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FURTHER EVIDENCE FOR A PHYCOBILISOME MODEL FROM SELECTIVE DISSOCIATION, FLUORESCENCE EMISSION, IMMUNOPRECIPITATION, AND ELECTRON MICROSCOPY

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SUMMARY

Phycobilisomes, isolated in 500 mM Sorensen's phosphate buffer pH 6.8 from the red alga, *Porphyridium cruentum*, were analyzed by selective dissociation at various phosphate concentrations. The results are consistent with a structural model consisting of an allophycocyanin core, surrounded by a hemispherical layer of R-phycocyanin, with phycoerythrin being on the periphery. Such a structure also allows maximum energy transfer.

Intact phycobilisomes transfer excitation energy ultimately to a pigment with a fluorescence emission maximum at 675 nm. This pigment is presumed to be allophycocyanin in an aggregated state. Uncoupling of energy transfer among the pigments, and physical release of the phycobiliproteins from the phycobilisome follow a parallel time-course; phycoerythrin is released first, followed by R-phycocyanin, and then allophycocyanin. In 55 mM phosphate buffer, the times at which 50 % of each phycobiliprotein has dissociated are: phycoerythrin 40 min, R-phycocyanin 75 min, and allophycocyanin 140 min.

The proposed arrangement of phycobiliproteins within phycobilisomes is also consistent with the results from precipitation reactions with monospecific antisera on intact and dissociated phycobilisomes. Anti-phycoerythrin reacts almost immediately with intact phycobilisomes, but reactivity with anti-R-phycocyanin and anti-allophycocyanin is considerably delayed, suggesting that the antigens are not accessible until a loosening of the phycobilisome structure occurs. Reaction with anti-allophycocyanin is very slow in *P. cruentum* phycobilisomes, but is much more rapid in phycobilisomes of *Nostoc* sp. which contains 6–8 times more allophycocyanin. It is proposed that allophycocyanin is partially exposed on the base of isolated intact phycobilisomes of both algae, but that in *P. cruentum* there are too few accessible sites to permit a rapid formation of a precipitate with anti-allophyocyanin.

Abbreviation: R-phycoerythrin, phycocyanin containing phycocyanobilin and phycoerythrobilin as chromophores.

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Phycobilisome dissociation is inversely proportional to phosphate concentration (500 mM to 2 mM), and is essentially unaffected by protein concentration in the range used (30–200 μg/ml). Phycobiliprotein release occurs in the same order (phycoerythrin > R-phycocyanin > allophycocyanin) in the pH range 5.4–8.0.

INTRODUCTION

Phycobilisomes (PBsomes) are multiprotein aggregates which serve as light harvesting antennae in red and blue-green algae. In vivo they are attached to the photosynthetic lamellae [1–3] to which they funnel the excitation energy. A major portion of the quanta used to drive photosynthesis is absorbed by phycobiliproteins [4, 5]. Chlorophyll fluorescence is sensitized by light absorbed by these pigments [6, 7], and the energy transfer from the phycobiliproteins to chlorophyll occurs with a high efficiency [8]. Thus, for maximum energy transfer phycobiliproteins can be expected to have an ordered arrangement consistent with the expected sequential energy transfer: phycoerythrin → phycocyanin → allophycocyanin.

We have suggested a structural model on data we obtained using phycobilisomes from the red alga *Porphyridium cruentum* [9, 10]. The essential features of the model are an allophycocyanin core which presumably is in physical contact with the lamellae, and with a hemispherical layer of R-phycocyanin. Phycoerythrin forms an outer layer in contact with R-phycocyanin (and possibly allophycocyanin) on one side, and the stroma on the other.

Phycobilisomes from this alga isolated in high ionic strength phosphate buffer have (a) a major fluorescence emission peak at 675 nm [9], (b) a 545 nm/620 nm (phycoerythrin/R-phycocyanin) absorbance ratio of 8.0, (c) a characteristic prolate shape when examined by electron microscopy, and (d) a particle size with a long axis of 45–50 nm and a short axis of about 32 nm [11]. They do not seem to vary in size or pigment composition with growth under different light intensity or wavelength; they can be stored for days in high salt and high protein concentrations without degradation or loss of energy transfer; and the constituent phycobiliproteins can be separated [10, 11] and quantitatively recovered. Phycobiliproteins account for the entire protein content of the phycobilisomes (84 % phycoerythrin, 11 % R-phycocyanin, and 5 % allophycocyanin). Two related types of phycoerythrin (b-phycoerythrin and B-phycoerythrin) exist in about equal amounts; because of difficulties in distinguishing them in a mixture, they have been referred to collectively as phycoerythrin.

In this paper we show that the physical release of the constituent phycobiliproteins occurs sequentially (phycoerythnin > R-phycocyanin > allophycocyanin), and that uncoupling of energy transfer follows a similar time course. These results together with those obtained with monospecific antisera are consistent with our proposed model. Preliminary accounts of this work were recently presented [12, 13].

MATERIALS AND METHODS

Phycobilisomes from the red alga, *Porphyridium cruentum*, were isolated in 500 mM Sorensen's phosphate buffer pH 6.8 as described previously [10, 11]. Phycobilisomes from the blue-green alga, *Nostoc* sp., were isolated in 750 mM sodium

phosphate buffer according to Gray et al. [14, 15]. *Nostoc* sp. Phycobilisomes were used only in the antisera reactivity tests (Table II).

