Properties of Subunits and Aggregates of Blue-Green Algal Biliproteins*

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ABSTRACT: A simple procedure is outlined for the purification of the biliproteins from a filamentous blue-green alga, *Fremyella diplosiphon*. Calibrated sodium dodecyl sulfate–acrylamide gel electrophoresis shows phycoerythrin and phycocyanin each to consist of two subunits having molecular weights of 20,000 and 18,300, and 17,600 and 16,300, respectively. Allophycocyanin consists of a single subunit with a molecular weight of 16,000. Disulfide linkages do not play a role in the subunit aggregation which occurs with each of the three biliproteins *in vitro* in the absence of denaturing agents. The heavy:light subunit stoichiometry of purified phycoerythrin is 1:1. Evidence is presented which indicates that the heavy and light phycoerythrin chains aggregate randomly into hexamers at pH 8.7 in 6 M urea during acrylamide gel electrophoresis. Alkylated phycoerythrin is resolved into two bands after gel electrophoresis at pH 3.5 in 8 M urea. The faster migrating band corresponds to the light band on sodium dodecyl sulfate gels, while the slower migrating band corresponds to the heavy band on sodium dodecyl sulfate gels. Absorption spectra (300–650 nm) of the two isolated alkylated phycoerythrin subunits are essentially identical in solutions containing 8 M urea, 0.2 M formic acid, and 0.08 M NaOH. Densitometric data suggest that two phycoerythrobilin molecules are bound to each heavy phycoerythrin subunit and that a single phycoerythrobilin molecule is bound to each light phycoerythrin subunit. Calibrated Sephadex chromatography indicates that the 10S phycoerythrin aggregate has a molecular weight of 180,000–210,000; it is either a decamer or a dodecamer. Dilution of concentrated phycoerythrin results in the diminution of an absorption band centered at 573–574 nm; it is shown that the 10S phycoerythrin aggregate has an absorption band centered at 573–574 nm with a high extinction relative to smaller aggregates.

In addition to chlorophyll and carotenoids, blue-green algae contain phycocyanin, allophycocyanin, and, in many cases, phycoerythrin. Action spectra for photosynthetic oxygen evolution and for the excitation of chlorophyll fluorescence indicate that these bile pigment–protein complexes function as accessory pigments (Haxo and Blinks, 1950; Duysens, 1952). The biliproteins occur *in vitro* as aggregates of various sizes, the relative proportions of which vary as a function of pH, ionic strength, and concentration (Ó hEocha, 1965). The molecular weights of the largest aggregates which are commonly observed *in vitro* have been estimated to be 278,000 for C-phycocyanin (Hattori *et al.*, 1965), 134,000 for allophycocyanin, and 226,000 for C-phycoerythrin (Hattori and Fujita, 1959). The biliproteins may aggregate more extensively *in vivo* to form discoid structures ("phycobilisomes") which assume a periodic spacing adjacent to the photosynthetic lamellae (Gantt and Conti, 1969).

No evidence has been available relating to the nature of the monomeric units from which the aggregate forms of C-phycoerythrin and allophycocyanin are constructed. On the other hand, conflicting estimates of the size of monomeric C-phycocyanin have appeared. The smallest C-phycocyanin aggregates in the ultracentrifuge have been assigned molecular weights of 28,000 (Scott and Berns, 1965) and 46,000 (Hattori *et al.*, 1965). Calibrated gel filtration experiments (Neufeld and Riggs, 1969) and calibrated sodium dodecyl sulfate–acrylamide gel electrophoresis (Kao and Berns, 1968) have indicated a monomer molecular weight of 30,000, whereas amino acid analyses of purified phycocyanin yield minimum molecular weights (assuming one histidine residue and one bilin chromophore per mole) of 15,700 (Berns *et al.*, 1963) and 13,200 (Raftery and Ó hEocha, 1965).

Weber and Osborn (1969) have demonstrated that many well-characterized aggregating proteins are converted into their monomeric forms after incubation in sodium dodecyl sulfate–mercaptoethanol solutions and that, over a wide molecular range, there is a linear relationship between the log of the monomer molecular weight and electrophoretic mobility in sodium dodecyl sulfate–acrylamide gels. A firmer theoretical basis for this log–linear behavior has recently been provided by Reynolds and Tanford (1970). These authors showed a variety of sodium dodecyl sulfate–protein complexes to have similar conformations and that an essentially constant mass of sodium dodecyl sulfate is bound per unit mass of protein at monomeric sodium dodecyl sulfate concentrations exceeding 5×10^{-4} M. In spite of its demonstrated value, the sodium dodecyl sulfate–gel technique must be employed with caution, since Pringle (1970) has pointed out that incubation of proteins in sodium dodecyl sulfate solutions can activate trace quantities of proteolytic enzymes which, in turn, can generate artifacts.

The objectives behind the experimental work presented in this communication were threefold: (1) to devise a simple procedure for the purification of phycoerythrin, phycocyanin, and allophycocyanin from a filamentous blue-green alga; (2) to establish the subunit composition of each biliprotein and to determine whether or not the protein components of the three biliproteins from a single blue-green alga are different; and (3) to examine several fundamental physical properties of C-phycoerythrin, including its subunit stoichiometry, the chromophore distribution between its subunits, and the way its visible absorption spectrum is influenced by the extent to which its subunits aggregate.

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All of the experimental results reported have been duplicated with two different batches of purified pigments.

Experimental Section

Sources and Cultivation of the Algae. Fremyella diplosiphon (strain 481) was obtained in axenic culture from the Indiana Culture Collection (Starr, 1964) and grown in blue-green medium "C" of Kratz and Myers (1955), which was adjusted to pH 7.0 with 1 N NaOH prior to autoclaving.

Large quantities of cells for biliprotein extraction were obtained by inoculating 14-l. New Brunswick Microferm fermenter vessels containing 11 l. of medium with 1 l. of fluorescent light illuminated dense culture. Fermenter vessels were illuminated with semicircular New Brunswick fluorescent light manifolds; cells were cultured at 32°, agitated at 300 rpm, aerated with 5 l. of air/min, and harvested after 1 week by vacuum filtration onto Whatman No. 1 filter paper.

Cultures (1 l.) of cells for preparation of crude extracts of *F. diplosiphon* and *Tolypothrix tenius* were grown on shakers at 30° in 2800-ml fernbach flasks illuminated with fluorescent lamps (Sylvania F30 T12-CW-RS) at an incident intensity of 250 ft-candles. Cell cultures (1 l.) were harvested by centrifugation. Phycoerythrin-less *F. diplosiphon* was obtained by interposing a red cast acrylic filter (Rohm & Haas Red Transparent 2423) between the fluorescent light source and the culture flasks. *T. tenuis* was the same strain as that used by Hattori and Fujita (1959).

Materials. Acrylamide and methylenebisacrylamide (MBA[1]) (Eastman) were recrystallized following the procedures of Loening (1967). *N,N,N',N'*-Tetramethylethylenediamine (Temed) (Eastman) was used without additional purification. Sodium lauryl sulfate (Fisher) and urea (Merck) were recrystallized from 80% ethanol. Guanidine hydrochloride was recrystallized from methanol.

Sephadex G-100 (lot TO 2692) was prepared according to instructions provided by Pharmacia. The procedure outlined in Whatman Data Manual 2000 was followed in the preparation of DEAE-cellulose (Microgranular DE 52). Brushite, prepared by the method of Siegelman *et al.* (1965) and subsequently washed with distilled water and incubated in 20 volumes of 10 mM K_2HPO_4, was kindly provided by Professor Winslow Briggs.

Catalase, β-amylase, fumarase, aldolase, LDH (type II), transferrin, bovine serum albumin, ovalbumin, deoxyribonuclease I, myoglobin, and cytochrome *c* were obtained from Sigma. Glutamate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase were obtained from Calbiochem. Ribonuclease A was a Worthington product, and papain was obtained from Nutritional Biochemicals.

