

The Characterization of C-Phycocyanin from an Extremely Halo-Tolerant Blue-Green Alga, *Coccochloris elabens*

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C-Phycocyanin was isolated and purified from a uni-algal culture of an extremely halo-tolerant blue-green alga, *Coccochloris elabens*. This alga can be grown under laboratory conditions in 25% (w/v) NaCl. Purified halophile phycocyanin was characterized by amino acid analysis and the measurement of sedimentation velocity, fluorescence polarization and immunodiffusion as a function of protein concentration, pH and ionic strength. The results were compared with those of studies of phycocyanin isolated from *Plectonema calothricoides* and from several other sources. The states of aggregation previously characterized as being present in other C-phycocyanins, monomer, trimer and hexamer, were present in halophile phycocyanin and were characterized as antigenically related to all C-phycocyanins tested. The equilibrium between 3S monomer and 11S hexamer at low concentrations in halophile phycocyanin was quantitatively similar to that for other phycocyanins. The effect of pH and ionic strength on the 6S (trimer) and 11S (hexamer) aggregation of halophile phycocyanin was markedly salt-dependent and the relative amount of each aggregate in the presence of 2M-NaCl was like that of C-phycocyanin from mesophiles, in the absence of additional salt. In antigenic relationship and aggregation properties, the phycocyanin from *C. elabens* appeared to be most closely related to that isolated from the thermophilic blue-green alga, *Synechococcus lividus*. Amino acid content of the halophile phycocyanin indicated the presence of a significantly larger number of acidic residues than that found in mesophiles. Explanations of the properties of the halophile protein require consideration of a strong contribution of hydrophobic forces and utilize both charge-shielding and salting-out effects.

The properties of the chromoprotein phycocyanin have been under careful study for a number of years (Berns, 1971). These studies have elucidated the step-wise aggregation of this protein and the forces involved in the aggregation process (Scott & Berns, 1965; MacColl *et al.*, 1971*b*). It seems logical to expect the eventual elucidation of the entire aggregation process leading to phycobilisome formation (Gantt & Conti, 1967; Edwards *et al.*, 1968; Gantt *et al.*, 1968). Phycobilisomes are the presumed sites of phycocyanin localization in Cyanophyta and Rhodophyta. Phycocyanins in blue-green and red algae are of increasing interest not only because of their established function as an energy-transfer pigment (Teale & Dale, 1970), but also of the recently introduced possibility that they function as an electron-directing agent in trans-membrane migration of electrons (Ilani & Berns, 1971, 1972). The Cyanophyta (all of which contain phycocyanin) are a group of micro-organisms, some of which are very resistant to environmental extremes. This characteristic leads to the use of this protein as an important probe in

studying the effect of wide variations in environmental conditions on the structure and function of this protein. The questions that can be logically asked are: (1) What changes in the aggregation process result from growth under environmental stress? (2) What are the gross changes in the balance of forces that explain the changes in the dependence on pH, ionic strength or temperature of the essential aggregation processes? (3) What changes in amino acid content and eventually in amino acid sequence explain the adjustments in protein structure and associated aggregation processes? (4) What, if any, unique characteristics of phycocyanin permit it to function under wide environmental extremes with minimal changes in structure?

We have reported the study of phycocyanin from a thermophilic blue-green alga, *Synechococcus lividus* (Berns & Scott, 1966), and have speculated on the causes for the observed changes in the aggregation process. In addition, cultures of several Cyanophyta grown in $^2\text{H}_2\text{O}$ have been used as a source of fully deuterated proteins. An examination of this

artificially produced environmental stress (Scott & Berns, 1967) has also led to important information about the structure and function of the protein.

We now report studies of phycocyanin from an extremely halo-tolerant blue-green alga. Field observations have indicated that blue-green algae are halo-tolerant or even halophilic (Hof & Fremy, 1933; Elazari-Volcani, 1943; Pillai, 1954) and one laboratory has reported the growth responses of blue-green algae to NaCl concentration (Batterton & Van Baalen, 1971). There are, however, no known studies with a protein from Cyanophyta cultured in the laboratory at greater than 15% (w/v) NaCl. Certainly, several studies are in the literature on proteins and particularly enzymes, from halophilic bacteria (Larsen, 1967; Lanyi & Stevenson, 1970). The unique aspect of the present investigation was its effort to study antigenically closely related proteins (Berns & Scott, 1966; Berns, 1967; Scott & Berns, 1967) isolated from Cyanophyta that grow under a wide variety of environmental stress (thermophiles, halophiles, psychrophiles and mesophiles) and those grown at acidic and basic pH values. Such studies should provide valuable information in our quest to understand the structural factors that help to determine the functional aspects of proteins.

Experimental

Algal isolation and culturing

A sample of a mixed ecosystem taken from a commercial salt pond at La Pargnera, Lajas, Puerto Rico, was kindly supplied by Dr. H. T. Odum (University of North Carolina) in January 1968. The NaCl concentration of the pond was about 30% (w/v) and the algae were growing about 1.5 cm below the surface of the soft mud bottom, where the temperature range was 26–36°C. Conventional plating and streaking techniques were used to isolate a uni-algal culture. The cultures were grown in Guillard's medium (Guillard & Ryther, 1962) enriched with nutrient broth (0.8 g/l) and supplemented with an appropriate amount of reagent-grade NaCl to a final concentration of 15% (w/v). The agar plates and slants were made up with the liquid medium plus 1.5% Bacto-agar (Difco, Detroit, Mich., U.S.A.).

The original culture containing several blue-green algae was received in this laboratory in 10% NaCl. Streaking on agar plates containing 15, 20 and 25% NaCl permitted the eventual isolation of a uni-algal culture. Efforts were made to free the culture from other micro-organisms by use of various amounts of penicillin, streptomycin, actinomycin, 5-fluorodeoxyuridine, actidione and Nystatin. It was not possible, however, to inhibit growth of 'contaminants' without inhibiting growth of the alga. Under the conditions normally employed for growth of the alga

in large batches (15–20% NaCl, w/v), the alga represented approx. 97% of the packed cells.

A sample of the uni-algal culture was sent to Dr. Francis Drouet of the Academy of Natural Sciences of Philadelphia, who was kind enough to confirm the uni-algal nature of the culture and identify it as *Coccochloris elabens*.

The growth of *C. elabens* was followed over a prolonged period of time with Guillard's medium and various concentrations of additional NaCl (0, 5, 10, 15, 17.5, 20, 22.5 and 25%, w/v). The cultures were grown in an incubator at 26°C on an Eberbach rotary shaker with an illumination of approx. 5380 lx. The growth was monitored by using absorption measurements similar to those of Kratz & Myers (1955). The absorption spectrum of the intact algal cells from approx. 730 nm to 400 nm was monitored with a Cary 14 spectrophotometer with opal-glass diffusing plates by the method of Shibata *et al.* (1954). Typical spectra of *C. elabens* and the filamentous Cyanophyta, *Lyngbya* sp., are shown in Figs. 1(a) and 1(b).

The growth of uni-algal cultures of *C. elabens* was greater in increasing NaCl concentrations and started to decline at 15% (w/v) NaCl. At very high salt concentrations, the growth curve indicates a substantial lag period (Fig. 2).

Purification of phycocyanin

Phycocyanin used in the present study was isolated from algae grown at 25°C in laboratory culture medium containing 15% (w/v) NaCl. Phycocyanin from *C. elabens* was isolated by lysing the cells with a large amount of lysozyme (approx. 3 mg/g of cells). The blue supernatant separated from cell debris was dialysed in sodium phosphate buffer, 1 0.1, pH 6.0, for 3 days at 4°C to remove large amounts of lipopolysaccharides. The polysaccharide precipitate was removed by centrifugation at 15000g for 20 min. The phycocyanin was then purified at pH 6.0 by the (NH₄)₂SO₄ method (Scott & Berns, 1965). The E_{620}/E_{280} ratio in a sodium phosphate buffer, pH 6.0, for all protein solutions was 4 or greater. A typical spectrum of purified phycocyanin from *C. elabens* is shown in Fig. 1(c).

