

Phycobilisomes from Blue-Green and Red Algae

ISOLATION CRITERIA AND DISSOCIATION CHARACTERISTICS¹

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ABSTRACT

A general procedure for the isolation of functionally intact phycobilisomes was devised, based on modifications of previously used procedures. It has been successful with numerous species of red and blue-green algae (*Anabaena variabilis*, *Anacystis nidulans*, *Agmenellum quadruplicatum*, *Fremyella diplosiphon*, *Glaucosphaera vacuolata*, *Griffithsia pacifica*, *Nemalion multifidum*, *Nostoc* sp., *Phormidium persicinum*, *Porphyridium cruentum*, *P. sordidum*, *P. aeruginum*, *Rhodospirillum rubrum*). Isolation was carried out in 0.75 molar K-phosphate (pH 6.8 to 7.0) at 20 to 23 C on sucrose step gradients. Lower temperature (4 to 10 C) was usually unfavorable resulting in uncoupling of energy transfer and partial dissociation of the phycobilisomes, sometimes with complete loss of allophycocyanin. Intact phycobilisomes were characterized by fluorescence emission peaks of 670 to 675 nanometers at room temperature, and 678 to 685 nanometers at liquid nitrogen temperature. Uncoupling and subsequent dissociation of phycobilisomes, in lowered ionic conditions, varied with the species and the degree of dissociation but occurred preferentially between phycocyanin and allophycocyanin, or between phycocyanin and phycoerythrin.

considered to possess an integrity which is close to that in the *in vivo* condition. Uncoupling of energy transfer between phycobiliproteins as measured by the fluorescence emission appears concomitant with physical dissociation. Fluorescence spectra of intact and partially dissociated PBS are included as indicators for assessing the integrity of PBS preparations.

MATERIALS AND METHODS

All of the unicellular algae were cultured in liquid media, supplied with 5% CO₂ and air, with shaking and continuous illumination with daylight fluorescent light (about 1,500 $\mu\text{w}/\text{cm}^2$). *Anabaena variabilis* was grown at 30 C (Kato and Gantt, in preparation), the other blue-green algae were grown at 37 C on media as referenced: *Agmenellum quadruplicatum* (20), *Anacystis nidulans*, *Nostoc* sp., and *Fremyella diplosiphon* (23). All red algae were grown at 18 C and the following were grown in artificial seawater developed by Jones *et al.* (15): *Porphyridium cruentum*, *Nemalion multifidum*, and *Rhodospirillum rubrum*. *Porphyridium sordidum* was grown on a dilute seawater medium (20), whereas *P. aeruginum* was grown on a freshwater medium (5). *Griffithsia pacifica* was a gift from Drs. R. and S. Waaland, *Glaucosphaera vacuolata* from Dr. J. Cain, and *Phormidium persicinum* from Drs. D. Berns and R. MacColl. Cultures of *F. diplosiphon* and *Nostoc* sp. were also grown under a red plastic filter (P-14, Gelatin Products, Glen Cove, N.Y.) with maximum transmission above 600 nm.

Phycobilisomes contain the phycobiliproteins which are major light-harvesting pigments in red and blue green algae (4). Isolation of PBS³ facilitates studies on characterization of their morphology, phycobiliprotein composition, and the interaction and reaggregation properties of the phycobiliproteins. PBS were first isolated without prefixation from the red alga *Porphyridium cruentum* in 0.5 M phosphate buffer on a sucrose step gradient at 4 C (6). The procedure was subsequently modified for some blue-green algae (11), but it was not generally applicable to other species, as judged from shorter wavelength fluorescence emission at 660 to 665 nm instead of 670 to 675 nm. Further modifications have been made and it was found that the most crucial condition to maintain PBS integrity, in addition to high ionic strength, is temperature. We have found that it is important that isolations be done at about 20 to 23 C, and that the phosphate buffer content be at 0.75 M (pH 6.8). The simplified procedure described here gives high PBS yields from red and blue-green algae thus far tried. These PBS exhibit the best energetic coupling of any obtainable, and are