Coomassie brilliant blue R 250 was obtained from Colab Laboratories, Inc., and fast green was obtained from Allied Chemicals.

Biliprotein Purification. All steps in the purification were carried out at approximately 4°. Cells (50 g wet wt) were suspended in 50 ml of 1 M sodium acetate (pH 5.0). Portions (20 ml) of the slurry were transferred to 60-ml Beckman No. 325609 cellulose nitrate tubes and each was sonicated (at maximum power output with the microtip of a Heat Systems Model W185D Sonifier cell disruptor) for a total of 6 min. The sonicates were pooled, stirred for 30 min, and centrifuged at 81,000*g* for 1 hr. The procedure was repeated with the pellets and the supernatants from both centrifugations were then pooled. (Crude extracts for sodium dodecyl sulfate gels were prepared by similar sonication and high-speed centrifugation of thick cell slurries in 0.1 M sodium phosphate, pH 7.0.)

The volume of the pooled supernatant solutions was adjusted by dilution with acetate buffer so that the final solution had an OD_{280} of 4.5. The solution was brought to 35% saturation with ammonium sulfate, left to stand for 90 min, and centrifuged at 16,000*g* for 15 min. (These centrifugation conditions were employed in all subsequent ammonium sulfate steps.) The supernatant was brought to 70% saturation with ammonium sulfate, left standing for 1 hr, and centrifuged. Both the phycoerythrin-rich pellet of the 0–35% ammonium sulfate fraction and the phycocyanin- and allophycocyanin-rich pellet of the 35–70% fraction were resuspended with 6 ml of 0.1 M acetate (pH 5.0) and dialyzed against 2 l. of the same buffer overnight.

The dialyzed 0–35 and 35–70% ammonium sulfate fractions were each passed through a 2.5 × 40 cm G-100 column which had been preequilibrated with 0.1 M acetate buffer (pH 5.0). (Under these solvent conditions, the biliproteins exist primarily as large aggregates which are eluted near the void volume, leaving smaller molecular weight contaminants behind.) After each run, the peak tubes from the early-eluting biliprotein bands were pooled. The phycoerythrin solution was brought to 30% saturation and the phycocyanin–allophycocyanin solution was brought to 70% saturation with ammonium sulfate, and both were allowed to stand for 1 hr prior to centrifugation. The pellets were resuspended in 0.005 M potassium phosphate (pH 7.0) and then dialyzed overnight against 4 l. of phosphate buffer.

The concentrated phycoerythrin solution from the pooled G-100 fractions was applied to a 2.2 × 48 cm DEAE-cellulose column, preequilibrated with 0.005 M potassium phosphate (pH 7.0). After elution with one column volume of starting buffer, the column was developed with a 550-ml linear 0.005–0.2 M, pH 7.0, potassium phosphate gradient. The peak tubes from the major phycoerythrin band eluting at about 0.05 M potassium phosphate were pooled, brought to 30% saturation with ammonium sulfate, permitted to stand for 30 min, and centrifuged. The pellet (purified phycoerythrin) was resuspended in a small volume of 0.1 M sodium phosphate (pH 7.0), 0.01% in sodium azide, and dialyzed against the same buffer.

The concentrated phycocyanin–allophycocyanin solution from the pooled G-100 fractions was applied to a 2.5 × 25 cm brushite column, preequilibrated with 0.005 M potassium phosphate (pH 7.0). One column volume of starting buffer was eluted, and then a 400-ml linear 0.005–0.1 M, pH 7.0, potassium phosphate gradient was developed. Under these conditions, phycocyanin is eluted from the column and allophycocyanin is retained at the top of the brushite bed. Peak tubes from the phycocyanin fractions were pooled, brought to 65% saturation with ammonium sulfate, and allowed to stand for 1 hr. That portion of the brushite bed containing allophycocyanin was removed with a spatula and extracted with 0.5 M potassium phosphate (pH 7.0). The allophycocyanin solution was then brought to 75% saturation with ammonium sulfate and left to stand overnight before centrifugation. The purified phycocyanin and allophycocyanin pellets were resuspended in small volumes of 0.1 M sodium phosphate (pH 7.0), 0.01% in sodium azide, and dialyzed against the same buffer. All of the purified biliproteins were stored at 4° in the dark.

Absorption Spectra. Absorption spectra were obtained with a Cary Model 14 recording spectrophotometer.

[1] Abbreviations used are: MBA, methylenebisacrylamide; Temed, *N,N,N',N'*-tetramethylethylenediamine; FMN, riboflavin 5'-phosphate (sodium dihydrate).

Sodium Dodecyl Sulfate–Acrylamide Gel Electrophoresis. The procedure of Weber and Osborn (1969) was employed with several modifications: (a) gels were polymerized with a 12-mg/ml ammonium persulfate solution, (b) the bromophenol blue tracking dye concentration was increased tenfold, (c) gels were run at 5 mA/tube to avoid bowing, and (d) gels were stained for 5 hr with an equivolume mixture of 0.25% coomassie blue in 7.5% acetic acid, 5% methanol, and of 0.25% coomassie blue in 5:5:1, acetic acid–water–methanol, both of which had been gravity filtered through Whatman No. 1.

Molecular weights of the biliprotein subunits were estimated from their measured mobilities and the equation for the least-squares line derived from the measured mobilities and known molecular weights of the standard proteins.

Acrylamide Gel Electrophoresis at pH 8.7 in 6 M Urea. Unpolymerized gel solutions contained 7.5% (w/v) acrylamide, 0.2% (w/v) MBA, 0.0675% (v/v) Temed, 6 M urea, 0.06 N HCl, 0.375 M Tris, 1.14×10^{-4} M potassium ferricyanide, and 1.5×10^{-5} M FMN. Gels (10×0.6 cm) were prepared by photopolymerizing the gel solution in 13×0.6 cm i.d. lime glass tubes with a Sylvania "cool white" fluorescent lamp (F-20T12-CW) at a distance of 4 cm for 30 min. Prior to and during photopolymerization, the tops of the gel solutions were layered with electrode buffer (0.01 M Tris—0.0767 M glycine) which was 5 M in urea. After polymerization, the gel tops were rinsed with 5 M urea–electrode buffer and the upper 3 cm of the tubes were filled with electrode buffer which was 6 M in urea.

Purified phycoerythrin in an 8 M urea–10 mM dithiothreitol solution was immersed in a boiling-water bath for 3 min. The solution (25 μl, cooled to 23°) was then layered on top of each of 24 gels, under the 6 M urea–electrode buffer solution. Electrophoresis (2.5 mA/tube) was conducted at 4° in the dark for 6 hr. After electrophoresis, several of the gels were fixed in 10% trichloroacetic acid, stained for 5 hr in 0.25% fast green in 7% acetic acid, and destained in 5% methanol–7.5% acetic acid solution. Colored bands from the remaining gels were excised with a razor blade and homologous bands were pooled into small beakers and eluted for 2 days at 4° in the dark into electrode buffer containing 8 M urea. The eluates were then dialyzed against distilled water and lyophilized. The lyophilized samples were analyzed on sodium dodecyl sulfate gels according to procedures already described.

Acrylamide Gel Electrophoresis at pH 3.5 in 8 M Urea. Unpolymerized gel solutions contained 7.5% (w/v) acrylamide, 0.25% (w/v) MBA, 0.5% (v/v) Temed, 8 M urea, 0.05 M formic acid, 0.01 N NaOH, and 1.5×10^{-5} M FMN. Gels (10×0.6 cm) were prepared by photopolymerization as described for the high pH urea gel system. Prior to and during polymerization, the tops of the gel solutions were overlayered with electrode buffer (0.2 M formic acid,–0.08 M NaOH) which was 6 M in urea. After polymerization, the gel tops were rinsed with 6 M urea–electrode buffer solution and the upper 3 cm of the tubes was filled with 7 M urea–electrode buffer solution.