The extinction coefficient of halophile phycocyanin was determined from a small amount of freeze-dried salt-free phycocyanin (e.g., 0.12 mg/ml) dissolved in pH 6.0 buffer. U.v. and visible spectra were measured with the Cary spectrophotometer. The absorbance of the protein solution was recorded at 620 nm with a 1 cm light-path cuvette. The extinction coefficient of 1% halophile phycocyanin was calculated as $E_{1\text{cm}}^{1\%} = 60$.

The concentration of all subsequent protein solutions was determined by measuring the absorbance at 620 nm with a Cary 14 or Gilford model 2400

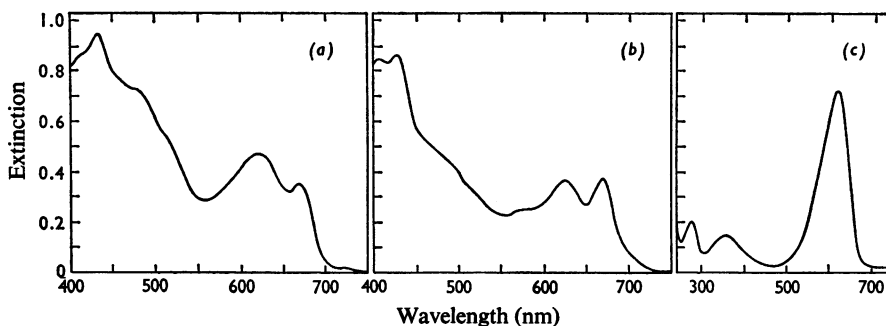


Fig. 1. Absorption spectra of (a) whole cell suspension of *C. elabens* grown in the presence of 17.5% (w/v) additional NaCl, (b) whole-cell suspension of *Lyngbya* sp. and (c) purified phycocyanin from *C. elabens* in sodium phosphate buffer, 1.0 M, pH 6.0

For experimental details see the text.

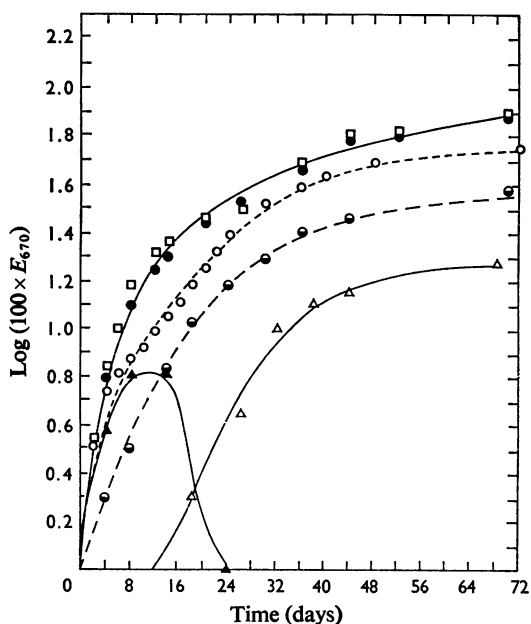


Fig. 2. Growth curves of *C. elabens* in the presence of various amounts of added NaCl

The log of 100 times the E_{670} is plotted versus time in days. All E_{670} measurements were made in a 1 cm path-length cell. NaCl added (w/v): ▲, 0%; ●, 5%; □, 10%; ○, 15%; ⊙, 17.5%; △, 20%.

spectrophotometer and assuming $E_{1\text{cm}}^{1\%} = 60$ for phycocyanin from *C. elabens*. The presence of 2M-NaCl in the pH 6.0 buffer had a negligible effect on the $E_{1\text{cm}}^{1\%}$ for phycocyanin from *C. elabens* at pH 6.0.

Ultracentrifuge studies with schlieren optics

Phycocyanin solutions above 5 mg/ml in concentration were studied by sedimentation velocity in the Spinco model E ultracentrifuge with schlieren optics. Type 1N Kodak spectroscopic plates and a Corning no. 5031 filter were required because of the intense blue absorption of the protein. A Nikon micro-comparator was used to measure the distance of each sedimentation peak. The relative percentage of each of the aggregates was determined from the area under each peak of the enlarged tracing of a sedimentation pattern (Scott & Berns, 1965).

Ultracentrifuge studies with absorption optics

Phycocyanin solutions of concentrations below approx. 2 mg/ml were studied by sedimentation velocity in the Spinco model E ultracentrifuge with absorption optics. Solutions of various concentrations were scanned at 278, 584, or 620 nm. Cells with a 12 mm double-sector centre piece and sapphire windows were used in an AN-F rotor for most of the phycocyanin solutions. At very low protein concentrations, a 30 mm double-sector centre piece and an AN-E rotor were employed.

The partial specific volume of *C. elabens* phycocyanin, calculated from amino acid composition by the method of Cohn & Edsall (1943), was 0.72.

Fluorescence polarization studies

Fluorescence spectra were recorded with a Baird Atomic fluorescence spectrophotometer SF-1 equipped with an R136 photomultiplier and Glan-Thompson prisms. The polarization results were obtained with fluorescence excitation spectra. The fluorescence emission was monitored at 650 nm. The

polarization (P) was calculated from: $P = (I_{vv} - GI_{vh}) / (I_{vv} + GI_{vh})$, where I_{vv} and I_{vh} are the intensities with analysers vertically and horizontally oriented, and G is a grating correction factor (Azumi & McGlynn, 1962). All measurements were obtained at room temperature.

The fluorescence polarization of several known solutions, such as Rhodamine B in glycerol-water (19:1, v/v), fluorescein in glycerol-0.1 M-NaOH (19:1, v/v), and riboflavin in glycerol-sodium phosphate buffer, I 0.1, pH 7.2 (9:1, v/v) was measured. These spectra were in very good agreement with those reported in the literature (Chen & Bowman, 1965).

Isoelectric focusing

The isoelectric point of purified phycocyanin was determined by isoelectric focusing in a sucrose-gradient column as a stabilizing system (Haglund, 1971). A narrow-range (pH 4.0-6.0) synthetic ampholyte (LKB Instruments, Bromme, Sweden) was used at an initial concentration of 7 mg/ml. The total solution volume was approx. 70 ml and the total amount of protein used on the 25 mm-diameter column was approx. 4 mg.

Amino acid analysis

The amino acid analysis was performed by the conventional method of Spackman *et al.* (1958) by using 6 M-HCl hydrolysis. The analysis was also performed by the toluenesulphonic acid method of hydrolysis (Liu & Chang, 1971). In this manner, it was possible to detect analytically the presence of tryptophan.

Immunodiffusion studies

Ouchterlony immunodiffusion studies were performed as previously described (Bernis, 1967) with rabbit antisera to phycocyanin from *Plectonema calothricoides*, *Phormidium luridum* and *Synechococcus lividus* and the phycocyanin from *C. elabens*, *S. lividus*, *Anabaena variabilis*, *Lyngbya* sp., *Cyanidium caldarium*, *Tolypothrix tenuis*, *Ph. luridum*, and *Pl. calothricoides*. All studies were made in 1.5% Bacto-agar containing 0.02% merthiolate. The agar was prepared with sodium phosphate buffers that were either pH 6.0, I 0.1, or pH 6.0+2 M-NaCl, or pH 7.0, I 0.1. The right-angle immunodiffusion method of Allison & Humphrey (1960) was also employed.

