JOURNAL OF BACTERIOLOGY, Feb. 1974, p. 866-881

Vol. 117, No. 2
Printed in U.S.A.

Phycobilisomes in Blue-Green Algae

RUTH B. WILDMAN AND C. C. BOWEN

Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa 50010

Received for publication 22 October 1973

Fifteen species of freshwater blue-green algae, including unicellular, filamentous, and colonial forms, were subjected to a variety of fixatives, fixation conditions, and stains for comparison of the preservation of phycobilisomes. Absorption spectra of the corresponding in vivo and released photosynthetic pigments, in 10 of the species that were maintained in culture, demonstrated the presence of phycocyanin in all 10 species and phycoerythrin in only 2 of them. Spectroscope and electron microscope evidence was obtained for localization of phycobiliproteins in phycobilisomes of *Nostoc muscorum*. Phycobilisomes were observed in all species examined in situ, strengthening the hypothesis that phycobilisomes are common to all phycobiliprotein-containing photosynthetic blue-green algae.

Gantt and Conti (16, 17) elegantly demonstrated the location of phycobiliproteins within small granules associated with the chloroplast lamellae of the red alga *Porphyridium cruentum* and named these structures phycobilisomes. They predicted that similar sites would be found on thylakoids of blue-green algae. The efficiency of the photosynthetic process in the *Cyanophyta* is dependent upon water-soluble phycobiliproteins whose chromophores (phycobilins) absorb wavelengths of light in the regions of the visible spectrum that are not absorbed by chlorophyll *a* and transfer the energy to chlorophyll (1, 3, 10, 13, 26). The action spectrum of photosynthesis corresponds closely to the absorption spectra of the phycobiliproteins and not to the absorption spectra of chlorophyll *a* and the carotenoids (4, 13, 26).

Quantitatively, the major phycobiliprotein in most blue-green algae is phycocyanin, but the presence of phycoerythrin and allophycocyanin has been reported for many species. Gantt and Lipschultz (20), based on their evidence that energy trapped by phycoerythrin and phycocyanin in the phycobilisomes of the red alga *P. cruentum* is transferred via allophycocyanin (also in the phycobilisomes) to chlorophyll in the chloroplast lamellae, have proposed that allophycocyanin plays an important role in photosynthesis.

Structures similar in appearance to the red algal phycobilisomes were evident in electron micrographs of the blue-green endosymbionts of *Glaucocystis nostochinearum* and *Cyanophora paradoxa* (5, 31). Subsequently, observations of structures presumed to be phycobilisomes have been reported for the free-living blue-green algae *Gloeocapsa alpicola* (8), *Tolypothrix tenuis*, and *Fremyella diplosiphon* (18); *Anacystis nidulans* (14, 41); *Synechococcus lividus* (11, 12, 28); and *Aphanizomenon flos-aquae*, *Arthrospira Jenneri*, *Microcoleus vaginatus*, *Nostoc muscorum*, *Spirulina major*, *Symploca muscorum*, and *Tolypothrix distorta* (41).

Cohen-Bazire and Lefort-Tran (8) found that glutaraldehyde fixation of intact cells of *Porphyridium* spp. and *Gloeocapsa alpicola* partly stabilized the association between phycobilisomes and the thylakoid membranes without greatly affecting the absorption spectra of the pigments. After disruption of these cells, 30 to 70% of the total phycobiliprotein could be sedimented on a sucrose gradient in association with fragments of thylakoids. Recently, Gantt and co-workers have succeeded in isolating intact phycobilisomes and identifying the component phycobiliproteins from the red alga *P. cruentum* (19, 20) and from two blue-greens, *Nostoc* sp. and *Anacystis nidulans* (23). Although these investigations have demonstrated that phycobilisomes represent localizations of phycobiliproteins in three blue-green species, the apparent absence of the phycobilisomes in ultrastructural studies of many thin-sectioned blue-greens is enigmatic. To support the hypothesis that phycobilisomes are cellular components common to all phycobiliprotein-containing blue-green algae, 15 species representing unicellular, filamentous, and colonial forms were subjected to a variety of fixatives and stains. Absorption spectra of the photosynthetic pigments were recorded to determine whether

any qualitative relationship exists between phycobiliprotein content and structure as observed in thin sections.

MATERIALS AND METHODS

Algal species and culture conditions. The blue-green algal species examined, their sources, and the dates of collection, where appropriate, are as follows: *Anabaena circinalis* Rabenhorst, Lake Cornelia, Wright County, Iowa, August 1972; *Anabaena cylindrica* Lemmerman, Culture Collection of Algae at Indiana University, Bloomington, Ind. (37) CCAIU 629; *Anabaena spiroides*, Cory's farm pond, Dickinson County, Iowa, June 1972; *Anabaena variabilis* Kütz., CCAIU 377 (37); *Anacystis nidulans*, CCAIU 625 (37); *Aphanizomenon flos-aquae*, Hickory Grove Lake, Story County, Iowa, September 1968; *Arthrospira Jenneri* (Hassall) Stizenberger, West Lake Okoboji, Dickinson County, Iowa, July 1967; *Calothrix* sp., liverwort, Iowa State University H-1; *Johannesbaptista pellucida* (21), Silver Lake fen, Dickinson County, Iowa, July 1972; *Microcoleus vaginatus* (Vauch.) Gom. (*per* Drouet) (9), West Lake Okoboji, Dickinson County, Iowa, July 1967; *Microcystis aeruginosa*, Izaak Walton League Lake, Story County, Iowa, June 1972; *N. muscorum*, Kaiser Research Foundation M-12.4.1; *Spirulina major*, CCAIU 552 (37); *Symploca muscorum* (Ag.) Gomont, CCAIU 617 (37); *Tolypothrix distorta* var. *symplocides* Hansgirg, CCAIU 424 (37). Table 1 summarizes the culture conditions for 10 species which were maintained in unialgal culture. The temperature was held at 22 C for all cultures, although it has been reported that *Anacystis nidulans* shows maximal growth at 41 C (30). The species isolated from natural waters were identified and freed from contaminating algae before fixation; of this group, those included in Table 1 were maintained in unialgal culture. *Anacystis nidulans* and *N. muscorum* (a lightly-sheathed strain) were grown in bacteria-free condition. It was not practical for the purposes of this study to maintain the heavily sheathed species in axenic culture, but aseptic transfers were made frequently enough to keep the bacterial growth minimal. Specimens were fixed (see below) at different ages of culture (1 to 55 days) for purposes of comparison.

TABLE 1. *Culture conditions for maintained species*

Medium	Species	Light[a] (1x)
Modified Chu 10 + 40 ml of soil extract per liter (22)	*Anabaena cylindrica*	1,076–2,152
	Aphanizomenon flos-aquae	538–1,076
	Arthrospira Jenneri	322.8–376.6
	Calothrix sp.	1,076
	Microcoleus vaginatus	322.8–376.6
	Nostoc muscorum	2,152 for 10 days, then 1,076
Kratz-Myers medium C (30)	*Anabaena variabilis*	1,076–2,152
	Anacystis nidulans	2,152–3,228
	Symploca muscorum	1,076
	Tolypothrix distorta	322.8–376.6

[a] Fluorescent (200 W) and incandescent (100 W); 12 h alternating light and dark.

Electron microscopy. Specimens were harvested in a refrigerated centrifuge at 7,000 to 9,000 × *g* for 10 min or aggregated on a transfer needle. Centrifugation was avoided for species containing arrays of gas vacuoles. All algae were washed with distilled water or buffer before further treatment.

Several fixation methods were employed, including 1% osmium tetroxide (OsO_4) in Veronal-acetate buffer (pH 6.1) by the method of Kellenberger et al. (29) and as modified by Pankratz and Bowen (35), 3 to 4% glutaraldehyde (36) in 0.1 M or 0.05 M phosphate buffer (pH 6.2, 6.8, or 7.3) followed by 1% OsO_4 in the modified Veronal-acetate buffer (35), unbuffered 1.5% potassium permanganate, and 2.5% glutaraldehyde-0.7% picric acid (0.1 M cacodylate buffer, pH 6.1) or 4% glutaraldehyde-0.7% picric acid (0.05 M phosphate buffer, pH 6.1) followed by 1% OsO_4 in the same buffer. To study the effects on cytological preservation, fixation times were varied from 1 to 16 h at a temperature of 4 or 23 C. Specimens were washed thoroughly with buffer (three changes over 1 to 12 h) between glutaraldehyde and OsO_4 fixations and once after the fixation before dehydration. A modification of a simultaneous fixation method (15) was carried out by mixing equal volumes of 4% glutaraldehyde and 2% OsO_4, each in 0.1 M phosphate buffer (pH 7.0) at 4 C. Specimens were fixed at 4 C for 1 h, washed with cold buffer (three changes over 30 min), and postfixed in 1% OsO_4 (0.1 M phosphate buffer, pH 7.0) for 2.5 h at 4 C. When necessary for convenient handling, cells were pelleted by low-speed centrifugation between steps of fixation and washing. The resulting pellets were embedded in 3% agar (held at 45 C in a constant temperature bath). On cooling, the solidified agar was cut into 1-mm cubes for further treatment. After dehydration through a graded ethanol series followed by propylene oxide (10 min/change), the specimens were infiltrated with Epon 812 or an Epon-Araldite mixture (33) in propylene oxide (1:3, 1:1, and 3:1) over 2 to 24 h and finally rotated in undiluted resin for 12 h before casting and polymerization.

