and chemical properties of proteins (reviewed, von Hippel & Schleich 1969) follows the Hofmeister, or lyotropic, series ($CNS^- > I^- > ClO_4^- > NO_3^- > Br^- > Cl^- > F^-$, for monovalent anions). There is evidence that the anions exert their influence by direct interaction with peptide and amide groups (Robinson & Jencks 1965); the different affinities of anions for these sites, attributed to differing interaction of the binding sites (anion $\longleftrightarrow$ peptide (amide) $\longleftrightarrow$ nearby hydrophobic substituents) with the water structure, is responsible for the ordering of the anions in the lyotropic sequence (Schrier & Schrier 1967; Nadi & Robinson 1972*a*, *b*; Hamabata & von Hippel 1973). The binding of anions to quaternary ammonium resins (Chu, Whitney & Diamond 1962) and to serum albumin (Scatchard & Yap 1964; Norne, Hyalmarsson, Lindman & Zeppezauer 1975) follows an identical order and has been similarly explained.

A number of enzymes are activated or inhibited by low concentrations of anions ($< 10^{-1}$ M). In these cases specific interaction with cationic groups, at one or more sites, is thought to be responsible for the effect of the anions on enzyme activity. For many (Massey 1953; Walaas & Walaas 1956; Fridovich 1963; Karniely & Benziman 1966; Packer & Stone 1967; Hersch & Jencks 1969; Ludewig *et al.* 1971; Kearney, Ackrell, Mayr & Singer 1974) the order of effectiveness of the anions on the activity follows, with some exceptions, the expected (lyotropic) order of affinity. On the other hand the order of effectiveness of anions in influencing the activity of others (Hayaishi 1962; Unemoto & Hayashi 1969; Rose & Liebowitz 1970; Craine, Daniels & Kaufman 1973; Kalish, Pineyro, Cooper & Gregerman 1974; Levitzki & Steer 1974; Lillehaug & Kleppe 1975) does not follow the Hofmeister series.

It has been proposed for *Clostridium* acetoacetate decarboxylase (Fridovich 1963) and for α-amylase (Levitzki & Steer 1974), and shown for carbonic anhydrase in X-ray crystallographic studies (Bergstén *et al.* 1972), that the anion-binding site is situated in a cleft on the surface of each enzyme. The specificity of the anion effect for these enzymes, and others, may arise from the additional selectivity, resulting from steric restriction, which cavities impose on the binding (Fridovich 1963; Levitzki & Steer 1974). For some of the enzymes, however, it is probably the extent to which the anions are able to change the configuration of their binding-site that governs the activity; the order of effectiveness of specifically bound anions in altering the activity of an enzyme need not, therefore, be necessarily the same as the order of relative

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affinities for the enzyme (cf. the relative activities of 2′,3′-cyclic phosphodiesterase in the presence of optimal activating concentrations of different anions (Unemoto & Hayashi 1969)).

The involvement of clusters of basic groups in the anion–protein interaction has been suggested for some proteins (serum albumin, Saroff & Healy 1959; ribonuclease, Saroff & Carroll 1962; Loeb & Saroff 1964; salmine, Carroll, Callanan & Saroff 1959) in which high binding constants for the anion have been observed. The high affinity chloride-ion binding site of α-amylase, however, includes a single lysine residue, probably activated by Ca^{2+}, within the substrate binding site of the enzyme (Lifshitz & Levitzki 1976).

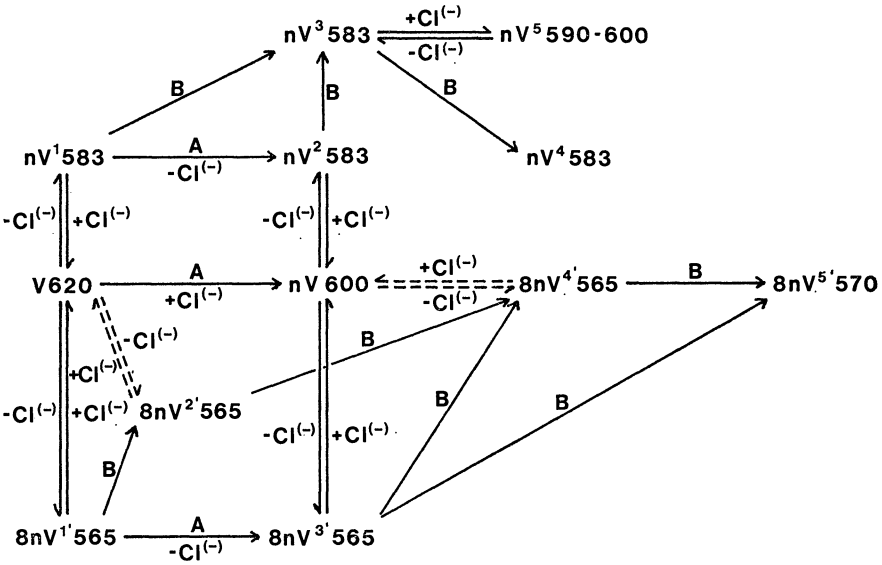

FIGURE 6. Scheme of the changes in tertiary and quaternary structure undergone by the *Velella* carotenoproteins during storage (A) or resulting from passage of halide-free solutions of the pigment through columns of Bio-Gel P300 (B).

The effect of specific anion-binding on the tertiary and quaternary structures of proteins has been infrequently studied. The effects of anion attachment on the conformations of α-amylase (Levitzki & Steer 1974) and dopamine β-hydroxylase (Craine *et al.* 1973) are localized to the regions of the anion-binding sites, without any major change in protein structure. In contrast, anion-binding induces configurational changes in the structures of succinate dehydrogenase (Kearney *et al.* 1974; Oestreicher, Hogue & Singer 1973), 2′,3′-cyclic phosphodiesterase (Unemoto & Hayashi 1969) and ribonuclease (Ginsburg & Carroll 1965). An effect of anion-binding on subunit interaction and quaternary structure has been inferred by Lillehaug & Kleppe (1975) for T4 polynucleotide kinase.

The properties of the *Velella* pigments are of interest in that they illustrate many of these features of anion–protein interactions. A scheme which accounts satisfactorily for the alterations in properties of V620 and V600 during both storage and the passage of halide-free solutions of the carotenoproteins through columns of Bio-Gel P300, is given in figure 6.