The entire procedure for PBS isolation was carried out at room temperature (20-23 C) in 0.75 M K-phosphate (pH 6.8-7.0). Cells were collected by centrifugation, sometimes after settling overnight, and rinsed twice in buffer. One to 2 g (wet weight) of cells were suspended in 10 ml buffer and then broken in a French pressure cell at 10,000 p.s.i. Triton X-100 was immediately added to the broken cells, to a final concentration of 2% (or more), and the mixture was incubated for 20 min with stirring. Large fragments and cell debris were removed by centrifugation for 30 min at 25,000g. To decrease Chl contamination, the supernatant was removed with a syringe from underneath the floating Chl layer. Supernatant samples were then layered (2-4 ml per tube) on a buffered step gradient with the following sucrose molarity: 2.0 M (6 ml), 1.0 M (4 ml), 0.5 M (4 ml), and 0.25 M (8 ml). The gradients were centrifuged for 3 h in an angle head rotor (Beckman 42.1 at 42,000 rpm) at 136,000g. PBS, during this time period, became concentrated in the 1.0 M sucrose layer, from which they were removed by suction through a flat tipped syringe needle. PBS were then stored in the suspending medium, or were diluted (3- to 4-fold) with buffer and concentrated by further centrifugation for 2.5 h at 254,000g. Cell breakage of some species such as *A. quadruplicatum* and *A. nidulans* was enhanced by pretreatment with lysozyme. Others such as *N. multifidum* required grinding with sand in a mortar and pestle. However, *G. pacifica* was broken very readily with two strokes in a glass homogenizer.

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³ Abbreviations and definitions: PBS: phycobilisomes; PE: phycoerythrin; PC: phycocyanin; APC: allophycocyanin; "dissociation": term used when fluorescence emission showed uncoupling of energy transfer, but when phycobiliprotein separation was not ascertained as in reference 8.

Table I. Phycobiliproteins in phycobilisomes of red and blue-green algae isolated by the method described.

Culture obtained from: (1) Dr. T. Katoh, Kyoto University, Japan; (2) Dr. J. Myers, U. Texas, Austin; (3,5) Dr. C. Van Baalen, U. Texas, Port Aransas; (4) Drs. J. Hauray and L. Bogorad, Harvard U., Cambridge; (6) Drs. D. Berns and R. MacColl, New York State Dept. of Health, Albany; (7) Dr. J. Cain, Illinois State U., Normal; (8) Drs. S. and R. Waaland, U. Washington, Seattle; (9) U. Texas Culture Collection of Algae (No. LB 745); (10) Dr. R. Stainier, Inst. Pasteur, Paris, France; (11) Dr. M. B. Allen, deceased; (12) Dr. F. Ott, Memphis State U., Memphis; (13) Dr. G. Giraud, Ecole Normal Superior, Paris, France.

Species (strain)	Phycobiliprotein types ^a	Identification of pigments
Cyanophytes:		
1. <i>Anabaena variabilis</i>	C-phycoerythrin, phycoerythrocyanin, allophycoerythrin	(3)
2. <i>Anacystis nidulans</i> (Tx20)	C-phycoerythrin, allophycoerythrin	U. O.
3. <i>Agmenellum quadruplicatum</i> (PR6)	C ₆₂₃ ⁻ , C ₆₃₅ ⁻ phycoerythrin, allophycoerythrin	(10)
4. <i>Fremyella diplosiphon</i>	C-phycoerythrin ^b , C-phycoerythrin, allophycoerythrin	(2,12)
5. <i>Nostoc</i> sp. (Mac)	C-I and C-II phycoerythrin ^b , C-phycoerythrin, allophycoerythrin	(11,12)
6. <i>Phormidium persicinum</i>	C-phycoerythrin, C-phycoerythrin, allophycoerythrin	U. O., (14)
Rhodophytes:		
7. <i>Glaucosphaera vacuolata</i>	R-phycoerythrin ^c , C-phycoerythrin, allophycoerythrin	U. O.
8. <i>Griffithsia pacifica</i>	R-phycoerythrin, R-phycoerythrin, allophycoerythrin	U. O.
9. <i>Nemalion multifidum</i>	R-phycoerythrin, R-phycoerythrin (?), allophycoerythrin	U. O.
10. <i>Porphyridium aeruginum</i>	C-phycoerythrin, allophycoerythrin	U. O.
11. <i>Porphyridium cruentum</i>	B-, b-phycoerythrin, R-phycoerythrin, allophycoerythrin	(4,18)
12. <i>Porphyridium sordidum</i>	B-phycoerythrin, C-phycoerythrin, allophycoerythrin	U. O.
13. <i>Rhodorus marinus</i>	B-, b-phycoerythrin, C-phycoerythrin, allophycoerythrin	U. O.