Results

pH-dependence of aggregation

Purified phycocyanin extracted from *C. elabens* was studied over a range of pH values from 4.7 to 8.0 in the presence and absence of 2 M-NaCl. In

general, the behaviour of the halophile phycocyanin in the presence of 2 M-NaCl is analogous to that of the non-halophile phycocyanin in the absence of added salt. In this study, we will assume that all 3S species are monomer, 6S, trimer, and 11S, hexamer, as established in our previous studies (Bernis, 1971; Kao *et al.*, 1971). Neufeld & Riggs (1969) have reported a dimeric species for 6S phycocyanin from *Anabaena nidulans* and the 6S halophile at this point cannot be unequivocally identified as not containing dimer. The effect of pH on the sedimentation-velocity properties of the halophile phycocyanin is shown in Figs. 3 and 4. In the absence of 2 M-NaCl at pH 4.7, which is close to the isoelectric point of this phycocyanin, the 11S species is predominant. At pH 6.0 and above, the 6S aggregate becomes the major species and at pH 8.0, the 6S species is the only one present in the system. In the presence of 2 M-NaCl, the 11S aggregate remains as the predominant species from pH 4.7 to 7.0. At pH 8.0, the 6S species is the major constituent of the system. In all these studies, the largest aggregate observed is the 19S species. The absence of larger aggregates (Lee & Bernis, 1968a) may in part be a result of the lengthy

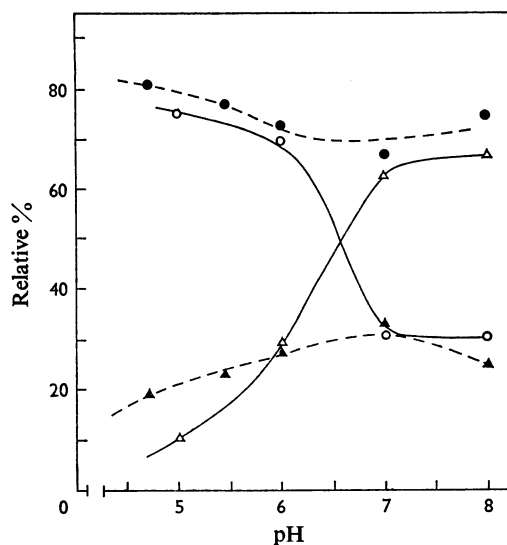


Fig. 3. Relative percentage of 6S and 11S aggregates of *C*-phycocyanin from *Pl. calothricoides* plotted as a function of pH in the presence and absence of 2 M-NaCl

All buffers are I 0.1. \blacktriangle , 6S species in 2 M-NaCl; \bullet , 11S species in 2 M-NaCl; \triangle , 6S species, no additional NaCl; \circ , 11S species, no additional NaCl. The results plotted are from analysis of sedimentation-velocity ultracentrifuge studies at a concentration of approx. 15 mg/ml.

purification procedure required for this phycocyanin. In the presence of high salt concentrations, the relative percentage of 19S aggregate remains approximately the same from pH 4.7 to 6.0 (Table 1). At pH 7.0, the amount of 19S species decreases sharply and finally disappears at pH 8.0. In the absence of added NaCl, the 19S aggregates were observed only in small amounts at pH 5.5 and 6.0. The $s_{20,w}^0$ values of 6.0 and 11.2 have been determined at pH 6.0 in the presence of 2M-NaCl. The plot of sedimentation coefficient versus concentration from 20mg/ml to approx. 1mg/ml exhibited the normal linear behaviour, with a negative slope. At pH 6.0 in the absence of additional salt, the extrapolated values of the sedimentation coefficient are essentially the same, except that the slope is close to zero.

Several non-halophile phycocyanins have been characterized in the absence of salt (Scott & Berns, 1965; Lee & Berns, 1968a,b; Berns, 1971; MacColl *et al.*, 1971b). For comparison, *Pl. calothricoides* phycocyanin was studied in the presence of 2M-NaCl (Fig. 3). Between pH 4.7 and 8.0, the 11S aggregate is the major species. However, in the absence of 2M-NaCl, the 11S aggregate is the major species only at pH values as high as 6.5. Above pH 6.5, there is a rapid decrease in the amount of 11S material and a corresponding increase in the amount of 6S species. This behaviour corresponds with that of the halophile phycocyanin in the presence of salt. As freeze-dried *Pl. calothricoides* phycocyanin was used for this study, no 19S or higher aggregate was present in the system (Lee & Berns, 1968a).

The reversible aggregation-disaggregation between 11S and 6S species was studied for the halophile phycocyanin by dialysing protein solutions from one pH value to another. The results were similar to those observed with the non-halophile phycocyanin reported by MacColl *et al.* (1971b). When the pH of the solution was changed from

pH 8.0 to 6.0 by dialysis, the relative quantities of 11S and 6S aggregates reverted to the values originally obtained at pH 6.0; however, the original amount of 19S species could not be recovered.

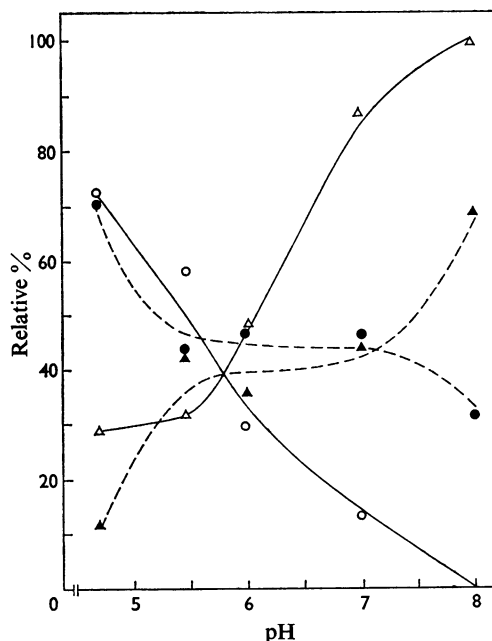


Fig. 4. Relative percentage of 6S and 11S aggregates of *C-phycocyanin* from *C. elabens* plotted as a function of pH in the presence and absence of 2M-NaCl

The study shown in Fig. 3 was repeated with phycocyanin from *C. elabens*. \blacktriangle , 6S species in 2M-NaCl; \bullet , 11S species in 2M-NaCl; \triangle , 6S species, no added NaCl; \circ , 11S species, no added NaCl.

Table 1. pH-dependence of the phycocyanin aggregates from *C. elabens* (halophile)

Buffers were of 1.0M. The percentage of each species was determined from schlieren patterns of samples containing approx. 15mg/ml. The relative area under each peak did not vary over the concentration range of approx. 5–20mg/ml, as reported for other C-phycocyanins (Scott & Berns, 1965; MacColl *et al.*, 1971b). For other details see the text.

Buffer and pH	Percentage present as species					
	With 2M-NaCl			Without NaCl		
	19S	11S	6S	19S	11S	6S
Acetate, 4.8	18	70	12	—	70	30
Phosphate, 5.5	14	44	42	10	58	32
Phosphate, 6.0	17	47	36	7	27	66
Phosphate, 7.0	10	46	44	—	13	87
Phosphate, 8.0	—	31	69	—	—	100

Concentration dependence

Solutions of halophile phycocyanin of very low concentrations were examined by sedimentation-velocity studies in an ultracentrifuge equipped with a photoelectric scanner. At sufficiently low concentrations, complete disaggregation from the hexamer (11S) to the monomer (3S) was observed in the

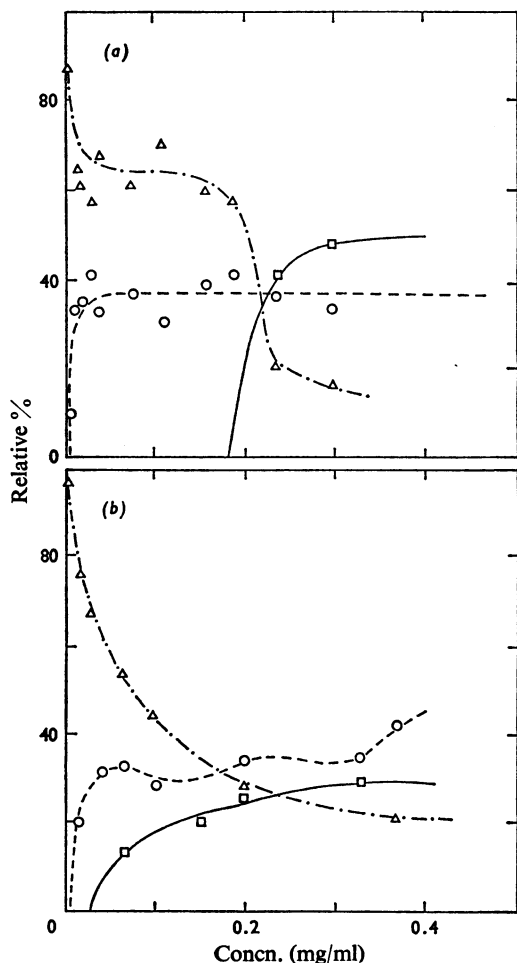


Fig. 5. Plot of relative amounts of monomer (3S), trimer (6S) and hexamer (11S) as a function of total concentration of phycocyanin

Data are from sedimentation-velocity experiments with absorption optics (620nm and 278nm). (a) Phycocyanin from *C. elabens* at I 0.1, pH6.0. (b) Phycocyanin from *Lyngbya* sp., I 0.1, pH6.0. Results for phycocyanin from *Ph. luridum* are almost identical. (These results are from MacColl *et al.*, 1971b.) Δ , 3S, \square , 6S; \circ , 11S.

presence or absence of 2M-NaCl. The concentrations of phycocyanin for complete disaggregation to monomer were not very sensitive to the salt concentrations employed in this study.