Pigment V620 dissociates in two possible ways on desaturation of the anion-binding sites, forming a pigment ($V^{1}583$) identical in size (mol. mass *ca.* 3.8×10^5) to V600 and a pigment ($V^{1\prime}565$) of lower (*ca.* 4.7×10^4) mol. mass. Pigment V600, formed by the irreversible dissociation of V620 in the presence of chloride ions, similarly forms, in the absence of halide ions, a pigment ($V^{2}583$) of unaltered molecular size and a pigment ($V^{3\prime}565$) identical in size to $V^{1\prime}565$.

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The native pigments (V620 and V600) are reformed if halide ions are restored immediately to halide-free solutions of the pigments. Plots of lg $((1-\bar{V})/\bar{V})$/lg $(\gamma[A^-])$ for the pigments (e.g. figures 4*a*, *b*) show the range of anion concentration over which the changes occur in the tertiary and quaternary structure of the proteins, with accompanying alterations in spectra. The plots are equivalent to Hill plots and the slope of such plots gives a measure of the interaction between the ligand-binding sites, rather than an estimate of their actual number (see Hammes & Wu 1974); the intercept on the lg $(\gamma[A^-])$ axis at lg $((1-\bar{V})/\bar{V}) = 0$ is a function of the equilibrium constants for anion-binding and protein association (equations (4) and (7)). A slope in Hill plots of less than 1 implies either that negative cooperativity exists between binding sites, or that they are intrinsically different (see Whitehead 1970). The slope of *ca.* 0.5–0.7 for both V620 and V600 with halide ions (table 1) may alternatively mean that the binding of successive anions (to sites of identical affinity) confers smaller increments on the 600 nm absorption. Comparison of the data from the Hill plots for Cl^- for V620 and V600 in 0.2 M phosphate buffer, and of the high and low molecular size components of these pigments, shows that the pigment fractions have a reduced affinity for the anion following gel filtration (table 1). The large-size protein fractions (V^3583) from both pigments appear to be identical (table 1). In addition to changes in the structure of the anion-binding site, the interaction between subunits (of the size of V570) in V^3583 must have altered since it no longer formed the small (4.7×10^4) size pigment after incubation in, and subsequent removal of, excess KCl ($V^3583 \longleftrightarrow V^5590$–600). The change in conformation of the anion-binding site is evidently irreversible, since the typical Hill plots for Cl^- of the original pigments (V620 and V600) were not restored by this treatment. To account for the increase in slopes and altered concentration ranges of Hill plots for Cl^- for the large-size pigment fraction, and for the variable position of the absorption maximum in the presence of excess KCl, it is suggested that not only the nature of the anion-binding sites but also the cooperativity between them (in altering the spectrum of the pigment) is altered; the anion sites themselves, or the interactions between carotenoid- and anion-binding sites, are gradually lost (V^4583).

The higher concentration range over which chloride ions are effective in Hill plots for Cl^- for the low molecular-size fractions ($V^{2\prime}565$ and $V^{4\prime}565$, figure 6, separated from V620 and V600 in halide-free Bio-Gel filtration) together with the increased slope of the plots, and the loss of the spectral changes for part of the pigment ($V^{5\prime}570$, figure 6) may be attributed to the combination of a number of possibly interrelated causes. These include: (1), alteration in the nature of the anion-binding sites (disorganization of basic residue clusters, see below) and their eventual ($V^{5\prime}570$) loss; (2), lowered association constants (K_p and K_p', equations (2) and (6), respectively) for the halide-dependent polymerization of the proteins; and (3), loss of negative cooperativity between anion-binding sites, or change in the interactions between some of the anion- and carotenoid-binding sites, resulting in spectral equivalence of the anion sites. The interaction between subunits in the aggregate formed in the presence of KCl from the low molecular-size fraction of V600 must be different from that in the latter pigment, since, on removal of chloride, the low- and high-molecular size fractions were formed in proportions different from those in the native pigment.

The low molecular mass fraction isolated from V620 in halide-free gel filtration is represented in scheme 6 as a mixture ($V^{2\prime}565$ (predominating), $V^{4\prime}565$ and $V^{5\prime}570$) of protein conformers; that from V600 is shown as a mixture of $V^{4\prime}565$ and $V^{5\prime}570$. The Hill plots for Cl^- at 24 °C for the low molecular mass component of V620 ($V^{2\prime}565 + V^{4\prime}565$) and of V600 ($V^{4\prime}565$) (figure 4;

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table 1) reveal the greater lability of the conformation of the anion-binding sites for the derivative of the latter pigment at this temperature, and explain the differing results obtained from V620 and V600 in Bio-Gel filtration experiments at low chloride ion concentration (figures 2 and 3). A mixture of V620, V600 and V570 ($V^{5'}570$) is formed when KCl is added to solutions of V620 in 0.2 M phosphate buffer left for prolonged periods at room temperature (represented in scheme 6 by a mixture of $V^{1-3}583$ and $V^{1'-5'}565$). Solutions of V600 incubated in 0.2 M phosphate buffer ($V^{3'-5'}565$ and $V^{2-4}583$) yield a mixture of V570 ($V^{5'}570$) and high molecular mass pigment of variable absorption maximum on readdition of KCl (a mixture of V600, $V^{5}590$–600 and $V^{4}583$, in figure 6). The longer the solutions remain in the absence of halide ions, and the higher the temperature of incubation, the greater the amounts of $V^{3,4}583$ and $V^{2,4,5}565$ (figure 6) formed.

No evidence was obtained in the halide-free gel filtration studies for the existence of reversible association–dissociation between the proteins $V^{1}583$ and $V^{1'}565$ and $V^{2}583$ and $V^{3}565$ respectively (figure 6). It is possible, however, that such equilibria would have been destroyed by the alterations in the tertiary structure of the pigments resulting from interaction with the gel matrix. The variation in E_{660}/E_{560} values of halide-free solutions of V620 and V600 ($V^{1,2}583$ and $V^{1',3'}565$) with temperature and ionic strength, although suggestive of equilibria between the components, may be explained by effects on the individual components of the mixture (cf. the sensitivity to these factors of the spectra of the separated components).

