Phycobilisomes of *Porphyridium cruentum:* Pigment Analysis†

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ABSTRACT: Phycobilisomes from the red alga *Porphyridium cruentum* were dissociated and the phycobiliproteins were purified by acrylamide gel electrophoresis and subsequently characterized. The entire protein content was accounted for by phycobiliproteins (84% phycoerythrin, 11% R-phycocyanin, and 5% allophycocyanin). Two types of phycoerythrin, designated as B- and b-phycoerythrin, were found to occur in about equal amounts. Calibrated Sephadex chromatography showed molecular weights of 265,000 for B-phycoerythrin, 55,000 and 110,000 for b-phycoerythrin, 127,000 for R-phycocyanin, and 120,000 for allophycocyanin. Subunit molecular weights of the purified pigments were determined by calibrated sodium dodecyl sulfate–acrylamide gel electrophoresis. It showed that allophycocyanin consisted of a single subunit with a molecular weight of 14,600. R-phycocyanin had two subunits, one blue in color at 16,400, and one red at 18,400. B-phycoerythrin had one major subunit band at 17,300 as well as a lesser band at 30,000 in a ratio of 6:1, while b-phycoerythrin had a single band at 17,200. The two phycoerythrins also differed spectrally in that B-phycoerythrin had a prominent shoulder at 495–500 nm, which correlated with the presence of the 492-nm-absorbing subunit (30,000 molecular weight). The 17,300 and 17,200 molecular weight subunits of B- and b-phycoerythrin had an absorption peak at 530 nm and may be identical. Owing to the spectral differences and the fact that *in vitro* disaggregation from B- to b-phycoerythrin, or aggregation of b- to B-phycoerythrin, was not observed, the phycoerythrins were regarded as separate pigments. The smaller size of b-phycoerythrin may facilitate closer spatial packing within the phycobilisome, but the functional significance of the two phycoerythrins within a phycobilisome is not known.

Phycobiliproteins may account for as much as 50% of the total protein in some algae (*e.g.*, the red alga *Porphyridium cruentum*, observations, our laboratory). The phycobiliproteins in red and blue-green algae are aggregated into phycobilisomes and function in harvesting light energy used in photosynthesis. Phycobilisomes in these algal groups (see references in Edwards and Gantt, 1971; Gantt and Lipschultz, 1972; Gray *et al.*, 1973; and Wildman and Bowen, 1974) are attached in a very regular pattern to the photosynthetic membranes. While phycobilisomes are released intact only with detergents, the highly water-soluble phycobiliproteins come off easily. Phycobilisomes have been isolated from one red alga (Gantt and Lipschultz, 1972) and three blue-green algae (Gray *et al.*, 1973). Allophycocyanin and phycocyanin are common to all phycobilisomes. Phycoerythrin may also be a major constituent as in organisms that contain phycoerythrin, such as *P. cruentum*.

As a result of investigations carried out in our laboratory in which dissociation of phycobilisomes, fluorescence transfer, and the ultrastructure were studied, a structural model of the phycobilisome has been proposed (Gantt and Lipschultz, 1973). The essential points of the model are an allophycocyanin (APC)[1] core which is in physical contact with the lamellae and with a hemispherical layer of R-phycocyanin (R-PC). Phycoerythrin forms an outer concentric hemispherical layer in contact with R-PC on one side and the stroma on the other. The possibility that phycoerythrin (PE) is in direct contact with the lamellae is not ruled out. Furthermore, the hemispherical layers are not necessarily regarded as solidly continuous for discontinuous areas may exist. Such a model is consistent with the overall energy-transfer scheme proposed from studies on whole cells (Duysens, 1952; French and Young, 1952; Mohanty *et al.*, 1972) and with data obtained from isolated phycobilisomes (Gantt and Lipschultz, 1973) which showed that allophycocyanin is the pigment through which light energy absorbed by other phycobiliproteins is transferred to the chlorophyll in the photosynthetic lamellae. In the process of testing the proposed phycobilisome model, it was first necessary to isolate and characterize the pigments of the phycobilisomes and to determine the amounts of each pigment present.

The methods and pigment characterization are described here. The results showed that: (1) only phycobiliproteins were detectable in phycobilisomes; and (2) two different phycoerythrins were observed in phycobilisomes.

Materials and Methods

Sephadex G-200 (lot 7884) was obtained from Pharmacia. Blue Dextran (lot 13C-0820), Coomassie Blue, and sodium lauryl sulfate were purchased from Sigma as were the following proteins: α-amylase, bovine serum albumin, β-galactosidase, carbonic anhydrase, catalase, α-chymotrypsin, α-chymotrypsinogen A, and myoglobin. Ferritin and hemoglobin were obtained from Nutritional Biochemicals, and trypsin from Calbiochem. Ultra Pure sucrose from Schwarz/Mann was used for the sucrose gradients. For electrophoresis ammonium persulfate was obtained from Fisher, whereas acrylamide, methylenebisacrylamide (Bis), and *N,N,N′,N′*-tetramethylethylenediamine (Temed) were from Eastman. Electrophoresis grade acrylamide and Bis for sodium dodecyl sulfate gels were purchased from Bio-Rad Labs.

