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Phycobilisomes from a Blue-Green Alga *Nostoc* Species

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Phycobilisomes were isolated from a *Nostoc* sp. strain Mac in phosphate buffer (pH 7.0) by treatment with 1% Brij 56 and centrifugation on discontinuous sucrose gradients (2.0, 1.0, 0.5, and 0.25 M in the proportions 6:4:4:10 ml, respectively). Absorption spectra of isolated phycobilisomes showed the presence of phycoerythrin, phycocyanin, and allophycocyanin. The phycobilisome pigments were partially resolved by electrophoresis on acrylamide gels. Stained gels demonstrated that each main protein band corresponded to a pigmented region. The phycobilisomes appeared compact with a rounded surface and flattened base (about 40-nm diameter) at the attachment site to the photosynthetic lamellae. Fixation in glutaraldehyde caused a significant reduction in total pigment absorption, as well as shifts in the absorption maxima, particularly that of phycoerythrin.

Phycobilisomes are multiprotein complexes composed of allophycocyanin, phycocyanin, and frequently phycoerythrin. They have been observed by electron microscopy in *Rhodophyta* (3, 11, 13) and *Cyanophyta* (9, 10, 14, 20) as discrete structures attached to the stroma surfaces of the lamellae. These phycobiliprotein aggregates function to harvest light energy and transfer it to chlorophyll (5, 16).

Studies of isolated phycobilisomes have been delayed due to instability of these protein complexes upon cell breakage in aqueous systems. Initially, phycobilisomes could only be recovered from cells if homogenates were treated with glutaraldehyde to stabilize these protein aggregates (12). However, glutaraldehyde fixation had several disadvantages. (i) It altered absorption characteristics of some phycobiliproteins. (ii) It resulted in the presence of attached membranes containing chlorophyll and carotenoids in addition to phycobilisomes (6, 10). (iii) It made phycobilisome dissociation impossible and further analysis difficult. For these reasons, phycobilisome isolation procedures were developed for the red alga, *Porphyridium cruentum*, that omitted glutaraldehyde fixation (15). Unfortunately, these procedures were not successful when applied to blue-green algae.

The present investigations were made to develop phycobilisome isolation procedures for blue-green algae. Criteria for successful isolation procedures were the following. Phycobilisome preparations had to be largely devoid of chlorophyll, membrane fragments, or other cellular components. The appearance of isolated phycobilisomes had to correspond to their appearance in vivo. Phycobilisomes had to be reasonably stable without pigment denaturation.

MATERIALS AND METHODS

Cultures. An axenic culture of an endophytic *Nostoc* sp. strain Mac, isolated previously (4), was provided by L. O. Ingram. Cultures were grown in liter batches on medium "CG 10" of Van Baalen (18, 19, 24) at 35 to 40 C in fermentation flasks bubbled continuously with 1% CO_2 and 99% air while being agitated at about 70 oscillations per min on a rotary shaker. Cultures were illuminated with Westinghouse F15 T8/CW cool, white, fluorescent lamps at an incident light intensity of about 500 foot candles (5,400 lux).

Spectrophotometric assays. Protein estimations were made by the Lowry method, with bovine serum albumin (fraction V, Calbiochem, Los Angeles, Calif.) as a standard (21). Chlorophyll was extracted with 80% acetone or methanol and estimated spectrophotometrically (25). Spectra obtained from a Cary 14 recording spectrophotometer were corrected by subtraction at all wavelengths of the scatter observed at 700 nm. Phycoerythrin was estimated from absorption spectra by determination of optical density at 553 nm.

Polyacrylamide gel electrophoresis. Disk gel electrophoresis was done in 5% polyacrylamide gels using a Buchler Polyanalyst Apparatus (Buchler Instruments, N.J.). Samples were applied in 10% sucrose solutions directly on running gels after 30 min of

pre-electrophoresis. Each gel was subjected to a constant 3 mA current for about 3 h. Gels were removed from tubes, stained 1 h in a 1% aniline blue-black 7% acetic acid solution, and destained in 7% acetic acid. In each experiment, material was eluted from excised regions of unstained gels by soaking in 0.01 M sodium potassium phosphate buffer (pH 7.0) containing 0.02% sodium azide. Gels subjected to electrophoresis at pH 4.0 were in the buffer system of Reisfeld et al. (23), those run at pH 7.3 were in the buffer system of Hedrick and Smith (17), and those at pH 8.3 were in the Davis buffer system (7).