Lyophilized alkylated phycoerythrin was dissolved in 8 M urea–electrode buffer to a concentration of 4 mg/ml and allowed to stand in the dark at room temperature for 4 hr. Twenty microliters was then layered on top of each gel, under the 7 M urea–electrode buffer solution. Electrophoresis (2.5 mA/tube, cathode at the bottom) was conducted in the dark at 4° for 20 hr. After electrophoresis, several of the gels were fixed, stained, and destained as described for sodium dodecyl sulfate gels. The two red bands of the remaining gels were excised. Homologous bands were pooled after quartering and then eluted for 24 hr at 4° in the dark into 8 M urea–0.08 M NaOH–0.2 M formic acid (6 ml for 22 gel disks). The eluates were removed with a Pasteur pipet and centrifuged at 4° for 10 min at 3000*g* to remove small acrylamide particles. The absorption spectra of the supernatants were determined, and the supernatants were then dialyzed against 0.2 M acetic acid, lyophilized, and analyzed on sodium dodecyl sulfate gels.

Fresh concentrated urea solutions were mixed with Amberlite MB-1 resin (Mallinckrodt) and filtered through Whatman No. 1 before use in the phycoerythrin alkylation and the high and low pH urea gel experiments.

Biliprotein Estimation. Biliprotein concentrations were determined by the method of Lowry *et al.* (1951). Standard curves were obtained with cytochrome *c*.

Alkylation of Phycoerythrin. Purified phycoerythrin was dialyzed against distilled water and lyophilized. The pH of an 8 M urea–10 mM mercaptoethanol solution was adjusted to 8.1 by the addition of 0.5 M Tris and the solution was used to resuspend the lyophilized phycoerythrin to a concentration of 2 mg/ml. The solutions were then capped and left to stand in the dark at room temperature for 5 hr. An 8 M urea solution containing 6.7 mg of iodoacetamide (Calbiochem)/ml was adjusted to pH 8.1 by the addition of 0.5 M Tris and 1 ml of this solution was added for each ml of the incubated phycoerythrin solution (8 M urea, pH 8.1 solution was added to unalkylated control samples). The mixtures were capped and placed in the dark at room temperature, and the reaction was allowed to proceed for 35 min. The solutions were then dialyzed exhaustively in the dark at 4° against 0.2 M acetic acid and the dialysates were lyophilized.

The above reagent ratios give a 60-fold molar excess of iodoacetamide over cysteine residues, assuming that *F. diplosiphon* phycoerythrin has the same amino acid composition as *P. cruentum* phycoerythrin. The amino acid composition of *P. cruentum* phycoerythrin has been determined by M. A. Raftery and C. Ó hEocha (1958).[2]

Densitometry of Acrylamide Gels. Densitometric analysis of stained acrylamide gels was performed with a Gilford Instruments linear transport device. Gels were scanned with a 0.05-mm slit in a solution having approximately the same refractive index (9% sucrose in 5% methanol–7.5% acetic acid) as the gels. Gels stained with coomassie blue were scanned at 620 nm and those stained with fast green were scanned at 630 nm. Unstained low pH urea gels of alkylated phycoerythrin were immersed in 8 M urea–0.2 M formic acid–0.08 M NaOH solutions and scanned at 545 nm.

Calibrated Sephadex Chromatography. Sephadex G-200 (lot 5563) was hydrated in 0.1 M sodium phosphate (pH 7.0)–0.04% in sodium azide and the fines were removed by decantation. A 70×0.9 cm column was poured and equilibrated with 0.1 M sodium phosphate (pH 7.0) 0.04% in sodium azide for 1 week. The column was equilibrated and run at room temperature (approximately 23°) under an 18-cm pressure head.

β-Amylase, aldolase, lactate dehydrogenase, transferrin, bovine serum albumin, and ovalbumin were detected by their absorbancies at 280 nm. Catalase and phycoerythrin were detected by their absorbancies at 406 and 560 nm, respectively. Fumarase was assayed by the method of Racker (1950). Void volumes were determined by monitoring the Blue Dextran absorbance at 620 nm. Blue Dextran was omitted in the bovine serum albumin run.

[2] Unpublished data cited in Ó hEocha (1960).

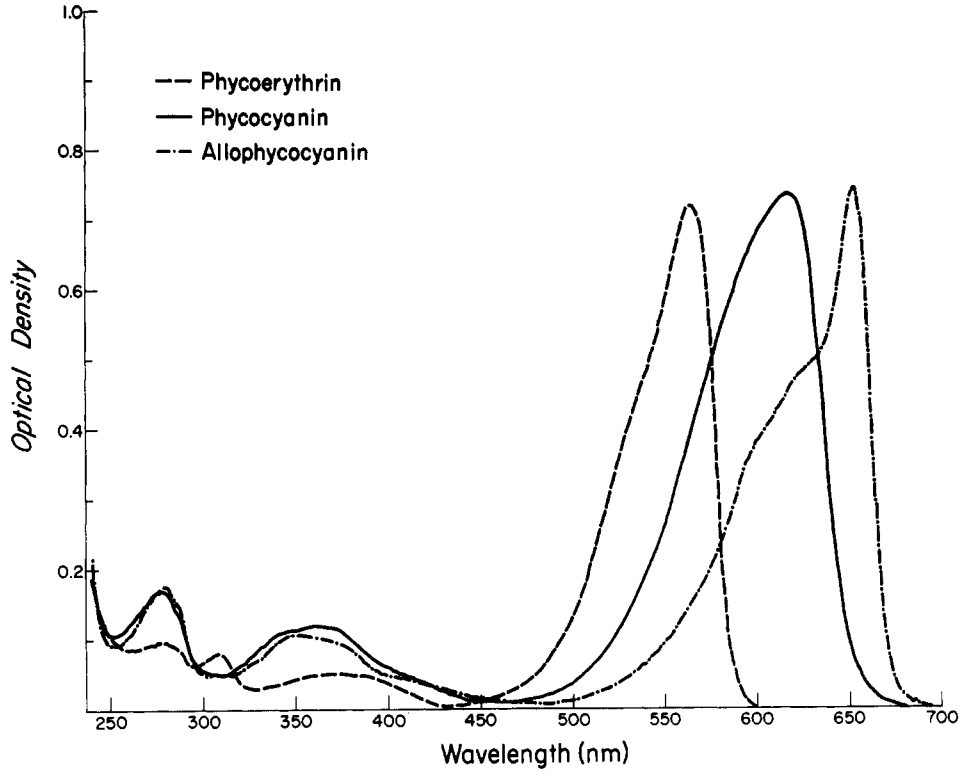

FIGURE 1: Absorption spectra of purified biliproteins of *F. diplosiphon* in 0.1 M sodium phosphate (pH 7.0). Path length for p hycoerythrin and phycocyanin is 1 cm; path length for allophycocyanin is 2 mm.

Data were treated according to Andrews (1964), although masses, rather than volumes, of column effluent were measured. The molecular weight of the large C-phycoerythrin aggregate was estimated from its V_e/V_0 ratio and the least-squares equation derived from the known molecular weights of the standard proteins and their measured V_e/V_0 ratios.

Difference Spectroscopy. For the dilute *vs.* concentrated difference spectra, base lines were set on the Cary spectrophotometer with 10 cm of buffer in the sample beam and a 1-mm cuvet with buffer in the reference beam. After the base line was set, concentrated phycoerythrin (2.24 mg/ml) in 0.1 M sodium phosphate (pH 7.0) was placed in the 1-mm cell, dilute phycoerythrin (0.0224 mg/ml) in 0.1 M sodium phosphate (pH 7.0) was placed in the 10-cm light path, and the visible difference spectrum was then determined.