U.O. - unpublished observations on pigments fractionated in our laboratory from PBS.

^a - Far emitting APC forms occur in all but have not been specifically purified from most species [9].

^b - Present in cultures grown in white light.

^c - A novel R-PC with a 563/615 nm ratio of 1.2.

Absorption spectra at room temperature were taken on a Cary 17 spectrophotometer. Fluorescence spectra were taken on an Aminco-Bowman fluorimeter equipped with an automatic corrected spectrum attachment. Emission spectra at liquid N₂ temperature were taken of samples frozen in an NMR tube (4 mm i.d.). For emission spectra at room and liquid N₂ temperatures the samples were diluted in the appropriate concentration of K-phosphate to a protein concentration of about 35 µg/ml which corresponds to an absorption of up to 0.1 A units for the predominant phycobiliprotein.

Chl estimations were made according to Arnon (1). Protein concentrations were determined by the method of Lowry *et al.* (19), using BSA as a standard.

Phycobiliproteins from PBS were partially or completely separated according to the methods previously used in this laboratory, or as identified by published spectra (Table I).

RESULTS

Isolation Conditions, and Characteristics of Intact PBS. In assessing the pigmented zones of the isolation gradient by absorption it was found, as previously reported (6, 11, 17), that Chl, carotenoids, and Cyt were on top of the 0.25 M sucrose layer. A mixture of loose phycobiliproteins was also in this layer, while PBS fragments extended down into the 0.50 M layer. Intact PBS were present in the 1 M sucrose layer. In many species about 85 to 90% of the total phycobiliproteins were present in the PBS layer. In some species more fragmentation occurred thus reducing recovery in the 1 M layer with increased pigment amounts in the upper layers.

PBS preparations were relatively free of contamination. Comparing the sum of the phycobiliprotein absorption peaks (λ max) with A at 275 nm a ratio of 4 or higher was obtained (Figs. 1A and 2A), and was typical for all PBS preparations. Upon PBS dissociation, the particle size decreased thus reducing light scattering in the UV region, and resulting in even higher ratios of $\frac{\gamma \text{ max}}{275 \text{ nm}}$. When the sucrose step gradients were heavily loaded, Chl contamination in the PBS preparation was as high as 1 µg