Sections 40 to 70 nm in thickness were cut with a DuPont diamond knife in an LKB Ultrotome III or a Reichert Om U2 ultramicrotome. The sections were collected on 400-mesh copper grids or 150-mesh Formvar-coated grids and stained for 25 min in 2% aqueous uranyl acetate, 10 min in 10% methanolic uranyl acetate (38) for Formvar-coated grids, or 5 min in 20% methanolic uranyl acetate for uncoated grids. Post-staining for 2 to 10 min in lead citrate (40) also enhanced the contrast. Specimens were viewed in an RCA EMU-3F or Hitachi HU-11C electron microscope. Micrographs were taken on DuPont Cronar litho film, developed in Kodak D-19, and printed on Kodak F2 or F3 Kodabromide paper.

In vivo absorption spectra of photosynthetic pigments. For each of the 10 species maintained in culture, the in vivo absorption spectrum was obtained by spreading or filtering algal cells on a Gelman (GF/C A) glass-fiber filter (39) which had been

balanced against a reference filter moistened with the corresponding medium but without cells. The absorption spectra were recorded from 750 to 350 nm with a Beckman DB-G spectrophotometer.

Absorption spectra of released pigments. Algal cells were harvested by centrifugation at 9,000 × *g*, washed with 0.2 M phosphate buffer (pH 7.0), suspended in cold buffer made 1% in Triton X-100, and broken in a French pressure cell at 15,000 to 18,000 lb/in^2. Each suspension of broken cells was chilled for 1 h and centrifuged in a Spinco model L ultracentrifuge at 100,000 × *g* for 1 h. The absorption spectra of the supernatants were recorded from 750 to 350 nm.

Disruption of N. muscorum for treatment with deoxycholate. Filaments of *N. muscorum* were suspended in cold 0.1 M phosphate buffer containing 17% sucrose and 20% Ficoll, and ruptured at 4,000 lb/in^2 in a French pressure cell. A fraction of the resulting thick suspension was treated with 0.2% deoxycholate at 4 C for 15 min, centrifuged, and washed. An untreated sample was processed similarly. Absorption spectra of the supernatants and the pellets (spread on glass-fiber filters) were recorded, and the pellets were fixed in 4% glutaraldehyde followed by 1% OsO_4 for electron microscopy.

Extraction of N. muscorum with methanol-acetone. Whole cells of *N. muscorum* were fixed in 4% glutaraldehyde, washed, and extracted with 80% methanol-20% acetone until no visible color appeared in the extracts. After centrifugation, absorption spectra of the extracts and of the remaining blue-gray pellet of extracted cells (spread on a glass fiber filter) were recorded, and the pelleted cells were postfixed in 1% OsO_4 for electron microscopy.

RESULTS

Periodically arranged, diffuse granules of moderate electron density frequently were observed in association with the photosynthetic thylakoids in specimens which had been fixed in OsO_4 (with or without prefixation in glutaraldehyde or picric acid-glutaraldehyde) and stained with uranyl acetate or uranyl acetate-lead citrate, but not in material fixed in potassium permanganate. Double staining accentuates these granules, which appear only on the exterior surfaces of the thylakoids. The granules are in rows and are either attached to or are partially embedded in the thylakoid membrane. In one plane of sectioning, such that the row is viewed "face on," the granule profiles are approximately semicircular and usually flattened at the region of association with the thylakoid as in Fig. 1, 2, 4, 5, 8, 19, and 20. In another plane of sectioning, such that the granules are viewed "edge on," the image at low magnification is one of rows of roughly parallel elements, oriented perpendicular to and in contact with the thylakoid as in Fig. 1, 7, 8, 12, 13, 14, 20, 23, 28, and 29. In Fig. 1 to 32, representative "face" profiles are indicated by arrows and "edge" profiles are indicated by arrowheads. Frequently, the plane of sectioning reveals arrays of granules in both profiles (Fig. 1, 3, 5, 6, 8, 9, 14-16, 20, 22, 23). The geometric form of these granules is best described as discoid.

The granules have been observed in every species of blue-green algae studied when fixed as described, although they are most conspicuous in micrographs of *Arthrospira Jenneri* (Fig. 1, 4), *Microcoleus vaginatus* (Fig. 19, 21, 22), *N. muscorum* (Fig. 8, 14, 16, 20), and *Symploca muscorum* (Fig. 17, 24, 25). The age of the culture (from 1 to 55 days) appears to have little influence on the quality of fixation or the appearance of the granules.

The granules are less frequently observed in material subjected to simultaneous fixation in cold glutaraldehyde-OsO_4 (Fig. 25-32), although either OsO_4 alone (Fig. 16-20) or glutaraldehyde and OsO_4 used in succession (Fig. 1-15) provides good preservation. Of the 15 species examined, only *Aphanizomenon flos-aquae* requires simultaneous fixation for good preservation of cell shape and ultrastructure, probably due to the many gas vacuoles and characteristically wide intrathylakoidal spaces (Fig. 31). In this species the granules are not conspicuous, although they have been observed (Fig. 18) after fixation with OsO_4. The best fixation schedule for the greatest number of species was found to be treatment with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h at 23 C, three washes (10 min each) in the same buffer and one wash in modified Veronal-acetate buffer, treatment with 1% OsO_4 in modified Veronal-acetate buffer (35) for 2 to 3 h at 23 C.

An accurate measurement of the size of the granules is difficult to obtain because they do not seem to have precise boundaries, especially in face view, and the diameter of the semicircular profile (face view) usually widens at the base. Several hundred granules were measured to compare sizes in relation to species, age of culture, and fixative. The diameter of the semicircular profile (face view) varies from 28 to 55 nm, with most granules in the range 35 to 38 nm. There is no strong correlation between granule size and species, age, or fixative; variation within the individual specimen accounts for as much variability as any other factor. The image of the granules in edge view is considerably sharper (e.g., Fig. 7) than in face view and is uniform at 9 to 10 nm regardless of species, age, or fixative. Rows of granules are generally parallel to each other with a variable center-to-center spacing (43 to 100 nm) in face view, but no correlation with species, age, or fixative was

discerned. The granules on adjacent thylakoids appear to interdigitate in some species (e.g., *Microcoleus vaginatus*; Fig. 19, 22) or to be spaced head-to-head on adjacent thylakoids as in *N. muscorum* (Fig. 8, 16), *Spirulina major* (Fig. 10), and *Symploca muscorum* (Fig. 24). When thylakoids are closely spaced (Fig. 2, 11, 16, 19, 22, 24, 27, 28, 30), phycobilisomes sometimes are nearly abutting.

Occasionally the image of the granule seems to have sub-unit structure (Fig. 20). When images were subjected to the technique of rotational reinforcement (32), we encountered most frequently a periodicity of six, but no conclusive evidence was found for fixed arrangement in face view. The uniform diameter and sharp image in edge view, however, suggest organization. Occasionally a face-view image is similar to a grouping of three or four edge-view profiles of varying lengths (Fig. 1, 5, 8). Phycobilisomes are more clearly contrasted and easier to visualize against a less-dense cytoplasmic background (Fig. 1, 4, 10, 17, 25) than when the cytoplasm contains many deeply stained ribosomes (e. g., Fig. 3, 5, 9, 12, 14). Another inclusion that appears between thylakoids and complicates discernment of phycobilisomes when sections are not carefully stained is the glycogen granule (G in Fig. 23, 24).

To establish the presence of the phycobiliproteins in structures observed and tentatively identified as phycobilisomes, the procedures used by Gantt and Conti (16) for the same purpose in the red alga *P. cruentum* were applied with slight modifications to the blue-green alga *N. muscorum*. Cells suspended in buffer containing sucrose and Ficoll were disrupted in a French pressure cell, and the resulting thick suspension was treated with deoxycholate and centrifuged. The granules which normally are seen associated with the thylakoids were absent from deoxycholate-treated material (Fig. 33) but present in the control (Fig. 34). The absorption spectrum of the supernatant from the deoxycholate treatment showed a maximum at 618 nm due to phycocyanin and a shoulder at 460 to 490 nm (carotenoids), but very little absorption due to chlorophyll *a*. Absorption spectra of the untreated control material indicated the presence of most of the phycocyanin in the pelleted, ruptured cells and very little in the supernatant. Deoxycholate treatment removed the phycocyanin pigment as manifested by the loss of the granules from the outer surfaces of the thylakoids and the presence of water-soluble phycocyanin in the aqueous supernatant.