Absorption spectra of phycoerythrin G-200 fractions in the 200,000, 100,000, and 45,000 molecular weight regions were obtained after the 200,000 and 100,000 molecular weight fractions were diluted with column buffer so that their 564-nm absorbancies were within 5% of that of the 45,000 molecular weight fraction. The spectra of the 100,000 and 45,000 molecular weight fractions were then normalized to that of the 200,000 fraction by multiplying them by the factor which was required to make their 564-nm absorbancies identical with that of the 200,000 molecular weight fraction. Difference spectra were computed manually by subtracting the spectrum of the 200,000 molecular weight fraction from normalized spectra of the 100,000 and 45,000 molecular weight fractions.

TABLE I: Subunit Molecular Weights of *F. diplosiphon* Biliproteins.

Biliprotein	Subunit	Mol Wt
Phycoerythrin	Heavy	20,000 ± 400
	Light	18,300 ± 400
Phycocyanin	Heavy	17,600 ± 400
	Light	16,300 ± 400
Allophycocyanin		16,000 ± 400

Results

Absorption Spectra. Absorption spectra of the purified biliproteins are shown in Figure 1. The 564/280, 620/280, and 650/280 absorption ratios of phycoerythrin, phycocyanin, and allophycocyanin were 7.5, 4.3, and 4.2, respectively, in 0.1 M sodium phosphate (pH 7.0). The allophycocyanin spectrum contains shoulders at approximately 598 and 629 nm which are not present in other published allophycocyanin spectra (Hattori and Fujita, 1959; Craig and Carr, 1968).

Absorption spectra of freshly prepared crude extracts of *F. diplosiphon* and *T. tenuis* in 0.1 M sodium phosphate (pH 7.0) are presented in Figure 2. Peaks or shoulders near the absorption maxima of phycoerythrin, phycocyanin, and allophycocyanin are evident in the *T. tenuis* and red *Fremyella* spectra. Blue-green *Fremyella* does not contain detectable quantities of phycoerythrin.

Sodium Dodecyl Sulfate. Figure 3a illustrates a coomassie blue stained sodium dodecyl sulfate gel containing phycoerythrin and several standard proteins. The results of calibrated sodium dodecyl sulfate–acrylamide gel electrophoresis are presented in Figure 3b, and molecular weight estimates for the biliprotein subunits are summarized in Table I.

Figure 4 illustrates a series of sodium dodecyl sulfate gels

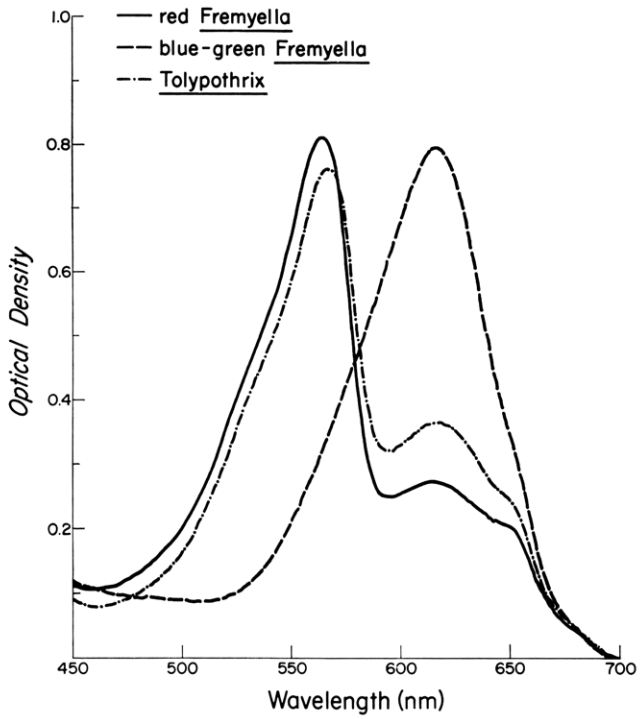

FIGURE 2: Absorption spectra of crude extracts of *F. diplosiphon* and *T. tenuis*. Red *Fremyella* and *T. tenuis* were grown under fluorescent illumination, whereas blue-green *Fremyella* was grown under a light source restricted to wavelengths above 595 nm in the visible region.

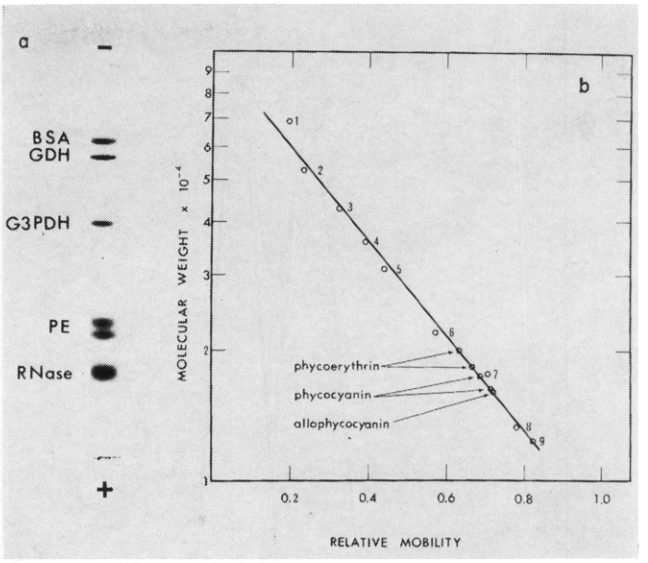

FIGURE 3: Studies with sodium dodecyl sulfate gel. (a) Sodium dodecyl sulfate gel containing several marker proteins and purified phycoerythrin from *F. diplosiphon*. Bands (from cathode to anode) are bovine serum albumin, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, heavy and light phycoerythrin subunits, and ribonuclease A. (b) Calibrated sodium dodecyl sulfate gel estimation of the subunit molecular weights of the biliproteins from *F. diplosiphon*. Standard proteins are: (1) bovine serum albumin, 69,000 (Tanford, 1969); (2) glutamate dehydrogenase, 53,000 (Eisenberg and Tomkins, 1968); (3) ovalbumin, 43,000 (Castellino and Barker, 1968); (4) glyceraldehyde 3-phosphate dehydrogenase, 36,000 (Davidson *et al.*, 1967); (5) DNase I, 31,000 (Lindberg, 1967); (6) papain, 22,100 (Light *et al.*, 1964); (7) myoglobin, 17,800 (Edmunson and Hirs, 1962); (8) RNase A, 13,700 (Smyth *et al.*, 1963); and (9) cytochrome *c*, 12,500 (Margoliash *et al.*, 1962).

which were run in parallel and which contain purified *F. diplosiphon* biliproteins and crude extracts of *F. diplosiphon* and *T. tenuis*. A three-banded pattern is obtained on stained gels of crude extracts of "red" *Fremyella* and *T. tenuis* and of mixtures of all three purified biliproteins because the sodium dodecyl sulfate gel system employed here is not capable of resolving the low molecular weight phycoerythrin subunit from the high molecular weight phycocyanin subunit or the low molecular weight phycocyanin subunit from the allophycocyanin subunit. Prior to staining, crude extracts of red *Fremyella* and *T. tenuis* and mixtures of all three purified biliproteins give a characteristic color pattern consisting of an upper (relatively slow migrating) red band, a faster migrating red band overlapping with a blue band, and a faster migrating blue band. It is evident that the biliprotein subunit numbers and molecular weights are the same in *F. diplosiphon* and *T. tenuis*. Unstained gels of crude extracts of blue-green *Fremyella* show two blue bands and after staining illustrate the absence of phycoerythrin.