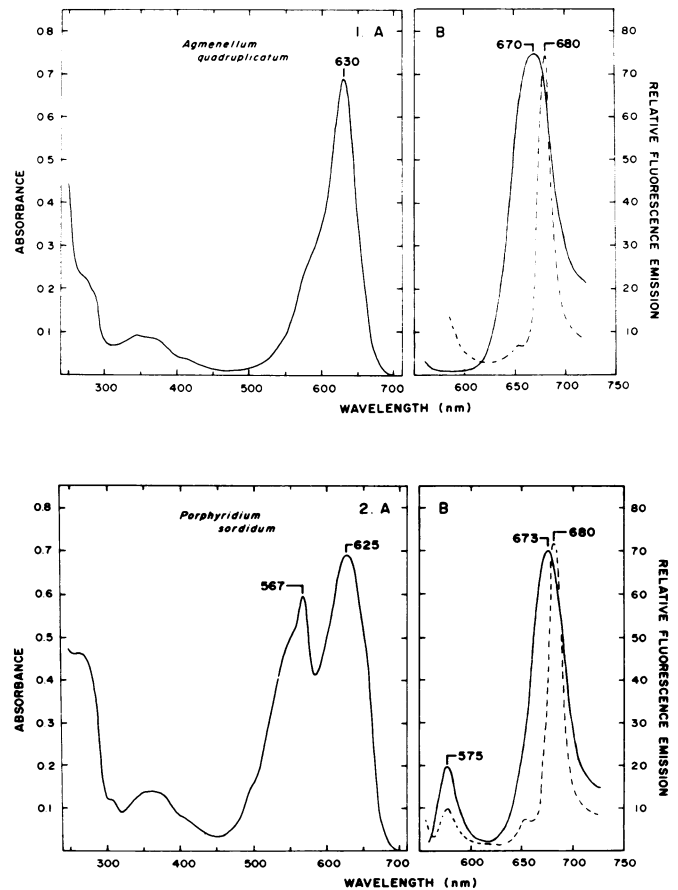


FIG. 1 and 2. PBS isolated from a blue-green algae (Fig. 1) and a red alga (Fig. 2) in 0.75 M K-phosphate (pH 6.8) at 23 C. A: Absorption spectra; B: fluorescence emission spectra with excitation at 545 nm. Relative emissions shown at 23 C (—) and at -196 C (---). Peak heights at -196 C were scaled down for ease of comparison. Pigment composition as in Table I.