Isolation of Phycobilisomes and Phycobiliprotein Purification. P. cruentum cultures were grown and phycobilisomes were isolated as previously described (Gantt and Lipschultz, 1972). Several small changes were made in the phycobilisome

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[1] Abbreviations used are: APC, allophycocyanin; R-PC, R-phycocyanin; PE, large (B-) and small (b-) phycoerythrin, Temed, *N,N,N′,N′*-tetramethylethylenediamine; Bis, methylenebisacrylamide.

isolation procedure: (a) the sucrose step gradient, on which the supernatant of the 27,000*g* centrifugation was layered, consisted of 2, 1, 0.75, 0.5, 0.25 M sucrose in proportions of 3:3:6:3:3 ml, respectively; (b) the total time of centrifugation on the above step gradient was 2–2.5 hr. With these changes the phycobilisome band was more concentrated and discrete.

After centrifugation, phycobilisomes in the 0.75–1 M sucrose were removed from the step gradient and diluted 5.5-fold with 0.5 M phosphate buffer (pH 6.8). The phycobilisomes were pelleted by centrifugation at about 250,000*g* for 1 hr. Phycobilisomes thus prepared could be stored for several days as a pellet or as a dense solution (in 0.5 M phosphate buffer), with little demonstrable dissociation as ascertained by monitoring diluted preparations by fluorescence (Gantt and Lipschultz, 1973) and absorption spectroscopy. To dissociate phycobilisomes into component proteins, the phycobilisomes were suspended in dilute (0.01 or 0.005 M) phosphate buffer and allowed to dissociate overnight in a dialysis bag by being dialyzed against the same buffer at 4°. Dissociated phycobilisome preparations were then centrifuged at 150,000*g* for 1 hr. A very small pellet was collected which had noticeable absorption peaks at 260, 440, 545, and 675 nm. Examination by electron microscopy showed that virus type particles were present which would account for the 260-nm absorption. Small membrane vesicles were also found and presumed to contain chlorophyll (440- and 675-nm absorption). The presence of B-PE (less than 0.5% of total) accounted for the 545-nm absorption. The supernatant from the 150,000*g* centrifugation was concentrated with an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.), using a PM-10 Diaflo membrane. Phycobilisome components were separated by (a) electrophoresis twice on 5% acrylamide gel, by (b) passage through a Sephadex G-200 column followed by gel electrophoresis, or by (c) sucrose gradient centrifugation.

Sephadex Chromatography. Hydrated Sephadex G-200 from which fines had been removed was poured to form a 2 × 62 cm column. It was equilibrated for 1 week at about 22° with 0.1 M Sorensen's phosphate buffer (pH 6.8)–0.02% (w/v) sodium azide. Preparations of dissociated phycobilisomes (4 mg/ml) were layered on the column in a volume of 0.8 ml (for molecular weight determination) or 1.5 ml (for pigment preparation). The flow rate was maintained at 8 ml/hr and 2-ml fractions were collected. Absorbance of fractions at 650, 620, 545, and 280 nm were recorded with a Cary 14 spectrophotometer. The content of each pigment was determined by making corrections for the overlapping absorptions at 650, 620, and 545 nm. For estimation of b- and B-PE, the B-PE peak was assumed to be symmetrial, because pure B-PE had been found to have a symmetrical elution peak.

The void volume of the column was determined by use of Blue Dextran. Molecular weights were calculated according to Andrews (1965). The following compounds were run individually: bovine serum albumin, catalase, ferritin, myoglobin, β-galactosidase, α-chymotrypsinogen A, and B-phycoerythrin (previously sized by several other methods). Bovine serum albumin, α-chymotrypsinogen A, catalase, ferritin, and β-galactosidase were detected by their absorbancies at 275–280 nm. Myoglobin and crystallized B-PE were detected by their absorbancies at 407 and 545 nm, respectively.

Sucrose Gradient Centrifugation. The following buffers were used: 0.1 M sodium citrate (pH 6.0), 0.034 M asparagine-Tris (pH 7.3), 0.1 M Sorensen's phosphate pH 6.8, and 0.1 M Sorensen's phosphate pH 8.0. Linear gradients from 0.1 to 1.0 M sucrose were prepared in each of the above buffers. Phycobilisomes were dissociated in 0.01 M of the respective buffers and concentrated. The dissociated phycobilisome sample (0.5 ml) was layered on each sucrose gradient tube and centrifuged in an SW 50.1 Beckman rotor at 130,000*g* for 16 hr. Fractions were collected by dripping (30 drops/tube) or by removal with a syringe, and scanned from 340 to 700 nm on a Cary 14 spectrophotometer.