Fluorescence measurements were made at room temperature with an Aminco-Bowman spectrofluorometer equipped with a 446 UR photomultiplier tube (Hamamatsu-TV) and corrected for the emission grating-phototube efficiency [9]. Fluorescence assays were made by suspending 10 λ of phycobilisomes (in 500 mM phosphate buffer pH 6.8) into 2 ml of the desired molarity (at a final protein concentration of 30–40 μg/ml). Results were expressed as percentages, where maximum fluorescence intensity (30 min in 2 mM phosphate) was equal to 100 %.

Time course dissociation of phycobilisomes was carried out at 4 °C. The phycobilisomes were again suspended in 500 mM Sorensen's phosphate buffer, pH 6.8; then 0.1 ml of the sample was added to 8.5 ml of buffer of chosen concentration to give a final protein concentration of 200 μg/ml. Dissociation time intervals ranged from 0 h (no dissociation) to 4 h. The released pigments were separated from the undissociated phycobilisome components by centrifugation for 15 min at 100 000 $\times g$. The undissociated portion of the phycobilisomes was recovered as a pellet and allowed to dissociate in 10 mM phosphate for 3–4 h. Absorbance of the supernatant fractions and dissociated pellet fractions was measured respectively in a 5 cm or 10 cm path length cuvette in a Cary 17 spectrophotometer. Individual pigment content was calculated using simultaneous equations derived for phycobiliproteins in solution [10]. Values for dissociation were expressed as percentages where the total pigment content recovered equalled 100 %. Caution is required to avoid exceeding the centrifugation forces or time, as doing such resulted in pelleting of free B-phycoerythrin. Degree of phycobilisome dissociation was essentially unaffected by protein concentration (30–500 μg/ml).

Phycobiliproteins, purified from phycobilisome of *P. cruentum* [10], served as antigens. Antisera were prepared by injection of New Zealand white rabbits. Three subcutaneous injections of these antigens in Freund's adjuvant and saline were made over a 4 week period. The rabbits were bled 2 to 4 weeks after the last injection, the sera collected, lyophilized, and stored at 4 °C until use. Antisera specificity was assayed on Ouchterlony plates (pH 6.8 Sorensen's phosphate buffer), and by precipitation in solution. The antisera were specific for their respective antigenic class, i.e., anti-B-phycoerythrin reacted only against phycoerythrin of red and blue-green algae, anti-R-phycocyanin reacted only against R- or cyanophytan phycocyanin, and anti-allophycocyanin reacted only against allophycocyanin whether derived from red or blue-green algae. Preliminary to precipitation reactions reported in Table II, serial dilutions of purified phycobiliproteins (and pigment mixtures derived from dissociated phycobilisomes) were made to determine the proper concentration of antisera and antigen to meet the equivalence zone. Protein concentrations of pigment mixtures from dissociated phycobilisomes in the range of 500 μg–2500 μg total protein/ml were sufficient to form a visible precipitate with the antisera used. Antisera were incubated at room temperature in an equal volume with intact or dissociated phycobilisomes, suspended in 500 mM and 750 mM phosphate buffer for *P. cruentum* and *Nostoc* sp. phycobilisomes, respectively. The reaction times given in Table II indicate the first positive identification of a precipitate in a small test tube viewed against an illuminated background. Although the incubations were always continued for a minimum of 24 h and a maximum of 72 h, a reaction with control sera was not observed. No significant

differences were observed in the specificity of reaction and approximate reaction times with antisera and gamma globulin fractions prepared from the sera by ammonium sulfate precipitation [16].

Protein concentrations were determined according to the method of Lowry et al. [17] using bovine serum albumin as standard.

For electron microscopy phycobilisomes were fixed in 4 % glutaraldehyde (1 h), and after extensive dialysis in 5 mM phosphate buffer were stained with 1 or 2 % phosphotungstic acid and examined in a Phillips EM-300.

All values expressed in the figures and tables represent the average of at least three experiments.

RESULTS

In intact phycobilisomes of *P. cruentum* light absorbed by phycoerythrin or R-phycocyanin is funneled to allophycocyanin, with a predominating fluorescence emission at 675 nm (Fig. 1). Maximum excitation for 675 nm fluorescence is at 545 nm (phycoerythrin); minor excitation peaks also occur at 620 nm (R-phycocyanin) and 650 nm (allophycocyanin). This fluorescence action spectrum is almost identical with the absorption spectrum [11] with the exception of the small shoulder at about 465 nm, of an unknown nature, which is not observed in the absorption spectrum.

Suspending intact phycobilisomes in lower phosphate concentrations (< 500 mM) results in an uncoupling of phycobiliproteins from the energy transfer sequence, and in dissociation (a physical release) of individual phycobiliprotein molecules from the phycobilisome aggregate; these usually occur concomitantly (Fig. 2). Uncoupling,

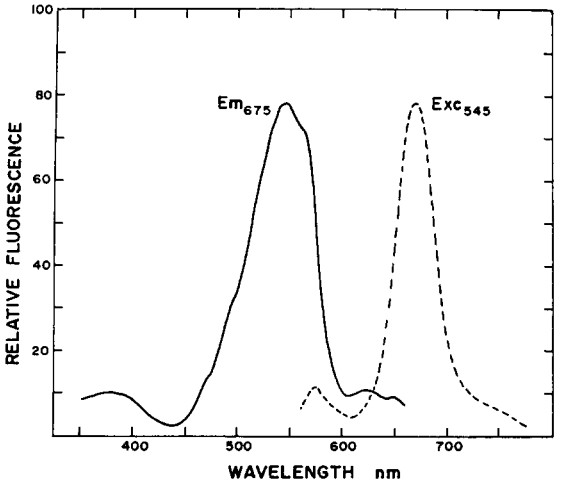

Fig. 1. Fluorescence spectra of intact phycobilisomes in 500 mM phosphate buffer pH 6.8, 23 °C. The excitation spectrum (—) has a maximum peak 545–563 nm (phycoerythrin) and lesser peaks at 620 nm and 650 nm (R-phycocyanin and allophycocyanin, respectively) (emission at 675 nm). The emission spectrum (– – – –) has a peak at 675 nm (excitation at 545 nm); the small peak at 575 nm is due to phycoerythrin.