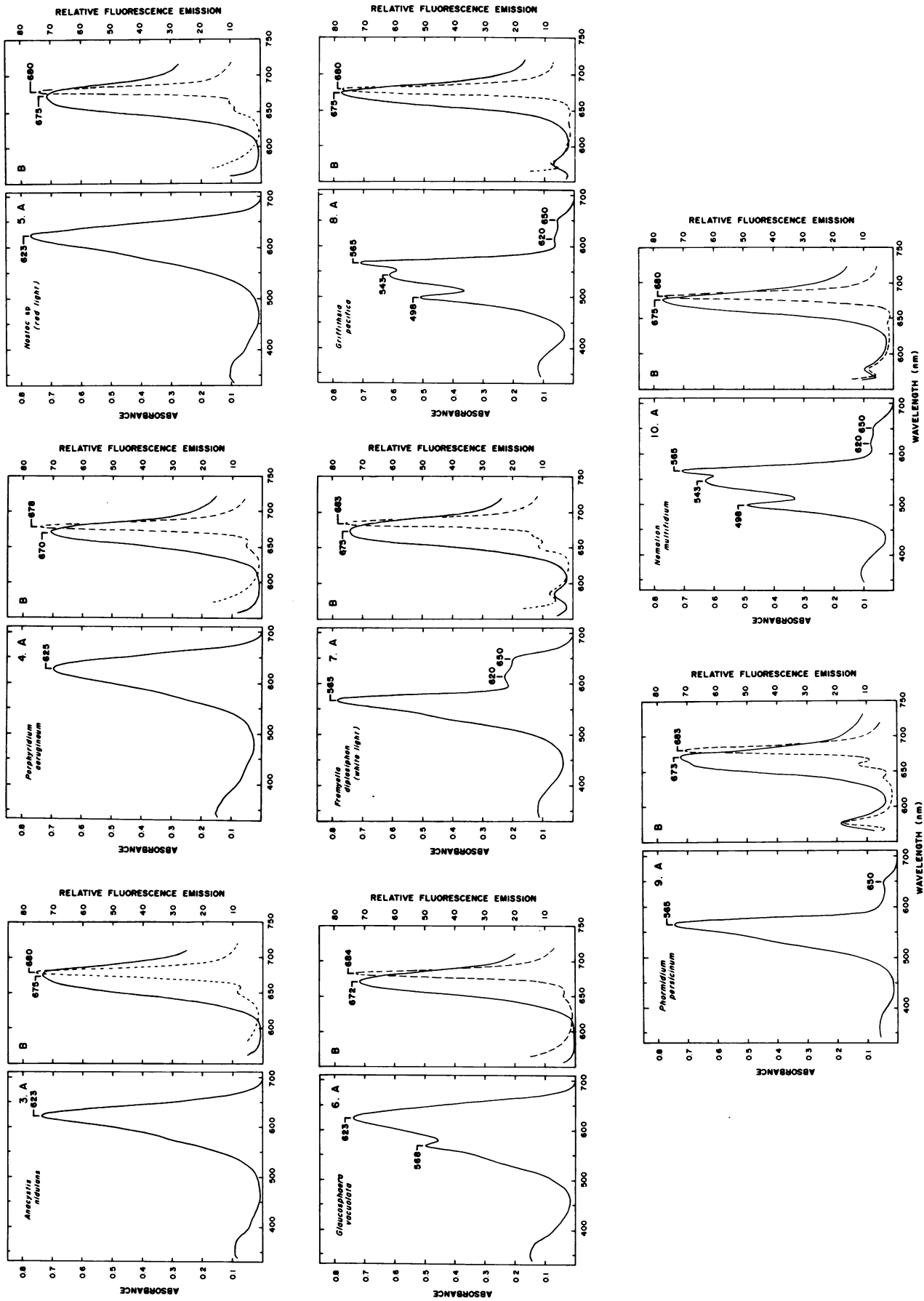


FIG. 3-10. Absorption spectra at room temperature (A), and fluorescence emission spectra (B) at room (—) and liquid N₂ temperature (---), with excitation at 545 nm of isolated PBS. From blue-green algae on the left and red algae on the right. *Nostoc* sp. (Fig. 5) was grown under a red filter. Pigment compositions as in Table I.

Chl/100 μ g protein in such blue-green algae as *Nostoc* sp. This contaminating Chl is not a functional constituent in PBS (8), and could be reduced by incubating the PBS preparation in 1 or 2% Triton X-100 overnight. After the second exposure to the detergent, the PBS preparations of *Nostoc* sp. had less than 1 μ g Chl/40 g protein, and about 1 μ g Chl/25 g protein in *P. cruentum*, which in the latter species has been calculated to be about 1 Chl molecule/10 PBS. Electron microscopic examination of negatively stained PBS preparations sometimes revealed other contaminants such as small fragments of cell wall and membranes, glycogen-type storage products, as well as pro-phage particles. These contaminants, which occur in small amounts and are hard to express quantitatively, can be further reduced by putting the PBS preparation through additional purification steps.

The absorption spectra of PBS recovered from the 1 M sucrose layer of the step gradient are shown in Figures 1A through 11A. A great diversity is exhibited from one species to another and reflects different phycobiliprotein types and proportions. The *A* of the principal phycobiliproteins are marked in each species with PE *A* ranging from 498 to 567 nm, PC from 610 to 630 nm, with overlaps of APC which peaks at about 650 nm. All species contained APC and PC, while some also contained PE. Table I gives a list of species from which PBS were isolated and their phycobiliprotein composition including some of the known types of PE and PC.

Presence of PE in *Nostoc* sp. and *F. diplosiphon* (2) was dependent on the predominant wavelength under which cultures were grown because these species readily undergo chromatic adaptation. In fluorescent white light, which has a high output in the green region, PE was formed (Fig. 7) whereas when both of the above algae were cultured through many changes in continuous red light, PE was not produced (Fig. 5). These were the only two species, of those listed in Table I, which were capable of complete adaptation to wavelength.

Fluorescence emission spectra of PBS from various algae are shown in Figures 1B through 11B. The major emission peak always occurred at about 670 to 675 nm at room temperature, and at 678 to 685 nm at liquid N₂ temperature. This was also the case in *P. cruentum* (8), in *A. variabilis*, and *Nostoc* sp. grown in white light (11). The emission wavelength of intact PBS is generally independent of the exciting wavelength. Tight energetic coupling between the phycobiliproteins of the PBS is also indicated by the relative lack of fluorescence loss from PC and PE. All preparations examined were well coupled by this criterion, but the most tightly coupled PBS appeared to be those from red algae. The major emission peak in the reds was generally more symmetrical than those of the blue-greens which often revealed shoulders at 660 nm, particularly notable in *A. nidulans* (Fig. 3B), and *P. persicinum* (Fig. 9B). Such asymmetrical emission peaks suggest the presence of several components with about equal fluorescence intensity. Examinations of the preparations at liquid N₂ temperature were made to try and resolve the components. Instead of two or more expected major peaks, only one or two minor additional peaks were sometimes revealed at 650 to 660 nm (Figs. 1B-7B, 9B, 11B). Additional minor peaks were not only found in those species with an asymmetrical peak, but also when the major peak was symmetrical (Figs. 2B, 4B, 6B). The apparent discrepancy of the emission spectra at two temperature extremes is not yet understood, but the results at -196 C may be due to PBS which are energetically more tightly coupled, or to the effect of freezing.