Acrylamide Gel Electrophoresis. Preparative gel electrophoresis was performed with an E-C slab gel apparatus (Milton Roy Co., Philadelphia, Pa.) with a 6-mm gel space. The gel was prepared by mixing: A (5.83 g of Tris and 0.46 ml of Temed per 100 ml, adjusted with HCl to pH 7.9), B (24 g of acrylamide and 0.8 g of Bis/100 ml), C (14 mg of ammonium persulfate/100 ml), 1:2:6.6. Aspargine buffer (0.034 M adjusted with Tris-base to pH 7.3) was the reservoir buffer. Preparations of dissociated phycobilisomes (up to 2 mg of protein) were layered in sucrose (15%) or glycerol (20%) on the gelled acrylamide, and electrophoresis was carried out at 325 V, at 10° until the pigment bands were clearly separated (about 2 hr). The pigment bands were cut out with a razor blade, diced, taken up with a small volume of 0.01 M phosphate buffer (pH 6.8)–0.02% (w/v) sodium azide, and subsequently dialyzed against the same buffer. The extracted pigments were then centrifuged to remove small pieces of acrylamide and concentrated for a second electrophoresis run if required, or stored. Purified pigments were stored in 0.1 M phosphate buffer (pH 6.8) and 0.02% sodium azide in the dark at about 4°.

The discontinuous acrylamide gel electrophoresis system of Hedrick and Smith (1968) was used to determine the molecular weights of the phycoerythrins and to differentiate between their size and charge isomers. Four concentrations were used ranging from 5 to 8%. To avoid excessively rapid gellation the ammonium persulfate was reduced so that in the final gel solution it was 4.7 mg/10 ml for 5% gel, and 2.8 mg/10 ml for 6, 7, and 8% gel. The final pH of the small pore solution was 7.9 and the large pore solution (stacking gel) was 5.7. The reservoir buffer (34 mM asparagine-Tris) was at pH 7.3. Each tube received about 30 μg of protein. Electrophoresis was at 10° (2 mA/tube initially and 4 mA after the Bromophenol Blue dye front passed into the small pore gel) for a total time of about 2 hr. After electrophoresis gels were treated in 15% (w/v) trichloroacetic acid at about 55° for 10 min, rinsed in water, and stained in Coomassie Blue (1% in 10% acetic acid) for 1–2 hr, then destained in 7% acetic acid by diffusion. The gel system was calibrated by using the following reference proteins: bovine serum albumin (monomer and dimer), ferritin, catalase, and α-amylase.

The Hedrick and Smith (1968) method was also used to assess the presence of unpigmented proteins in phycobilisomes. For this purpose phycobilisome samples (30–50 and 75 μg of protein per tube) were electrophoresed on four gel concentrations (5–8%). After electrophoresis the migration distance of all visible bands was measured and then the gels were fixed and stained. Location of unstained and stained bands were compared to determine if unpigmented bands were present.

Sodium Dodecyl Sulfate–Acrylamide Gel Electrophoresis. The procedure of Weber and Osborn (1969) was used with the following modifications: (a) the gel solution containing the ammonium persulfate (final concentration 2.5 mg/10 ml) was degassed for 5 min immediately after which the Temed was added and the gels were poured; (b) the samples were incubated in 1% (w/v) sodium dodecyl sulfate and 1% (v/v) β-mercaptoethanol for 5 min in a boiling-water bath, and (c) subsequent to electrophoresis the gels were treated for 10 min in 15% trichloroacetic acid at about 55° and rinsed in water before and after staining in Coomassie Blue. The following proteins were

TABLE I: Extinction Coefficients (mg/ml at 1 cm) of Phycobiliproteins from Phycobilisomes of *Porphyridium cruentum* in 0.1 M Sorensen's Buffer (pH 6.8).[a]

Pigments	Extinction Coefficients		
	545 nm	620 nm	650 nm
Phycoerythrin[b]	5.26	—	—
R-phycocyanin	2.27	4.16	0.44
Allophycocyanin	0.67	3.33	5.00

[a] Based on the maximum absorption peaks of the respective pigments and their protein content as determined by the Lowry method (Lowry *et al.*, 1951). [b] B- and b-phycoerythrin were regarded as the same for these calculations because the relative concentration of each phycoerythrin cannot be ascertained in a mixture.

used as molecular weight calibration markers: bovine serum albumin, carbonic anhydrase, trypsin, myoglobin, hemoglobin, and α-chymotrypsin.

Acrylamide Gel Densitometry. Acrylamide gels were scanned before and/or after staining in Coomassie Blue, on a Gilford spectrophotometer 240 equipped with a linear gel transport device and a 6040 recorder (Gilford Instrument Labs., Oberlin, Ohio). Gels stained with Coomassie Blue were scanned at 620 nm with a 0.05-mm slit. To estimate the amount of B- and b-PE unstained gels were scanned at 545 nm (0.05-mm slit). Then the area under each peak was traced onto heavy paper, cut out, and weighed.

Phycobiliprotein Estimation. Solutions of purified PE (B- and b-), R-PC, and APC in 0.1 M phosphate buffer (pH 6.8) were measured at their respective absorption maxima at 545, 620, and 650 nm. Extinction coefficients for each biliprotein were determined by measuring the protein content of solutions of known absorption at the various absorption maxima. Protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The extinction coefficients for the purified pigments are given in Table I. Content (mg/ml of protein) of individual pigments, when present in mixtures such as dissociated phycobilisomes, can be determined by using the following equations which correct for spectral overlap: phycoerythrin = $[A_{545} - 0.572(A_{620}) + 0.246(A_{650})]/5.26$, R-phycocyanin = $[A_{620} - 0.666(A_{650})]/3.86$, and allophycocyanin = $[A_{650} - 0.105(A_{620})]/4.65$.