The dissociation of halophile phycocyanin at very low concentration is similar to that of non-halophile ones reported previously (MacColl *et al.*, 1971b).

When the concentration of halophile phycocyanin was below 0.4mg/ml in the absence of salt at pH6.0, the 6S species began to disappear rapidly and the 3S monomer became the predominant species (Fig. 5). The relative percentage of 11S species remained about 35% at concentrations from 0.02 to 0.4mg/ml. When the concentration of phycocyanin was lower than 0.02mg/ml, the 11S species began to disappear rapidly. At sufficiently low phycocyanin concentrations (0.01mg/ml or lower), only the 3S species could be detected in the system.

In the presence of 2M-NaCl, pH6.0, at protein concentrations of 0.4mg/ml and lower, the halophile phycocyanin contained only 11S and 3S species, with 3S being the major species (Fig. 6). This behaviour demonstrated that at pH6.0, the 11S species probably was the last to disaggregate to 3S species in both the presence and absence of 2M-NaCl. In previous studies of the dissociation of phycocyanin aggregates at very low concentrations (MacColl *et al.*, 1971b), the 11S hexamer was also reported to be the last species to disaggregate to monomer (3S).

The association constant, K , at pH6.0 and 23°C, for the interconversion of 6 monomer \rightleftharpoons 1 hexamer for phycocyanin from *C. elabens* was estimated to be approx. 10^{31} . This estimate, by using data from Fig. 5, assumes a monomer molecular weight of 30000 and a hexamer molecular weight of 180000. In 2M-NaCl, pH6.0 and 23°C, the equilibrium constant K

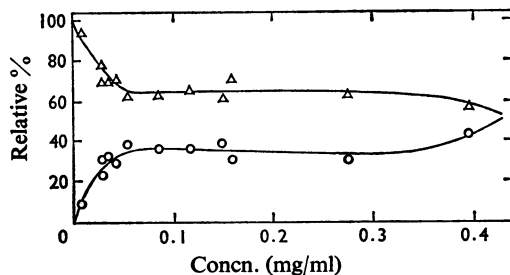


Fig. 6. Plot of relative amounts of monomer (3S) and hexamer (11S) as a function of total concentration of phycocyanin in the presence of 2M-NaCl

A similar concentration study to that shown in Fig. 5(a) was done, except that these results are for phycocyanin from *C. elabens* in the presence of sodium phosphate buffer, I 0.1, pH6.0, plus 2M-NaCl at approx. 25°C. Δ , 3S; \circ , 11S.

for *C. elabens* was about 10^{29} . The magnitudes of these equilibrium constants obtained for halophile phycocyanin are in good agreement with the K value, 10^{30} , reported for other non-halophile phycocyanins (MacColl *et al.*, 1971b).

Immunodiffusion studies

Ouchterlony immunodiffusion studies were performed to determine the antigenic relationship of phycocyanin from *C. elabens* to phycocyanins isolated from *Ph. luridum*, *Pl. calothricoides*, *Lyngbya* sp., *Cy. caldarium*, *A. variabilis*, *T. tenuis* and *S. lividus*. In addition to these antigens, rabbit antisera to phycocyanin from *Ph. luridum*, *Pl. calothricoides* and *S. lividus* were tested. All antigens tested exhibited precipitin lines of partial identity with phycocyanin from the halophile. The most intense antibody-antigen reaction of the halophile phycocyanin occurred with antiserum to *S. lividus* phycocyanin [compare Plate 1(a) and (b)]. Analysis of the Ouchterlony immunodiffusion studies demonstrates that the amount of spurring of precipitin lines between heterologous antigens was dependent on the rabbit antiserum employed. In Plate 1(a), the spurring of precipitin lines indicates that although *C. elabens* phycocyanin is antigenically related to *Ph. luridum* phycocyanin, they are antigenically quite distinct. The rabbit antiserum is anti-(*Pl. calothricoides* phycocyanin), and *Ph. luridum* is antigenically very closely related to *Pl. calothricoides* (Berns, 1967). When antiserum to *S. lividus* is used (Plate 1b), the spurring of precipitin lines is considerably less, and *Ph. luridum* and halophile phycocyanin appear to be antigenically much more closely related. In general, phycocyanin from *C. elabens* appeared to be closest in antigenic relationship to *S. lividus*, *Cy. caldarium* and *A. variabilis*. All the other phycocyanins tested showed partial identity, but to a lesser degree. All precipitin lines fluoresced red under a long-wavelength u.v. lamp and definitely represented phycocyanin-anti-phycocyanin precipitates.

The Ouchterlony plates (Plates 1 and 2) demonstrate the presence of multiple antigen-antibody lines for phycocyanin from *Ph. luridum*, *S. lividus* and *C. elabens*. By using serial dilutions and right-angle plates (Plates 3a, 3b, 4a-4d), we were able to correlate these lines with the several aggregates observed in the dilute solution sedimentation-velocity studies. Phycocyanin from *S. lividus* at 25°C has been reported to contain principally trimer (6S) and slower-sedimenting species at pH 6.0 and higher pH values (Berns & Scott, 1966). Antiserum to this phycocyanin consequently contains very little antibody to hexameric phycocyanin. *Pl. calothricoides* phycocyanin at 25°C contains hexamer, trimer and monomer in the pH 6.0-7.0 region (Scott & Berns, 1965) and antiserum to this protein contains

significant amounts of antibody to all these species.

The reaction of *C. elabens* phycocyanin with antisera to *Pl. calothricoides* phycocyanin at pH 6.0 (Plate 2a) produces two, possibly three, precipitin lines, whereas the reaction of phycocyanin from *Ph. luridum* on the same plate exhibits three definite lines. At a concentration of 1 mg/ml and under these conditions, sedimentation data indicate (Fig. 5) a sizeable trimer concentration in the halophile phycocyanin and considerable trimer and hexamer in the *Ph. luridum* phycocyanin. Studies by Berns & Morgenstern (1968) have indicated the aggregation properties of *Pl. calothricoides* and *Ph. luridum* are identical. Decreasing the concentration of both antigens results in fading of the outer precipitin lines, which are probably associated with trimer and hexamer, and retention of the innermost line, which is almost certainly associated with monomer. In Plate 4(a), right-angle immunodiffusion plates demonstrate that the *Ph. luridum* phycocyanin has significant amounts of material diffusing at approx. 4×10^{-7} and 7×10^{-7} cm²/s, which are diffusion coefficients previously shown to be associated with hexamer and trimer, respectively. The phycocyanin from *C. elabens* exhibits only a fast diffusion coefficient quite difficult to determine precisely ($>10 \times 10^{-7}$ cm²/s), but nevertheless indicative of the absence of any large amount of hexamer. If these studies are done at pH 6.0 + 2M-NaCl (Plates 2b, 4b), sizeable amounts of slower-diffusing material are present in both *Ph. luridum* and *C. elabens* and right-angle plates demonstrate the predominance of a precipitin line associated with hexameric phycocyanin ($D = 4 \times 10^{-7}$ cm²/s). This observation correlates well with the sedimentation data in 2M-NaCl at pH 6.0 which indicate sizeable increases in hexamer in *C. elabens*. The same studies were performed with antisera to *S. lividus* phycocyanin, which has little antibody to hexameric phycocyanin (Plates 2d, 3a, 4c). Serial dilution of *C. elabens* phycocyanin at pH 7.0 (Plate 3a) demonstrates the presence of two intense precipitin lines. The outer precipitin line begins to disappear at a concentration of approx. 0.12 mg/ml and the *S. lividus* phycocyanin exhibits a single precipitin line with partial identity with both *C. elabens* precipitin lines. The right-angle plates at this pH value demonstrate that the predominant species in both *S. lividus* and *C. elabens* has a diffusion coefficient most characteristic of the monomer ($>10 \times 10^{-7}$ cm²/s). At pH 6.0, the results are quite similar (Plate 4d), except that two precipitin lines are now evident for the halophile phycocyanin. The lighter precipitin line is interpreted as representing the trimer (6S). Sedimentation-velocity studies (Fig. 5) indicate that trimer disappears below 0.3 mg/ml and indeed, on serial dilution in Plate 3(b), the outer precipitin line representing trimer does disappear in this concentration region (H2 to H4). A