It is not possible to determine unequivocally for V620 and V600 the number of halide ion binding sites, or values for their association constants, from the equations (4) and (7) in which these are related to the halide-dependent spectral changes of the pigments. However, the low concentrations of halide ions over which Hill plots for anions apply indicate a high affinity of the sites for anions. In halide-free solutions of V600 apparent binding constants for chloride ions of 660 and 170 at 4 and 24 °C, respectively, were obtained for the large-size fragment (see table 7).

The ^{36}Cl measurements for V600 at 2 °C (table 6) show that at 3.26×10^{-3} M ($\gamma[Cl^-]$), when the halide-dependent change is *ca.* 45 % complete, about two chloride ions are bound per apoprotein. The limited data for the chloride ion binding by V600 may be accounted for (providing certain assumptions are made, see above) by the possession of three identical anion-binding sites per apoprotein of high apparent association constant, *ca.* 8×10^{2} and 3.5×10^{2} for chloride at 2 and 22 °C, respectively. Although the large standard errors for bound chloride (table 6) leave some uncertainty in these assignments, there is reasonable agreement between the values of the chloride association constants and those obtained for the large-size fraction of halide-free solutions of V600 from the Hill plots for Cl^-.

Although the number of halide ion binding sites for V620 was not estimated, the apparent association constants of the sites must be of a similar magnitude to those of V600, since Hill plots for anions cover nearly the same anion concentration range for the two pigments. Pigment V600 is formed irreversibly from V620 even in the presence of 1 M KCl. This dissociation, and the accompanying changes in the interactions between anion sites and carotenoid sites, or in the nature of the carotenoid–protein linkage, is therefore more likely to have arisen through changes in the tertiary structure of the subunits of pigment V570 size (thereby affecting the binding sites between V600 size units) than through loss of halide ion binding sites. The latter possibility cannot, however, be discounted. The properties of the polymers formed by addition of excess KCl to the low molecular mass fractions (indicated tentatively as V620 and V600, dotted line

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figure 6) were not studied in detail. The polymer formed from the low molecular mass fraction derived from V600 gave both the large and small-size fragments on removal of chloride and rechromatography.

It is evident from the increased curvature in the Hill plots for Cl^- at the higher halide concentrations (e.g. figure 4) that the structural changes in the pigments, assisted by interaction with the gel matrix in Bio-Gel filtration, also occur at room temperature in halide-free solution, though more slowly.

The high affinity of the pigments for anions may be adequately explained by the involvement of clusters of basic residues at the binding sites. It is proposed that in the absence of bound anion the configuration of the clusters is gradually deformed by an unfolding of the protein structure in this region, leading first to a lower affinity for anions (cf. the smaller apparent chloride-binding constants following passage through Bio-Gel, table 7) (forms $V565^{2'-5'}$ and $V^{3,4}583$, figure 6), and finally to a complete loss of affinity (V^4583 and $V^{5'}570$, figure 6).

The thermodynamic data for chloride binding and for the halide-dependent association of the pigment subunits (table 7) establishes that the concomitant alterations in polypeptide interactions are relatively limited. The sign and magnitude of the enthalpy and entropy changes for chloride binding by the large-size pigment fraction present in halide-free solutions of V600 ($\Delta H = -27$ to -46 kJ/mol (-6.5 to -11.0 kcal/mol), $\Delta S = -46$ to -113 J K^{-1} mol^{-1}) are the same as those for chloride inhibition of *Clostridium* acetoacetate decarboxylase (Fridovich 1963), assigned to changes in the structure of water coincident to the formation of the anion-enzyme complex. The high affinity chloride site of serum albumin, however, is saturated with a positive ΔS (Scatchard & Yap 1964). The measurements of ΔH and ΔS thus plainly represent the net effect of the halide attachment on water structure and protein configuration. If the whole of the ΔH and ΔS values are attributed to hydrogen-bond formation between peptide groups ($\Delta H \approx -6.3$ kJ/mol (-1.5 kcal/mol), $\Delta S = 18$ J K^{-1} mol^{-1} and $\Delta S/\Delta H = 2.8$, Scheraga 1963), then the attachment of a single chloride is accompanied by the formation of not more than 7 such bonds. The more positive values of ΔS and ΔH for the pigment fraction following passage through Bio-Gel columns indicates that this treatment reversibly disrupts electrostatic or hydrophobic interactions within the protein (Kauzmann 1959). The halide-dependent polymerization of the small-size fraction present in halide-free solution of V620 mainly involves an increase in hydrogen-bond formation; the value for the ratio $\Delta S/\Delta H$ (2.2) implies only a small contribution of hydrophobic and/or electrostatic bonding to the association (Kauzmann 1959). If the production of hydrogen-bonds between peptide groups is solely responsible for the ΔS value ($-24\,000$ J K^{-1} mol^{-1}) then $< 5\,\%$ of the amino acid residues ($\sim$ 700 residues per V620 of *ca.* 1.47×10^4 amino acid residues) would be involved in the polymerization; reversible breakage of hydrophobic and/or electrostatic bonding is evidently caused by gel filtration of the small-size fragment, as seen in the more positive values for ΔS and ΔH.

The values of ΔH and ΔS (-3200 kJ/mol (-770 kcal/mol) and -9500 J K^{-1} mol^{-1} respectively) for the halide-dependent association of the small-size fragment, isolated from V600 in halide-free Bio-Gel filtration, may be accounted for by the formation of new peptide group hydrogen-bonds between 14 % of the amino acid residues of the polymer. The similarity of the circular dichroism spectrum of V600 in the far ultraviolet in the presence and absence of chloride ions (see below), and the lower range of anion concentration of the Hill plots for anions for the isolated pigment fractions, suggests that gel filtration causes considerable

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unfolding of the protein structure. A reversible dissociation of part of the β-pleated structure of the pigment fraction (see below) in the absence of halide ions and passage through Bio-Gel is consistent with these observations. The β-structure of the small-size fragment of V620 appears to be more resistant to disruption in halide-free gel filtration. The stabilization by chloride of the tertiary and quaternary structures of V600 is reflected in the ability of the anion to decrease the area occupied at fixed surface pressure at an air–water interface. The magnitude of the effect (8 % decrease in area over the chloride concentration range 10^{-5}–10^{-1} M) is consistent with the small enthalpy and entropy changes accompanying anion-binding.

The unaltered spreading of the separated large- and small-size fragments of the pigment in the presence and absence of chloride ions is related to the lower affinity of these proteins for the anion.