Electron microscopy. Cells were fixed at 20 to 25 C in 2% glutaraldehyde and 0.1 M potassium phosphate buffer (pH 6.8) for 1 h and postfixed in 0.5% OsO_4 for 3 h. Fixed cells were suspended in 1% agar and handled as small blocks for dehydration in ethanol and embedding in Epon. Sections were stained by methods indicated in Fig. 1-3. Phycobilisomes or vesicle preparations were fixed in 4% glutaraldehyde for 1 h at ambient temperature before being dialyzed against distilled water to remove glutaraldehyde, sucrose, and phosphate buffer. After dialysis, preparations were stained with sodium silicotungstate or phosphotungstic acid. All electron micrographs were made with a Phillips 300 electron microscope.

Phycobilisome isolation procedure. For the phycobilisome isolation procedure, cells of 6- to 8-day-old cultures were collected by centrifugation, rinsed twice in 0.5 M ammonium phosphate buffer (pH 7.0), and resuspended in the same buffer at a concentration of 1.0 g (wet wt) per 10 ml. Cells were broken in a French pressure cell at 16,000 to 20,000 lb/in^2, and the homogenate was incubated 20 min at ambient temperature in 1% Brij 56. The homogenate was centrifuged for 30 min at 40,000 $\times$ g_{max}. The orange layer at the top was removed and discarded, and the red supernatant fluid below was collected to the region of the loose green pellet. The remaining green 40,000 $\times$ g pellet was generally discarded. Supernatant fluid was centrifuged for 30 min at 100,000 $\times$ g_{max}, and red supernatant was collected again as above. The remaining 100,000 $\times$ g_{max} pellet was generally discarded. A 1.0-ml amount of the 100,000 $\times$ g_{max} red supernatant fluid was layered on each sucrose gradient made with 0.75 M sodium and potassium phosphate buffer, pH 7.0. The sucrose gradients were 2.0, 1.0, 0.5, and 0.75 M in the proportions: 6, 4, 4, and 10 ml, respectively. Sucrose gradients were centrifuged for 6 h in a Beckman 50.1 rotor at 40,000 rpm. A purple phycobilisome layer was collected from the region extending from the 0.5 and 1.0 M interface into the 1.0 M layer. Phycobilisomes were pelleted by centrifugation at 350,000 $\times$ g_{max} for 4 h at 20 C.

Vesicle preparations were made by following the above procedure, except that treatment with Brij 56 was omitted and the centrifugation at 40,000 rpm (Beckman rotor 50.1) was reduced to 2 h. Each step of either isolation procedure was done at 4 to 8 C unless otherwise indicated.

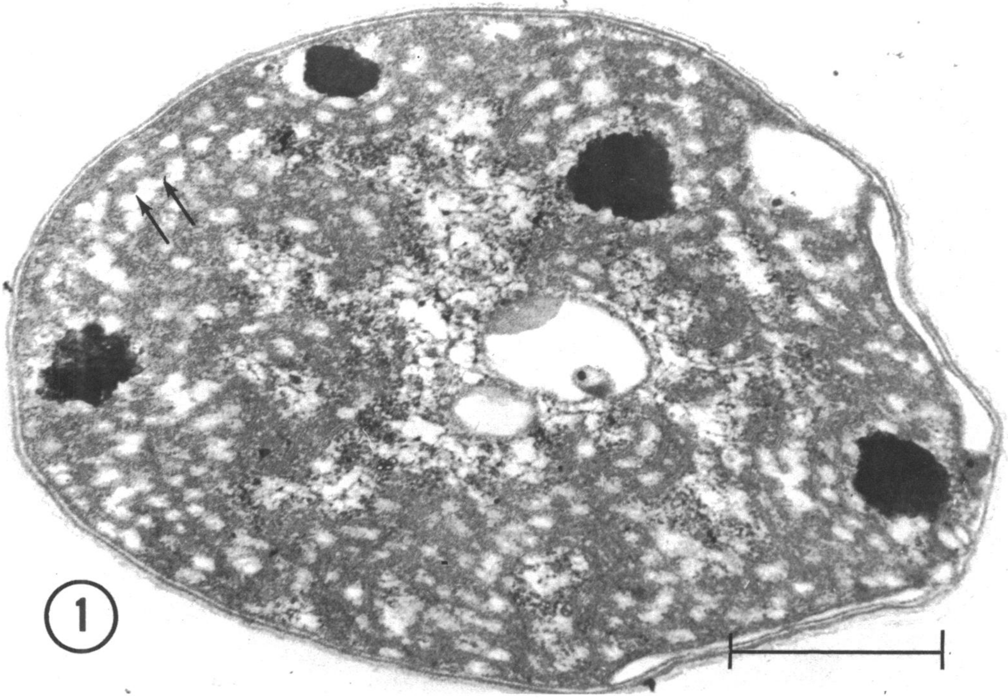
Detergents tested for cell homogenate incubations were Brij 56 and Tween 80 obtained from Atlas Chemical Industries, Wilmington, Del; Triton X-100 from Rohm and Haas Co., Philadelphia, Pa.; and Ammonyx-LO, a gift from the Onyx Chemical Co., Jersey City, N.J.