Table 2. *Fluorescence polarization of phycocyanins*

Values were determined by using a sample with an extinction at 620 nm of 0.2. The procedures used in compiling these results are identical with those reported by MacColl *et al.* (1971a).

pH	P_{620}			
	<i>C. elabens (halophile)</i>		<i>Pl. calothricoides</i>	
	With 2M-NaCl	Without NaCl	With 2M-NaCl	Without NaCl
4.8	0.092	0.070	0.149	0.172
5.5	0.118	0.101	0.137	0.124
6.0	0.157	0.156	0.212	0.157
7.0	0.159	0.138	0.211	0.163
8.0	0.191	0.175	0.249	0.233

Table 3. *Amino acid composition (weight %) of phycocyanin from C. elabens*

Results are averages of five determinations (three 24h, one 48h and one 72h hydrolysate). Serine and threonine values are extrapolated to zero time from 24, 48, and 72h hydrolysates.

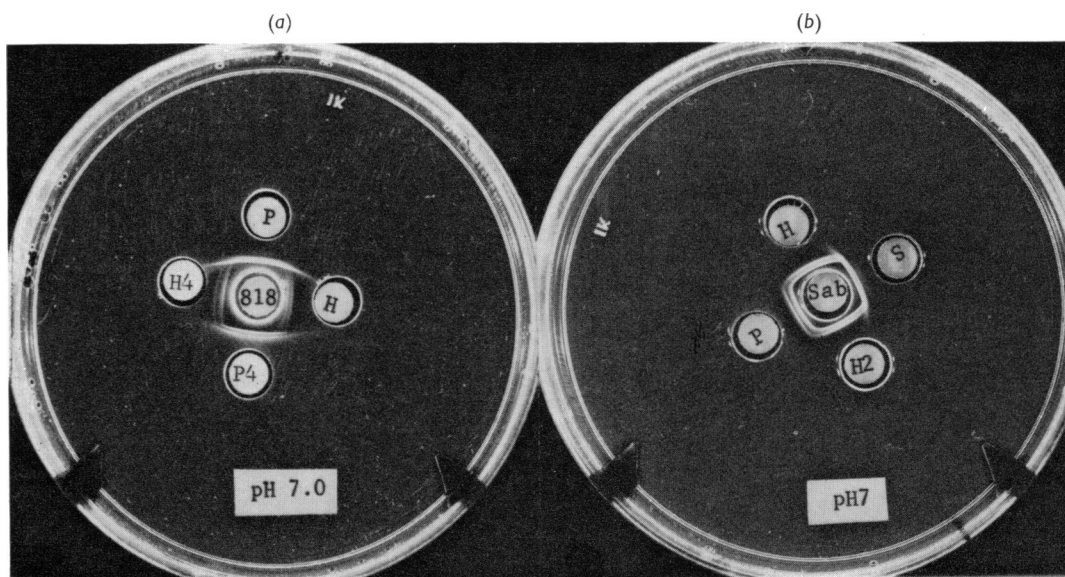
	Weight % (average)
Asp	12.44 ± 0.29
Thr	5.60
Ser	4.34
Glu	13.12 ± 0.30
Pro	2.80 ± 0.19
Gly	4.23 ± 0.11
Ala	8.21 ± 0.25
Cys	0.53 ± 0.34
Val	5.41 ± 0.12
Met	3.67 ± 0.40
Ile	3.98 ± 0.35
Leu	8.70 ± 0.17
Tyr	6.66 ± 0.35
Phe	3.48 ± 0.16
Lys	4.17 ± 0.31
His	1.13 ± 0.11
Arg	9.38 ± 0.51
	97.85%

precipitin line to hexamer does not appear in these studies (Plates 3b, 4d), since there is very little antibody to hexamer in anti-(*S. lividus*) serum. Sedimentation-velocity studies (Fig. 6) indicate that in the presence of 2M-NaCl at pH 6.0 in halophile phycocyanin, there are only monomer and hexamer, and in the absence of substantial antibody to hexamer, a single precipitin line for monomer would be predicted at all concentrations; indeed, in Plate 3c, serial dilution of *C. elabens* phycocyanin exhibits only one line. Right-angle immunodiffusion plates (Plate 4d)

also indicate that the predominant species is probably monomer.

Fluorescence polarization

Fluorescence polarization of halophile phycocyanin was studied in buffer solutions of various pH values in the presence or absence of 2M-NaCl. The results of fluorescence polarization at 620 nm (P_{620}) are summarized in Table 2. The extinction at 620 nm of the protein solution used was 0.2. The polarization of halophile phycocyanin increases with increasing pH of the solution. The magnitude of the fluorescence polarization of phycocyanin, P_{620} , has been demonstrated to be inversely related to the molecular size (MacColl *et al.*, 1971a; Vernotte, 1971) with the limiting P_{620} for the monomer equal to 0.39. For halophile, the lowest P_{620} value exists at pH 4.75. Sedimentation-velocity studies indicate that under these conditions at a concentration of approx. 0.05 mg/ml the sample is a mixture of hexamer (11S) and monomer (3S). As the pH of the sample is increased, the amount of hexamer is decreased and the amounts of monomer and trimer are substantially enhanced. Greater polarization is predicted on the basis of the presence of decreasing amounts of larger aggregates. The correlation between P_{620} values in the presence and absence of 2M-NaCl is not simple. These fluorescence measurements are made at very low concentrations and we have not determined the concentration dependence of aggregation under all these conditions. Correlations with Table 1 are inappropriate since these measurements are performed at 50 to 100 times the concentration of the fluorescence measurements. The general trend of increase in polarization with decreasing amounts of hexamer is consistent with the several published studies on phycocyanin (MacColl *et al.*, 1971a; Vernotte, 1971). The qualitative relationship cannot be translated into a quantitative relationship until results on the absorption and



EXPLANATION OF PLATE I

Ouchterlony double-diffusion studies of phycocyanin antigens from several algal sources

Immunodiffusion studies were done as described in the Experimental section in 1.5% Bacto-agar containing sodium phosphate buffer, *I* 0.1. Antigens from several algae, as well as antisera prepared with different antigens, were used. These experiments demonstrate that phycocyanin from *C. elabens* is antigenically related to phycocyanins from a unicellular thermophile (*S. lividus*) and a filamentous mesophile (*Ph. luridum*). Antigens P and P4 are phycocyanin from *Ph. luridum* at concentrations of 1 and 0.25 mg/ml respectively. Antigens H, H2 and H4 are phycocyanin from *C. elabens* at concentrations of 1, 0.5 and 0.25 mg/ml respectively. S is phycocyanin from *S. lividus* at a concentration of 1 mg/ml. In (a) the central well, 818, is antiserum to phycocyanin from *Pl. calothricoides* and in (b) the central well, Sab, is antiserum to phycocyanin from *S. lividus*.