The specificity shown for the anion in effects on the structures and spectra of the pigments, and the order of their effectiveness ($Br^- > I^- > Cl^- > CNS^-, NO_3^-$), implies that the clusters of basic residues are situated in clefts of specific geometry and that these impose stereospecific restrictions on the anion binding. It is concluded, from the result (figure 5) with the amino acid blocking agent methylglyoxal, that the anion-binding sites consist of clusters of lysine residues. Acetimidation of the lysine residues of halide-free V600 leaves the charge on the residues unaltered, but affects both the position of the final absorption maximum on readdition of excess chloride and the slope of the $\lg((1-V)/\bar{V})/\lg(\gamma[Cl^-])$ plot (figure 5). The pigment has sufficient lysine (16 residues/apoprotein) to account for the proposed three anion-binding sites/apoprotein if each site contains three basic residues, by analogy with the anion-binding clusters suggested for ribonuclease and salmine. If arginine residues also contribute to the clusters, these are inaccessible to the specific blocking agent (phenylglyoxal). The lack of effect of amidation on the halide-dependent spectral changes of the pigments must mean that carboxyl groups, if accessible to the carbodiimide reagents, are not essential for the maintenance of the basic residue clusters. They may, however, compete with chloride for interaction with the clusters at low pH as demonstrated for ribonuclease (Loeb & Saroff 1964). The sulphydryl groups do not appear to be essential for maintenance of the anion-binding sites or for interactions between subunits of V570 size.

No difference in the accessibility of the sulphydryl groups of V600 was detected whether the anion-binding site was saturated or unsaturated. Sulphydryl groups, partially protected in V620 from reaction with NEM, do not have a rôle in the aggregation of the pigment, since the NEM-treated halide-free protein reforms the native pigment in the presence of KCl.

It is not clear whether the clusters of basic residues occur within apoprotein units or, as for one of the sulphate ion binding sites of lactate dehydrogenase (Adam, Lilyas & Rossman 1973), between subunits of apoprotein or V570 size.

If the sites are situated between the subunits of molecular mass 4.7×10^4, the anion association constants for the small-size components of the halide-free pigments would be of lower magnitude than those of the larger fragments. Non-identity of the anion-binding sites is one possible inference from the lower than unity value of the slopes of Hill plots for anions.

The inability of CNS^- to effect changes in the quaternary structures of the low molecular mass components ($V^{1\prime}565$ and $V^{3\prime}565$) of halide-free V620 and V600 suggests either that the geometry of the anion-binding clefts differs in the small- and large-size components derived from these pigments, or that the large anion is unable to bring about the prerequisite change in tertiary structure for association of the smaller fragment. The large anions (CNS^- and NO_3^-)

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evidently cause distortion of the anion-binding sites (cf. α-amylase, Levitzki & Steer 1974) with altered site–site interaction between carotenoid and anion sites, since a complete recovery of the spectra of the native pigments was not achieved with these ions. The distinctive charge distribution of the complex anions, or the production of different disorganization of the protein tertiary structures, are presumably responsible for the contrasting slopes of Hill plots for anions for V620 and V600 with thiocyanate, nitrate and halide ions. The dimensions of the anion-binding cleft restricts the association of larger anions; the ineffectiveness of fluoride for the plots of $\bar{V}/(\gamma[F^-])$ requires that the diameter of the cleft must be less than that of the hydrated fluoride ion (0.61 nm, Hindman 1962; Padova 1964).

The lack of influence of chloride ions on the spreading of V570 ($V^{5'}570$) at an air–water interface, and the absence of measurable protein-bound chloride at low chloride ion concentration (table 6), requires that it has no halide ion binding sites of high affinity. Changes in the tertiary structure of the pigment may result in loss of the specific anion-binding clusters of basic groups, rather than simply disrupt the interaction between anion- and carotenoid-binding sites.

(*c*) *Circular dichroism*

The large maximal molar ellipticity values for astaxanthin in the *Velella* pigments may stem from extrinsic stereospecific twisting of the chromophore, proposed as an explanation for the strong optical activity of bound astaxanthin in crustacyanin (Buchwald & Jencks 1968*b*), or from a Kirkwood-type coupled oscillator mechanism (Zagalsky 1976).

The position (215 nm) of the negative maximum of the *Velella* pigments V620, V600 and V570 and the mean residue ellipticity value of V600 at this maximum are those of alkaline denatured poly-L-lysine, often used as a reference in computing the content of β-structure in proteins (Greenfield & Fasman 1969). The unsuitability of this form of poly-L-lysine as a standard for the evaluation of β-structure has been emphasized (Li & Spector 1969); sodium dodecylsulphate-denatured poly-L-lysine, considered to be a more reliable standard of β-structure (but see Woody 1969), has a lower mean residue ellipticity value at the negative maximum. It is possible that the higher value for the mean residue ellipticity of the *Velella* pigment at 215 nm is due to an influence of the prosthetic group chromophores on the circular dichroism spectrum of the protein in the far ultraviolet (see Adler, Greenfield & Fasman 1973). Although uncomplexed astaxanthin, derived from crustacyanin, shows no optical rotary dispersion at 217 nm (Bartlett *et al.* 1969), the chromophore may have optical activity in the far ultraviolet when present in carotenoproteins, where it is presumed to be twisted about the polyene double bonds (Buchwald & Jencks 1968*b*; Zagalsky 1976). The amino acid composition of the protein is consistent with a tertiary structure of low helical content and high content of β-structure (Zagalsky 1976). A mode of binding of the astaxanthin prosthetic groups in astaxanthin-proteins has been proposed involving, with additional interactions, hydrogen bonding of the 4- and 4′-keto groups of the polyene to imide groups of peptide bonds of sections of β-structure in the apoprotein (Zagalsky 1976). It has been suggested for crustacyanin that twisting of the β-ionone rings of an astaxanthin prosthetic group about the double bonds of the polyene chain not only results in the strong optical activity in the visible region but is also responsible for the bathochromic shift in the absorption band of the prosthetic group (Buchwald & Jencks 1968*b*). The order of the maximal molar ellipticity values of the *Velella* pigments in the visible region (V620 > V600 > V570) may be ascribed to a progressive decrease in the extent of