The absorption spectra of methanol-acetone extracts of *N. muscorum* showed peaks at 664 nm (chlorophyll *a*) and 478 nm (carotenoids). The absorption spectrum of the residual blue-gray pellet of extracted cells indicated the absence of chlorophyll and the presence of phycocyanin. Examination of this material in the electron microscope showed a major loss in the image of the normally conspicuous thylakoid system but a retention of many of the granules associated with the surfaces of the thylakoids (Fig. 35). The combined spectrophotometric and electron microscope data provide additional evidence that the granules are sites of phycobilin pigments and appropriately can be called phycobilisomes.

In vivo absorption spectra were desired to better correlate the pigment content with the ultrastructural image of the phycobilisomes. The difficulties involved in obtaining a well-resolved spectrum on a reasonably heavy cell suspension are well known. The most successful technique, especially for spectra of heavily sheathed, filamentous species that cannot be evenly suspended in liquid, involves using glass-fiber filters (39). The absorption spectra obtained by this method indicated phycocyanin as the major phycobiliprotein present in vivo in each of these specimens (Table 2). Maxima in the ranges 385 to 395, 420 to 425, and 675 to 679 nm are due to chlorophyll *a*; 435 to 440 and 480 to 490 nm, to carotenoids; 585 to 595 and 625 to 630 nm, to phycocyanin; 562 nm, to phycoerythrin; and 645 to 650 nm, to allophycocyanin. Peaks representing small quantities of pigments (e.g., allophycocyanin) may be obscured by the lack of sharp resolution between maxima.

Cells were broken in a French pressure cell, releasing pigments into 0.2 M phosphate buffer (pH 7.0) containing 1% Triton X-100. After centrifugation at 100,000 $\times$ *g* for 1 h, improved resolution of maxima for phycocyanin and phycoerythrin was obtained on the supernatant fractions (largely due to removal of much of the chlorophyll), but the expected allophycocyanin peak around 650 nm did not emerge. Phycoerythrin was detected as a major pigment in the strains of *Calothrix* and *Symploca* studied but not in the other species examined (Table 2). Peak heights indicate that phycocyanin is present in larger concentrations than phycoerythrin in these strains of *Calothrix* and *Symploca*. Maxima of released pigments roughly corresponded to in vivo maxima, but several peaks appear at slightly shorter wavelengths in solution, i.e., chlorophyll *a* (415 to 420 and 665 to 668 nm), carotenoids (433 to 435 nm), phyco-

FIG. 1-32. *Phycobilisomes in cells representing 15 species of blue-green algae. For orientation, sample phycobilisomes in face view are indicated by arrows; in edge view, by arrowheads; and groupings of edge-view profiles of varying lengths, by wavy arrows. Additional arrays of phycobilisomes can be discerned by careful observation. Bars represent 0.1 μm.*

FIG. 1-4. *Blue-green algae fixed in 4% glutaraldehyde followed by 1% OsO_4 and stained with methanolic uranyl acetate and lead citrate.*

FIG. 1. *Arthrospira Jenneri. Both face and edge views of phycobilisomes are prominent.*

FIG. 2. *Anacystis nidulans. Phycobilisomes can be seen on peripheral thylakoids.*

FIG. 3. *Anabaena cylindrica. Careful inspection reveals arrays of phycobilisomes in both profiles although they show little contrast in density against the cytoplasmic background.*

FIG. 4. *Arthrospira Jenneri. A less-dense cytoplasmic background permits easy visualization of interdigitating phycobilisomes in face view.*

FIG. 5-10. *Blue-green algae fixed in 4% glutaraldehyde followed by 1% OsO_4 and stained with methanolic uranyl acetate and lead citrate.*

FIG. 5. *Anabaena variabilis. Arrays in edge view appear on both outer surfaces of a thylakoid. O, Osmiophilic body (β-granule).*

FIG. 6. *Anabaena variabilis. Phycobilisomes in face view interdigitate in close interthylakoidal spacing.*

FIG. 7. *Anabaena variabilis. Higher magnification shows the uniform diameter and sharp image of phycobilisomes in edge view.*

FIG. 8. *Nostoc muscorum. Images of phycobilisomes in face view occasionally appear similar to groupings of edge-view images of varying lengths. P, Polyhedral body.*

FIG. 9. *Anabaena variabilis. Plane of sectioning shows phycobilisomes in face, edge, and tangential views.*

FIG. 10. *Spirulina major. Phycobilisomes on adjacent thylakoids appear head to head, sometimes nearly abutting.*

FIG. 11-15. *Blue-green algae fixed in 4% glutaraldehyde followed by 1% OsO_4 and stained with methanolic uranyl acetate and lead citrate.*

FIG. 11. *Tolypothrix distorta. Thylakoids characteristically are convoluted and closely spaced. Numerous phycobilisomes in edge view are faintly contrasted.*

FIG. 12. *Spirulina major. Edge-view phycobilisomes are difficult to discern against the dense (with ribosomes) cytoplasmic background. The nucleoplasm (N) is less dense.*

FIG. 13. *Anabaena circinalis. Edge-view phycobilisomes appear on thylakoids adjacent to gas vacuoles (V) in longitudinal and cross-sectional views.*

FIG. 14. *Nostoc muscorum. Many arrays of phycobilisomes appear in both views.*

FIG. 15. *Anabaena variabilis. These edge-view phycobilisomes appear as units with dense vertical boundaries and less dense interiors. Face-view phycobilisomes have broadly flattened bases.*

FIG. 16-20. *Blue-green algae fixed in 1% OsO_4 only and stained with methanolic uranyl acetate and lead citrate.*

FIG. 16. *Nostoc muscorum. Face-view phycobilisomes appear head to head on adjacent thylakoids. Edge-view phycobilisomes appear in arrays of uniform length and diameter.*

FIG. 17. *Symploca muscorum. Although this strain contains both phycocyanin and phycoerythrin, the phycobilisome image is not conspicuously different from that in species containing no phycoerythrin. P, Polyhedral body.*

FIG. 18. *Aphanizomenon flos-aquae. Phycobilisomes are difficult to show in this species; several groups in edge view can be seen. Gas vacuoles (V) are abundant and wide intrathylakoidal spaces (IT) are characteristic.*

FIG. 19. *Microcoleus vaginatus. Massive arrays of closely packed, interdigitating phycobilisomes such as this are not frequently encountered.*

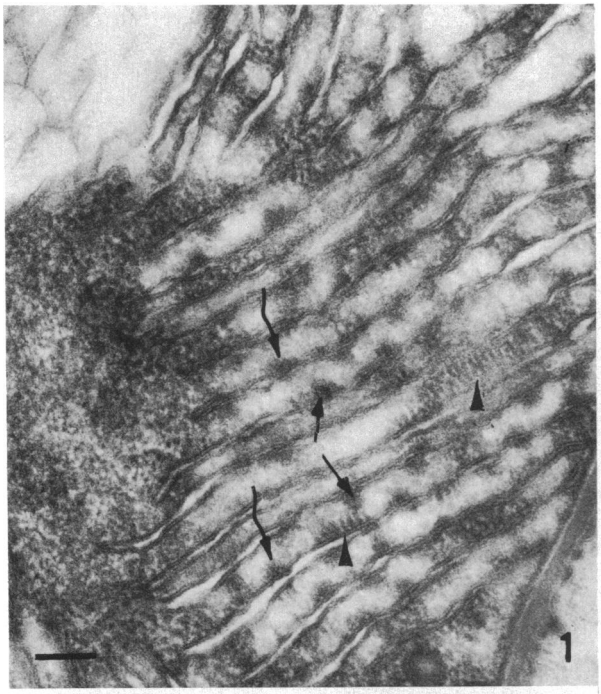
FIG. 20. *Nostoc muscorum. Face-view phycobilisomes appear to have sub-unit structure. A periodicity of six is most frequently obtained when these images are subjected to rotational reinforcement.*

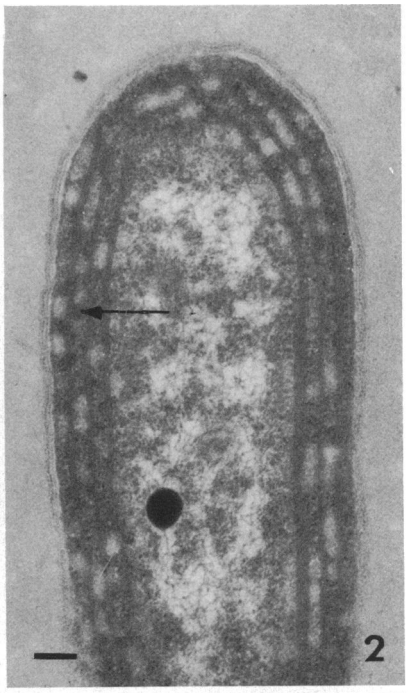
FIG. 21-24. *Blue-green algae fixed in 4% glutaraldehyde-0.7% picric acid (0.05 M phosphate buffer) followed by 1% OsO_4 (same buffer) and stained with methanolic uranyl acetate and lead citrate.*