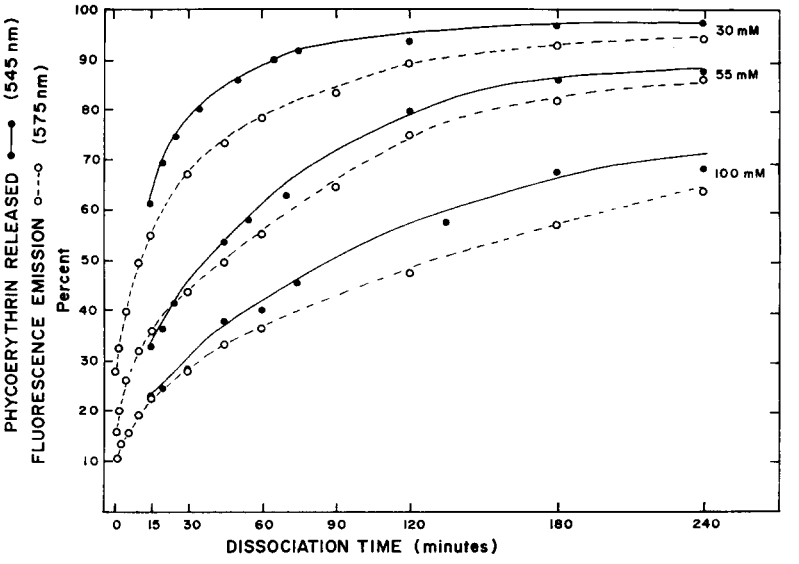

Fig. 2. A comparison of the time dependence of phycoerythrin release and increase in its fluorescence emission from phycobilisomes, at three buffer concentrations. Fluorescence of phycoerythrin at 575 nm (- - - -) was assayed directly on the dissociating mixture by excitation at 545 nm. Phycoerythrin released as free pigment (—) was determined from the supernatant fraction as described in Methods.

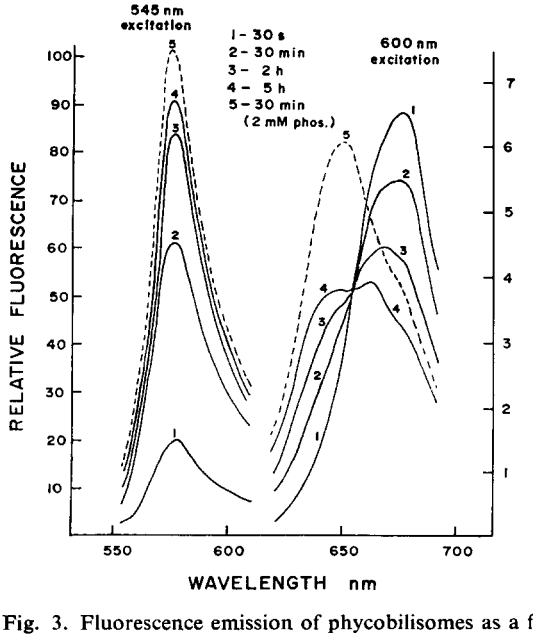

Fig. 3. Fluorescence emission of phycobilisomes as a function of time in 30 mM phosphate buffer pH 6.8 (—), and of phycobilisomes (- - - -) in 2 mM phosphate (30 min). Scale on left is for excitation at 545 nm, and scale on right for excitation at 600 nm. The protein concentration of each sample was 35 μg/ml.

as measured by fluorescence, results in a decrease in energy transfer among the pigments with a diminution of fluorescence at 675 nm and an increase in the relative fluorescence emission peaks of the individual pigments (575 nm phycoerythrin, 636 nm R-phycocyanin, 660 nm allophycocyanin) (Fig. 3).

A comparison of uncoupling of phycoerythrin from the energy transfer sequence, and its physical release from the phycobilisome shows a similar time course (Fig. 2). Suspension of phycobilisomes in 100 mM phosphate buffer results in 50 % dissociation of phycoerythrin at 100-120 min. Release of phycoerythrin is faster in lower ionic strengths, with half-times being 40 and 12 min (extrapolated) in 55 mM and 30 mM phosphate, respectively. As seen in Fig. 2, the percentages of phycoerythrin uncoupled at all three ionic strengths are lower than the percentages of phycoerythrin dissociated. This apparent discrepancy may be due to the fact that the supernatant fraction, in addition to the free phycoerythrin, also contained small pieces of phycobilisomes (not yet uncoupled) which would not be distinguishable from the phycoerythrin by absorption measurements.