Results identical with those pictured in Figure 4 are obtained (a) when the purified biliproteins are incubated in sodium dodecyl sulfate in the absence of 2-mercaptoethanol and 2-mercaptoethanol is eliminated from the application solution, and (b) when the purified pigments and crude extracts are injected into 6 M guanidine hydrochloride at 100°, dialyzed into 8 M urea, and subsequently processed by the standard sodium dodecyl sulfate gel technique. These results strongly argue against: (1) the involvement of disulfide linkages in the subunit aggregation of phycoerythrin, phycocyanin, or allophycocyanin; (2) degradation of the pigments during their purification; and (3) a sodium dodecyl sulfate induced proteolytic origin for the multiple subunits of phycoerythrin and phycocyanin. Pringle (1970) has shown guanidine hydrochloride pretreatment of protein solutions to be an effective deterrent to sodium dodecyl sulfate induced proteolytic activity.

Sodium Dodecyl Sulfate Gel Densitometry. A densitometer trace of the phycoerythrin bands on a coomassie blue stained

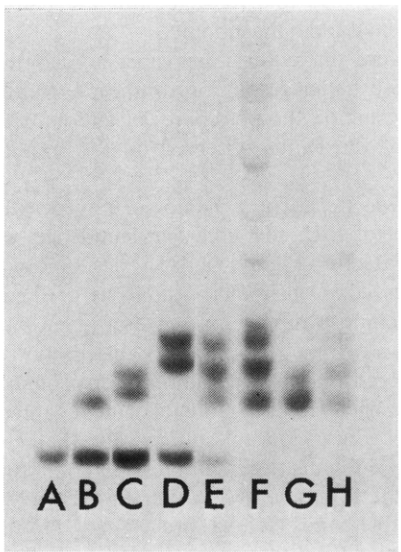

FIGURE 4: Sodium dodecyl sulfate gels of purified *F. diplosiphon* biliproteins and blue-green algal crude extracts: A–E: cytochrome *c* marker; B: allophycocyanin; C: phycocyanin; D: phycoerythrin; E: mixture of all three biliproteins; F: red *Fremyella* crude extract; G: blue-green *Fremyella* crude extract; H: *T. tenuis* crude extract.

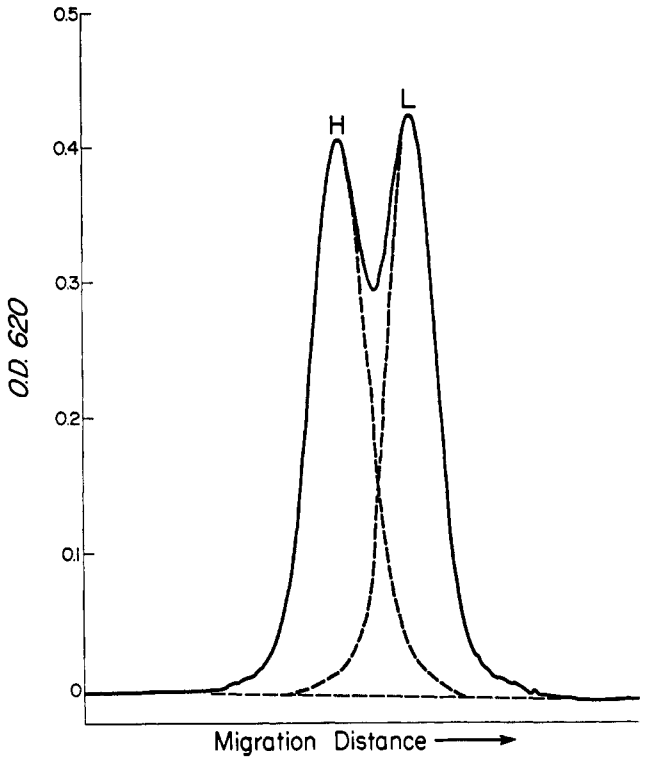

FIGURE 5: Densitometric scan of the phycoerythrin bands on a sodium dodecyl sulfate gel.

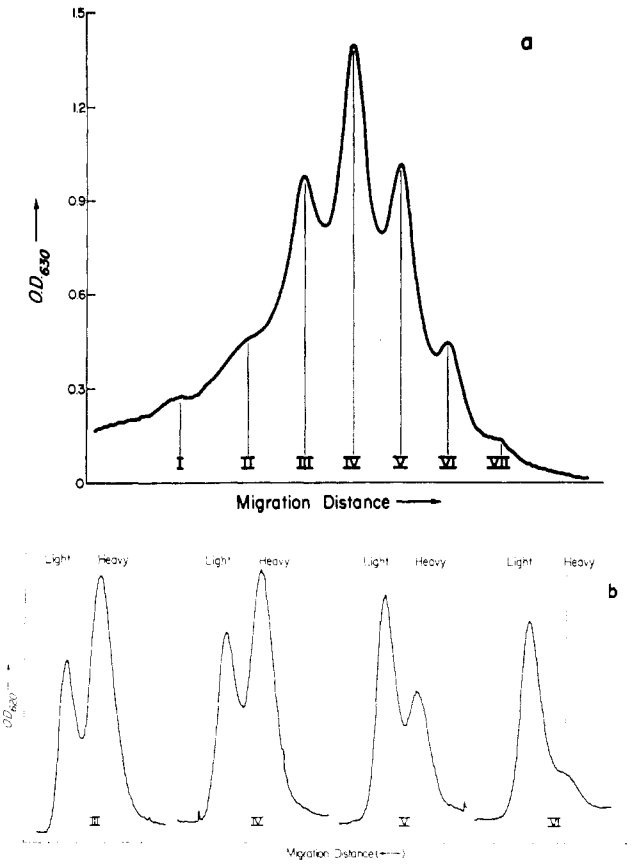

FIGURE 6: Densitometric scans. (a) At 630 nm of the phycoerythrin bands on a fast green stained pH 8.7, 6 M urea–acrylamide gel. Migration was toward the anode. (b) At 620 nm of coomassie blue stained sodium dodecyl sulfate gels containing the isolated phycoerythrin contents of bands III through VI from the pH 8.7, 6 M urea gels.

sodium dodecyl sulfate is presented in Figure 5. It is evident that the two bands are not totally resolved, although they appear to be present in roughly equivalent amounts. It was assumed that there was no overlap on the high molecular weight half of the heavy subunit peak or on the low molecular weight half of the light subunit peak. These regions of the peaks were cut out above the base line and integrated by weighing, and the ratio of their areas was determined after multiplying the heavy peak area by 0.935 to compensate for the molecular weight difference between the two chains. The heavy:light ratio varied from 0.93 to 0.96, suggesting a 1:1 stoichiometry between the heavy and light subunits.

Attempts were made to utilize urea–acrylamide gel systems to achieve better resolution of the phycoerythrin subunits and to examine the possibility of charge heterogeneity within each of the bands observed on the sodium dodecyl sulfate gels.

High pH Urea Gels. The behavior of phycoerythrin under the conditions of high pH–urea acrylamide gel electrophoresis is illustrated in Figure 6a. A series of evenly spaced bands is observed. Densitometric analysis of the fast green stained gels demonstrates the presence of seven bands which are present in a symmetrical intensity distribution. Since the bands are colored red prior to staining for protein, it is possible to cut out each band, to elute its protein contents, and to determine on sodium dodecyl sulfate gels whether or not it contains heavy subunits, light subunits, or a mixture of both heavy and light subunits. When the sharpest and most accurately excisable bands (III–VI) are examined in this fashion (Figure 6b), it is found that they *all* contain both heavy and light subunits, but that the heavy:light ratio decreases progressively with increasing electrophoretic mobility.