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extrinsic stereospecific twisting of the prosthetic groups, or to a loosening of fit between chromophores and protein with disaggregation of the carotenoprotein. The presence of two carotenoids per apoprotein, and the narrower half-band width of the visible absorption band of V620 and V600 compared with free astaxanthin (Buchwald & Jencks 1968*b*; Zagalsky 1976), suggests that carotenoid–carotenoid interaction may also contribute to the spectral properties of the pigments. The lack of significant exciton splitting in the circular dichroism (c.d.) spectra of V600 and V570 may mean either that the carotenoids are situated too far apart for detectable interaction, or that the relative orientation of the chromophores is such that one of the exciton transitions is forbidden (see Kasha, Rawls & El-Bayoumi 1965). It seems reasonable to suppose, by analogy with considerations of carotenoid interactions in crustacyanin (Buchwald & Jencks 1968*b*), that the degenerate exciton interaction, responsible for the splitting in the circular dichroism spectrum of V620 in the visible region, is between pairs of carotenoids, either within apoprotein units or between adjacent apoprotein units of the polymer. Dissociation of the pigment, effected by desaturation of the anion-binding sites, abolishes the double peak of the c.d. spectrum. Unless the exciton splitting arises solely through carotenoid interactions between subunits of V600 size, occupation of the anion-binding sites of the carotenoprotein must alter either the relative orientation of the prosthetic groups or their distance apart. A similar protein association-dependent splitting has been reported in the c.d. spectrum of the chromophore prosthetic groups of phycoerythrin (Fujimori & Pecci 1970). The less dramatic difference in the c.d. spectrum of V600 in the presence and absence of chloride ions may also be ascribed to changes in the relative orientation or separation of carotenoids between, or within, subunits. The weaker optical activity in the visible region shown by V570, and the inability of chloride ions to alter the c.d. spectrum, is consistent with a looser carotenoid-protein attachment and loss of the anion-binding sites.

A comparison of the c.d. spectra of pigments V620, V600 and V570 between 200–300 nm in the presence and absence of chloride ions did not reveal any major alteration in protein conformation resulting from anion binding. The configurational rearrangements in V620 and V600 which take place on desaturation of the anion-binding sites, evident in differences in the spectra and quaternary structures, are likely therefore to be confined to minor variations at the anion-, carotenoid-binding and subunit-interaction sites and/or to changes in relative orientation of polypeptide structures. Extensive changes in the nature of polypeptide interactions would be inconsistent with the thermodynamic data for the processes accompanying chloride binding.

The lack of exciton splitting under the short-axis polarized *cis* peak (at 345 nm) of V620 and V600 is evidence that, as in crustacyanin (Gardiner & Thomson, quoted in Zagalsky 1976), the directions of short-axis transition moments of interacting carotenoid molecules are parallel. The planes containing the long axis of each carotenoid must therefore also be parallel. If exciton interaction contributes to the bathochromic shift in the absorption band of the protein-bound astaxanthin in V620, then the positive component of the c.d. spectrum of the pigment should have the greater oscillator strength; this may only occur if the angle between the long-axis transition moments is $> 90°$ (see Van Holde, Brahms & Michelson 1965). The angle between the long-axis transition moments of the interacting carotenoids in crustacyanin has been estimated at 77° (Gardiner & Thomson, quoted in Zagalsky 1976). In this case the negative component of the c.d. spectrum, which is inverted compared with that of the *Velella* pigment (cf. Buchwald & Jencks 1968*b*), must have the greater oscillator strength.

The maintenance of the unique quaternary structures of V620 and V600 is dependent both on

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saturation of the anion sites and on occupation of the carotenoid-binding positions. The apoprotein, which behaves as a single polypeptide in SDS-electrophoresis, remains unassociated in the presence of 1 M KCl. The identity in the electrophoretic patterns given by the apoprotein in the presence and absence of 0.01 M KCl provides evidence that the high affinity halide-ion binding clusters become disordered on removal of the carotenoid chromophores. The dimerization of the apoprotein on the restoration (in the absence of chloride ions) of astaxanthin prosthetic groups is accompanied by the recovery (either between or within apoprotein units) of the anion-binding sites. This is shown by the further association and the change in the visible absorption spectrum of the protein in the presence of chloride ions. The ability of carotenoid to effect specific changes in the tertiary and quaternary structures of the apoproteins of astaxanthin-proteins is well documented for this group of pigments (Cheesman, Lee & Zagalsky 1967; Zagalsky 1976). It is the reciprocity between the anion-binding sites, positions of carotenoid attachment and loci of subunit association, however, which determines the quaternary structures of the *Velella* pigments. The astaxanthin-protein of the related chondrophore *Porpita* has been shown to be identical in size and closely related in composition (see Zagalsky 1976) to the *Velella* pigment V600 (Zagalsky & Herring, unpublished observations). The *Porpita* pigment also undergoes reversible changes in spectrum in the visible region in the presence or absence of chloride. The reason for the longer wavelength position of the carotenoid absorption of the *Porpita* pigment, at 650 nm, must reside in differences in the specific carotenoid–protein associations (?degree of twist about the polyene double-bonds) or in the carotenoid–carotenoid interactions between the pigments.

The properties of the crustacean astaxanthin-proteins have not been shown to depend on any specific interactions with anions. The quaternary structure and visible absorption spectrum of the lobster pigment crustacyanin, and other crustacean carotenoproteins, alter with ionic strength, but the changes are independent of the nature of the anion (Cheesman *et al.* 1966; Zagalsky *et al.* 1970). The hypodermal carotenoprotein of the copepod, *Anomalocera patersoni*, similar to the *Velella* pigments in having two astaxanthin prosthetic groups per apoprotein (of mol. mass 1.9×10^3), remains unaltered in size (4.3 nm) and spectrum in 0.1 M phosphate buffer, pH 7, in the presence and absence of 1 M KCl (Zagalsky & Herring, unpublished observations). The blue canthaxanthin-lipovitellin of the fresh water anostracan *Branchipus stagnalis* (*L.*) undergoes partially reversible changes in spectrum in the presence and absence of halide ions, and may possess specific anion-binding sites (Zagalsky & Gilchrist, in press).