RESULTS

Electron micrographs of thin sections prepared from intact or disrupted *Nostoc* sp. cells show that phycobilisomes are located on the outside (i.e., the stroma) surfaces of photosynthetic lamellae (Fig. 1-3). These phycobilisomes appear to be distributed in a regular, rather than a random, array on the lamella (Fig. 1, 2). They can be most easily distinguished in partially disrupted cells where stromal material has been loosened or removed (Fig. 3). Each phycobilisome has a broad base with an average diameter of about 40 nm and a rounded surface with apparent substructure.

Phycobilisomes were recovered from the single, purple band which extended from the 0.5 and 1.0 M interface into the 1.0 M sucrose gradient region. Pigmented zones from sucrose gradients (Fig. 4) contained the following when examined spectroscopically. Zone I, the brownish orange layer floating on top, was rich in carotenoids and chlorophyll; zone II, a turquoise band, was rich in allophycocyanin; zone III, a red layer, contained phycoerythrin and some phycocyanin; zone IV, a purple band, had all three phycobiliproteins. The purple band (zone IV) contained phycobilisomes based on the following criteria. All three phycobiliproteins were present in the 1.0 M sucrose layer, whereas free allophycocyanin, phycoerythrin, and phycocyanin were retarded in less-dense sucrose layers under the sedimention conditions employed. Structures were seen in zone IV (Fig. 5) that were similar in size and shape to those visible in thin sections of *Nostoc* sp. cells (Fig. 3). Vesicle preparations (Fig. 6) also contained structures attached to membranes which were similar in size and shape to those visible in both thin sections of *Nostoc* sp. cells (Fig. 3) and negatively stained zone IV preparations. The phycobilisome fraction recovered from zone IV (Fig. 5) contained, in addition to 40- to 50-nm phycobilisomes, smaller structures which may be the result of fragmentation upon release from the membranes. About 60% of the phycoerythrin present in cell homogenates could be recovered in the phycobilisome fraction (zone IV) (Table 1). This pigment served as a measure of phycobilisome recovery. Only about 1.0 μg of chlorophyll per 3,500 μg of protein could be detected in phycobilisome preparations. This represents less than 0.01% of the total chlorophyll present in the cell homogenate.

The phycobilisome fraction (zone IV) had twin absorption maxima at 553 and 571 nm in the phycoerythrin-absorbing region (Fig. 7).