Densitometric analyses of purified phycoerythrin on sodium dodecyl sulfate gels suggest a 1:1 stoichiometry for the heavy and light phycoerythrin chains. Two electrophoretically distinct species assorted randomly into stable hexameric combinations would give rise to seven electrophoretically distinct species. If the mobilities of the aggregate species were determined solely by their net charge, they would be evenly spaced after electrophoresis. Furthermore, if the molar ratio of the monomeric combining species were 1:1, the binomial expansion would predict a symmetrical 1:6:15:20:15:6:1 intensity distribution for the hexameric aggregates, a 1:1 stoichiometry in the central band (IV), and a mirror-image relationship between bands III and V. The relative peak heights above background in Figure 6a are very close to the intensity distribution predicted by the binomial expansion and bands III and V form a reasonably good mirror image relationship. Although the heavy:light ratio in band IV in this experiment was somewhat greater than 1:1, in several other experiments it was very close to 1:1. Since the bands are not totally resolved, cross-contamination does occur, and judging where one band ends and another begins during band excision is to a certain extent subjective; these factors can obviously result in deviation from ideality. The bulk of the evidence favors the interpretation that the banding pattern observed on high pH urea gels is due to the random aggregation of heavy and light phycoerythrin subunits, present in a 1:1 stoichiometry, into hexamers. It is not suggested that this random association characterizes the normal *in vitro* and *in vivo* subunit aggregation, but rather that it is a curious artifact of this system which may be found to occur with other aggregating polypeptide chains.

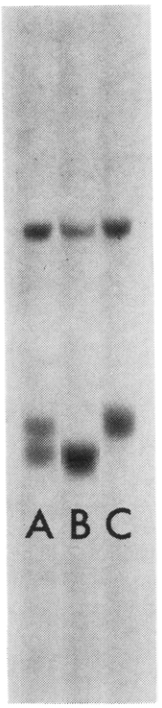

FIGURE 7: Coomassie blue stained sodium dodecyl sulfate gels of whole alkylated phycoerythrin and the isolated fast- and slow-migrating alkylated phycoerythrin bands from the low pH urea gels. A: (top to bottom) glyceraldehyde 3-phosphate dehydrogenase (GPDH), alkylated heavy chain, alkylated light chain; B: GPDH, phycoerythrin component from fast-migrating band; C: GPDH, phycoerythrin component from slow-migrating band.

Low pH Urea Gels. Vaughan (1964), who first proposed a multiple subunit structure for any biliprotein, was able to separate R-phycoerythrin from *Ceramium rubrum* into two distinct electrophoretic components with the low pH urea starch gel system of Edelman and Poulik (1961). Alkylated phycoerythrin from *F. diplosiphon* also gives rise to two well-resolved components on electrophoresis in a low pH urea–acrylamide gel system. Electrophoresis in urea solution of unalkylated phycoerythrin, on the other hand, gives rise to a smeared pattern which shows three major peaks when scanned with a densitometer. The alkylated phycoerythrin bands are highly colored and they can be accurately excised and eluted for analysis with the sodium dodecyl sulfate gel system. Figure 7 shows the results of electrophoresis on sodium dodecyl sulfate gels of alkylated phycoerythrin and of the contents of the isolated low pH urea gel bands. In contrast to the bands from the high pH urea gel system with unalkylated phycoerythrin, each of the low pH urea gel bands gives rise to a single band on sodium docecyl sulfate gels; the fast-migrating band on the low pH urea gels corresponds to the light phycoerythrin subunit, and the slow-migrating band corresponds to the heavy phycoerythrin subunit. Thus, each of the two phycoerythrin bands observed on sodium dodecyl sulfate gels is homogeneous and different with respect to charge, as well as size.

Densitometry of Low pH Urea Gels. The heavy and light alkylated phycoerythrin chains are completely resolved after low pH urea gel electrophoresis. Densitometer scans of these gels prior to and after staining for protein (Figure 8) confirm the 1:1 stoichiometry of the heavy and light subunits and show there to be different numbers of chromophores on the two subunits. The areas under the heavy and light peaks scanned at 620 nm after staining for protein with coomassie blue have a

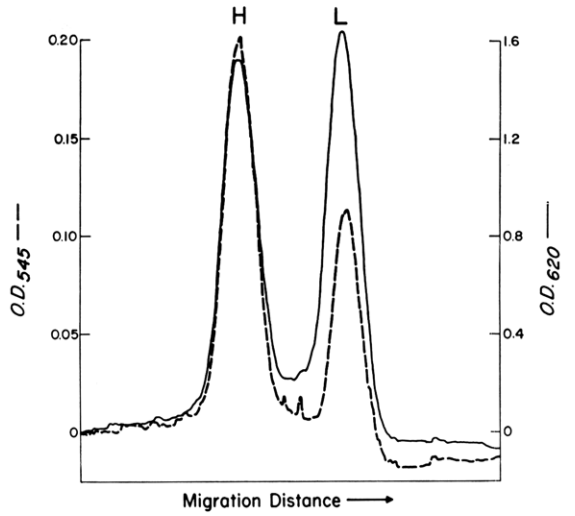

FIGURE 8: Densitometric scan of a low pH urea gel with alkylated phycoerythrin before (545 nm) and after (620 nm) staining for protein with coomassie blue.

ratio which varies from 0.93 to 0.97. The areas under the heavy and light peaks scanned at 545 nm before staining for protein, however, have a ratio which varies from 1.80 to 1.87. These results strongly suggest that there are twice as many chromophores on the heavy chain as there are on the light chain.

Absorption Spectra of Isolated Alkylated Subunits. As is shown in Figure 9, the absorption spectra of the isolated alkylated subunits in 8 M urea–0.2 M formic acid–0.08 M NaOH are essentially identical. Peaks at 552 nm and about 385 and 316 nm are present in the spectra of both subunits, indicating that the basic structures of the chromophores on the heavy and light subunits are identical. This is a point worth establishing, since the structure of phycoerythrobilin has been determined with chromophores cleaved from phycoerythrin by methanolysis (Siegelman *et al.*, 1968), and there is no evidence that methanolysis cleaves the chromophores from both of the subunits. Peaks at 564, 380, and 310 nm characterize the absorption spectrum of native phycoerythrin in 0.1 M sodium phosphate (pH 7.0). The most drastic spectral change brought about by the alkylation of phycoerythrin and its exposure to the low pH urea solvent conditions is a very large drop in the α band extinction; the extinctions of the excitations in the

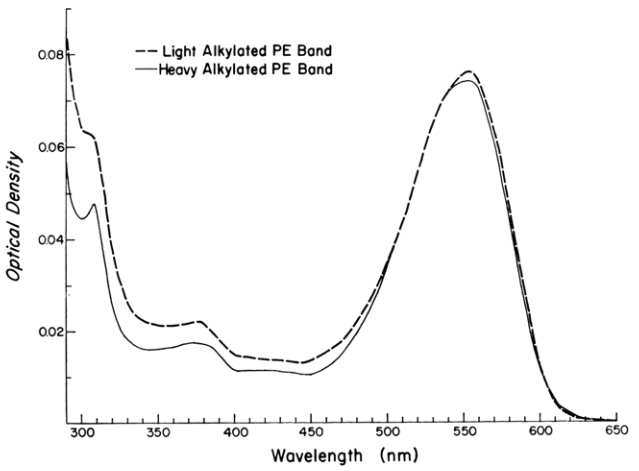

FIGURE 9: Absorption spectra of isolated, alkylated heavy and light phycoerythrin chains in 8 M urea–0.2 M formic acid–0.08 M NaOH.