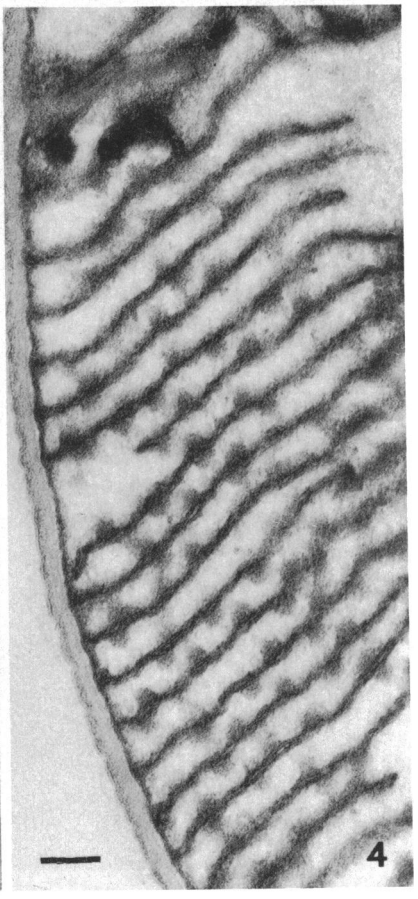
FIG. 21, 22. *Microcoleus vaginatus. Phycobilisomes in face and edge views; face profile is almost circular. Denser staining osmiophilic bodies also appear between thylakoids. Nature of paracrystalline body in Fig. 21 is unknown.*

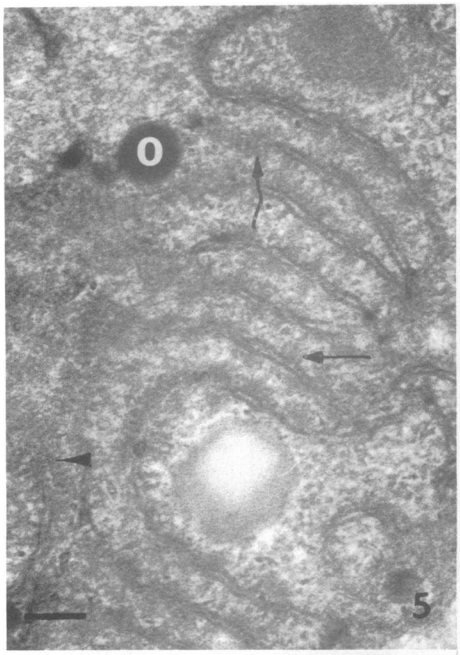
FIG. 23. *Nostoc muscorum. Face-view is broadly flattened at site of attachment to thylakoid. G, Glycogen granule.*

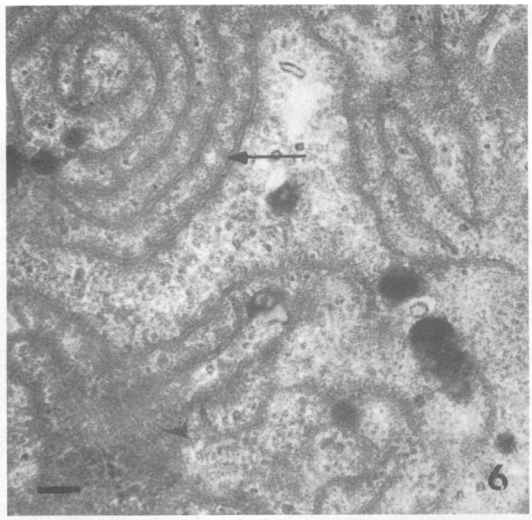
FIG. 24. *Symploca muscorum. Interthylakoidal glycogen granules (G) are distinguished from darker staining phycobilisomes attached to thylakoids. C, Cylindrical body (35).*