PBS' integrity is affected by temperature. Their isolation at 4 to 10 C was not possible in most species, and only phycobiliprotein aggregates were recovered in the upper or middle sucrose layers. They were not tightly coupled, as shown by emission loss through PE and PC, and sometimes APC (at 660-665 nm). An unusually clear example is provided by *R. marinus*, where PBS isolated at room temperature (Fig. 11) had a full pigment complement and emission at 675 nm. A preparation isolated at 4 C under the same

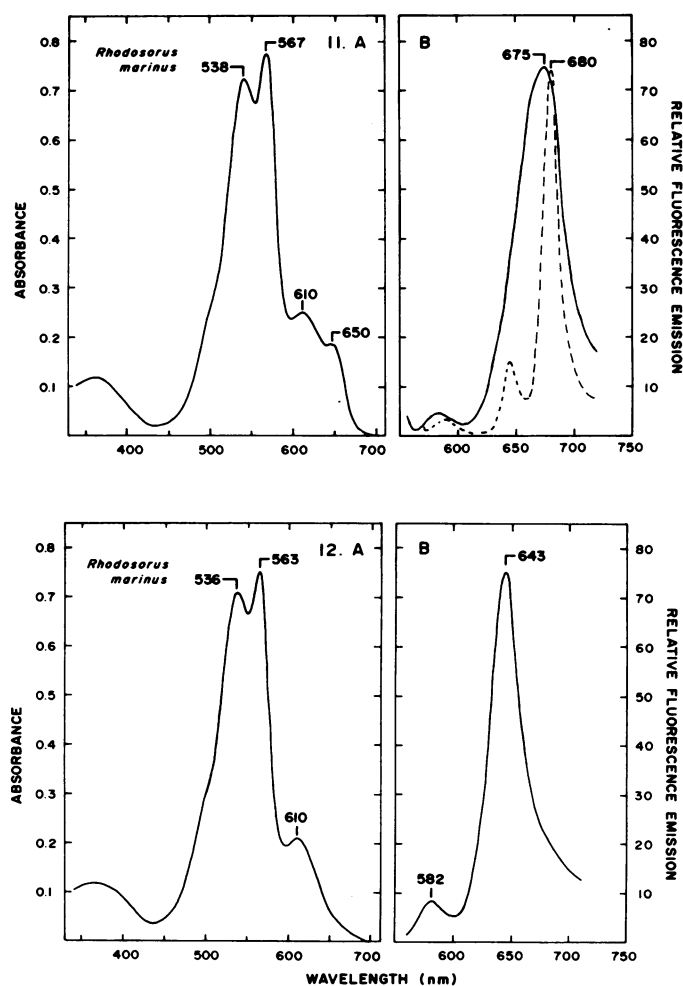
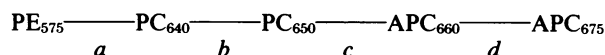


FIG. 11 and 12. Absorption (A) and fluorescence emission (B) spectra of PBS isolated from *Rhodospirillum rubrum* at 23 C (Fig. 11), and of phycobiliprotein aggregates at 4 C (Fig. 12).

conditions yielded a PE-PC complex; it showed a substantial reduction in 650 nm absorption (Fig. 12) and an enhanced emission at 643 nm, both indicating the loss of APC.