Results

Spectral Characteristics. Complete separation of the phycobiliproteins obtained from dissociated phycobilisomes was attained by acrylamide gel electrophoresis, and the absorption spectra of the four pigments thus obtained are shown in Figure 1A. APC has a peak at 650 nm and shoulders at 630 and 600 nm. R-PC has distinct peaks at 555 nm and at 617 nm but no peak at 310 nm. The 555:617 nm ratio of 0.63 is similar to that obtained by Haxo *et al.* (1955). The two phycoerythrins are spectrally distinct. B-PE, which has been described by Airth and Blinks (1956) as being characteristic of the red algal order Bangiales, has a double peak at 545 and 563 nm, and a prominent shoulder at about 495–500 nm. The second phycoerythrin has been designated here as b-PE, thus observing the conventional nomenclature where the prefix "b" denotes its taxonomic origin, while the lower case gives recognition to its smaller size. It has a major peak at 545 nm and a shoulder at 563 nm, but lacks the 495–500-nm shoulder. Both phycoerythrins had a

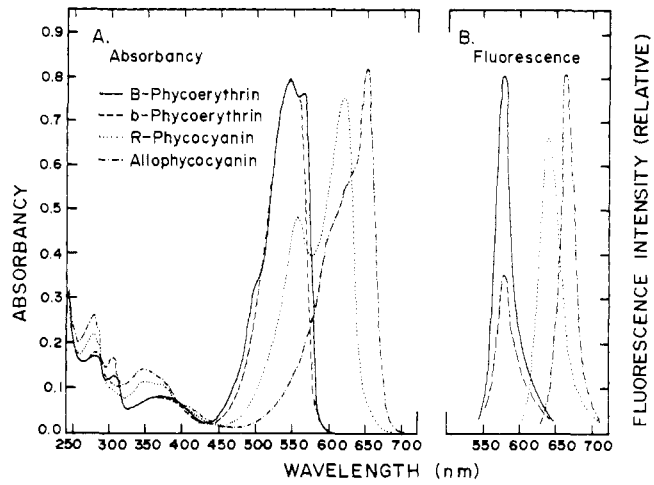

FIGURE 1: Spectra of purified phycobiliproteins from phycobilisomes of *P. cruentum* in 0.1 M Sorensen's phosphate buffer (pH 6.8) at about 25°. (A) Absorption spectra in a 1-cm path-length cell with Cary 14 spectrophotometer. (B) Fluorescence emission spectra were made within an absorbance range of 0.02–0.1 at the pigment maxima (545, 620, or 650 nm). This range was linear, thus avoiding peak shifts or concentration quenching. Each pigment was excited at its maximum absorption peak in an Aminco-Bowman spectrofluorometer. B- and b-PE each had an absorbance at 545 nm of 0.1. The spectrofluorometer was equipped with an off axis ellipsoidal mirror condensing system and an R 136 Hamamatsu TV photomultiplier tube. The band pass on the excitation side was 11 nm, and 2.7 nm on the emission side. Spectra were corrected after determining the lamp output and the emission grating-phototube efficiency.

small characteristic peak at 310 nm. Fluorescence spectra made at room temperature are shown in Figure 1B. The phycoerythrins have an emission at 575 nm; however, the fluorescence yield of b-PE is only about 45% that of B-PE. R-PC emission was at 637 nm whether excited at 555 or 617 nm. The APC fluorescence emission was at 660 nm.

Separation of Phycobilisome Components. The separation of the pigments from dissociated phycobilisomes by electrophoresis occurred according to their size as well as charge with a pattern as shown in Figure 2 where b-PE migrated the greatest distance from the origin, then B-PE, APC, and R-PC. For complete separation of the pigments, the phycobiliproteins were eluted from the gel and reelectrophoresed. It was found that all protein bands corresponded to phycobiliprotein bands, when the migration distance of visible phycobiliprotein bands was compared with the bands stained by Coomassie Blue.

Fractionation of dissociated phycobilisomes on Sephadex G-200 can be seen in Figure 3. Absorption in the ultraviolet region (280 nm) coincided with the elution profile of the colored fractions. Of the protein layered, 95% was removed in the pooled pigmented fractions (40–83). This indicates that the phycobilisomes are mainly composed of phycobiliproteins, especially since other protein bands were not detectable upon electrophoresis (Figure 2). It seems unlikely that unpigmented proteins would have migrated with phycobiliproteins on Sephadex (a size fractionation) as well as on acrylamide gel electrophoresis (a size and charge separation).

The fastest moving pigmented fractions on Sephadex G-200 consisted almost entirely of B-PE, while the slower eluting pigments were R-PC, APC, and b-PE. The b-PE was found to be composed of a disaggregating system. B-PE disaggregation was not observed.