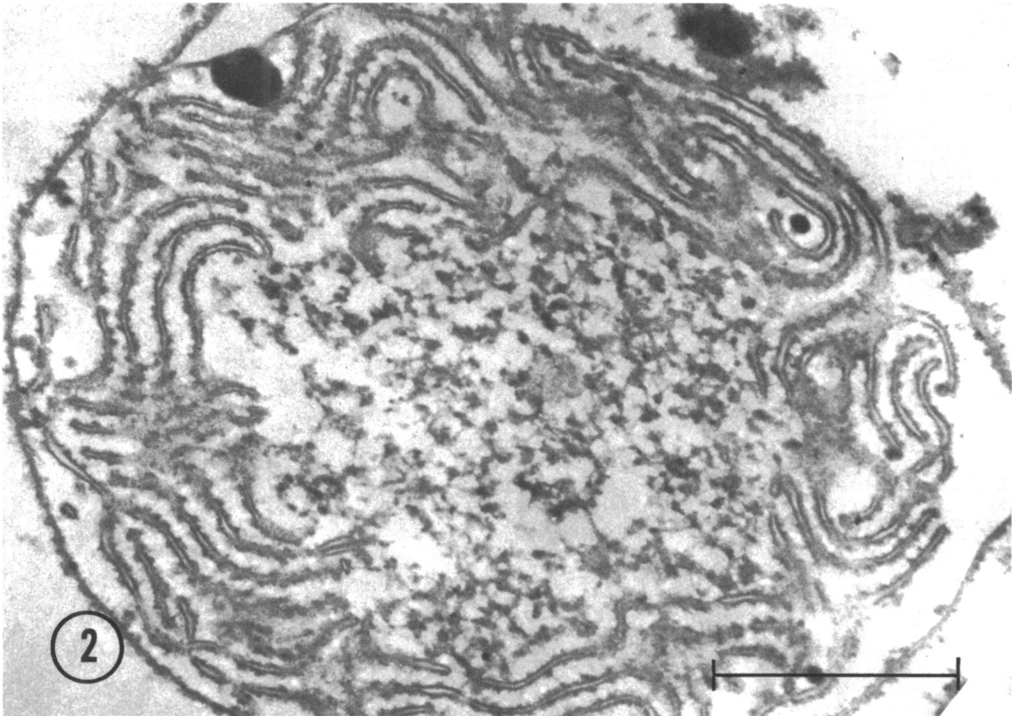


FIG. 1. *Section of a Nostoc sp. cell stained for 5 min in a saturated solution of uranyl acetate in 60% methanol. The cell components were not stained selectively. A suggestion of phycobilisome structures (arrows) can be seen attached to membranes. The clear areas may contain carbohydrate storage products (α-granules). The bar represents 500 nm.*

FIG. 2. *Section of a Nostoc sp. cell that has lysed releasing cellular contents. Remnants of phycobilisome structures are attached along the stroma side of the photosynthetic lamellae in a regular array. The section was stained with a saturated aqueous solution of uranyl acetate for 10 h. The bar represents 500 nm.*