(*d*) *Specificity of carotenoid attachment*

A direct cross-linking of apoprotein subunits by astaxanthin, involving attachment of the β-ionone rings to sites in separate protomer units, is unlikely since dimerization of the apoprotein is obtained with 15-15′-dehydro-8′-apo-β-carotene-8′-al-3,4-dione. The carotenoid prosthetic groups evidently lie between or within (but not across) individual apoprotein units. The carotenoid effects dimerization of the apoprotein in the presence of a 4-keto group in one ring and the presence, in either ring, of an additional hydrophilic substituent (e.g. 4′-hydroxy-β,β-caroten-4-one; β,β-carotene-3,4-dione; 2-nor-β-carotene,β-carotene-3,4-dione; 3,3′,4′-trihydroxy-β,β-caroten-4-one). An unsubstituted β-ionone ring can apparently be anchored to the polypeptide, by hydrophobic interaction of the 5-methyl group with an amino acid side chain (see Zagalsky 1976), provided the other ring is firmly bound.

A stronger binding (or greater twisting about the polyene double bonds) when 4-keto groups

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are present in both rings can be inferred from the longer wavelength position of the apoprotein dimer constituted by canthaxanthin as compared with that formed with euglenanone.

Both 4- and 4′-keto groups and additional hydrophilic substituents in the carotenoid structure are prerequisites for the further polymerization (in the presence of KCl) of the protein. Although astaxanthin combines most efficiently with the apoprotein, pigment in a state of high aggregation may be obtained with any one of a number of carotenoids, differing from the natural prosthetic group in the nature of the substituents, shape and size of end rings and in the structure of the polyene chain (e.g. 15-15′-dehydro-2-nor-β-carotene,β-carotene-3,4,4′-trione and 15-15′-dehydro-2,2′-dinor-β,β-carotene-3,3′,4,4′-tetraone). The position at shorter wavelengths of the absorption maxima of apoprotein dimers also formed with these carotenoids in the presence of chloride ions, reflects the synergism between the degree of polymerization of the pigment and the strength of carotenoid–protein and/or carotenoid–carotenoid interactions.

The rather broad specificity of the carotenoid–protein combination can be accounted for if the polypeptide at a carotenoid-binding site has a certain flexibility. The exact nature of the fit between polypeptide and carotenoid evidently determines the extent of subunit–subunit association. Chloride ions have no influence on the visible absorption spectra of the dimeric apoprotein pigments obtained with carotenoids substituted with a 4-keto group in a single ring (e.g. 2-nor-β-carotene, β-carotene-3,4-dione; 15,15′-dehydro-8′-apo-β-carotene-8′-al-3,4-dione). By contrast the visible absorption bands of the dimers containing carotenoids with both 4- and 4′-keto groups (e.g. 2,2′-dinor-β,β-carotene, 3,3′,4,4′-tetraone; β,β-carotene-3,3′,4-4′-tetraone; 15-15′-dehydro-2-nor-β-carotene, β-carotene-3,4,4′-trione) are bathochromically shifted in the presence of chloride ions. It is likely, therefore, that the proposed twisting about the carotenoid double-bonds can be enhanced (by a reorientation of the polypeptide-β-ionone ring attachment points) on saturation of the anion-binding sites only when the β-ionone rings are bound to the protein by both 4- and 4′-keto groups. Alternatively, the formation of the anion sites may depend on the precise change of the polypeptide configurations achieved by the carotenoid attachment.

The proposed mode of attachment of the astaxanthin prosthetic groups of the *Velella* pigments (and of other astaxanthin-proteins) is consistent with the necessity of keto groups at one or both ring positions for carotenoid-dependent association of the apoprotein. A detailed comparison of the specificity of carotenoid combination for the *Velella* pigment and other astaxanthin-proteins has been made elsewhere (Zagalsky 1976).

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References

Ackers, G. K. & Thompson, T. E. 1965 Determination of stoichiometry and equilibrium constants for reversibly associating systems by molecular sieve chromatography. *Proc. natn. Acad. Sci. U.S.A.* **53**, 342–349.

Ackers, G. K. 1967 A new calibration procedure for gel-filtration columns *J. biol. Chem.* **242**, 3237–3238.

Adam, M. J., Lilyas, A. & Rossman, M. G. 1973 Functional anion binding site in dogfish M_4 lactate dehydrogenase. *J. molec. Biol.* **76**, 519–531.

Adler, J., Greenfield, M. J. & Fasman, C. D. 1973 Circular dichroism and optical rotatory dispersion of proteins and polypeptides. *Meth. Enzym.* **27**, 675–735.