Each of the two phycoerythrins consisted of various isomers which appeared as separate bands upon electrophoresis. B-PE usually had two bands, sometimes three (the bands of B-PE were masked in Figure 2 due to the unavoidable overloading

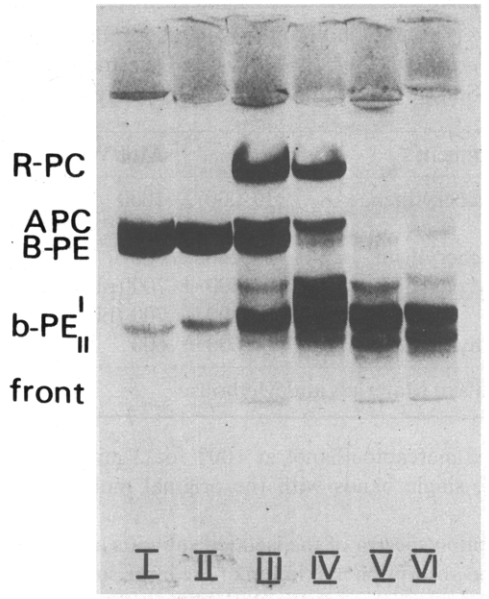

FIGURE 2: Separation of phycobiliproteins by electrophoresis on 6% acrylamide from fractions of a Sephadex G-200 column as shown in Figure 3. Roman numbers indicate pooled fractions: I (42–46), II (49–52), III (56–60), IV (62–65), V (68–72), and VI (73–83). Pigment bands are designated on left: R-phycocyanin as R-PC, allophycocyanin as APC, B-phycoerythrin as B-PE, and b-phycoerythrin as b-PE$_I$ (110,000 molecular weight range), b-PE$_{II}$ (55,000 molecular weight range). Some phycoerythrin bands are charge isomers and some are size isomers. Front was faint pink prior to staining, as was the very faint band below the stacking gel.

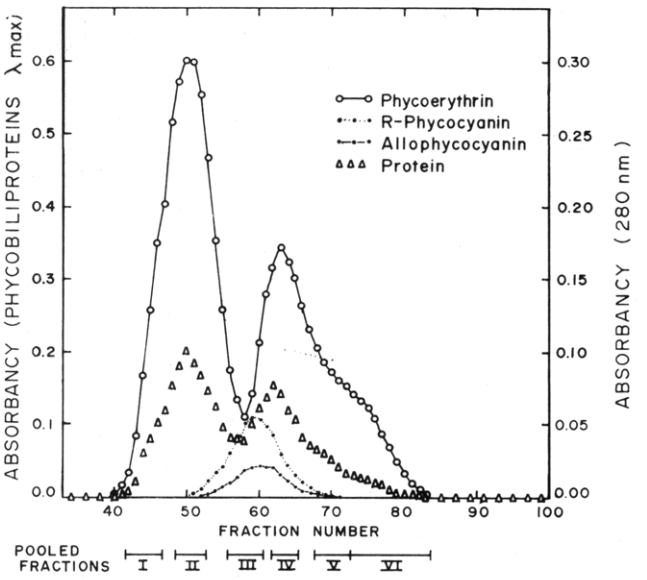

FIGURE 3: Elution profile of phycobilisome pigments from *P. cruentum* in 0.1 M Sorensen's phosphate (pH 6.8) and 0.02% sodium azide on a Sephadex G-200 column. Each fraction was scanned on a Cary 14 spectrophotometer from 700 to 240 nm. Values expressed as absorbance at: 280 nm for protein, 545 nm for phycoerythrins, 620 nm for R-phycocyanin, and 650 nm for allophycocyanin. Horizontal bars indicate pooled fractions which were combined for analysis by electrophoresis (Figure 2). Details are given in Materials and Methods.

necessary to reveal the other pigments present), and b-PE usually had three bands. The gel system of Hedrick and Smith (1968) was utilized to determine if the various PE bands were being resolved on the basis of charge or size differences. When the log of the protein mobility relative to the dye front is plotted against gel concentration, a group of nonparallel lines intersecting around 0% gel concentration indicates that the proteins are size isomers. But proteins of similar size and different charge give parallel lines. On this basis it has been decided that the bands of B-PE are charge isomers. However, of the three b-PE bands (Figure 2 gels V and VI) the one nearest the front labeled as b_{II} [with a molecular weight of about 60,000 (Table III)] was a size isomer as was b_I (with a molecular weight of 110,000). The exact b-PE species of the dark middle band has not been identified; it appears to consist of charge isomers (perhaps of b_{II}) and size isomers (between b_I and b_{II}). The presence of two molecular weight species of b-PE was also consistent with data obtained from Sephadex filtration. It should be noted that the multiple gel bands of B-PE had identical absorption and fluorescence peaks. Furthermore, no spectral distinctions could be made between the b-PE bands upon elution and examination of the pigments in dilute solutions.

The Hedrick and Smith (1968) gel system was also employed to determine protein molecular weights. Table III demonstrates that similar molecular weight estimates for the *P. cruentum* phycobiliproteins were obtained by calibrated Sephadex chromatography and by gel electrophoresis.

Phycobilisomes of *P. cruentum*, under the growth conditions used, consisted mainly of PE (84%) and lesser amounts of R-PC (11%) and APC (5%). The phycobiliproteins appear to account for the major, or even entire, protein content of phycobilisomes (Table II). Determinations of total protein by the Lowry method were essentially the same as those calculated from the absorption spectra by using the extinction coefficients given in Table I. The absence of unpigmented protein bands after electrophoresis of dissociated phycobilisomes also suggests that other proteins were not present.