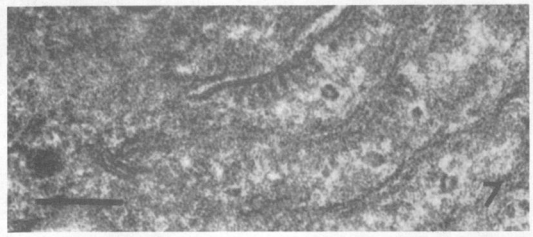


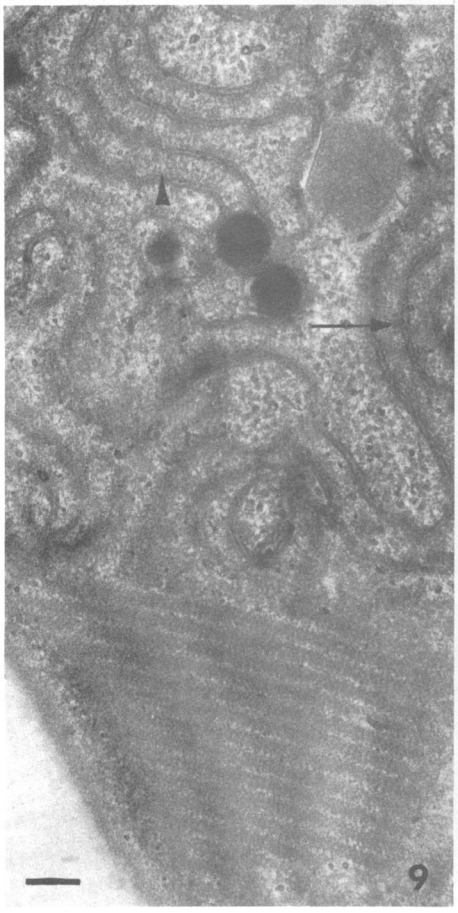


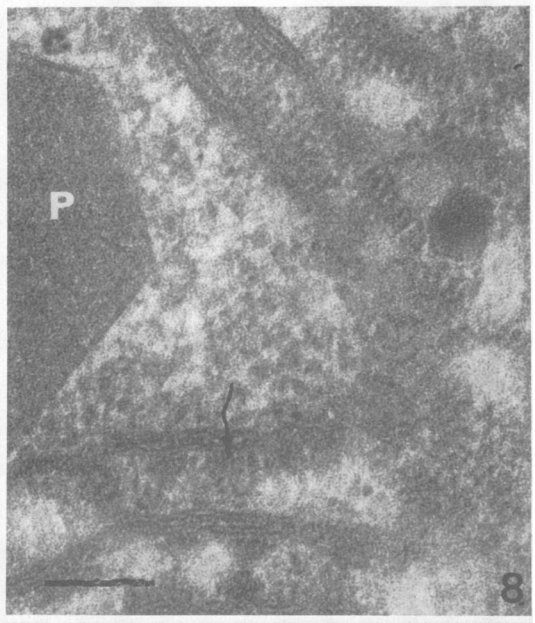


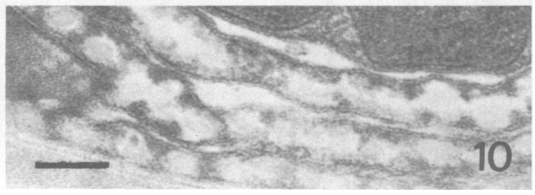

O
5

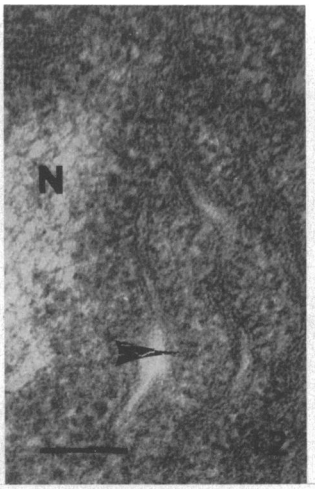

6

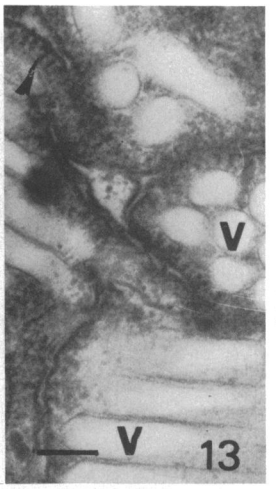

7

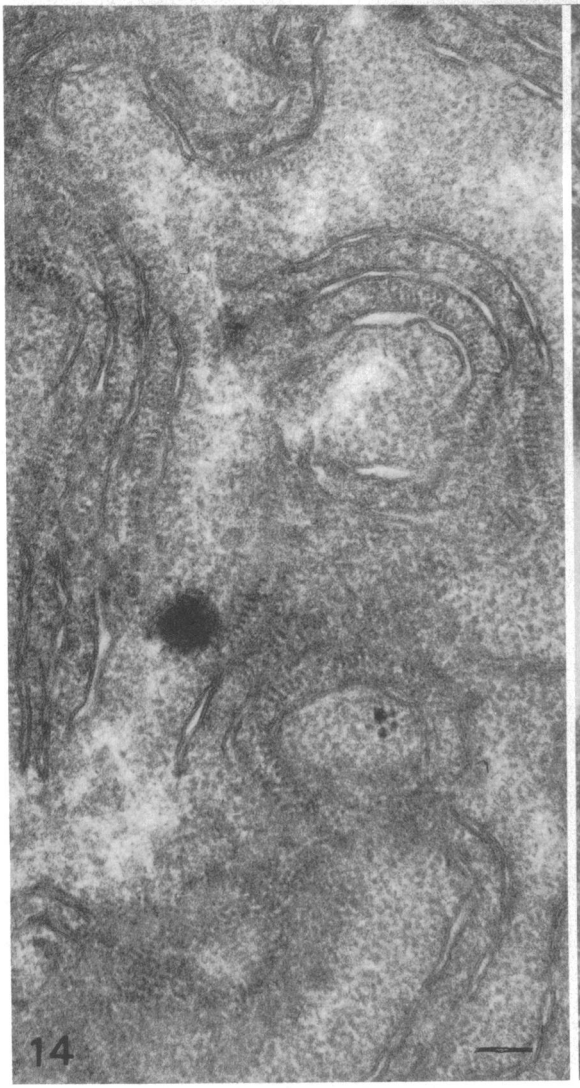

9

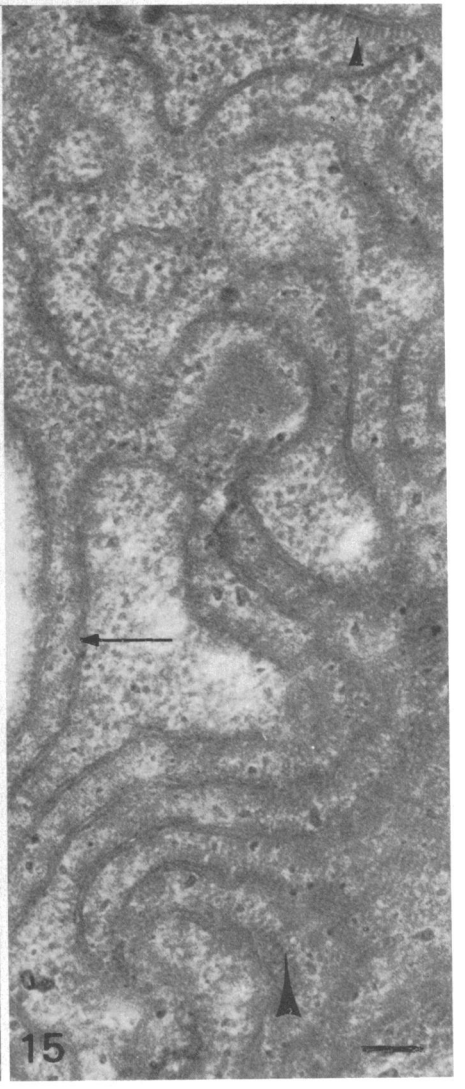

P
8

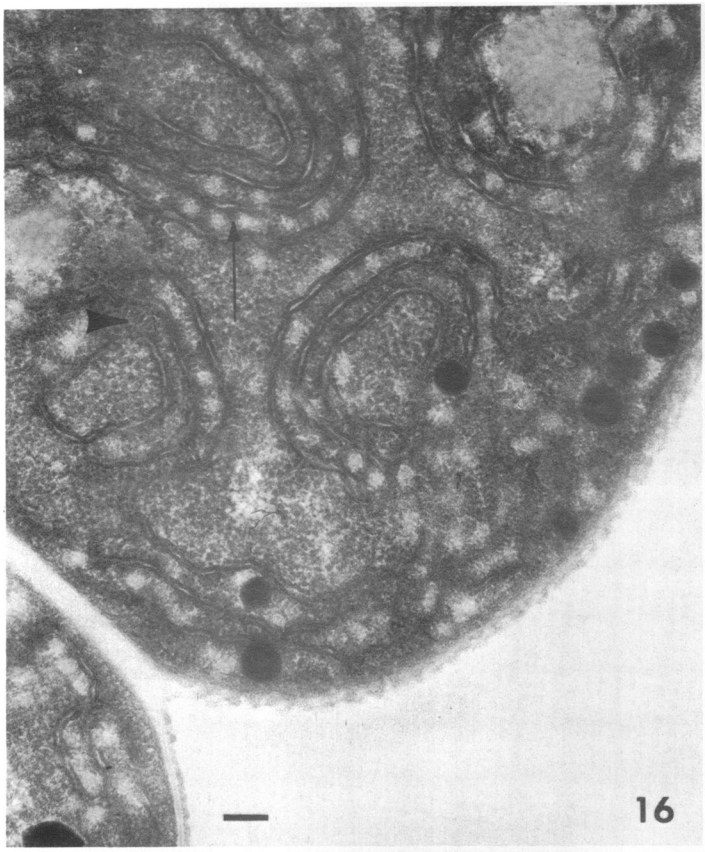

10

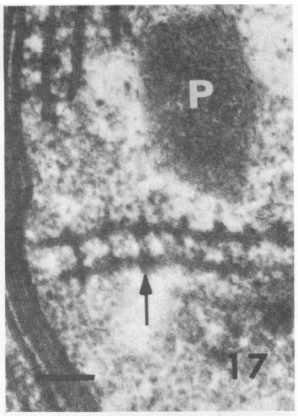

N

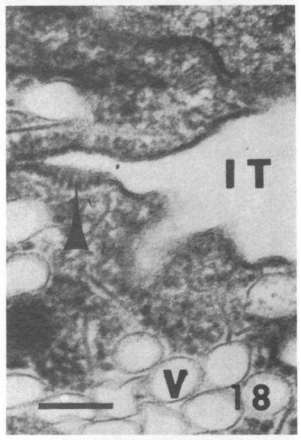

V
V
13

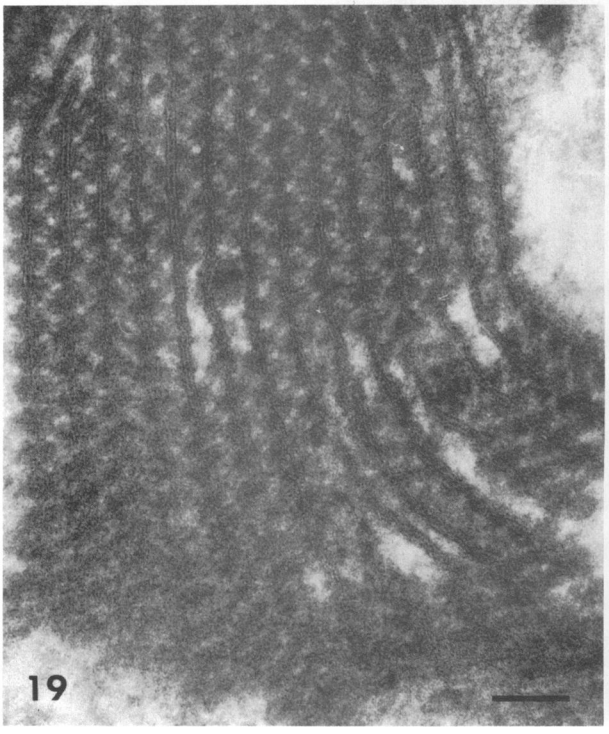

14

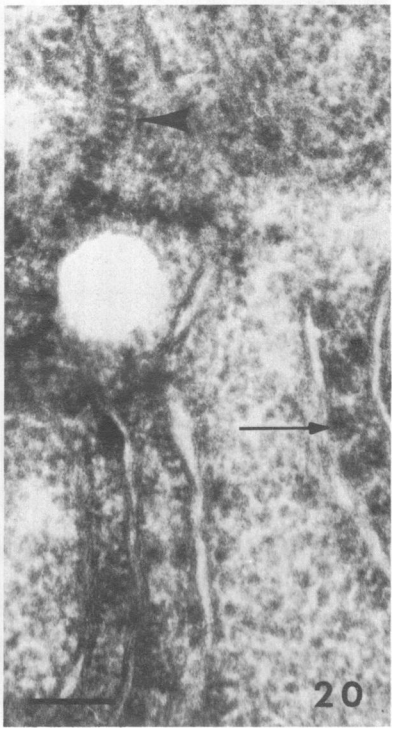

15




P
17


IT
V
18


19


20

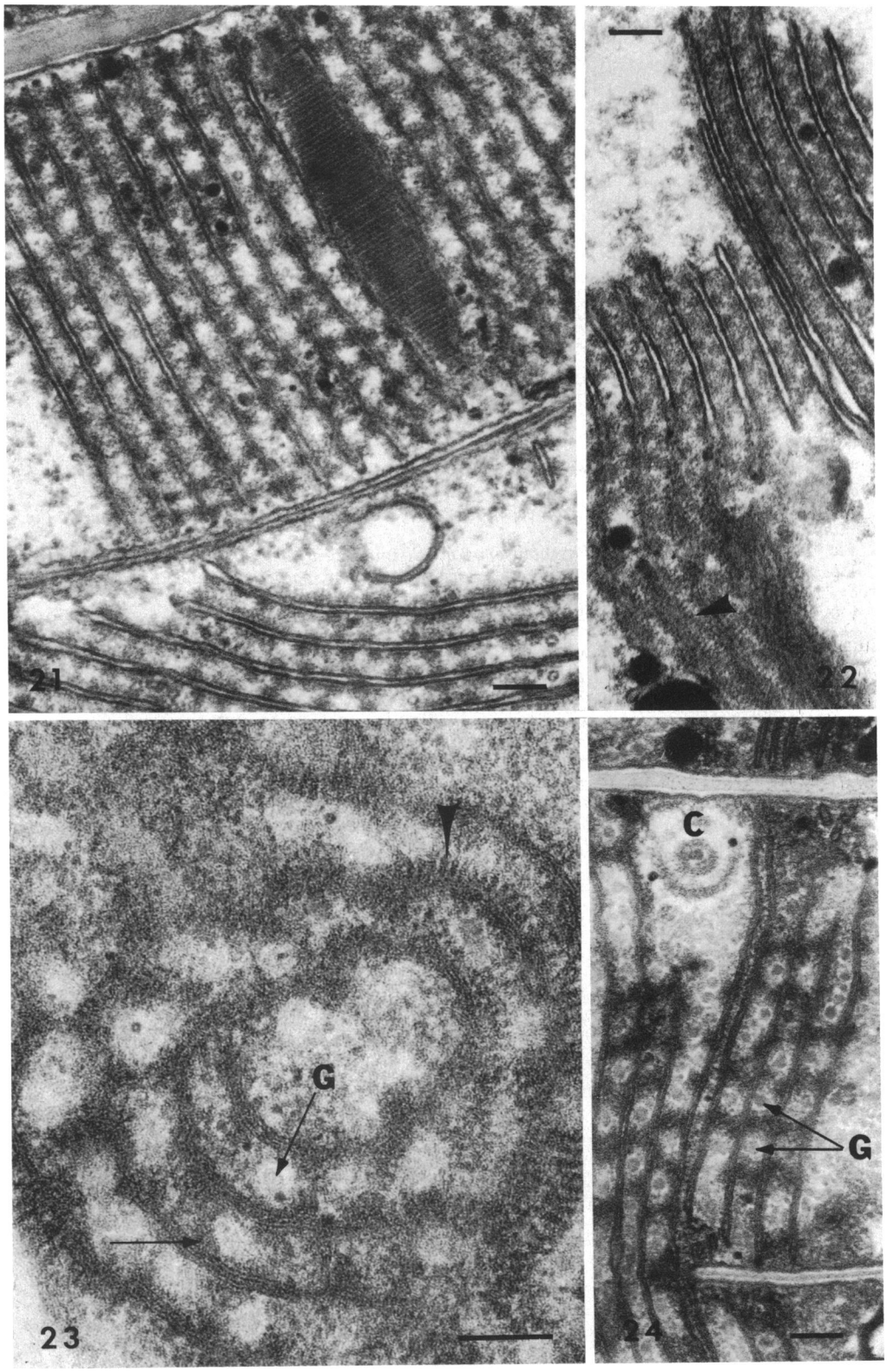

21
22
23
G
C
G
24

FIG. 25–32. *Blue-green algae fixed simultaneously in glutaraldehyde and OsO_4 and stained with methanolic uranyl acetate and lead citrate.*

FIG. 25. *Symploca muscorum. Profiles of phycobilisomes appear more triangular than in Fig. 17. O, Osmiophilic body.*

FIG. 26. *Symploca muscorum. Phycobilisomes are distinguished from glycogen granules (G).*

FIG. 27. *Calothrix sp. Phycobilisomes in face view are regularly spaced on parallel thylakoids. P, Polyhedral body.*