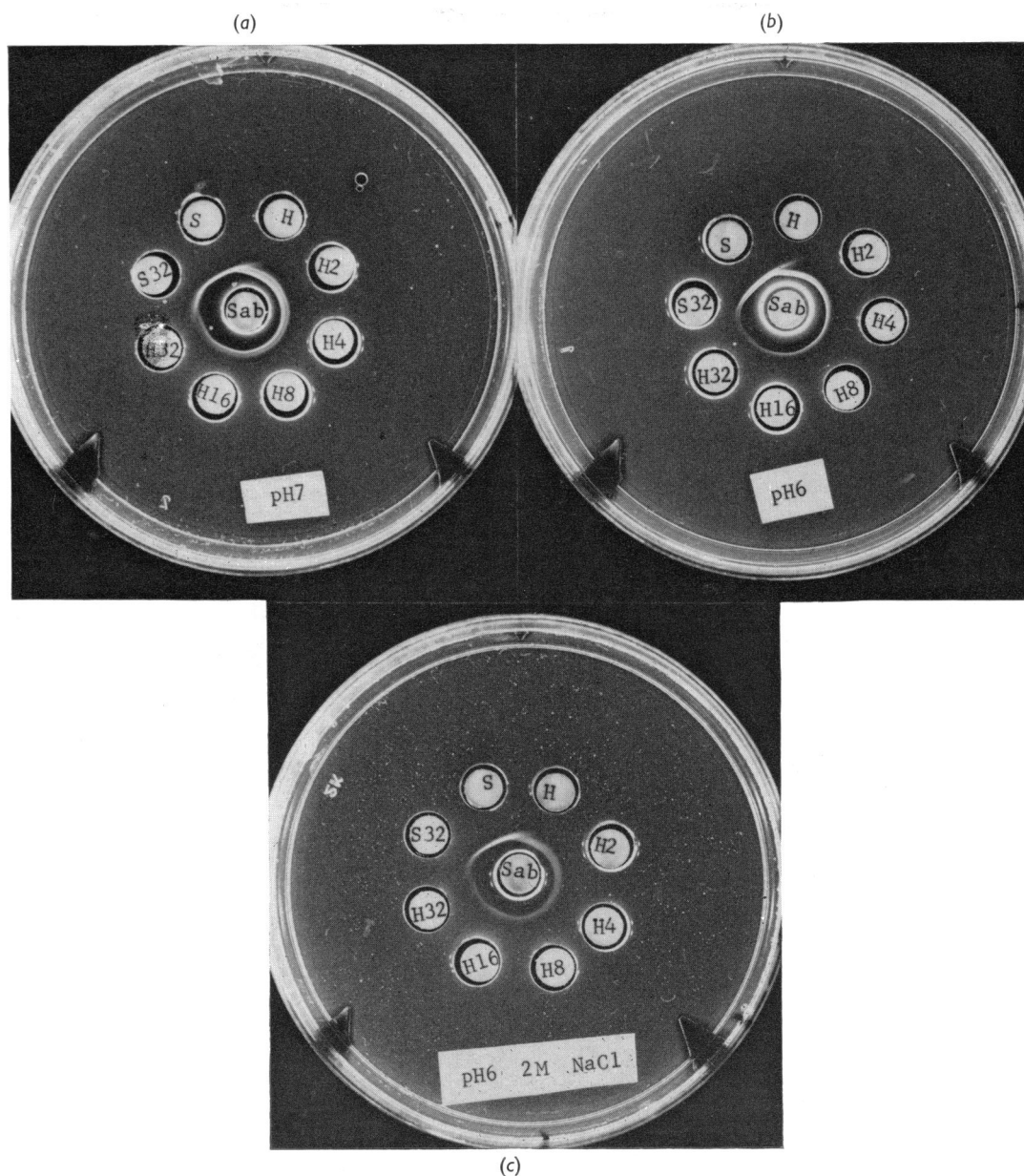


EXPLANATION OF PLATE 2

Ouchterlony double-diffusion studies as a function of pH and ionic strength

For details see the Experimental section. To test the presence of the several aggregating species (hexamer, trimer and monomer) in phycocyanin from *C. elabens*, and the relationship of these aggregates to aggregates in other phycocyanins, experiments were performed as a function of pH and ionic strength with protein concentrations in the range of those used in the sedimentation experiments of Figs. 5 and 6. In (a) the agar contains sodium phosphate buffer, *I* 0.1, pH6.0; in (b) and (c) sodium phosphate buffer, pH6.0, plus 2M-NaCl; in (d) sodium phosphate buffer, *I* 0.1, pH7.0. Sab is antiserum to *S. lividus* phycocyanin and pab is antiserum to *Pl. calothricoides* phycocyanin. Antigens H and H8 are phycocyanin from *C. elabens*, 1 and 0.12mg/ml respectively. Antigens P and P8 are phycocyanin from *Ph. luridum*, 1 and 0.12mg/ml respectively. Antigens P, H, H2 and S are identical with those in Plate 1(b). Phycocyanin from *S. lividus* usually contains less hexamer than phycocyanin from *Pl. calothricoides* and *Ph. luridum* (Berns & Scott, 1966) and antiserum to *S. lividus* contains less antibody to hexameric phycocyanin and more to monomer and trimer. This is demonstrated explicitly in Plate 4.

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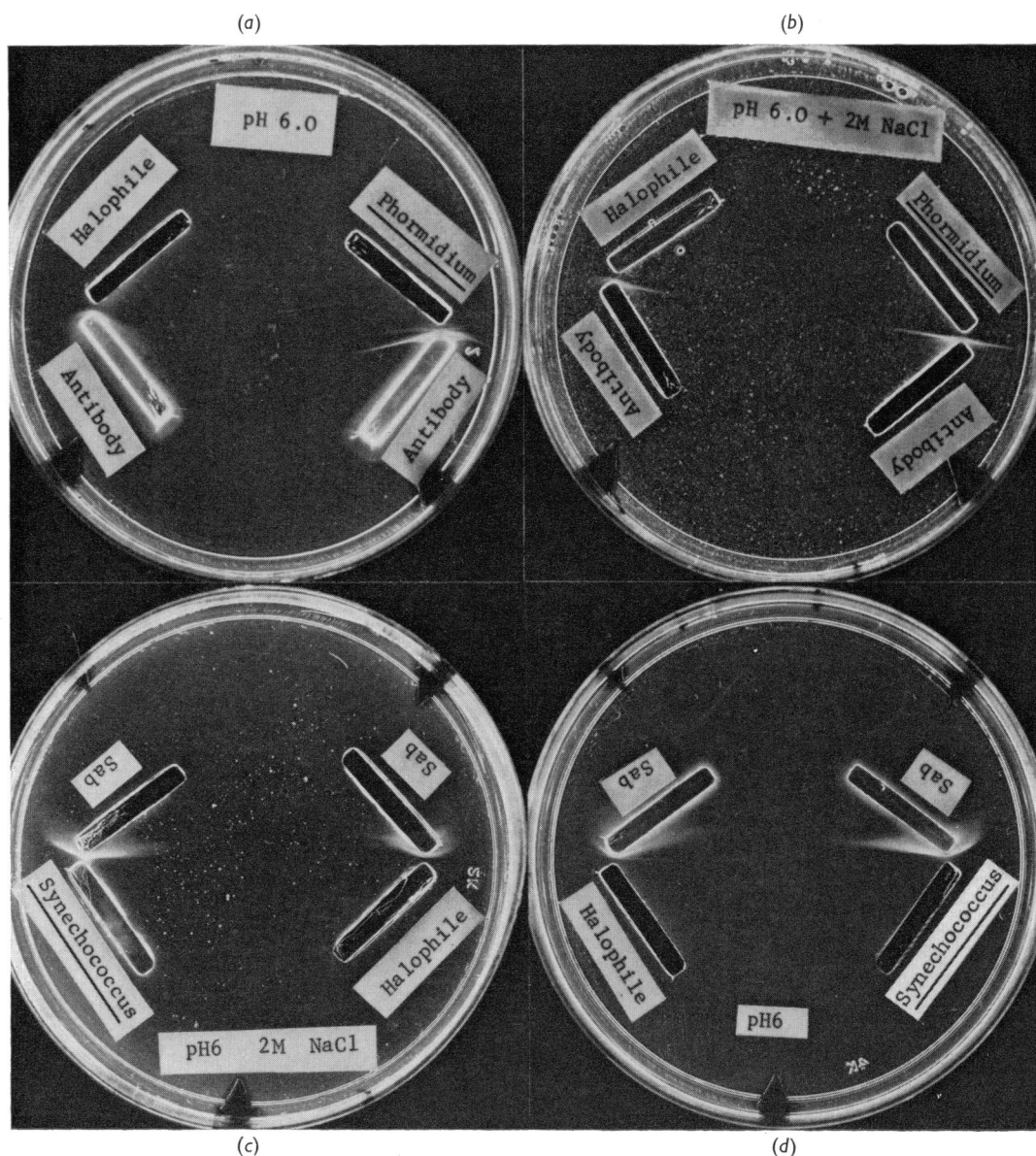