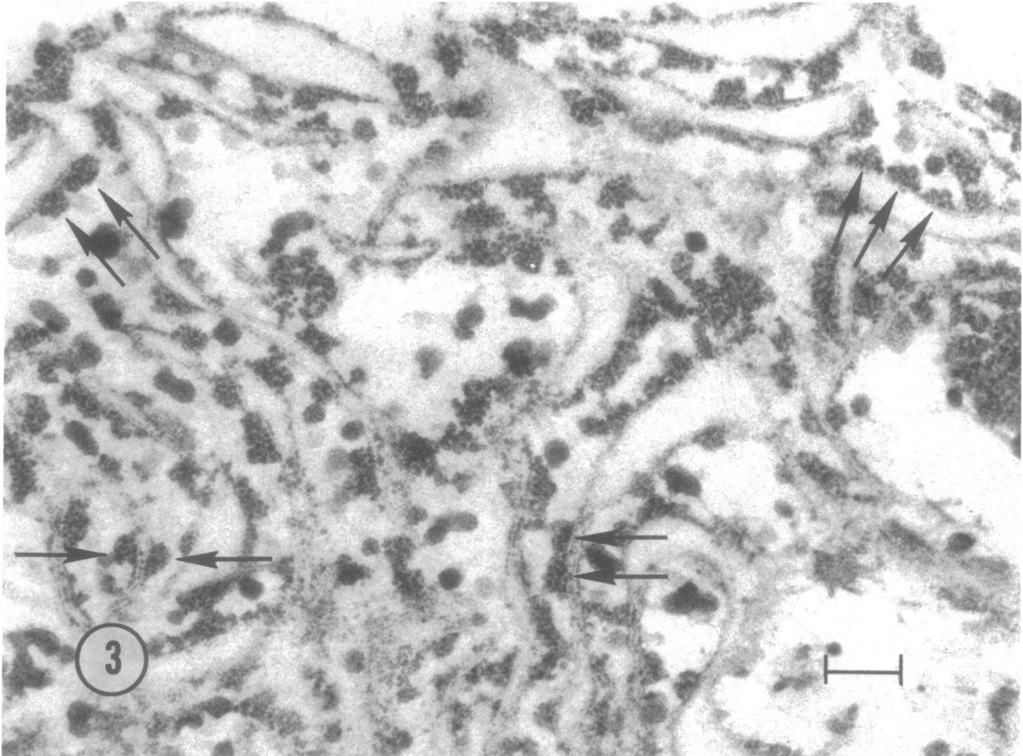


FIG. 3. *Section of a Nostoc sp. cell that had lysed releasing cellular contents. Phycobilisomes (arrows) are attached with their broad base (about 40 nm) to the photosynthetic lamellae. Their granular appearance makes them clearly distinguishable from the α-granules still present. The section was stained in 1% aqueous vanadyl sulfate for 2 h and poststained for 2 min in lead citrate. The bar represents 100 nm.*

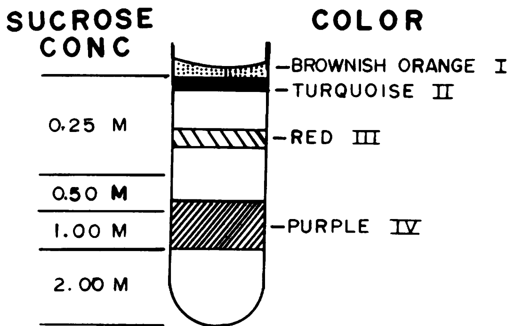
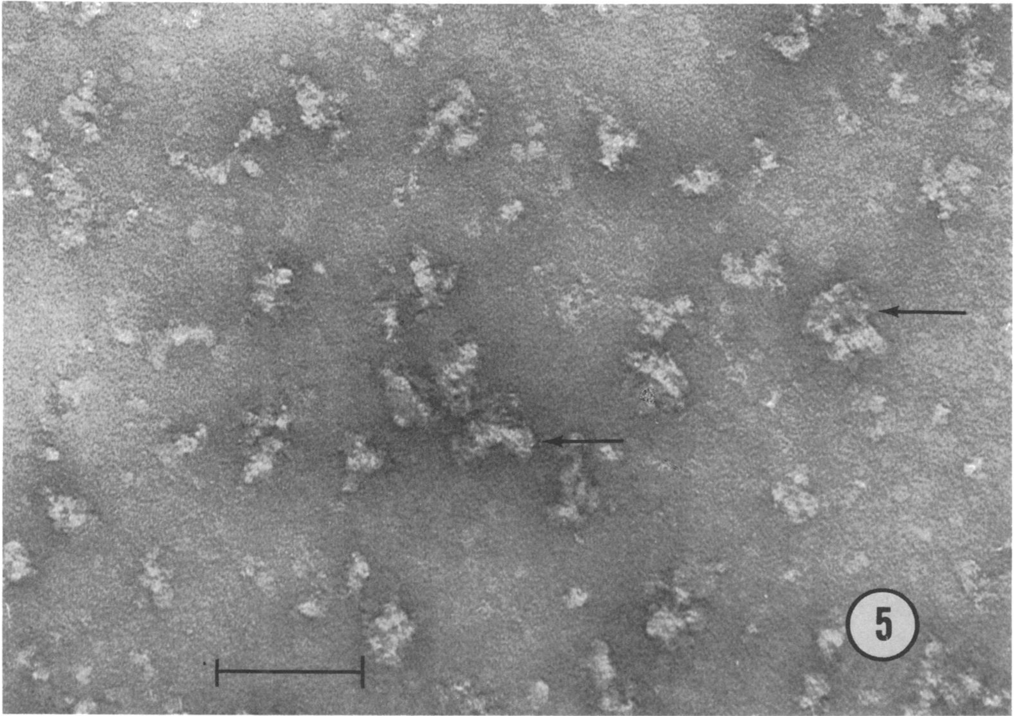