Andrews, P. 1964 Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **91**, 222–232.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Armstrong, J. M. & McKenzie, H. A. 1967 A method for modification of carboxyl groups in proteins: its application to the association of bovine β lactoglobulin A. *Biochim. Biophys. Acta* **147**, 93–99.
Banker, G. A. & Cotman, C. W. 1972 Measurement of free electrophoretic mobility and retardation coefficient of protein-sodium dodecyl sulfate complexes by gel filtration. *J. biol. Chem.* **247**, 5856–5861.
Bartlett, L., Klyne, W., Mose, W. P., Scopes, P. M., Galasko, G., Mallams, A. A., Weedon, B. C. L., Szabolcs, J. & Toth, G. 1969 Optical rotary dispersion of carotenoids *J. chem. Soc.* C. III, 2527–2544.
Bergstén, P-C., Waara, I., Lövgren, S., Lilyas, A., Kannan, K. K. & Bengtsson, U. 1972 In *Oxygen affinity of hemoglobin and red cell acid base* (eds P. Astrup & M. Roth). New York: Academic Press.
Buchwald, M. & Jencks, W. P. 1968*a* Optical properties of astaxanthin solutions and aggregates. *Biochemistry* **7**, 834–843.
Buchwald, M. & Jencks, W. P. 1968*b* Properties of the crustacyanins and the yellow lobster shell pigment. *Biochemistry* **7**, 844–859.
Carroll, W. R., Callanan, M. J. & Saroff, H. A. 1959 Physical and chemical properties of protamine from the sperm of salmon (*Oncorhynchus tschawytscha*). II. Anion binding characteristics. *J. biol. Chem.* **234**, 2314–2316.
Cheesman, D. F., Lee, W. L. & Zagalsky, P. F. 1967 Caroteno-proteins in invertebrates. *Biol. Rev.* **42**, 131–160.
Cheesman, D. F., Zagalsky, P. F. & Ceccaldi, H. J. 1966 Purification and properties of crustacyanin. *Proc. R. Soc. Lond.* B **164**, 130–151.
Chu, B., Whitney, D. C. & Diamond, R. M. 1962 On anion-exchange resin selectivities. *J. inorg. Nucl. Chem.* **24**, 1405–1415.
Craine, J. E., Daniels, G. H. & Kaufman, S. 1973 Dopamine hydroxylase. The subunit structure and anion activation of the bovine adrenal enzyme. *J. biol. Chem.* **248**, 7838–7844.
Diez, M. J. F., Osuga, D. T. & Feeney, R. E. 1964 The sulfhydryls of avian ovalbumins, bovine β-lactoglobulin, and bovine serum albumin. *Archs biochem. Biophys.* **107**, 449–458.
Diezel, W., Kopperschläger, G. & Hofmann, E. 1972 An improved procedure for protein staining in polyacrylamide gels with a new type of coomassie brilliant blue. *Analyt. Biochem.* **48**, 617–620.
Fairclough, G. F. (Jr) & Fruton, J. S. 1966 Peptide-protein interaction as studied by gel filtration. *Biochemistry* **5**, 673–683.
Fridovich, I. 1963 Inhibition of acetoacetic decarboxylase by anions. The Hofmeister series. *J. biol. Chem.* **238**, 592–598.
Fujimori, E. & Pecci, J. 1970 Circular dichroism of single- and double-peaked phycoerythrin: mercurial induced changes. *Biochim. biophys. Acta* **221**, 132–134.
Gammack, D. B., Raper, J. H., Zagalsky, P. F. & Quarmby, R. 1971 The physical properties of the lobster carapace carotenoprotein, crustacyanin. *Comp. Biochem. Physiol.* **40**B, 295–300.
Ginsburg, A. & Carroll, W. R. 1965 Some specific ion effects on the conformation and thermal stability of ribonuclease. *Biochemistry* **4**, 2159–2174.
Greenfield, N. & Fasman, G. D. 1969 Computed dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8**, 4108–4116.
Hamabata, A. & Von Hippel, P. H. 1973 Model studies in the effects of neutral salts on the conformational stability of biological macromolecules. II. Effects of vicinal hydrophobic groups on the specificity of binding of ions to amide groups. *Biochemistry* **12**, 1264–1271.
Hammes, G. G. & Wu, C-W. 1974 Kinetics of allosteric enzymes. *A. Rev. Biophys. Bioeng.* **3**, 1–31.
Hayaishi, O. 1962 Kynurenine hydroxylase. *Meth. enzym.* **5**, 807–809.
Herring, P. J. 1971 Stability of the blue pigment of *Velella* and *Porpita* (Coelenterata: Siphonophora). *Comp. Biochem. Physiol.* **39**B, 1039–1043.
Hersch, L. B. & Jencks, W. P. 1969 3 Ketoacid CoA-transferase. *Meth. Enzym.* **13**, 75–81.
Hindman, J. C. 1962 Nuclear magnetic resonance effects in aqueous solutions of 1–1 electrolytes. *J. chem. Phys.* **36**, 100–1015.
Hunter, M. J. & Ludwig, M. L. 1962 The reaction of imidoesters with proteins and related small molecules. *J. Am. Chem. Soc.* **84**, 3491–3594.
Kalish, M. I., Pineyro, M. A., Cooper, R. & Gregerman, R. I. 1974 Adenyl cyclase activation by halide anions other than fluoride. *Biochem. Biophys. Res. Commun.* **61**, 781–787.
Karniely, Y. & Benziman, M. 1966 Solubilisation of the NAD-linked malic dehydrogenase of *Actobacter xylinum*. Assay and some properties of the soluble enzyme. *Israel J. Chem.* **4**, 75p.
Kasha, M., Rawls, H. R. & El-Bayoumi, M. A. 1965 The exciton model in molecular spectroscopy. *Pure appl. Chem.* **11**, 371–392.
Kauzmann, W. 1959 Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* **14**, 1–63.
Kearney, E. B., Ackrell, B. A. C., Mayr, M. & Singer, T. S. 1974 Activation of succinate dehydrogenase by anions and pH. *J. biol. Chem.* **249**, 2016–2020.
Keilin, D. & Hartree, E. F. 1937 On the mechanism of the decomposition of hydrogen peroxide by catalase. *Proc. R. Soc. Lond.* B **124**, 397–405.
Kuntz, I. D. 1971 Hydration of macromolecules. III. Hydration of polypeptides. *J. Am. Chem. Soc.* **93**, 514–516.
Laemmli, U. K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **227**, 680–685.