EXPLANATION OF PLATE 3

Ouchterlony double-diffusion dilution study

For details see the Experimental section. Decreasing the protein concentration decreases the relative amount of trimeric and hexameric phycocyanin in all phycocyanins. Immunodiffusion studies at decreasing phycocyanin concentrations demonstrate that the slower-diffusing antigens (hexamer and trimer) are not present at low concentrations and the fastest diffusing species (monomer) is predominant at low concentrations. Antiserum Sab is the same as those in Plates 1*b* and 2*d*. Antigens H, H2, H4, H8, H16 and H32 are all phycocyanin from *C. elabens* at concentrations of 1, 0.5, 0.25, 0.12, 0.06 and 0.03mg/ml respectively. Antigens S and S32 are phycocyanin from *S. lividus* at 1 and 0.03mg/ml respectively. The agar in (a) and (b) contains sodium phosphate buffer, 1 0.1, pH6.0; in (c) the agar contains sodium phosphate buffer, plus 2M-NaCl, pH6.0.

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EXPLANATION OF PLATE 4

Right-angle Ouchterlony double-diffusion studies

For details see the Experimental section. The determination of the diffusion coefficient of the major antigenic species in phycocyanin is an excellent indication of the size of the major species present. Right-angle immunodiffusion as a function of pH and ionic strength is used to determine these species. The diffusion coefficient is a function of the tangent of the angle the precipitating line makes with the antigen trough (Allison & Humphrey, 1960). The antigen ('Halophile') is phycocyanin from *C. elabens*; 'Phormidium' is phycocyanin from *Ph. luridum*; and 'Synechococcus' is phycocyanin from *S. lividus*. The antisera are Sab, which is antibody to *S. lividus* phycocyanin, and 'Antibody,' which is antibody to *Pl. calothricoides*. In (a) and (d), the agar contains sodium phosphate buffer, I 0.1, pH 6.0; in (b) and (c), sodium phosphate buffer, plus 2M-NaCl, pH 6.0. In (b), it is well documented that the major antigenic constituents in *C. elabens* phycocyanin and *Ph. luridum* phycocyanin have similar diffusion coefficients at pH 6.0, plus 2M-NaCl. This species in *C. elabens* is not demonstrated in (c) under identical conditions since antiserum to *S. lividus* phycocyanin does not contain appreciable amounts of antibody to hexamer.

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Table 4. *Amino acid composition of some phycocyanins*

All residue contents are based on molecular weight of approx. 30000 for the phycocyanin monomer (protein plus two chromophores). Values for *Plectonema calothricoides* and *S. lividus* are from Berns *et al.* (1964).

	<i>Pl. calothricoides</i>	<i>S. lividus</i>	<i>C. elabens</i> (halophile)
Polar residues			
Acidic			
Asp	28	28	30
Glu	18	30	29
	46	58	59
Basic			
Lys	10	11	9
His	2	2	2
Arg	14	19	17
	26	32	28
Hydroxy acids			
Thr	14	14	15
Ser	16	10	14
	30	24	29
Non-polar residues			
Aliphatic			
Gly	20	10	21
Ala	40	26	32
Val	16	17	15
Ile	14	16	10
Leu	22	23	22
Pro	8	10	8
Met	8	8	8
Cys	1	1	1
	129	111	117
Aromatic*			
Tyr	10	16	11
Phe	8	10	7
	18	26	18
Total residues	249	251	251

* Amino acid analysis by the toluenesulphonic acid (Liu & Chang, 1971) method of hydrolysis by J. Harris of this laboratory indicates that these phycocyanins have 1 tryptophan residue per 30000 molecular-weight unit.

fluorescence properties of monomer, trimer, hexamer and dodecamer are obtained.

Amino acid composition

The amino acid composition of halophile phycocyanin (*C. elabens*) was compared with a non-halophile (*Pl. calothricoides*) and a thermophile (*S. lividus*). The results are listed in Tables 3 and 4. Although the halophile phycocyanin contains a greater number of acidic and basic residues than that of *Pl. calothricoides*, 87 as against 72, the number of residues is comparable with that of the thermophilic *S. lividus*, 87 as against 90. The number of non-polar

residues in thermophile and halophile phycocyanin is comparable and is lower than that found in *Pl. calothricoides*. If the weight percent of ammonia (approx. 2% in both proteins) is taken as a measure of the amide content of both proteins, there is no detectable difference in amide groups.

Isoelectric focusing

The isoelectric point of the halophile phycocyanin was determined to be pH4.6. The pH gradient-absorption plot for this isoelectric focusing experiment is shown in Fig. 7. The sample collected from the pH gradient was examined immediately in the

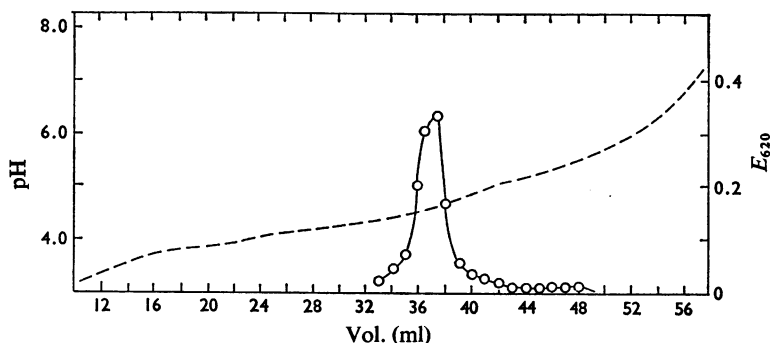


Fig. 7. Isoelectric focusing of phycocyanin from *C. elabens*

Details are given in the Experimental section. ----, pH of samples; —, E_{620} of samples. Both are plotted as a function of sample volume taken from the focused column.

analytical ultracentrifuge with the photoelectric scanner at 620 nm. The sedimentation results indicated the presence of 3S, 11S and 19S material at this pH value (4.6) and after dialysis with several changes of buffer to minimize the presence of sucrose, the same species were present. Phycocyanin from *Ph. luridum* also has an isoelectric point of pH 4.6 for hexamer (11S), but the monomer has an isoelectric point at a distinctly higher pH value, i.e., 4.8.

Discussion

The results given in the present study establish that the aggregates of C-phycocyanin from *C. elabens* are related to those reported for other C-phycocyanins (Bernis, 1971). The immunochemical results (Plates 1-4) demonstrate that C-phycocyanin from *C. elabens* is antigenically related to C-phycocyanin from a large number of other algae, and the aggregates in this C-phycocyanin are similar to, if not identical with, those reported for other C-phycocyanins. The spectral characteristics of this phycocyanin are also identical with those reported for other C-phycocyanins. The fluorescence polarization results from *C. elabens* (Table 2) are also analogous to those from other C-phycocyanins (MacColl *et al.*, 1971a; Vernotte, 1971) and reflect the relative amounts of aggregates determined from immunochemical and sedimentation experiments. It therefore seems quite logical to state that essentially the same states of aggregation exist for *C. elabens* phycocyanin as for other C-phycocyanins (Bernis, 1971), i.e., 3S (monomer), 6S (trimer), 11S (hexamer) and 19S (dodecamer). These stages of aggregation associated with these sedimenting species are consequently used in all discussions of the experimental results. It is conceivable that high salt concentrations could cause