FIG. 4. *Diagram of visible material present in sucrose gradients following centrifugation at 40,000 rpm in a Beckman 50.1 rotor. Zones are designated by Roman numerals beside their respective colors. A 1.0-ml amount of 100,000 × g supernatant fluid was layered on the gradient before centrifugation.*

The absorption spectrum also had a maximum at 620 nm and a shoulder at 645 nm, characteristic of phycocyanin and allophycocyanin, respectively (2). The absorption maxima of phycoerythrin did not correspond exactly to those of the isolated pigment and, therefore, may reflect additive absorption of all three phycobiliproteins in the protein complexes, as well as a peak shift due to phycoerythrin aggregation as proposed by Bennett and Bogorad (2). Glutaraldehyde fixation of a phycobilisome fraction (zone IV) resulted in significant decreases in total absorption in the visible region, replacement of absorption maxima at 553 and 571 nm by a single maximum at 547 nm, and an increase in the absorption in the ultraviolet region, as well as a shift of the maximum from 276 to 268 nm.

Polyacrylamide gel electrophoresis of phycobilisomes resulted in partial resolution of each phycobiliprotein, although none of the polyacrylamide gel electrophoresis systems tested completely resolved the three classes. However, the pigmented bands corresponded to protein bands observed by staining with aniline blue-black (Fig. 8). Only one faint non-pigmented protein band about 1 cm in front of the phycocyanin region was present in the pH 8.3 gels. In the pH 4.0 gels only one faint band was present near the origin.



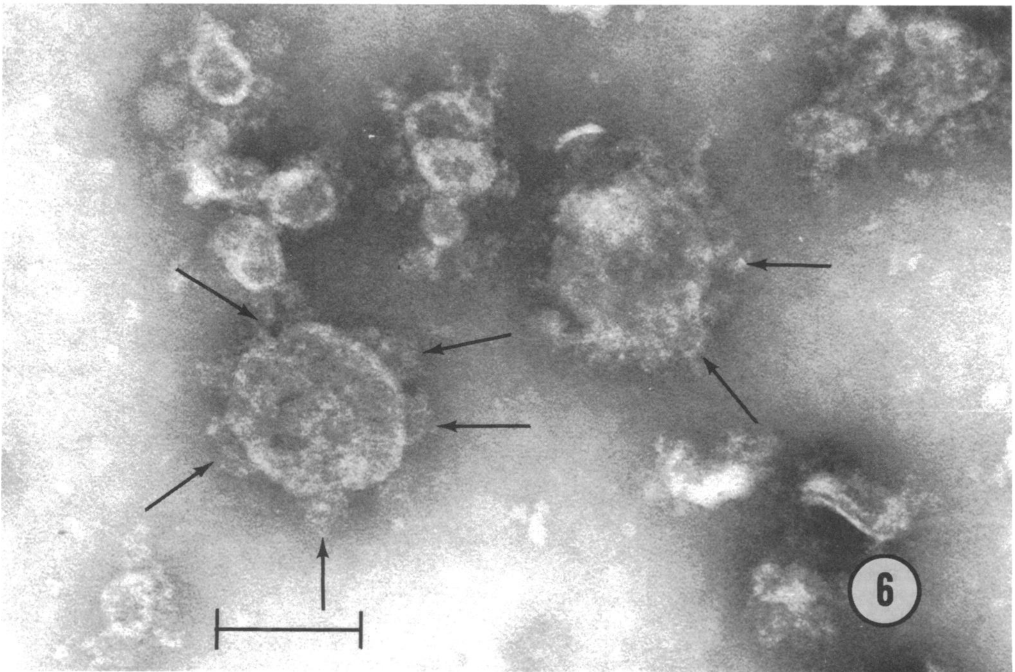


FIG. 5. *Electron micrograph of phycobilisomes recovered from sucrose gradients (zone IV). The phycobilisome subunits are readily observed because of the generally loose structure. Smaller structures in the background are presumed to be subunits of dissociated phycobilisomes. Occasionally phycobilisomes are in clumps as in the center and pair at the right (arrows). The bar represents 100 nm.*

FIG. 6. *Electron micrograph of vesicles recovered from sucrose gradients. Vesicles were prepared by omitting Brij 56 incubation during isolation. Arrows point to attached phycobilisomes. The bar represents 100 nm.*

TABLE 1. *Percentages of phycoerythrin and chlorophyll found in fractions made during phycobilisome isolations*[a]

Cell fraction	Phycoerythrin in fraction (%)	Chlorophyll in fraction (%)
Cell homogenate	100	100
40,000 × *g* supernatant	85	40
40,000 × *g* pellet	15	60
100,000 × *g* supernatant	77	18
100,000 × *g* pellet	8	22
Phycobilisomes	59	>0.01

[a] Data presented are averages of four separate isolation procedures.