Dissociation Patterns of PBS. Uncoupling of PBS can occur between two or more pigments, with the rate being affected by the susceptibility of the pigments to lowered ionic strength. Examination of fluorescence emissions of dissociating PBS has revealed multiple peaks suggesting uncoupling at one or several points. The following emission forms and points of uncoupling can be noted in the various algae:



Several general types of dissociation are suggested, although a more thorough analysis by our means is complicated by unavoidable emission overlaps and nonequivalent excitation of each constituent pigment. States of uncoupling in dissociating conditions (0.05 M phosphate) are illustrated in Figures 13 through 16. In the first type, most evident is the initial drop in the 673 nm emission peak and an increase of PE fluorescence (575 nm) (Fig. 13) which resulted from the probable uncoupling at *a*; continuation of the dissociation revealed subsequent uncoupling between the other pigments (8). Another common pattern was characterized by the initial shift in emission from 673 to 650 nm (Figs. 14 and 15) which represents uncoupling at *c*, and probably *d*. The most complicated is represented in Figure 16 where in 30 s the peak had already shifted from 673 nm to 650 nm. By 1 h a substantial

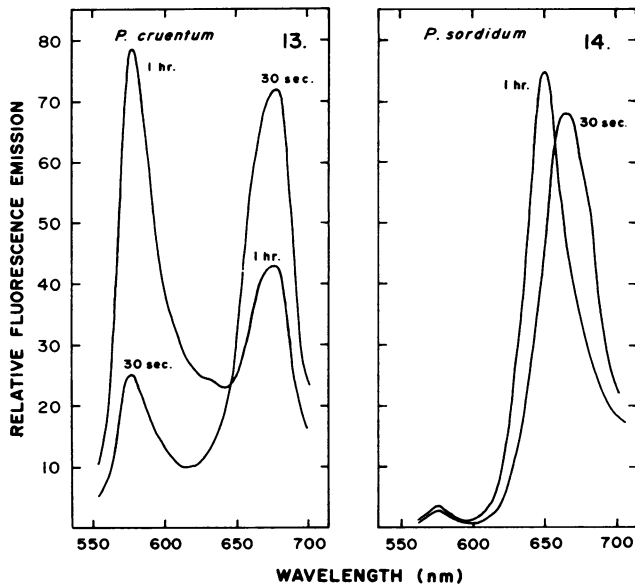


FIG. 13 and 14. PBS in dissociating conditions: 50 mM K-phosphate (pH 6.8). Emission spectra with excitation at 545 nm, made at 23 C. Figure 13: *Porphyridium cruentum*; Figure 14: *Porphyridium sordidum*.

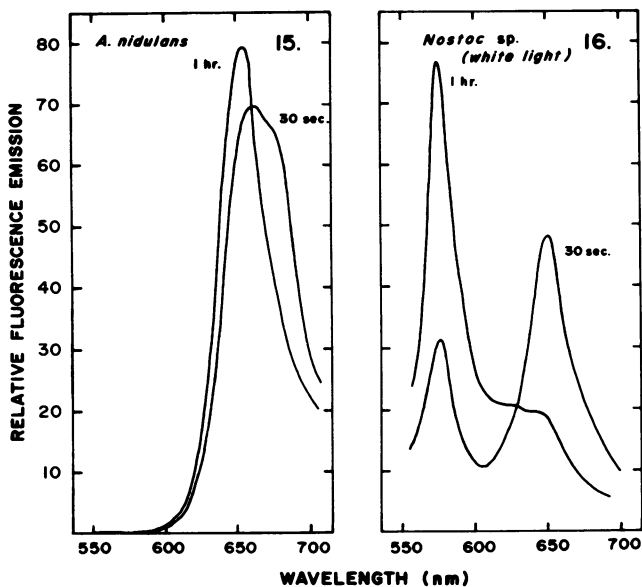


FIG. 15 and 16. PBS in dissociating conditions: 50 mM K-phosphate (pH 6.8). Emission spectra with an excitation at 545 nm, made at 23 C. Figure 15: *Anacystis nidulans*; Figure 16: *Nostoc* sp. grown in white light.

uncoupling had occurred at *b*, as suggested by the shoulder at about 635 nm, and at *a* with an increased peak at 575 nm and resulting in the lack of transfer indicated by the decrease from 635 to 650 nm. This sequence of dissociation when carried out at higher phosphate buffer concentrations (0.25 M) (not shown) revealed that the uncoupling at *d* occurred first, followed by *c*, and lastly by *a*.