Similar sequential events can be observed for all pigments in both the uncoupling (Fig. 3) and the physical release (Fig. 4 and 5). Phycoerythrin uncoupling (575 nm emission) is faster than uncoupling of R-phycocyanin and allophycocyanin (Fig. 3). Excitation of R-phycocyanin and allophycocyanin at 600 nm during dissociation in 30 mM phosphate shows a drop in 675 nm fluorescence emission which is followed by a shift in the emission to shorter wavelengths. Emission from the R-

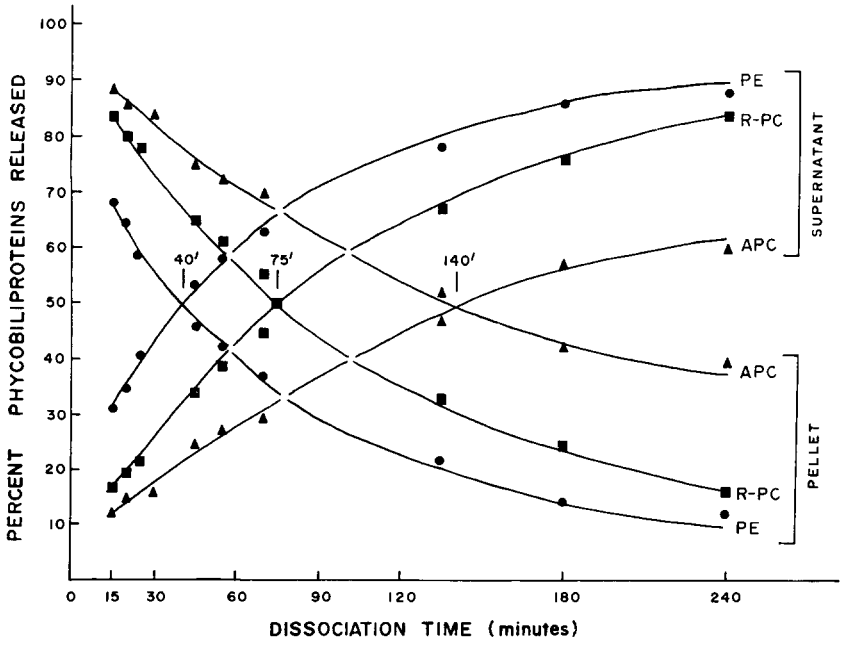

Fig. 4. Time course of phycobiliprotein release from phycobilisomes in 55 mM phosphate buffer from the supernatant fractions and pellet fractions (100 000 × *g*). The 50 % release points are: 40 min phycoerythrin, 75 min R-phycocyanin, and 140 min allophycocyanin.

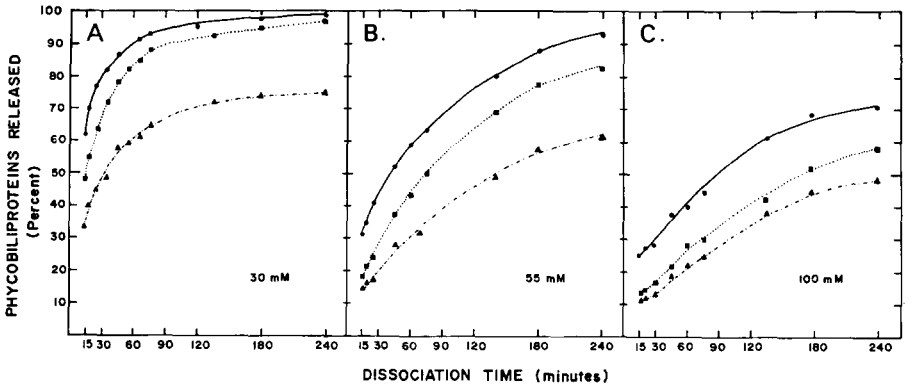

Fig. 5. Time course of pigment release from phycobilisomes in (A) 30 mM, (B) 55 mM, and (C) 100 mM phosphate buffer pH 6.8. phycoerythrin, ● — ●; R-phycocyanin, ■ . . . ■; allophycocyanin, ▲–·–▲.

phycocyanin and allophycocyanin is not detected until about 4–5 h in 30 mM phosphate, when shoulders can be resolved at 640 nm, 660 nm, and 675 nm. In 2 mM phosphate (for 30 min) phycoerythrin is totally uncoupled and a greatly increased emission peak at 650 nm results from allophycocyanin and R-phycocyanin. Even under these conditions an uncoupled "core" portion, as indicated by the 670 nm shoulder still exists and may be due to a small amount of aggregated allophycocyanin. A quantitative recovery of dissociating phycobilisomes is shown in Fig. 4 in 55 mM phosphate buffer. This buffer concentration is convenient for most experimental purposes, as the 50 % "cross-over" points can be obtained within a reasonable period of time; the sequential pigment release occurs with the following 50 % dissociation points: phycoerythrin 40 min, R-phycocyanin 75 min, and allophycocyanin 140 min.

Time course dissociation studies on phycobilisomes at three phosphate concentrations (Figs. 4 and 5) show that dissociation of all the phycobilisome pigments is inversely related to ionic strength. In all cases tested, sequential pigment release is the same. Dissociation of phycobilisomes is probably not a simple unlayering but consists rather of at least two events. The early dissociation rate constants are greater than the latter dissociation rate constants (Discussion). A comparison of the dissociation curves in Fig. 5 (A–C) reveals that at 30 mM phosphate concentration R-phycocyanin dissociation follows that of phycoerythrin much more closely than at 55 mM or 100 mM. At 100 mM phosphate the opposite occurs in that R-phycocyanin more closely follows the release of allophycocyanin. An explanation for this phenomenon is presently not readily apparent. In 100 mM phosphate, R-phycocyanin and allophycocyanin release is retarded, especially in the early dissociation points (15–40 min).