significant changes in such factors as the partial specific volume; however, the immunochemical evidence indicates we are still dealing with the same state of aggregation of phycocyanin reported in previous studies (Bernis, 1971). The qualitative aspects of the aggregation process are relatively unaffected by the environmental stress of high salt concentration. The quantitative nature of the phycocyanin aggregation process is definitely affected in the halophilic organism. The general behaviour of the halophile phycocyanin in the presence of 2M-NaCl is analogous to that observed for non-halophile phycocyanin in the absence of salt (Figs. 3 and 4). *C. elabens* is an extremely salt-tolerant alga. Its maximal growth is not found at salt concentrations above 15%, but it does grow at higher salt concentrations (Fig. 2). The organism will also grow at low salt concentrations and at temperatures as high as 45°C. The behaviour of phycocyanin from *C. elabens* in the absence of added salt is similar to that observed for the thermophile phycocyanin from *S. lividus* (Bernis & Scott, 1966) and significantly different from *Pl. calothricoides* (Figs. 3 and 4). The pH-dependence of the halophile phycocyanin has shown that the higher aggregates (11S and 19S) are more stable in the presence of salt (Table 1). At pH 6.0, the relative percentage of 11S aggregate increases from 27 to 47% with the addition of 2M-NaCl to the buffer solution. The effect of salt is greater at pH 7.0, where the addition of 2M-NaCl increases the amount of 11S aggregate from 13 to 46%. The difference in dependence on pH and ionic strength of the aggregation process of phycocyanin in *Pl. calothricoides* and *C. elabens* can be examined in relation to possible changes in protein structure. The simplest indication of changes in protein structure is the gross amino acid analysis of the protein. If the stabilization of 11S hexamer is at least partially due to hydrophobic

interactions, as suggested by Scott & Berns (1965), Berns (1971), MacColl *et al.* (1971*a,b*), and Berns & Morgenstern (1968), the presence of large amounts of electrolyte in the vicinity of charged side chains decreases the competing electrostatic repulsion among them and also causes a decrease in solubility of non-polar groups in water (Kauzmann, 1959). Both factors tend to result in enhancement of the hydrophobic interaction responsible for formation of larger aggregates. At higher pH values, the acidic and basic side chains of all proteins are mostly in the charged state with a maximum effect of charge repulsion. With the non-halophile protein from *Pl. calothricoides*, the competition of charge-repulsion forces, resulting from the ionized group interaction, with the hydrophobic forces maintaining the hexamer structure, is not sufficient to perturb the hexamer structure. The results in Fig. 4 indicate that, in the presence of 2M-NaCl, the 11S hexamer is the dominant species up to at least pH 8.0. In the absence of added NaCl, the 6S trimer is dominant above pH 6.5. In contrast, in the halophile phycocyanin (Fig. 3) in the absence of added salt, the 6S trimer becomes dominant above pH 5.5 and the addition of 2M-NaCl only maintains the hexamer as the dominant species up to a pH of about 7.0. A larger number of charged groups in *C. elabens* phycocyanin (Table 4) could explain the decreased effectiveness of 2M-NaCl in maintaining the phycocyanin hexamer at high pH values. The number of glutamic acid and aspartic acid residues that are in fact glutamine and asparagine have not been determined, and for purposes of this discussion, we are essentially ignoring the potential effect of the amide groups and assume that no large differences in amide content exist between *Pl. calothricoides* and *C. elabens* phycocyanin.

Phycocyanin from non-halophilic *Pl. calothricoides* contains a large amount of 11S aggregates in the presence and absence of additional salt. Since this phycocyanin apparently has fewer acidic groups than the halophile phycocyanin (Table 4), the influence of charge repulsion on the quaternary structure is attenuated. Reistad (1970) found that proteins of the extremely halophilic bacteria have high contents of aspartic acid and glutamic acid and low contents of lysine and alanine compared with their non-halophilic counterparts. The number of aspartic acid and glutamic acid residues in the halophile phycocyanin is about 30% higher than in that of its non-halophile counterpart, *Pl. calothricoides* (Table 4). The increase in acidity of halophile protein could require more salt to neutralize its negative charges as well as to salt out hydrophobic groups. The isoelectric point of the halophile phycocyanin is not appreciably lower than the isoelectric point (approx. 4.6) for the hexamer (11S) species of several other phycocyanins. The important difference is that in *C. elabens* phycocyanin the isoelectric points of the

3S, 11S and 19S species are either identical or extremely close to one another. Phycocyanin from *Ph. luridum* exhibits an isoelectric point of 4.6 for 11S hexamer and 4.8 for monomer. The isoelectric points of the monomeric and higher aggregates are probably a more valuable measure of charge groups than the simple cataloguing of amino acid content.

One explanation for the lowering of the isoelectric point of monomeric phycocyanin is the presence of sizeable numbers of acidic amino acids. If the addition of acidic groups is accompanied by a small number of arginine residues, which would tend to minimize the change in the isoelectric point, the presence of a significant number of charged residues in specific locations is sufficient to cause greater electrostatic repulsion in the halophile protein.

A variety of enzymes of halophiles require a high concentration of Na⁺ (or other cations) for activity (Larsen, 1967; Lanyi & Stevenson, 1970). Some experimental evidence has shown that the intracellular salt concentration of the extreme halophiles is very high and reaches values comparable with that in the medium in which the cells are grown (Larsen, 1967). Therefore the cell must be faced with the problem of avoiding the precipitation of its intracellular proteins. Halophile proteins cannot be precipitated by ammonium sulphate in the presence of NaCl. This observation was reported for the enzyme malate dehydrogenase, but is also true for most of the proteins of the cell (Baxter, 1959; Holmes & Halvorson, 1965*a,b*). Our experimental evidence seems to agree with the suggestion that the halophile proteins have a higher negative charge and in this way avoid the precipitating effect of the salt (Brock, 1969).

Phycocyanin from the thermophilic alga, *S. lividus*, is similar to the halophile phycocyanin in content of acidic and basic amino acids residues and many aspects of the pH-dependence of aggregation (Scott & Berns, 1967). The immunochemical results also indicate a close antigenic relationship and confirm the similarity in aggregation processes. The information currently available is insufficient to suggest that the mechanism of thermophilic and halophilic adaptation for proteins is related, particularly since phycocyanin from *S. lividus* has a significantly greater number of aromatic residues. The growth of laboratory cultures of *C. elabens* at temperatures as high as 45°C has stimulated further investigation of possible similarity of the mechanism of adaptation for proteins derived from thermophilic and halophilic organisms.

The study of the concentration dependence of aggregation of the halophile protein in the presence of 2M-NaCl demonstrates that only monomer (3S) and hexamer (11S) are present at concentrations below 0.4 mg/ml (Fig. 6). In the absence of added salt at phycocyanin concentrations of 0.3–0.4 mg/ml, the 6S (trimer) is the dominant species. The addition

of salt completely inhibits the formation of trimer. This fact would appear to be another piece of evidence that under physiological conditions the hexamer is the basic aggregate that participates in formation of phycobilisomes.

There have been at least two attempts to explain the properties of enzymes isolated from halophilic sources (Baxter, 1959; Lanyi & Stevenson, 1970). The analysis of the properties of C-phycocyanin isolated from a halo-tolerant Cyanophyta provides results consistent with essential features of either explanation. Baxter (1959) emphasizes the importance of electrostatic interactions and the need for the high concentration of salt to neutralize repulsive forces, whereas Lanyi & Stevenson (1970) ascribe a greater significance to the salting-out effect of the high concentration of NaCl and its enhancement of hydrophobic interactions, and are specifically critical of shielding effects as the dominant contribution in the presence of 2M-NaCl. In the present work with C-phycocyanin, there is ample evidence that hydrophobic forces are of central importance in stabilizing the 11S hexamer structure (Scott & Berns, 1965; Berns, 1971; MacColl *et al.*, 1971b). It is important to consider that the dominant effect to be overcome in stabilizing the hexameric structure is that of charge repulsion, and at higher pH values the hydrophobic forces become less effective in this respect. Lieberman & Lanyi (1972), in investigating threonine deaminase from an extremely halophilic bacterium, have come to the conclusion that the effect of salt on the stability of the enzyme cannot be readily divided into charge-shielding and salting-out effects. The comparison of dependence of phycocyanin aggregation on pH and ionic strength in mesophile and halophile proteins also leads to this conclusion.

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