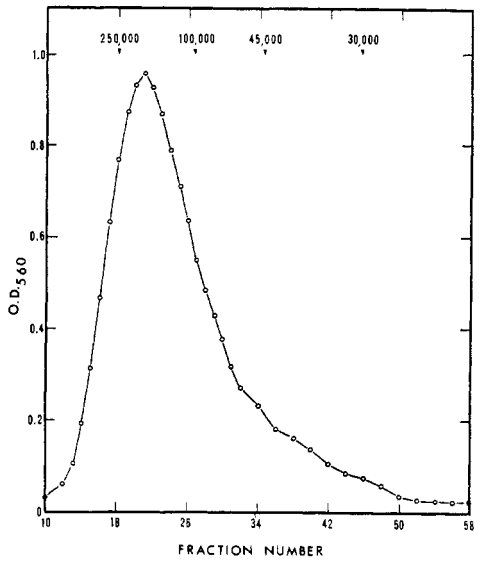

FIGURE 10: Purified phycoerythrin elution profile from a calibrated G-200 column. Standard proteins for calibration included: (1) catalase, 240,000 (Samejima and Yang, 1963); (2) β-amylase, 215,000 (Thoma *et al.*, 1963); (3) fumarase, 196,000 (Kanarek *et al.*, 1964); (4) aldolase, 160,000 (Kawahara and Tanford, 1966); (5) lactate dehydrogenase, 135,000 (Schwert and Winer, 1963); (6) transferrin, 74,000 (Roberts *et al.*, 1966); (7) bovine serum albumin, 69,000 (Tanford, 1969); and (8) ovalbumin, 43,000 (Castellino and Barker, 1968).

380- and 310-nm regions are relatively unaffected.

Calibrated Sephadex Chromatography. The largest aggregate of purified *T. tenuis* phycoerythrin observed in the ultracentrifuge in the neutral pH region has a sedimentation coefficient of 10.3 S (Hattori and Fujita, 1959). These workers calcuated the diffusion coefficient of phycoerythrin and estimated the molecular weight of the 10.3S aggregate to be 226,000. An elution diagram for purified *F. diplosiphon* phycoerythrin from a calibrated Sephadex G-200 column is shown in Figure 10. A molecular weight estimate of 191,500 ± 15,000 was obtained for the large, early-eluting phycoerythrin aggregate, which presumably corresponds to the 10.3S component of *T. tenuis* phycoerythrin. Association–dissociation effects and overlap with smaller components probably make 191,500 ± 15,000 a low estimate for the molecular weight of the large *F. diplosiphon* aggregate.

The sodium dodecyl sulfate gels (Figure 4) indicate that the phycoerythrin subunit molecular weights in *F. diplosiphon* and *T. tenuis* (20,000 and 18,300) are identical, and densitometric measurements (Figure 8) indicate that the subunit stoichiometry in *F. diplosiphon* is 1:1. The *Fremyella* and *Tolypothrix* data can be combined in order to make an estimate of the size and subunit composition of the 10S phycoerythrin aggregates. An aggregate composed of five of each of the subunits would have a molecular weight of 191,500, whereas an aggregate composed of six of each of the subunits would have a molecular weight of 229,800. Literal interpretation of the Sephadex data suggests that the large phycoerythrin aggregate is a decamer. Because the value for Sephadex filtration is probably low and a weight of 226,000 has been obtained by ultracentrifugal methods, it seems more likely that the large phycoerythrin aggregate is a dodecamer which is composed of six heavy and six light subunits.

Difference Spectroscopy. During several experiments with purified phycoerythrin, it was observed that dilution of the biliprotein resulted in a greater loss of absorption on the long-

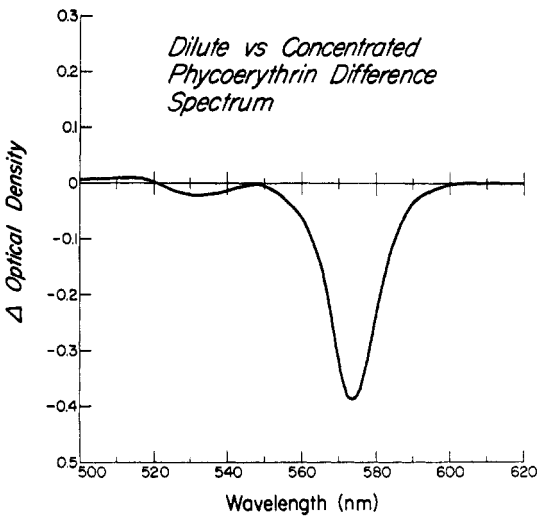

FIGURE 11: Dilute *vs.* concentrated difference spectrum for purified *F. diplosiphon* phycoerythrin. Procedure described in Experimental Section.

wavelength side than on the short-wavelength side of the 564-nm absorption peak. Figure 11 shows a dilute *vs.* concentrated difference spectrum for phycoerythrin. (The optical density at 564 due to phycoerythrin in the reference beam was 1.85.) There is a band centered at 573–574 nm, the extinction of which is diminished as a consequence of dilution.

For simple self-associating systems, dissociation is usually a consequence of dilution. One explanation for the observed spectral differences is that dissociation of the phycoerythrin aggregates occurs with dilution. Larger aggregates would contain an excitation band centered at 573–574 nm which would be diminished in extinction or eliminated in the small aggregates produced as a consequence of dissociation.

The absorption spectra of phycoerythrin Sephadex fractions in the 200,000, 100,000, and 50,000 molecular weight regions (fractions 18, 30, and 40 from Figure 10) were normalized, and the difference spectra between the 200,000 molecular weight fraction and the smaller fractions were computed (Figure 12). It is evident that the large phycoerythrin aggregate contains an absorption band centered at about 573 nm which is enhanced relative to the smaller phycoerythrin aggregates. This observation supports the dissociation–spectral change argument which was invoked above to explain the dilute *vs.* concentrated difference spectrum. The loss in absorption in the 573-nm region appears to be correlated with dissociation of the 10S phycoerythrin aggregate.

Discussion

The only other filamentous blue-green alga from which all three biliproteins have been purified is *Tolypothrix tenuis*. From published spectra of the *Tolypothrix* biliproteins (Hattori and Fujita, 1959), 565/280, 620/280 and 650/280 absorption ratios were calculated to be 8.9, 4.5, and 3.8 for phycoerythrin, phycocyanin, and allophycocyanin, respectively. Corresponding values for the purified *Fremyella* biliproteins in 0.1 M sodium phosphate, pH 7.0, are 7.5, 4.3, and 4.2. Hattori and Fujita did not report the buffer molarity or pH at which their spectra were obtained, and changes in these parameters are well known to have an effect on the visible extinction of the biliproteins. The slight differences in the absorption ratios between the pigments of the two organisms could therefore be due to solvent effects.

The monomer molecular weight estimates presented here are in good agreement with other recent calibrated sodium dodecyl sulfate gel experiments with biliproteins. Rice (1971) has observed two phycocyanin subunits with molecular weights of 17,200 and 15,100 and a single allophycocyanin subunit having a molecular weight of 15,300 with biliproteins purified in this laboratory from *Plectonema boryanum* (Indiana Culture Collection strain 581). Ó Carra (1970) has claimed, without presenting experimental evidence or citing biliprotein sources, subunit molecular weights of 20,500 and 18,500 for C-phycocyanin and of 22,000 and 19,700 for C-phycoerythrin. Glazer and Cohen-Bazire (1971) have estimated subunit molecular weights of 22,000 and 20,000 for phycoerythrin, 20,000 and 16,000 for phycocyanin, and 17,500 and 15,500 for allophycocyanin from work with the unicellular blue-green algae *Anacystis nidulans* and *Aphanocapsa* sp. Why we and Rice observe a single allophycocyanin subunit and Glazer and Cohen-Bazire find two subunits is not fully understood. All of the above results are in conflict with those of Kao and Berns (1968), who found a single C-phycocyanin subunit having a molecular weight of about 30,000 on calibrated sodium dodecyl sulfate gels.