Crucial to a correct understanding of the phycobilisome structure is the interpretation of the dissociation results. One has to assume that the three phycobiliprotein types have similar dissociation constants at a given pH and at certain ionic strengths of the suspending buffer. Phycocyanin aggregation has been shown to be influenced by pH and various ions [18]. One might expect that disaggregation of phycobilisomes would be similarly affected; therefore, to investigate whether the individual pigments were released differently, a dissociation series was carried out in 55 mM Sorenson's

TABLE I

PERCENT PHYCOBILIPROTEINS RELEASED FROM PHYCOBILISOMES IN 55 mM SORENSEN'S PHOSPHATE BUFFER pH 5.4–8.0. DISSOCIATION TIME 80 MIN AT 4 °C

pH	Phycoerythrin	R-Phycocyanin	Allophycocyanin
5.4	54	28	24
5.9	67	43	27
6.6	76	54	38
6.8	71	53	36
7.4	66	48	34
8.0	65	50	31

phosphate with the pH ranging from 5.4 to 8. As seen in Table I, regardless of pH, the three phycobiliprotein types were released in the usual sequence. It should be noted that other buffers were also investigated (citrate, acetate and Tris), but were generally less desirable because allophycocyanin degradation occurred more readily.

Evidence for b-phycoerythrin release is obtained from difference spectra (Fig. 6) of intact versus dissociating phycobilisomes. The b-phycoerythrin absorption peak narrows, with minima at 545 and 573 nm, as it changes from an aggregated or concentrated state to an unaggregated or more dilute state [10]. Such striking absorption changes have not been observed with B-phycoerythrin, therefore, the absorption differences at 545 and 573 nm with time are attributed to the release of b-phycoerythrin. Changes in absorption are also observable in the R-phycocyanin and allophycocyanin region seen as a decrease at 640 nm and about 660 nm. The difference peaks of

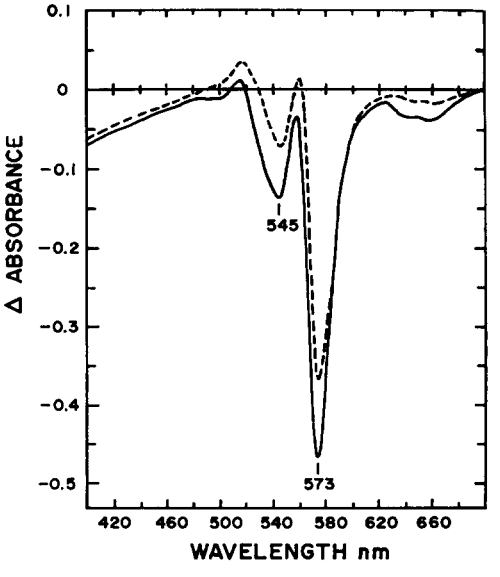

Fig. 6. Difference spectra of intact phycobilisomes in 500 mM phosphate (in reference compartment) vs. rapidly dissociating phycobilisomes in 2 mM phosphate buffer (in sample compartment of Cary 17 spectrophotometer). Dissociation times: 10 min (– – –), and 30 min (—).

phycoerythrin remain essentially unchanged after the initial 30 min, but the R-phycocyanin and allophycocyanin regions continue to increase. This delay in R-phycocyanin and allophycocyanin dissociation is similar to the delayed uncoupling of the energy transfer (Fig. 3). Upon dissociation of phycobilisome the particle size also decreases, which is reflected by a decrease in scatter at wavelengths shorter than 480 nm (Fig. 6).

Accessibility of the phycobiliproteins in phycobilisome of *P. cruentum* was also probed with monospecific antisera. As seen in Table II the reaction times of the three antisera with their respective antigens range, from an immediate reaction to 1.25 h in dissociated phycobilisome when all pigments are readily accessible. In intact phycobilisome the reaction with anti-phycoerythrin is again immediate, but reaction with anti-R-phycocyanin and anti-allophycocyanin are retarded. Therefore, phycoerythrin is readily accessible and is presumed to be present on the surface. To test if the delayed reaction with anti-allophycocyanin in intact *P. cruentum* phycobilisomes was possibly due to a scarcity of reaction sites large enough to form a precipitate, intact phycobilisomes from another alga, containing a larger amount of allophycocyanin were used. The phycobilisomes of *Nostoc* sp. [14, 15] used (Table II) contain the three basic phycobiliprotein types, but in about equal amounts. With these phycobilisomes the anti-allophycocyanin and anti-phycoerythrin reactions were equally fast.

In high phosphate buffer concentrations, after some time, a loosening of phycobilisomes occurs (but not yet a dissociation) which seems to allow the precipitation of the phycobiliproteins with their respective antisera. It has been observed that the final behavior of the precipitates in *P. cruentum* is not identical with the different antisera. When intact phycobilisomes precipitate with anti-phycoerythrin, the precipitate sinks to the bottom and a clear supernatant remains. Even incubation of the anti-phycoerythrin-phycobilisome precipitate in dilute phosphate buffer for 48–72 h does not result in the release of the companion pigments. However, when phycobilisomes react with anti-R-phycocyanin or anti-allophycocyanin, the unreacted companion pigments phycoerythrin and allophycocyanin, or phycoerythrin and R-phycocyanin, respectively, are released into the supernatant. The reaction of anti-phycoerythrin with phycobilisomes and the prevention of further dissociation, i.e.