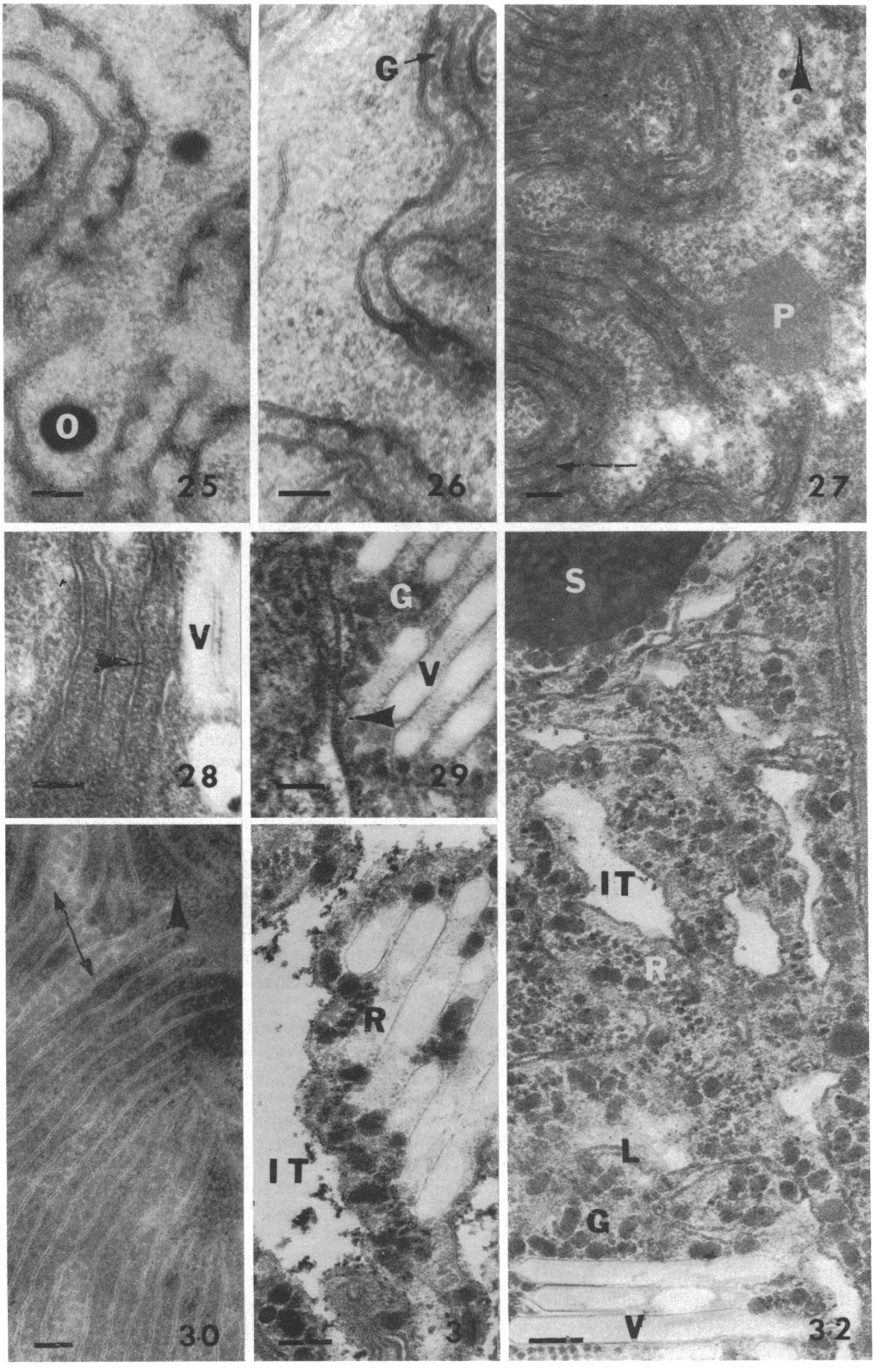
FIG. 28. *Microcystis aeruginosa. Edge-view phycobilisomes are barely visible against dense cytoplasmic background. V, Gas vacuoles.*

FIG. 29. *Anabaena spiroides. Numerous glycogen granules (G) and gas vacuoles (V) surround the phycobilisome-laden thylakoids. Glycogen granules are as deeply stained as the phycobilisomes.*

FIG. 30. *Johannesbaptista pellucida. Same methods used for Fig. 25–32; negative stain effect seen only for this species. Phycobilisomes in face view appear medium gray attached to lighter gray thylakoids.*

FIG. 31. *Aphanizomenon flos-aquae. Outer surfaces of lamellae bounding characteristically wide intrathylakoidal spaces (IT) appear free of phycobilisomes. R, Polyribosomes.*

FIG. 32. *Immature Aphanizomenon flos-aquae akinete. Intrathylakoidal spaces (IT) are narrowing to a lamellar configuration (L) more commonly seen in blue-green species, but no evidence of phycobilisomes can be discerned. Gas vacuole (V); polyribosomes (R), structured granule (S), and glycogen granules (G) stain quite densely.*