Subunit Molecular Weights. On calibrated sodium dodecyl sulfate–acrylamide gels APC had one band with an apparent molecular weight of 14,600 (Table IV). R-PC resolved into two bands, which were more easily distinguishable prior to staining because of their color differences; the smaller band (16,400 molecular weight) was blue, whereas the larger band (18,400

TABLE II: Phycobiliprotein Content of Phycobilisomes from *Porphyridium cruentum*.

		Calculated Phycobiliproteins (Table I)						
Expt	Total Protein (Lowry/BSA[a]) (μg/ml)	Total Phycobiliprotein (μg/ml)	Phycoerythrin μg/ml	%	R-phycocyanin μg/ml	%	Allophycocyanin μg/ml	%
1	210	203	171	85	23	11	9	4
2	209	216	182	84	21	10	13	6
3	155	160	134	84	18	11	8	5
4	150	149	125	84	16	11	8	5
5	100	106	88	83	13	12	5	5

[a] BSA = bovine serum albumin.

TABLE III: Molecular Weights of Phycobiliproteins from *Phycobilisomes*.

Method[a]	B-PE	b-PE$_I$	b-PE$_{II}$	R-PC	APC
Sephadex G-200	265,000	110,000	55,000	125,000	120,000
Acrylamide gel electrophoresis	280,000	110,000	60,000	ND[b]	ND

[a] Details in Materials and Methods. [b] ND = not determined.

molecular weight) was purplish-pink. b-PE exhibited only one band (17,200 molecular weight) although its leading boundary was somewhat diffuse. B-PE had two bands; the major band had the same mobility as that of b-PE with an apparent molecular weight of 17,300. The second B-PE band had a molecular weight of about 30,000.

Comparison between B- and b-phycoerythrin. The two phycoerythrins from dissociated phycobilisomes appear to be distinct pigments and not merely an aggregation series as one might presume by the differences in their molecular weights.

Difference spectra (Figure 4) between B- and b-PE demonstrated that, per unit of phycobiliprotein weight, b-PE has less absorption at 570 nm and at 495–500 nm than B-PE. An absorption band centered at about 570 nm appears to be generated by aggregation of b_{II} to form b_I because the extinction of this band is reduced when b-PE is diluted. This phenomenon has also been observed in C-PE, another dissociating system, by Bennett and Bogorad (1971). In contrast, equivalent dilution of B-PE produces no such diminution in absorption around 570 nm, and it is well known that B-PE is comprised of very stable aggregates.

On sodium dodecyl sulfate–acrylamide gels only B-PE contained a 30,000 molecular weight band in addition to the 17,300 molecular weight band. A densitometer scan of an sodium dodecyl sulfate gel containing B-PE is shown in Figure 5. The two bands observed were present in the same proportion (approximately 1:6, 30,000:17,300) whether the chromoprotein was incubated at 37° for 2 hr or in a boiling-water bath for 2–5 min before electrophoresis. If the individual protein bands were eluted (0.1% sodium dodecyl sulfate–0.1 M sodium phosphate) from excised gel discs, reincubated in 1% sodium dodecyl sulfate–1% β-mercaptoethanol at 100° for 2 min and reelectrophoresed, single bands with the original mobilities were obtained.

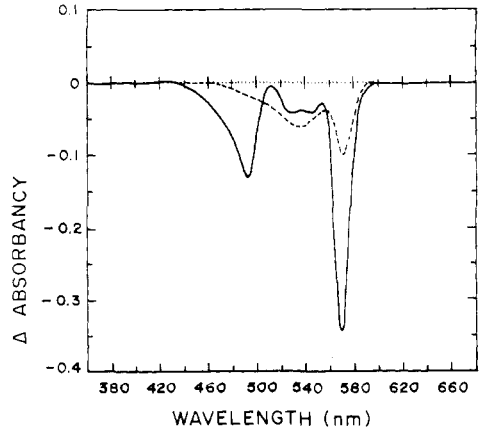

FIGURE 4: Difference spectra between B- and b-phycoerythrin in concentrated (2.2 mg/ml) and dilute solutions (22 μg/ml) in 0.1 M phosphate buffer (pH 6.8). One sample was placed in the sample compartment and another in the reference compartment of the spectrophotometer in the following combinations: concentrated B-PE *vs.* dilute B-PE (. . . .); dilute b-PE *vs.* concentrated b-PE (- - - -); and concentrated b-PE *vs.* concentrated B-PE (—). Path length for concentrated samples was 1 mm; that for dilute samples was 10 cm.

TABLE IV: Subunit Molecular Weights of Phycobiliproteins from *P. cruentum* Phycobilisomes as Determined by Sodium Dodecyl Sulfate–Acrylamide Gel Electrophoresis.[a]

Pigment	Mol Wt
B-phycoerythrin	30,000 ± 1000
	17,300 ± 600
b-phycoerythrin	17,200 ± 600
R-phycocyanin	18,400 ± 700 (purplish-pink)
	16,400 ± 700 (blue)
Allophycocyanin	14,600 ± 600

[a] Details in Materials and Methods.