TABLE II

PRECIPITATION REACTION OF ANTISERA WITH PHYCOBILISOMES FROM *P. CRUENTUM* AND *NOSTOC* SP.

		Time of visible precipitate (h)	
		Undissociated phycobilisomes	Dissociated phycobilisomes
P. cruentum:	Anti B-phycoerythrin	Immediate reaction	Immediate reaction
	Anti R-phycocyanin	1.5	0.75
	Anti allophycocyanin	12.0	1.25
	Control serum	No reaction	No reaction
Nostoc sp.:	Anti B-phycoerythrin	0.5	1.3
	Anti R-phycocyanin	4.3	1.5
	Anti allophycocyanin	0.6	0.9
	Control serum	No reaction	No reaction

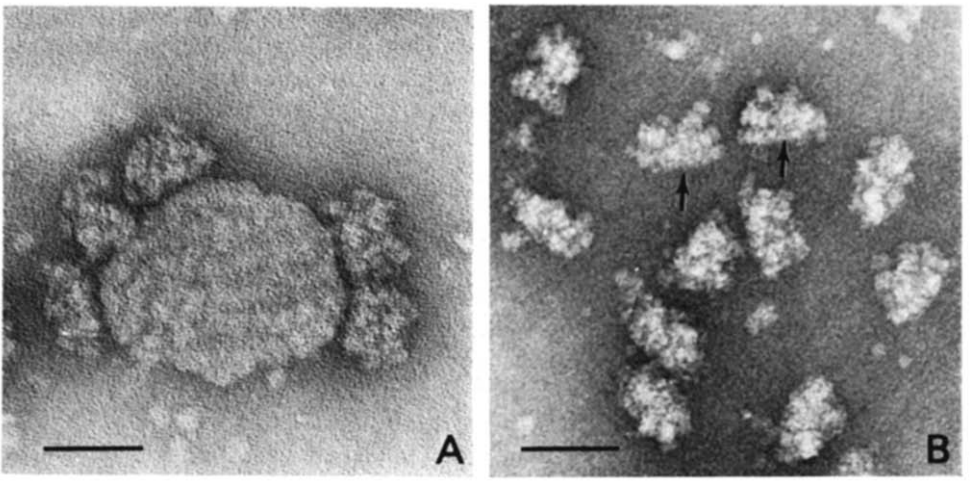

Fig. 7. Phycobilisomes stained with phosphotungstic acid. A, Attached to small thylakoid vesicle. B, Free phycobilisomes (prepared by Triton X-100 treatment) with defined substructures. Arrows indicate the flattened face normally adjacent to the thylakoid membrane. Bar indicates 50 nm.

release of R-phycocyanin and allophycocyanin, further suggests that phycoerythrin is on the outside.

DISCUSSION

The proposed structure of a phycobilisome from *P. cruentum* is shown in a cut-away model in Fig. 8. It is supported by structural data from electron microscopy, time course dissociation studies on the sequential release of the phycobiliproteins, antisera precipitation reactions with intact and dissociated phycobilisomes, and the relative amounts of the phycobiliprotein constituents; it is also an arrangement predicted for maximum energy transfer.

Results from electron micrographs of negatively stained phycobilisomes (Figs. 7A and 7B) are used for the major morphological model features. Negatively stained phycobilisomes have a prolate shape with a long axis of 45–50 nm, a height of 32 nm and a presumed width of 32–35 nm. The phycobilisome surface, adjacent to the lamellae, is flattened (Fig. 7A and Fig. 2A of ref. 19), whereas the surface normally facing into the stroma is rounded. Within each phycobilisome, substructures are discernible which are comparable in size and shape to isolated phycobiliproteins [20–22]. Stacks of the aggregates appear to radiate toward a center (Fig. 7B). The aggregated phycobiliproteins do not appear to be as closely packed as phycobiliprotein crystals, nor do they have as regular an appearance as crystals.

A phycobilisome can spatially accommodate the equivalent of 70–80 B-phycoerythrin molecules (265,000–280,000 daltons per molecule), assuming a close, but not a crystalline packing. B-phycoerythrin of this size is disc-shaped with a diameter of about 10 nm and width of 5 nm, as shown in electron micrographs of isolated pigments [20]. The preferred phycobiliprotein aggregation state in vivo is unknown, but judging from B-phycoerythrin, any number of configurations are possible [20]; in

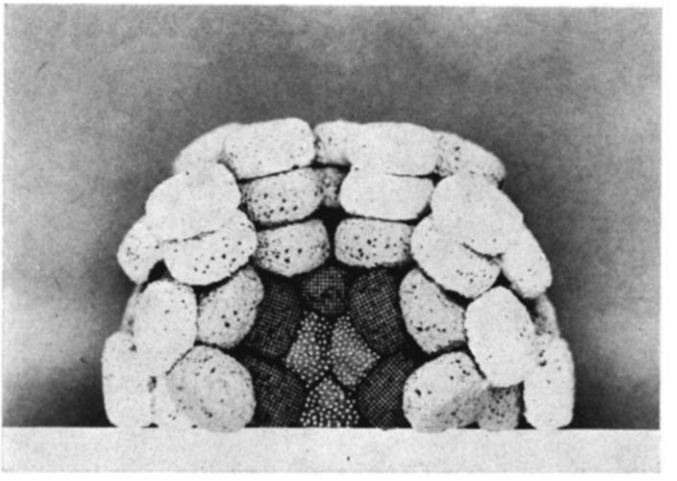

Fig. 8. A phycobilisome as envisioned in the red alga, *Porphyridium cruentum*. The allophycocyanin core (white stipples) is shown to contact the underlying photosynthetic membrane. R-phycocyanin (black stipples) is adjacent to the core, surrounded by phycoerythrin on the outer surface (stroma surface). The core would not normally be exposed, as in this cut-away model, but would be covered by R-phycocyanin and phycoerythrin.

vitro various aggregation states have been known to occur, i.e., attachment of molecules along the 10 nm faces, joining of the 10 nm face with 5 nm faces, and joining of several molecules along the 5 nm faces.