O
25
G
26
P
27
V
28
G
V
29
S
IT
R
L
G
V
32
R
IT
31
30

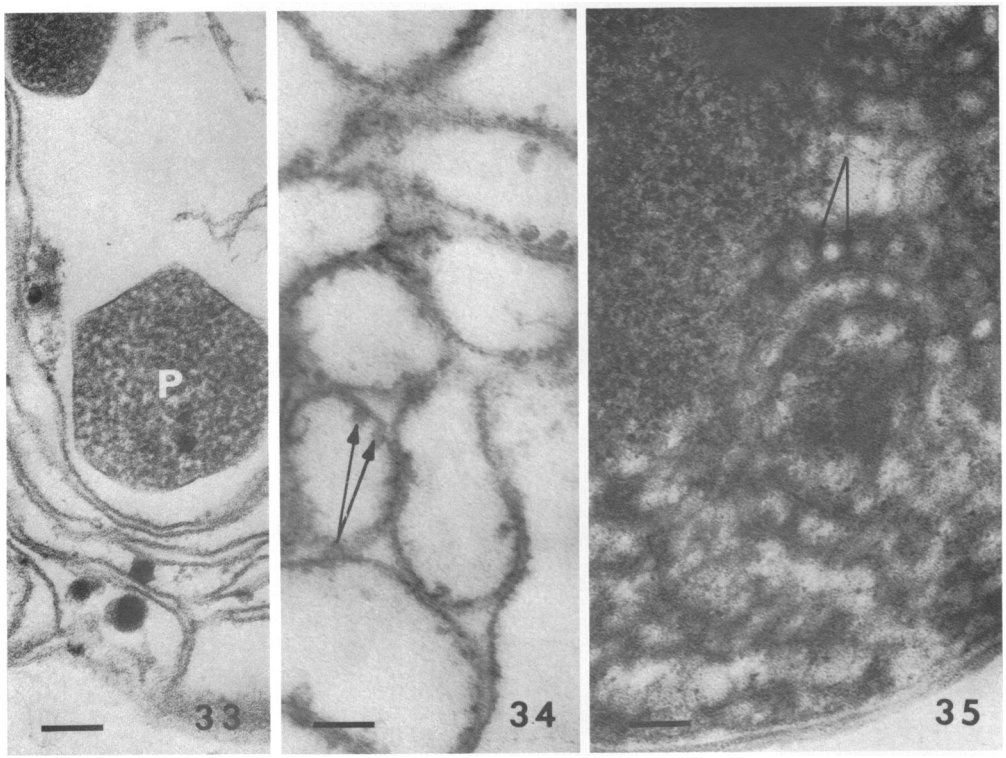


FIG. 33. *Thin section from a pellet of Nostoc muscorum cells which were broken in a French press, treated with deoxycholate, and fixed. Lamellae are free of phycobilisomes. P, Polyhedral body.*

FIG. 34. *Same as Fig. 33 but without deoxycholate. Whole phycobilisomes and fragments remain attached to lamellae.*

FIG. 35. *Thin section from a pellet of Nostoc muscorum cells which were fixed in glutaraldehyde, extracted with methanol-acetone, and post fixed in OsO_4. Extraction of chlorophyll and carotenoids has largely removed lamellar images, but phycobilisomes remain in position.*

cyanin (615 to 620 nm), and phycoerythrin (553, 560 nm).

DISCUSSION

Realizing that the ubiquity of phycobilisomes in blue-green algae is not yet generally accepted, we offer ample evidence for phycobilisomes in each of fifteen diverse species. Visual delineation of phycobilisomes seems to depend on a combination of technical factors. Figures 1 to 24 demonstrate that phycobilisomes are dependably preserved by fixation in glutaraldehyde followed by OsO_4, glutaraldehyde-picric acid followed by OsO_4, or OsO_4 alone. Early reports on the difficulties of preserving phycobilisomes indicated that glutaraldehyde was essential, but Fig. 16 to 20 demonstrate that OsO_4 alone is sufficient for good preservation. Phycobilisomes cannot be visualized, however, in permanganate-fixed specimens. It is also perplexing to note that in images of blue-green algae simultaneously fixed in glutaraldehyde and OsO_4 (Fig. 25–32) phycobilisomes are inconspicuous or completely missing, although excellent fixation of glycogen granules (6), polyribosomes, gas vacuoles, structured granules, and membranes is apparent.

We conclude that a second factor of importance for consistent visualization of phycobilisomes is the method of staining. Methanolic uranyl acetate provides much better contrast than does aqueous uranyl acetate. Staining time must be carefully controlled to avoid an overstaining of cytoplasmic contents that muddies the background against which phycobilisomes must be observed. A fine balance must be achieved to stain selectively for optimal quality of the phycobilisome image. Membranes often appear densely stained, but glycogen granules usually stain less intensely than phycobilisomes and can be easily distinguished if the proper conditions are employed.

The phycobiliproteins found in the blue-green

TABLE 2. *Absorption maxima[a] of blue-green algal photosynthetic pigments*

Alga	Absorption maxima of in vivo pigments (nm)	Absorption maxima of released pigments (nm)
Anabaena cylindrica	385-390, 420-425, 438, 480-490, 590-600, 630, 679	375-380, 415, 433, 480-485, 580-585, 618, 667
Anabaena variabilis	385-395, 420-425, 438, 480-490, 585-595, 628, 677	375, 415, 433, 480-485, 580-590, 618, 665
Anacystis nidulans	385-395, 420-425, 435, 485-490, 625, 675	380-390, 415-420, 435, 485, 580-590, 620, 668
Aphanizomenon flos-aquae	385-395, 420-425, 438, 480-490, 515-525, 590-610, 635, 678	375-380, 410-415, 430, 480-490, 580-590, 618, 668
Arthrospira Jenneri	385-395, 420-425, 435, 480-490, 595-605, 630, 678	380-390, 415-420, 433, 485, 585-590, 618, 668
Calothrix sp	385-395, 420-425, 438, 495-500, 540, 562, 600-605, 625, 645-650, 678	380-390, 415-420, 435, 485, 560, 618, 668
Microcoleus vaginatus	385-395, 420-425, 440, 480-490, 590-595, 630, 678	380-390, 415-420, 434, 485, 580-590, 618, 668
Nostoc muscorum	385-395, 420-425, 435, 480-485, 625, 678	385-390, 415-420, 435, 485, 580-590, 618, 668
Symploca muscorum	385-395, 420-425, 437, 490-495, 562, 625, 678	375-380, 415-420, 430-435, 485-495, 553, 618, 665
Tolypothrix distorta	385-390, 415-420, 435, 465-470, 485-490, 595, 627, 678	380-390, 415-420, 433, 480-490, 615, 667

[a] Single wavelengths indicate peaks; ranges indicate shoulders.

algae vary among species and have even been reported to differ in type within the same species depending upon habitat (27). We wanted to determine whether any correlation exists between the types of phycobiliproteins present in a species and the size and/or shape of the phycobilisome image. Spectral properties of the phycobiliproteins and their chromophores, the phycobilins, have served as the major means of identification and classification of these pigments. The literature is full of confusing and conflicting reports concerning absorption maxima in the visible spectrum, partially due to the drastic methods that were used in early isolations and attempted purifications that may have led to formation of pigment artifacts (34). Furthermore, Hattori et al. (24) found that phycocyanin from the blue-green *Plectonema calothricoides* associates or dissociates reversible to a monomeric, trimeric, or hexameric state under various conditions of pH, ionic strength, and protein concentration, with resultant shifts in absorption maxima. There are conflicting reports on the size of monomeric C-phycocyanin; Bennett and Bogorad (2) have published the lowest molecular weights for component subunits. The absorption maxima included in this study (Table 2) are reported for the purpose of identifying the major phycobiliproteins (phycocyanin and/or phycoerythrin) in species that were examined with an electron microscope. No attempt was made to isolate the individual pigments or to determine states of aggregation.

Although phycocyanin is the major phycobiliprotein in most blue-green species, Hattori and Fujita (25) observed levels of phycoerythrin slightly higher than phycocyanin in strains of *Tolypothrix tenuis*, *Anabaena variabilis*, *N. muscorum*, and *Aphanothece sacrum*. Hirose et al. (27) reported that two blue-green algal species did not contain phycocyanin. For their isolation of intact phycobilisomes, Gray et al. (23) used a blue-green algal strain that originally had been isolated and identified as an endophytic *Nostoc* species. They demonstrated the presence of both phycocyanin and phycoerythrin. The strain of *N. muscorum* used in our study appears to lack phycoerythrin, based on absorption spectra of in vivo and released pigments. According to early literature, allophycocyanin was not considered a common phycobiliprotein in blue-green algae but, with improved methods of isolation, its presence has been reported to be widespread with levels up to 50% of that of phycocyanin (25). Spectral identification of allophycocyanin usually depends upon isolation of the individual phycobiliproteins, and consequently it was not detected as a separate peak in vivo or in the spectra of crude aqueous extracts in our study except for *Calothrix*.

The size and shape of the blue-green phycobilisome appears to be qualitatively independent

of the nature of the phycobiliproteins present, at least at the level of measuring electron micrograph images. Phycobilisomes in species containing little or no phycoerythrin do not differ in appearance in thin sections from phycobilisomes containing both phycocyanin and phycoerythrin (Fig. 17, 24-27). More variation in sizes in phycobilisome images was found within individual specimens than could be related to species, age, or method of fixation. The diameter of the edge view of phycobilisomes in the specimens examined was more uniform in size than was the diameter of the profile in face view. To some extent the shape of the phycobilisome is responsible for the considerable range in diameters reported for the face view: many phycobilisomes have a broad base at the point of attachment to the thylakoid, whereas others appear nearly semicircular or even roughly triangular (Fig. 10, 17, 25, 26) in profile.