Absorption spectra of the isolated subunits are shown in Figure 6. The absorption maximum of the large subunit is at about 492 nm; that of the small subunit is at about 530 nm. The small B-PE subunit (17,300) and the single b-PE subunit (17,200) may be identical, since they also have the same absorption spectrum in sodium dodecyl sulfate reservoir buffer (0.1% sodium dodecyl sulfate–0.1 M sodium phosphate).

Since the possibility existed that b-PE could be a disaggregation product or an enzymatically generated fragment of B-PE, as has been suggested to occur with phytochrome (Gardner *et al.*, 1971), phycobilisomes were dissociated under various conditions of temperature and pH. Their b-PE:B-PE ratios were then determined after the two phycoerythrins were resolved by either gel electrophoresis, G-200 Sephadex chromatography, or centrifugation in linear sucrose gradients. As shown in Table V, the two phycoerythrins were always present in a ratio between 1B:1b and 3B:2b.

On linear sucrose gradients (0.1–1.0 M sucrose), in the various buffers ranging in pH from 6.0 to 8.0, B-PE was completely separated from the other pigments by centrifugation for 16 hr at 130,000*g*. B-PE was present as a discrete band near the bottom of the gradient, whereas b-PE, APC, and R-PC were found in another band near the top of the gradient. It is worth noting that in 0.034 M asparagine-Tris at pH 7.3 the phycobilisomes dissociated much more quickly than in phosphate or citrate buffer. In the Tris-asparagine buffer system there was also a relative increase of b_{II} to b_I-PE.

Neither time nor temperature (Table V) had any significant

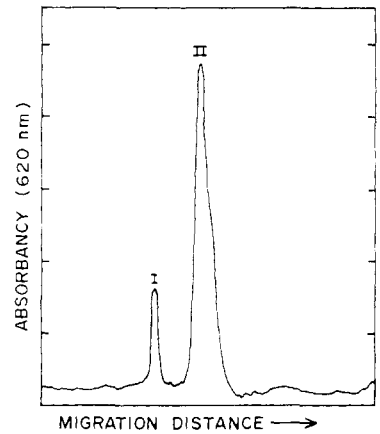

FIGURE 5: Densitometric scan at 620 nm of Coomassie Blue stained sodium dodecyl sulfate–acrylamide gel of B-PE. The large subunit of 30,000 molecular weight is designated by I and the small subunit of 17,300 as II. Details are as given in Materials and Methods.

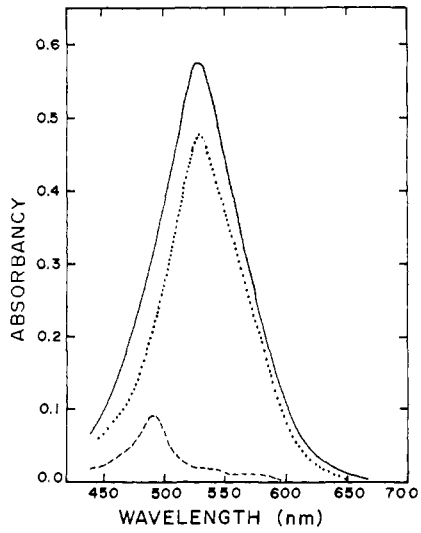

FIGURE 6: Absorption spectra (in 0.1% sodium dodecyl sulfate–0.1 M sodium phosphate buffer) of subunits from B-PE and b-PE derived after sodium dodecyl sulfate treatment and electrophoresis. The large molecular weight subunit of B-PE (- - - -) had an absorption peak at about 492 nm. The absorption peak of the small B-PE (—) and of the b-PE subunit (. . . .) was at about 530 nm.

effect on the B:b ratio. Therefore, enzymatic action does not seem to be a factor. The reduced b-PE percentage (to about 40%) obtained with Sephadex chromatography and gel electrophoresis is attributed to a small decrease in b-PE absorption, which is known to occur with time, especially when dilute.

Discussion

The phycobiliproteins B-PE, R-PC, and APC isolated from crude cell extracts of *P. cruentum* by Haxo *et al.* (1955) are spectrally indistinguishable from those derived from isolated phycobilisomes in the present investigation. We have described b-PE and further characterized these pigments by their subunit molecular weights, fluorescence emission spectra, and aggregation.

R-PC is unique among algal biliproteins because it contains both phycoerytherobilin and phycocyanobilin chromophores (Chapman *et al.*, 1967), which are responsible for the absorption peaks at 555 and 617 nm, respectively. Fluorescence emission in this pigment occurred only at 637 nm which is attributed to the phycocyanobilin chromophore. On sodium dodecyl sulfate gels, R-PC gives rise to two subunit molecular weights of 18,400 and 16,000. The heavy subunit is purplish-pink and the light subunit, blue. These findings are in accord with the observations of O'Carra (1970) who first demonstrated two subunits on sodium dodecyl sulfate gels and suggested that phycoerythrobilin was bound only to the heavy subunit and phycocyanobilin only to the light subunit. The molecular weight of R-PC on a calibrated Sephadex G-200 column was 127,000 at pH 6.8. This corresponds to about half the value of that obtained by Eriksson-Quensel (1938) for a single R-PC component at $s_{20,w}$ of 11.4 S (273,000 molecular weight).