The smallest C-phycocyanin species which have been observed in ultracentrifugal and gel filtration experiments have molecular weights of 30,000 (Scott and Berns, 1965; Neufeld and Riggs, 1969) and 46,000 (Hattori *et al.*, 1965). Since the C-phycocyanin subunits have molecular weights of approximately 17,600 and 16,300, in good agreement with the minimum molecular weights for phycocyanin calculated on the basis of its amino acid composition (Berns *et al.*, 1963; Raftery and Ó hEocha, 1965), it is apparent that the 30,000 and 48,000 molecular weight moieties would correspond to dimeric and trimeric aggregates of the protomeric subunits. The results of the sodium dodecyl sulfate gel experiments without mercaptoethanol indicate that the strong associative tendencies of the smallest phycocyanin aggregates are not due to disulfide linkages between the subunits.

The 2.2S (Hattori and Fujita, 1959) and 2.9S (Pecci and Fujimori, 1968) C-phycoerythrin species observed in the ultracentrifuge are also evidently aggregates whose strong associations are not due to interchain disulfide bonds. The 2.2S, irreversibly dissociated *T. tenuis* phycoerythrin species observed by Hattori and Fujita (1959) was obtained by exposing the purified pigment to pH 11.6. *F. diplosiphon* phycoerythrin dialyzed against 0.1 M glycine–NaOH (pH 10.5) for 24 hr at 23° in the dark has a molecular weight of 44,000, based on Sephadex G-200 chromatography (A. Bennett and L. Bogorad, unpublished data). This suggests that C-phycoerythrin dissociates into dimers upon exposure to strongly alkaline conditions.

The unstained sodium dodecyl sulfate gels indicate that each of the phycoerythrin subunits contains at least one red bilin chromophore and that each of the phycocyanin subunits and the allophycocyanin subunit contains at least one blue bilin chromophore. With this information, the actual number of chromophores on each subunit can be determined from the known molecular weights of the chromophores and the fraction of the total weight of the biliproteins which is contributed by the chromophores. The molecular weight of phycoerythrobilin and phycocyanobilin (as free acids) is 584 (Siegelman *et al.*, 1968). Crespi *et al.* (1968) have demonstrated the chromophore content of C-phycocyanin from *Synecchococcus lividus* and *Phormidium luridum* to be 4.0%, which (assuming one phycocyanobilin chromophore per subunit) yields a minimum molecular weight of 14,600. Since the subunit molecular weights of C-phycocyanin are about 17,600 and 16,300, it can be concluded that each subunit contains one chromophore. Purified phycocyanin from the cryptophyte *Cyanidium caldarium* gives two blue bands in the 15,000–17,000 molecular weight region on unstained SDS gels (A. Bennett and L. Bogorad, unpublished data). Troxler and Lester (1968) have elegantly demonstrated that the phycocyanobilin content of *C. caldarium* phycocyanin is 3.6% from which a minimum molecular weight of 16,200 can be calculated. It is evident that each of the phycocyanin subunits from *C. caldarium* also contains one chromophore. It has not been established that methanolysis cleaves the chromophores from both of the phycocyanin subunits. Spectral analysis of denatured C-phycoerythrin from *Phormidium persicinum* (Ó Carra, 1962) has indicated a chromophore composition of 6.46%, from which a minimum molecular weight of 9050 can be calculated. The simplest interpretation of this result would be that the C-phycoerythrin subunits (mol wt 20,000 and 18,300) each contain two chromophores. Assuming roughly equivalent extinction coefficients for phycoerythrobilin and phycocyanobilin in phycoerythrin and phycocyanin, the extinction coefficients (A. Bennett and L. Bogorad, unpublished) of phycoerythrin at 565 nm and of phycocyanin at 620 nm in 0.1 M sodium phosphate (pH 7.0) (9.13 and 5.18 ml/(mg cm), respectively) suggest a higher chromophore content in phycoerythrin than in phycocyanin. The simplest empirical interpretation of our densitometric data with alkylated phycoerythrin on low pH urea gels is that there are two phycoerythrobilin molecules on the heavy chain and a single chromophore on the light chain. Estimates of the chromophore percentage composition of allophycocyanin have not been published.

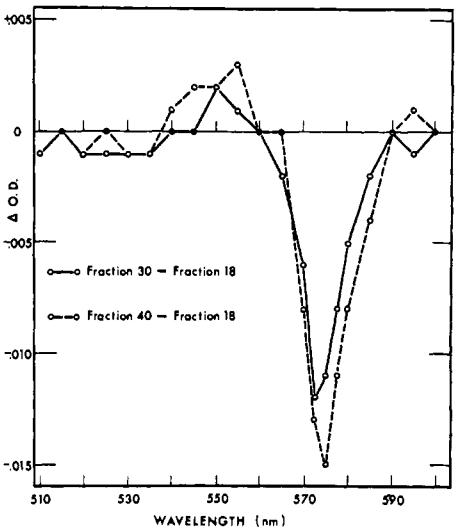

FIGURE 12: Computed difference spectra for large and small molecular weight phycoerythrin fractions obtained by G-200 chromatography. Procedure described in Experimental Section. Fraction numbers correspond to those in Figure 10.

Our dilute *vs.* concentrated and Sephadex fraction difference spectra are consistent with ultracentrifugal and circular dichroic measurements on C-phycoerythrin (Pecci and Fujimori, 1968; Fujimori and Pecci, 1970) which indicate an absorption band on the long wavelength side of the "single-peaked" C-phycoerythrin absorption maximum which is generated in highly aggregated forms of the chromoprotein. A relationship probably exists between this absorption band and the "fluorescing" type of phycoerythrobilin proposed by Dale

and Teale (1970); see also Teale and Dale (1970).

C-phycoerythrin is antigenically unrelated to ether C-phycocyanin or allophycocyanin from the same organism (Berns, 1967; A. Bennett and L. Bogorad, unpublished data). The reports on the antigenic relationship between C-phycocyanin and allophycocyanin are in conflict. Berns (1967) has observed some cross-reaction between rabbit anti-*Plectonema calothricoides* allophycocyanin and purified *P. calothricoides* phycocyanin and between anti-*Synecchococcus lividus* phycocyanin and an allophycocyanin-rich fraction from *S. lividus*. Insufficient purity of the phycocyanin and allophycocyanin preparations and chromophore determinants as potential sources of cross reaction could not be excluded in Berns' experiments. Bogorad (1965), however, observed no detectable reaction between antiserum to phycocyanin from *C. caldarium* and allophycocyanin from this organism. Anti-*F. diplosiphon* phycocyanin does not cross-react with *F. diplosiphon* allophycocyanin, and *vice versa* (A. Bennett and L. Bogorad, unpublished data). In addition, Glazer and Cohen-Bazire (1971) have established that phycocyanin and allophycocyanin from *A. nidulans* are antigenically unrelated. M. A. Raftery and C. Ó hEocha (1958)[2] have found significant differences in the amino acid compositions of phycocyanin and allophycocyanin from the blue-green alga *Arthrospira maxima*.

The biliprotein subunit molecular weights which have been presented here indicate that the protein components of phycoerythrin, phycocyanin, and allophycocyanin from a single blue-green alga are different; polypeptides of five different molecular weights have been discerned. It is not known at present whether the protein components of each of the phycoerythrin chains and each of the phycocyanin chains have any amino acid sequence similarities. Siegelman, Crespi, and their coworkers (Siegelman *et al.*, 1968; Crespi *et al.*, 1967) have shown that there are two structurally distinct chromophores distributed among the blue-green algal biliproteins. The relative intracellular level of the three biliproteins is altered in reponse to the wavelengths of light under which the blue-green algae are cultivated. The information available at present indicates that such "complementary chromatic adaptation" involves regulation over the synthesis and assembly or the degradation of several or all of five different polypeptide chains and two different chromophores.

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