Glycogen granules, which are dumbbell-shaped (averaging 31 nm in width and 65 nm in length) in profile or spherical (31 nm), depending upon the plane of sectioning, are located between the photosynthetic thylakoids (6). They have been mistaken for phycobilisomes by some investigators (14), leading to the erroneous conclusion that some phycobilisomes may be located free from attachment to thylakoid membranes. Such a possibility is unlikely since close spatial association of the component phycobiliproteins and thylakoidal chlorophyll is essential for the efficient energy transfer that occurs during photosynthesis in blue-green algae. Gantt and Lipschultz (20) have proposed a structural model for the phycobilisome of the red alga *P. cruentum* in which a core of allophycocyanin in direct contact with the photosynthetic lamella transfers energy from peripheral phycoerythrin and phycocyanin to chlorophyll *a* and to the reaction center. A similar arrangement should prove to be functional in blue-green algae.

Isolation of phycobilisomes (8, 23) and identification of the component pigments have established phycobilisomes as the structural locations of the photosynthetically active phycobiliproteins in three blue-green algal species. Evidence based on studies of freeze-etched preparations (7, 28) and thin-sectioned material has extended the number of species in which phycobilisomes have been observed. To this list, our work adds 15 species of blue-green algae varying in form and pigment content but all containing clearly recognizable phycobilisome structures.

ACKNOWLEDGMENTS

We are grateful to Judy Lehman and Lillian Johnson for typing the manuscript.

LITERATURE CITED

1. Arnold, W., and J. R. Oppenheimer. 1950. Internal conversion in the photosynthetic mechanism of blue-green algae. J. Gen. Physiol. **33**:423-435.
2. Bennett, A., and L. Bogorad. 1971. Properties of subunits and aggregates of blue-green algal biliproteins. Biochemistry **10**:3625-3634.
3. Bergeron, J. A. 1963. Studies of the localization, physicochemical properties, and action of phycocyanin in *Anacystis nidulans*. *In* Photosynthetic mechanisms of green plants. Nat. Acad. Sci. Nat. Res. Counc. Pub. **1145**:527-536.
4. Blinks, L. R. 1954. The photosynthetic function of pigments other than chlorophyll. Annu. Rev. Plant Physiol. **5**:93-114.
5. Bourdu, R., and M. Lefort. 1967. Structure fine, observée en cryodécapage, des lamelles photosynthétiques des Cyanophycées endosymbiotiques: *Glaucocystis nostochinearum* Itzigs, et *Cyanophora paradoxa* Korschikoff. C. R. Acad. Sci. Ser. D **265**:37-40.
6. Chao, L., and C. C. Bowen. 1971. Purification and properties of glycogen isolated from a blue-green alga, *Nostoc muscorum*. J. Bacteriol. **105**:331-338.
7. Cohen-Bazire, G., and M. Lefort-Tran. 1968. Structure granulaire du chromatoplasma des Cyanophycées, p. 395-396. 4th Eur. Reg. Conf. Electron Microscopy, Rome.
8. Cohen-Bazire, G., and M. Lefort-Tran. 1970. Fixation of phycobiliproteins to photosynthetic membranes by glutaraldehyde. Arch. Mikrobiol. **71**:245-257.
9. Drouet, F. 1968. Revision of the classification of the Oscillatoriaceae. *In* Academy of Natural Sciences of Philadelphia monogr. no. 15. Fulton Press, Lancaster, Pa.
10. Duysens, L. N. M. 1951. Transfer of light energy within the pigment systems present in photosynthesizing cells. Nature (London) **168**:548-550.
11. Edwards, M. R., D. J. Berns, W. C. Ghiorse, and S. C. Holt. 1968. Ultrastructure of the thermophilic blue-green alga, *Synechococcus lividus* Copeland. J. Phycol. **4**:283-298.
12. Edwards, M. R., and E. Gantt. 1971. Phycobilisomes of the thermophilic blue-green alga *Synechococcus lividus*. J. Cell Biol. **50**:896-900.
13. Emerson, R., and C. M. Lewis. 1942. The photosynthetic efficiency of phycocyanin in *Chroococcus*, and the problem of carotenoid participation in photosynthesis. J. Gen. Physiol. **25**:579-595.
14. Evans, E. L., and M. M. Allen. 1973. Phycobilisomes in *Anacystis nidulans*. J. Bacteriol. **113**:403-408.
15. Franke, W. W., S. Krien, and R. M. Brown, Jr. 1969. Simultaneous glutaraldehyde-osmium tetroxide fixation with postosmication. Histochemie **19**:162-164.
16. Gantt, E., and S. F. Conti. 1966. Granules associated with the chloroplast lamellae of *Porphyridium cruentum*. J. Cell Biol. **29**:423-434.
17. Gantt, E., and S. F. Conti. 1966. Phycobiliprotein localization in algae. *In* Energy conversion by the photosynthetic apparatus. Brookhaven Symp. Biol. **19**:393-405.
18. Gantt, E., and S. F. Conti. 1969. Ultrastructure of blue-green algae. J. Bacteriol. **97**:1486-1493.
19. Gantt, E., and C. A. Lipschultz. 1972. Phycobilisomes of *Porphyridium cruentum*. I. Isolation. J. Cell. Biol. **54**:313-324.
20. Gantt, E., and C. A. Lipschultz. 1973. Energy transfer in phycobilisomes from phycoerythrin to allophycocyanin. Biochem. Biophys. Acta **292**:858-861.
21. Gashwiler, K., and J. D. Dodd. 1961. Algae from the warm pools of Silver Lake fen. Proc. Iowa Acad. Sci. **68**:129-131.
22. Gerloff, G. C., G. P. Fitzgerald, and F. Skoog. 1950. The isolation, purification, and culture of blue-green algae. Amer. J. Bot. **37**:216-218.

23. Gray, B. H., C. A. Lipschultz, and E. Gantt. 1973. Phycobilisomes from a blue-green alga *Nostoc* species. J. Bacteriol. **116**:471-478.
24. Hattori, A., H. L. Crespi, and J. J. Katz. 1965. Association and dissociation of phycocyanin and the effects of deuterium substitution on the processes. Biochemistry **4**:1225-1238.
25. Hattori, A., and Y. Fujita. 1959. Spectroscopic studies on the phycobilin pigments obtained from blue-green and red algae. J. Biochem. **46**:903-909.
26. Haxo, F. T. 1960. The wavelength dependence of photosynthesis and the role of accessory pigments, p. 339-360. *In* M. B. Allen (ed.), Comparative biochemistry of photoreactive systems. Academic Press Inc., New York.
27. Hirose, H., S. Kumano, and K. Madono. 1969. Spectroscopic studies on phycoerythrins from Cyanophycean and Rhodophycean algae with special reference to their phylogenetical relations. Bot. Mag. Tokyo **82**:197-203.
28. Holt, S. C., and M. R. Edwards. 1972. Fine structure of the thermophilic blue-green alga *Synechococcus lividus* Copeland. A study of frozen-fractured-etched cells. Can. J. Microbiol. **18**:175-181.
29. Kellenberger, E., A. Ryter, and J. Sechaud. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671-678.
30. Kratz, W. A., and J. Myers. 1955. Nutrition and growth of several blue-green algae. Amer. J. Bot. **42**:282-287.
31. Lefort, M. 1965. Sur le chromatoplasma d'une Cyanophycée endosymbotique: *Glaucocystis nostochinearum* Itzigs. C. R. Acad. Sci. Ser. D **261**: 233-236.
32. Markham, R., S. Frey, and G. J. Hills. 1963. Methods for the enhancement of image detail and accentuation of structure in electron microscopy. Virology **20**:88-102.
33. Mollenhauer, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technol. **39**:111-114.
34. OhEocha, C. 1963. Spectral properties of the phycobilins. I. Phycocyanobilin. Biochemistry **2**:375-382.
35. Pankratz, H. S., and C. C. Bowen. 1963. Cytology of blue-green algae. I. The cells of *Symploca muscorum*. Amer. J. Bot. **50**:387-399.
36. Sabatini, D. D., K. Bensch, and R. J. Barrnett. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17**:19-58.
37. Starr, R. C. 1964. The culture collection of algae at Indiana University. Amer. J. Bot. **51**:1013-1044.
38. Stempak, J. G., and R. T. Ward. 1964. An improved staining method for electron microscopy. J. Cell Biol. **22**:697-701.
39. Trüper, H. G., and C. S. Yentsch. 1967. Use of glass fiber filters for the rapid preparation of in vivo absorption spectra of photosynthetic bacteria. J. Bacteriol. **94**:1255-1256.
40. Venable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**:407-408.
41. Wildman, R. B., and C. C. Bowen. 1972. Phycobilisomes in blue-green algae, p. 1-5. 1st Internat. Symp. Taxon. Biol. Blue-green Algae, Madras. *In* T. V. Desikachary (ed.), Taxonomy and biology of blue-green algae. University of Madras Centre for Advanced Study in Botany, Madras.