The aggregation state of R-PC seems to be pH dependent because when phycobilisomes were dissociated at pH 5.5 (observations, our laboratory) some R-PC sedimented on sucrose gradients with B-PE.

We regard B-PE and b-PE as two distinct, but related biliproteins because: (a) B-PE contains an absorption shoulder at 495–500 nm, which is not present in b-PE; (b) B-PE also has a larger aggregated molecular weight (about 280,000) than b-PE (about 110,000 and 60,000); and because (c) we have not observed any interconversions between B- and b-PE *in vitro*.

TABLE V: Content of B-phycoerythrin (B-PE) and b-phycoerythrin (b-PE) from Phycobilisomes Dissociated in Various Buffers and Separated by Electrophoresis, Sucrose Density Centrifugation, and on Sephadex G-200.[a]

Method of Separation	% of Total Phycoerythrin	
	B-PE	b-PE
Sucrose gradients		
0.1 M Sorensen's phosphate (pH 6.8)	51	49
0.034 M asparagine-Tris (pH 7.3)	57	43
0.1 M sodium citrate (pH 6.0)	57	43
0.1 M Sorensen's phosphate (pH 8.0)	57	43
Sephadex G-200		
0.1 M Sorensen's phosphate (pH 6.8)	60	40
Acrylamide electrophoresis		
Dissociated at 4° in 0.01 M phosphate (pH 6.8)	62	38
Dissociated at 20° in 0.01 M phosphate (pH 6.8)	62	38
Dissociated at 4° in 0.01 M phosphate (pH 6.8) and stored 12 hr at 30°	64	36

[a] Details in Materials and Methods.

The 30,000 molecular weight subunit of B-PE obtained by sodium dodecyl sulfate–gel electrophoresis was stable upon sodium dodecyl sulfate reelectrophoresis and, therefore, does not appear to be simply a dimer of two 17,300 molecular weight subunits. The adsorption peak at 492 nm of the large subunit is presumed to be responsible for the distinctive 495–500-nm shoulder which characterizes B-PE. A subunit with a similar absorption (500 nm) had also been obtained from B-PE treated with *p*-chloromercuribenzoate by Fujimori and Pecci (1967). They suggested that the 500-nm absorption is due to a phycourobilin chromophore. Presently, however, some doubt exists about this chromophore because Siegelman *et al.* (1968) have not found any variants to phycoerytherobilin and phycocyanobilin, which they regard as the two "native" chromophores.

A small PE, which we designate b-PE, has been noted in several red algae and has been described with various degrees of confidence (Tiselius *et al.*, 1956; Airth and Blinks, 1957; Vaughn, 1964; Neufeld, 1966; Nolan and O'hEocha, 1967; Mieras and Wall, 1968; Teale and Dale, 1970; Van der Velde, 1973). The existence of b-PE has often been completely overlooked, as in our earlier work (Gantt, 1969). We had naively assumed that *P. cruentum* contained only B-PE. In retrospect we know that b-PE was denatured and discarded in the 1-butanol treatment which was part of the isolation procedure for purification of B-PE. Airth and Blinks (1957) were the first to partially separate the two phycoerytherins from *Porphyra naiadum*. Their fresh extracts contained more b-PE (= PE II, their designation) than B-PE (= PE I). Since the B-PE percentage increased upon standing, they assumed that the small PE was an intermediate to large PE. Van der Velde (1973) drew a similar conclusion from his observations with *Acrochaetium virgatulum*. Neufeld (1966), however, regarded b-PE as a possible breakdown product of B-PE. The constant B-PE:b-PE ratio (1.0), obtained from dissociated *P. cruentum* phycobilisomes upon analysis on sucrose gradients, shows that neither aggregation nor disaggregation of B-PE occurs in isolated phycobilisomes. However, it does not exclude the possibility that aggregation or disaggregation could occur in crude cell extracts.

Our results on *P. cruentum* phycoerythrins differ from those reported for *Acrochaetium virgatulum* by Van der Velde (1973) in two major respects. We did not observe a 495–500-nm shoulder on b-PE as Van der Velde did on PE II. On sodium dodecyl sulfate gels both phycoerythrins of *A. virgatulum* had two subunits (19,800 and 22,500 molecular weights), whereas in *P. cruentum* b-PE had only one subunit (17,200 molecular weight) and B-PE had two (17,300 and 30,000 molecular weights). The differences may reflect variability between different algal species. The discrepancies in the spectra might also be attributed to a slight contamination of one phycoerytherin with another, because as is shown in Figure 2, Sephadex G-200 chromatography does not separate the two phycoerythrins completely.

Although we regard B- and b-PE as two physically distinct aggregated chromoproteins they may be related. They may share a 17,300 molecular weight subunit (see Table IV) and thus be structurally related at this level. The functional importance of the two phycoerythrins is not known. Both are presumed to function in absorbing light used in photosynthesis; however, they may have different ratios of sensitizing and fluorescing chromophores (Dale and Teale, 1970). Perhaps b-PE also aids in the close packing of the phycobilisome structure, thus facilitating maximum energy transfer.

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