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Levitzki, A. & Steer, M. L. 1974 The allosteric activation of mammalian α-amylase by chloride. *Eur. J. Biochem.* **41**, 171–180.
Li, L-K. & Spector, A. 1969 The circular dichroism of poly-L-lysine. *J. Am. Chem. Soc.* **91**, 220–222.
Lifshitz, R. & Levitzki, A. 1976 Identity and properties of the chloride effector binding site in hog pancreatic α-amylase. *Biochemistry* **15**, 1987–1993.
Lillehaug, J. R. & Kleppe, K. 1975 Effect of salts and polyamines on T4 polynucleotide kinase. *Biochemistry* **14**, 1225–1229.
Loeb, G. I. & Saroff, H. A. 1964 Chloride- and hydrogen-ion binding to ribonuclease. *Biochemistry* **3**, 1819–1826.
Ludewig, M., Lasch, J., Kattmann, U., Frohne, M. & Hanson, H. 1971 The influence of anions on enzymic activity and metal cation binding of leucine aminopeptidase. *Enzymologia* **41**, 59–67.
Marchalonis, J. J. & Weltman, J. K. 1971 Relatedness among proteins: a new method of estimation and its application to immunoglobulins. *Comp. Biochem. Physiol.* **38B**, 609–625.
Massey, V. 1953 Studies on fumarase. 2. The effects of inorganic anions on fumarase activity. *Biochem. J.* **53**, 67–71.
McKerrow, J. H. & Robinson, A. B. 1971 Deamidation of asparaginyl residues as a hazard in experimental protein and peptide procedures. *Analyt. Biochem.* **42**, 565–569.
Nadi, P. K. & Robinson, D. R. 1972 The effects of salts on the free energies of nonpolar groups in model peptides. *J. Am. Chem. Soc.* **94**, 1308–1316.
Norne, J-E., Hyalmarsson, S-G., Lindman, B. & Zeppezauer, M. 1975. Anion binding properties of human serum albumin from halide ion quadrupole relaxation. *Biochemistry* **14**, 3401–3407.
Oestreicher, G., Hogue, P. & Singer, T. P. 1973 Regulation of succinate dehydrogenase in higher plants. II. Activation by substrates, reduced coenzyme φ, nucleotides, and anions. *Pl. Physiol., Lancaster* **52**, 622–626.
Packer, Y. & Stone, J. T. 1967 The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalysed hydrolysis of *p*-nitrophenyl acetate. *Biochemistry* **6**, 668–678.
Padova, J. 1964 Solvation approach to ion solvent interaction. *J. Chem. Phys.* **40**, 691–694.
Patterson, L. S. & Greene, R. C. 1965 Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. *Analyt. Chem.* **37**, 854–857.
Peacock, A. C. & Dingman, C. W. 1968 Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry* **7**, 668–674.
Righetti, P. & Secchi, C. 1972 Preparative acrylamide gel electrophoresis. *J. Chromat.* **72**, 165–175.
Robinson, D. R. & Jencks, W. P. 1965 The effect of concentrated salt solutions on the activity coefficient of acetyltetraglycine ethyl ether. *J. Am. Chem. Soc.* **87**, 2470–2479.
Rose, Z. B. & Liebowitz, J. 1970 2,3-Diphosphoglycerate phosphatase from human erythrocytes. General properties and activation by anions. *J. biol. Chem.* **245**, 3232–3241.
Saroff, H. A. & Carroll, W. R. 1962 The binding of chloride and sulphate ions to ribonuclease. *J. biol. Chem.* **237**, 3384–3387.
Saroff, H. A. & Healy, J. W. 1959 The binding of chloride ions to alkyl amines *J. phys. Chem. Wash.* **63**, 1178–1181.
Sasakawa, S. & Walter, H. 1972 Partition behaviour of native proteins in aqueous dextran-poly(ethyleneglycol) phase systems. *Biochemistry* **11**, 2760–2765.
Scatchard, G., Scheinberg, I. H. & Armstrong, S. H. 1950 Physical chemistry of protein solutions. IV. The combination of human serum albumin with chloride ion. *J. Am. Chem. Soc.* **72**, 535–540.
Scatchard, G. & Yap, W. T. 1964 The physical chemistry of protein solutions. XII. The effects of temperature and hydroxide ion on the binding of small anions to human serum albumin. *J. Am. Chem. Soc.* **86**, 3434–3438.
Scheraga, H. A. 1963 Intermolecular bonds in proteins. II. Noncovalent bonds. In *The proteins* (ed. H. Neurath), pp. 477–594. New York: Academic Press.
Schrier, E. E. & Schrier, E. B. 1967 The salting-out behaviour of amides and its relation to the denaturation of proteins by salts. *J. phys. Chem. Wash.* **71**, 1851–1860.
Scotchler, J. W. & Robinson, A. B. 1971 Deamidation of glutaminyl residues: dependence on pH, temperature and ionic strength. *Analyt. Biochem.* **42**, 319–322.
Silhavy, T. J., Szmelcman, S., Boos, W. & Schwartz, M. 1975 On the significance of the retention of ligand by protein. *Proc. natn. Acad. Sci., U.S.A.* **72**, 2120–2124.
Smith, I. 1968 *Chromatographic and electrophoretic techniques*. Second edition. Bath: Pitman Press.
Takahashi, K. 1968 The reaction of phenylglyoxal with arginine residues in proteins. *J. biol. Chem.* **243**, 6171–6179.
Tanford, C. 1967 In *Physical chemistry of macromolecules*. New York: John Wiley.
Unemoto, T. & Hayashi, M. 1969 Chloride ion as a modifier of 2′,3′-cyclic phosphodiesterase purified from halophilic *Vibrio alginolyticus*. *Biochim. biophys. Acta* **171**, 89–102.
Van Holde, K. E., Brahms, J. & Michelson, A. M. 1965 Base interactions of nucleotide polymers in aqueous solutions. *J. molec. Biol.* **12**, 726–739.
Von Hippel, P. H. & Schleich, T. 1969 In *Structure and stability of biological macromolecules* (eds S. F. Timasheff & G. B. Fasman) vol. 2. New York: Marcel Dekker.

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Walaas, E. & Walaas, O. 1956 Kinetics and equilibria in flavoprotein systems. V. The effects of pH, anions and partial structural analogues of the coenzyme on the activity of D-aminoacid oxidase. *Acta Chem. Scand.* **10**, 122–133.

Whitehead, E. 1970 The regulation of enzyme activity and allosteric transition. *Prog. Biophys. & molec. Biol.* **21**, 323–397.

Wilchek, M., Frensdorff, A. & Sela, M. 1967 Modification of the carboxyl groups of ribonuclease by attachment of glycine or alanylglycine. *Biochemistry* **6**, 247–252.

Winzor, D. J. & Scheraga, H. A. 1964 Studies of chemically reacting systems on Sephadex. II. Molecular weights of monomers in rapid association equilibrium. *J. phys. Chem.* **68**, 338–343.

Woody, R. W. 1969 Optical properties of polypeptides in the β-conformation. *Biopolymers* **8**, 669–683.

Zagalsky, P. F. 1976 Carotenoid-protein complexes. *Pure appl. Chem.* **47**, 103–120.

Zagalsky, P. F., Ceccaldi, H. J. & Daumas, R. 1970 Comparative studies on some decapod crustacean carotenoproteins. *Comp. Biochem. Physiol.* **34**B, 579–607.

Zagalsky, P. F. & Gilchrist, B. M. 1976 Isolation of a blue canthaxanthin–lipovitellin from the yolk platelets of *Branchipus stagnalis* (L.) (Crustacea: Anostraca). *Comp. Biochem. Physiol.* **55**B, 195–200.

Zagalsky, P. F. & Herring, P. J. 1972 Studies on a carotenoprotein isolated from the copepod, *Labidocera acutifrons* and its relationship to the decapod carotenoproteins and other polyene-binding proteins. *Comp. Biochem. Physiol.* **41**B, 397–415.

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