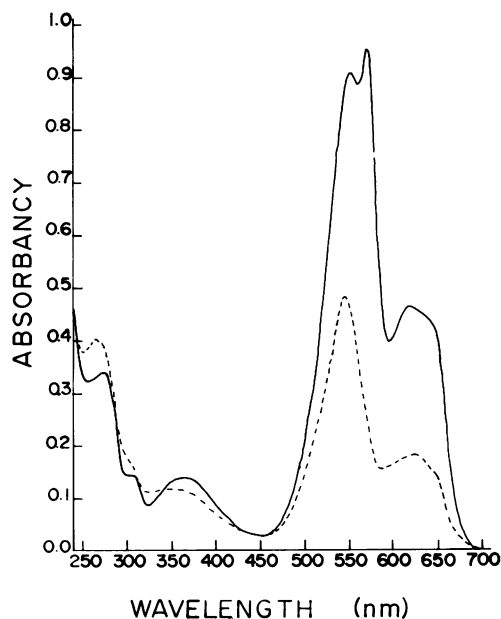


FIG. 7. *Absorption spectra of phycobilisomes (solid line) and glutaraldehyde-fixed phycobilisomes (broken line). The untreated phycobilisome absorption spectrum has maxima at 276, 360, 553, 571, and 619 nm with shoulders at about 308 and 645 nm. The 276-nm absorption peak resulted from absorption of all three pigments, as well as from scatter, because a 30% peak reduction can be caused by dissociation of the phycobilisome preparation. The spectrum of glutaraldehyde treated phycobilisomes (----) has absorption maxima at 268, 350, 547, and 622 nm with shoulders at about 308 and 650 nm. Each spectrum was made with solutions having equal protein concentrations (about 175 μg of protein/ml).*

Modification of conditions outlined in the isolation procedure significantly altered sucrose gradient profiles. Phycobilisome yield was decreased by substitutions of Ammonyx LO or

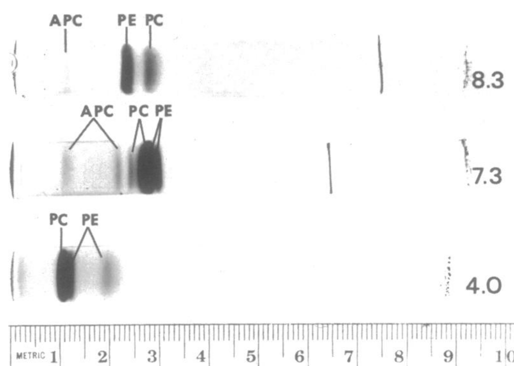


FIG. 8. *Photograph of polyacrylamide gels subjected to electrophoresis in buffers with the pH values listed. The metric ruler is graduated in centimeters. Each gel was loaded with the phycobilisome sucrose solution at the zero centimeter mark then subjected to electrophoresis. Pigmented bands eluted from unstained gels corresponded to the stained bands listed: APC, allophycocyanin; PC, phycocyanin; PE, phycoerythrin. The sharp lines nearest the ends of pH 7.3 and 8.3 gels mark a bromophenol blue band.*

Tween 80 for Brij 56 as the incubation detergent, reduction of either the ammonium phosphate or sodium potassium phosphate concentrations used during isolation, or substitution of sodium potassium phosphate buffer for ammonium phosphate during detergent incubation. Omission of detergent treatment failed to release phycobilisomes from photosynthetic membranes. When such preparations were centrifuged on discontinuous sucrose gradients, the top three pigmented bands (zones I, II, and III, Fig. 5) were reduced, and a brown band was present in zone IV (vesicle preparation, Fig. 6). Absorption spectra showed that vesicle preparations contained all three phycobiliproteins, chlorophyll and carotenoids.