PE release does not always progress in the sequence indicated either in *P. cruentum* (Fig. 13), or white-light-grown *Nostoc* sp. (Fig. 16). In *P. sordidum* (Fig. 14), where the PBS contain the same proportion of PE as that in *Nostoc*, this pigment is more tightly coupled in low phosphate than in high phosphate (Fig. 2) and exists as a complex with PC. This complex has been isolated (7) and found to undergo reversible dissociation and thus far represents the only recombinable *in vitro* system.

DISCUSSION

This study showed that PBS can be isolated from all blue-green and red algae tried, whether freshwater or marine. It is an improvement over the original procedure and its modifications (6, 11, 17, 18, 21, 22). The main criterion used in assessing well coupled PBS is the fluorescence emission at 670 to 675 nm at room temperature, and 678 to 685 nm at -196°C . This emission was obtained in all 13 species, providing that the entire isolation was carried out at room temperature, including the centrifugation on the sucrose-phosphate step gradient. It has been noted that the sucrose step gradient can be omitted (21), but this depends on the intended purpose of the PBS preparation. The step gradient, and additional subsequent purification steps are required for removal of free phycobiliproteins, soluble Cyt, adhering proteins, Chl, nucleic acids, and carbohydrate storage products (particularly in the blue-green algae). For example, small amounts of Chl can easily account for the reported PC and APC variations between PBS from early and late log phase cultures (21).

The 670 to 675 nm emission (23 C) of intact PBS arises from far-red-emitting APC forms. One of these, designated as APC-B, has been isolated from various blue-green and red algae (9, 13, 18), another form designated as APC-I has thus far been purified from one blue-green alga (24). Whether emitting at 660 or 675 nm, it is the APC forms and their energetic coupling which show the greatest lability at low temperature, but they are largely recoverable in the zone of free pigments at the top of the gradient. Some are probably also degraded, because we have noted that isolated APCs stored at 4 C were generally less stable than at 23 C.

The interpretations of the dissociation patterns are based partly on analysis of *P. cruentum* PBS (8) which revealed a sequential physical release of phycobiliprotein with concomitant uncoupling as confirmed from fluorescence emission. The order of release was: PE first, followed by R-PC, and finally by APC. The basis for the concept of uncoupling stages (under "Results") includes changes observed in isolated PC forms (11) and the PE:PC complex (7) where the initial longer-wavelength-absorbing (627–637 nm) and -emitting (650–653 nm) forms gradually revert to shorter-wavelength-absorbing (615–623 nm) and -emitting (640–643 nm) forms. Similar observations have been made also in our laboratory with APC complexes. There seems to be also considerable size transformation such as has been observed by Kessel *et al.* (16) who compared PC complexes from fresh cell extracts and highly purified pigment preparations. They found, however, that these changes in size were not accompanied by changes in the fluorescence emission.

Interpretation of the dissociation patterns cannot be made with the precision desired because several factors exist which make reconstructions of the mixture of absorption and emission spectra difficult. (a) Only some of the several varieties of the phycobiliproteins have been isolated and spectrally characterized. (b) Certain forms of the pigments are affected by degree of aggregation, but they are not necessarily those of monomeric protein in dilute solution. (c) Further, interacting prosthetic groups may perturb the emission characteristics.

Although the determination of the morphology of the isolated PBS was not part of this study, it should be noted that the PBS of the different algae exhibit variation in size and shape when examined by electron microscopy. The PBS of *P. cruentum* had a distinct oblate shape and uniform size in sections, as well as when isolated and negatively stained (6, 8). *Rhodella violacea* examined by Koller *et al.* (17) had definitive disc-shaped PBS. In most species examined, the PBS were small and generally disc-shaped. In negatively stained preparations the disc-shaped PBS were less compact and somewhat amorphous, often with spoke-like structures radiating from a center.

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