In constructing the model, it has been assumed that discrete phycobiliprotein molecules exist within the phycobilisome, because the dissociation products of B-phycoerythrin, b-phycoerythrin, R-phycocyanin, and allophycocyanin have a finite size with respective molecular weights (Sephadex G-200 values) of 280 000, 110 000 (and 55 000) 127 000 and 120 000 [10]. These molecular sizes can be considered as the minimum, if not the actual in vivo species. Results from difference spectra (Fig. 6) suggest that b-phycoerythrin, R-phycocyanin, and allophycocyanin exist in a more highly aggregated state in the intact than in the dissociated phycobilisome; upon dissociation of phycobilisomes, the b-phycoerythrin disaggregation, manifested by the narrowing of the phycoerythrin absorption band, is particularly striking. The validity of this assumption is strengthened by the fact that it is a reversible reaction and has been shown to occur in vitro with purified b-phycoerythrin [10]. In a concentrated solution (2.2 mg protein/ml), where the aggregation state is presumed to be greater, the absorption peak is broader than in a dilute solution (22 μg protein/ml). Similar effects have also been observed in phycobiliproteins from blue-green algae [15, 23].

The proposed pigment arrangement within the phycobilisome is directly supported by the sequential release of the phycobiliproteins (phycoerythrin fastest, followed by R-phycocyanin, then allophycocyanin). Since the order of release is the same at various buffer concentrations (Fig. 5) and over a range of pH (Table I), the interpretation of the pigment location can be made with a greater degree of confidence. Additionally, there is also a close correlation between physical release of the pigments and the resultant uncoupling expressed as decrease in energy transfer (Figs. 2 and 3).

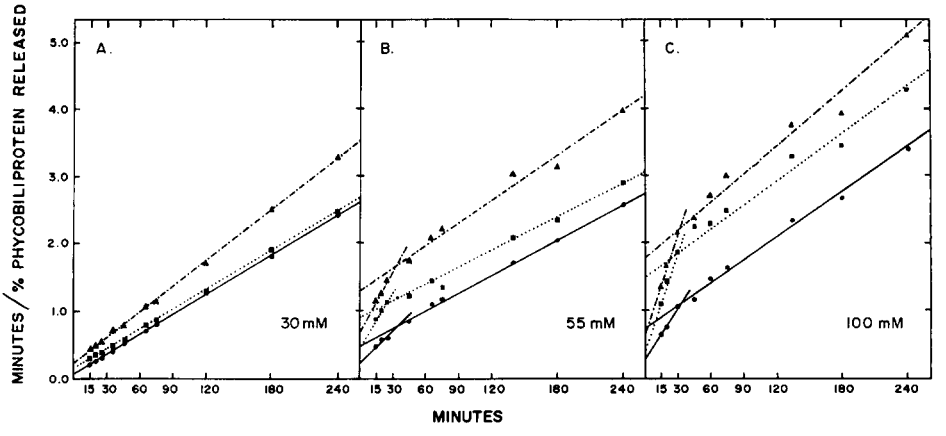

Fig. 9. Rectification of dissociation data from Fig. 5A. 30 mM phosphate, B. 55 mM phosphate, C. 100 mM phosphate. See text for explanation. Phycoerythrin, ●–●; R-phycoerythrin, ■–■; allophycocyanin, ▲–▲.

Results obtained with the anti-sera leave little doubt that phycoerythrin is located on the outside. Localization of allophycocyanin by this method is complicated by the small amount of this pigment in the phycobilisome of *P. cruentum*. The results obtained with *Nostoc* sp. Phycobilisomes, which are assumed to have the same basic phycobiliprotein arrangements, give support to the idea that allophycocyanin is at the phycobilisome base and provides a connection with the photosynthetic lamellae. Although phycobilisomes of *P. cruentum* are presumed to have an exposed allophycocyanin site, the evidence is indirect, and it is possible that the allophycocyanin core is completely covered by the companion pigments and, therefore, could not be in direct contact with the photosynthetic lamellae.

Analysis of the dissociation data from Fig. 5 suggests two classes of dissocia-

TABLE III

INITIAL VELOCITY OF PIGMENT RELEASE FROM PHYCOBILISOMES IN THREE SORENSEN'S PHOSPHATE BUFFER CONCENTRATIONS, pH 6.8.