DISCUSSION

Phycobilisomes had been shown to be present in blue-green algae (9, 10, 14, 20), but identification of the membrane-attached structures as phycobilisomes was based on comparison with analogous structures that had been observed in red algae and subsequently isolated from *P. cruentum* (3, 11, 12, 13, 15). Evidence presented in this paper reinforces the validity of the previous hypothesis that the structures seen in vivo in the blue-green algae are, in fact, phycobilisomes.

Selective staining of phycobilisomes in sections from blue-green algal cells has been difficult and cells of *Nostoc* sp. strain Mac were no exception. Phycobilisomes were either poorly

stained in sections of intact cells (Fig. 1) or masked by the densely staining stroma material. Distortion of phycobilisome shapes in intact cells could be caused by close contact with the abundant α-granules which compete for the same stromal space (22). For these reasons, the best resolution of these protein aggregates was achieved in broken or lysed cells using aqueous vanadyl sulfate prestaining and lead citrate poststaining (Fig. 3). With this procedure the phycobilisomes appeared granular whereas the α-granules appeared smooth. Without this identifying feature the α-granules can be easily mistaken for phycobilisomes (10). Phycobilisomes observed by this method had an average diameter of about 40 nm at the base, but there appeared to be greater size variation than had been observed in sections of *P. cruentum* (13) or *Synechococcus lividus* (9).

Isolated phycobilisomes also exhibited some variation in size and shape (Fig. 5). Several explanations are possible for this. The phycobilisomes may be heterogeneous in pigment composition, and this may be reflected in their morphology as has been suggested for two pigment variants of *Porphyridium* (12). Furthermore, isolated phycobilisomes from *Anacystis nidulans*, which lack phycoerythrin, were rod-shaped and smaller than those from *Nostoc* sp. strain Mac. Fragmentation of phycobilisomes during isolation probably accounts for some of the smaller units observed in isolation preparations. However, while these may not be morphologically intact, the basic pigment complex was intact because the energy transfer, as measured by fluorescence (16), was unimpaired (unpublished results).

Absorption spectra of phycobilisomes have maxima characteristic of allophycocyanin, phycocyanin, and phycoerythrin. The presence of each of these proteins has been further verified by partial resolution of each phycobiliprotein by polyacrylamide gel electrophoresis. Twin absorption maxima in the phycoerythrin-absorbing spectral region may be due to two types of phycoerythrin and/or to aggregation as suggested above. Whereas we have indications of more than one spectral type of phycoerythrin from gel electrophoresis at pH 4.0, the complete separation (now in progress) of all pigments is necessary before this question can be resolved. All three phycobiliproteins have overlapping absorbance bands. These pigments are combined in close proximity in the phycobilisome which is attached to the chlorophyll-containing membrane. Thus, they constitute a system which can efficiently transfer energy (1, 5, 8, 16) from pigments absorbing at the shortest wavelength (phycoerythrin), to phycocyanin, to allophycocyanin, and to chlorophyll where it is translated to chemical energy.

The effect of glutaraldehyde fixation on absorption spectra of phycobilisomes indicates alteration of chromophores or chromophore protein interactions or conformational changes in quaternary structure of the protein complexes. Similar, though less striking, glutaraldehyde-induced spectral pertrubations have been observed in other blue-green algae (6).

The isolation procedure described here has allowed us to isolate phycobilisomes from two other blue-green algae, *Anacystis nidulans* and *Agmanellum quadruplicatum*, in addition to *Nostoc*. Its application to other species of *Cyanophyta* should also prove effective though some modifications may be required.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Excellent electron micrographs showing phycobilisomes in sections prepared from 15 species of blue-green algae appear in a paper recently submitted to the Journal of Bacteriology by R. Wildman and C. C. Bowen (phycobilisomes in blue-green algae). The authors wish to thank R. Wildman for making this paper and her doctoral dissertation available to them. We have recently determined that substitution of Triton X-100 for Brij 56 resulted in the isolation of more intact phycobilisomes as judged from their morphological appearance, uniformly large size, and pigment composition.

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