Phosphate Concentration	Pigment	Initial velocity [% Pigment released / min]	
		Early	Late
30 mM:	phycoerythrin	–	12.50
	R-phycocyanin	–	6.25
	allophycocyanin	–	4.54
55 mM:	phycoerythrin	4.35	2.00
	R-phycocyanin	2.17	1.11
	allophycocyanin	1.43	0.77
100 mM:	phycoerythrin	4.16	1.39
	R-phycocyanin	2.50	0.66
	allophycocyanin	1.66	0.55

tion aggregates (early and late) in the 55 mM and 100 mM phosphate solutions, while only one class (late) is apparent in the 30 mM buffer. When the dissociation data are plotted as time divided by percent dissociation versus time (x/y vs x), a hyperbola of the form $y = x/bx+a$ is converted to a linear function (Fig. 9 A–C). Fig. 9A demonstrates that the experimental data for the 30 mM phosphate solution closely fit this equation while the data from higher phosphate concentrations seem to consist of two equations. However, the initial velocity calculated by setting the first derivative $dy/dx = a/(bx+a)^2$ to zero and substituting the intercept on the y axis for a, resulted in the initial velocities of both the early and late equations to have the order phycoerythrin > R-phycocyanin > allophycocyanin (Table III). The interpretation of these two classes is not clear. It could represent two types of phycobilisomes, or that the dissociation constants of the proteins which dissociate early are different from those which are measured later. A third possibility is that the early rapidly dissociating components are an artifact of isolation and measuring technique.

A second anomaly observed from the data of the late classes (55 mM and 100 mM) is that phycoerythrin and R-phycocyanin reach the same maximum percent dissociation, as measured by the asymptote of the hyperbola, whereas allophycocyanin is substantially lower. The asymptote is $y = 1/b$ where b is the slope of the line; if the slopes are parallel, the asymptotes are the same, while if the slope increases, the asymptote decreases. It can be seen that allophycocyanin never reaches the same percentage dissociation that phycoerythrin and R-phycocyanin reach, particularly at the lower buffer concentrations. It is possible that allophycocyanin remains in some aggregate, or it may be that allophycocyanin is lost by degradation with time.

Chlorophyll molecules have not been included in the phycobilisome model because it has not yet been possible to ascertain if chlorophyll is a structural component. The 675 nm fluorescence emission peak has been attributed to an aggregated form of allophycocyanin [9]. Fluorescence emission of allophycocyanin in whole cells of *Cyanidium caldarium* [24] has been reported to be at 670 nm, which is close to the 675 nm in *P. cruentum*. Since allophycocyanin in solution has an emission peak at 660 nm, it is necessary and reasonable to assume that in an allophycocyanin aggregate there would be a peak shift. Suggestions for a peak shift from aggregated allophycocyanin in vitro will be discussed in a forthcoming paper. The possibility, that chlorophyll or an unknown pigment is responsible for the 675 nm emission, cannot be ruled out but is unlikely for reasons to be described in the paper mentioned above.

Location of allophycocyanin in the photosynthetic membranes has been of particular interest; if allophycocyanin is the connecting link between the membrane and phycobilisome, it may exist in both structures. Thus far, our attempts at locating allophycocyanin in photosynthetic membranes have been inconclusive, firstly because membranes give a nonspecific precipitation reaction with control rabbit sera, and secondly because fluorescence assays are difficult to interpret (because of possibly overlapping chlorophyll *a* forms), and because of possible allophycocyanin degradation.

The *P. cruentum* phycobilisome model is consistent with the data to date. It is one of several logically possible models and was chosen for its simplicity but may be modified as future data dictate.

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REFERENCES

1 Gantt, E. and Conti, S. F. (1966) Brookhaven Symp. Biol. 19, pp. 393–405
2 Cohen-Bazire, G. and Lefort-Tran, M. (1970) Arch. Mikrobiol. 71, 245–257
3 Wildman, R. and Bowen, C. C. (1974) J. Bact. 117, 866–881
4 Jones, L. W. and Myers, J. (1965) J. Phycol. 1, 6–13
5 Brody, M. and Brody, S. S. (1962) in Physiol. and Biochem. of Algae (Lewin, R. A., ed.), pp. 3–23, Academic Press, New York
6 Duysens, L. N. M. (1952) Ph. D. Thesis, Utrecht
7 French, C. S. and Young, C. K. (1952) J. Gen. Physiol. 35, 873–890
8 Tomita, G. and Rabinowitch, E. (1962) Biophys. J. 2, 483–499
9 Gantt, E. and Lipschultz, C. A. (1973) Biochim. Biophys. Acta 292, 858–861
10 Gantt, E. and Lipschultz, C. A. (1974) Biochemistry 13, 2960–2966
11 Gantt, E. and Lipschultz, C. A. (1972) J. Cell Biol. 54, 313–324
12 Gantt, E. (1975) Bioscience 25, 781–788
13 Gantt, E., Lipschultz, C. A. and Zilinskas, B. (1975) Plant Physiol. Suppl. to 56, 51, 10
14 Gray, B. H., Lipschultz, C. A. and Gantt, E. (1973) J. Bacteriol. 116, 471–478
15 Gray, B. H. and Gantt, E. (1975) Photochem. Photobiol. 21, 121–128
16 Goldman, M. (1968) Fluorescent Antibody Methods, pp. 94–95, Academic Press, Inc., New York
17 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
18 Scott, E. and Berns, D. (1965) Biochemistry 4, 2597–2606
19 Clement-Metral, J. D. and Lefort-Tran, M. (1971) FEBS Lett. 12, 225–228
20 Gantt, E. (1969) Plant Physiol. 44, 1629–1638
21 Kessel, M., MacColl, R., Berns, D. S. and Edwards, M. R. (1973) Can. J. Microbiol. 19, 831–836
22 Eiserling, F. A. and Glazer, A. N. (1974) J. Ultrastruct. Res. 47, 16–25
23 Bennett, A. and Bogorad, L. (1971) Biochemistry 10, 3625–3634
24 Mohanty, P., Braun, B. Z., Govindjee and Thornber, J. P. (1972) Plant Cell Physiol. 13, 81–91