GRANULES ASSOCIATED WITH THE CHLOROPLAST LAMELLAE OF *PORPHYRIDIUM CRUENTUM*

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ABSTRACT

Small granules with a diameter of approximately 350 A are attached to the chloroplast lamellae of the red alga *Porphyridium cruentum*. To some extent, their size depends on the culture conditions and the age of the cell. It was possible to preserve the granules only with aldehyde prefixation. It can be seen that fixed or negatively stained granules are comprised of smaller subunits. The granules are arranged regularly on the lamellae in repeating rows with a center-to-center granule distance of 400 to 500 A. Attempts at characterization of these chloroplast granules revealed that they are resistant to hydrolysis by ribonuclease and appear to be structurally unaffected by methanol-acetone extraction. Because of their close association with the chloroplast lamellae, they are considered as possible sites of phycobilin concentration. This possibility is supported by two observations: when the phycobilins are removed, the granules disappear; and, when the chlorophyll and stainable membrane portions are selectively removed, the phycobilins and granules are still present. It was found that all other marine red algae examined had granules which were associated with the chloroplast lamellae.

In a previous study on the ultrastructure of the red alga *Porphyridium cruentum* (6, 7), we found that small granules were associated with the chloroplast lamellae. The granules are on only one side of each chloroplast membrane, i.e., on the side facing away from its own membrane pair, and are absent from both the inner and outer chloroplast limiting membranes. Grazing sections over several chloroplast lamellae revealed that the granules have a highly ordered spatial arrangement on the lamellar membranes. Since the granules are larger than ribosomes in size, and preliminary results indicated that they are resistant to digestion by ribonuclease, they may not be ribosomes.

Brody and Vatter (4) have inferred, from observations with the light microscope and from the use of appropriate interference filters, that both the chlorophyll and phycobilins are located in the chloroplast lamellae of *P. cruentum*. Giraud (8, 9), who has worked extensively with red algae, particularly *Rhodosorus marinus*, believes that the phycobilins are in the stroma of the chloroplast. His contention is based primarily on the changes of fluorescence spectra of cells exposed to various ionic conditions and on the observation that the phycobilins are released immediately from cells disrupted in aqueous media. Location of the phycobilins, in or on the chloroplast lamellae, satisfies the generally accepted view that in order for resonance transfer of excitation energy between chlorophyll and phycobilins to be efficient, the distance between them must be no more than 50 A (4, 9).

We have considered the granules which are associated with the chloroplast lamellae of *P. cruentum* as possible sites of phycobilin concentration and, because of their close association with the lamellae, they have the required proximity for efficient energy transfer. The purpose of this study was to investigate this possibility. We have ex-

amined chloroplasts of various algae and vascular plants, especially those of red algae, for the presence of similar granules on the chloroplast lamellae.

MATERIALS AND METHODS

GROWTH CONDITIONS: *P. cruentum* Naegeli was grown in artificial sea water medium (11) in 1 liter quantities in fermentation flasks. Cultures were continuously illuminated with "cool white" fluorescent light (incident light intensity of 400 ft-c) and maintained at 18° to 22° C by continuous shaking in a New Brunswick Incubator-Shaker. We passed a continuous stream of 5% CO_2 in 95% air through the medium to aerate the cultures.

FIXATION FOR ELECTRON MICROSCOPY: After incubation for 6 to 10 days, cells were harvested by centrifugation (4,000 *g*) and fixed by procedures similar to those described by Sabatini et al. (16). Unless specifically stated, all material pictured in the illustrations was fixed with 4% glutaraldehyde (Eastman Organic Chemicals, Rochester, New York), in 0.1 M phosphate buffer (pH 6.8) for 2 hr, rinsed several times in the same buffer, and postfixed for 1 hr or sometimes 2 hr in 1% osmium tetroxide in 0.1 M phosphate buffer. The material was passed through a graded ethanol dehydration series and embedded in Epon in a manner similar to that described by Luft (13). Sections were cut with a diamond knife on an LKB ultrotome, stained with 1% uranyl acetate and basic lead hydroxide (14) or lead citrate (19), and examined in a Philips 200 electron microscope.

Various other fixation procedures were tried, and their effect on the preservation of the granules was noted. The treatment and results are given in Table I.

DISRUPTION OF CELLS FOR RIBONUCLEASE OR DEOXYCHOLATE TREATMENT: Cells were collected by centrifugation, rinsed, and finally suspended in cold 0.1 M phosphate buffer (pH 6.8). The cell suspension was transferred to a cold French pressure cell and subjected to a pressure of 8000 to 10,000 lb. per sq in. The broken cell material was kept at 4° C and centrifuged in a refrigerated Sorvall RC 2 centrifuge at 5000 *g*.

RIBONUCLEASE TREATMENT: Using a method similar to that of Jacobson et al. (10), we treated whole cells and cell fragments with ribonuclease in order to determine whether or not the chloroplast granules are ribosomes. Unfixed and glutaraldehyde-fixed intact cells, as well as unfixed broken cells, were incubated in ribonuclease with a final concentration of 0.35 mg/ml (2 hr), 0.7 mg/ml (3 hr), and 0.7 mg/ml (30 min), respectively. Controls were similarly incubated except that ribonuclease was absent. Both the ribonuclease-treated and untreated material was then subjected to extraction with 5% trichloroacetic acid at 4° C for 30 min. Fixation was then completed for electron microscopy.

DEOXYCHOLATE TREATMENT: Unfixed broken cell material was collected in a pellet by centrifugation at 5000 *g*. The pellet was divided into halves and kept at 4° C. One-half was set aside as a control in 0.1 M phosphate buffer, while the other half, also in phosphate buffer, was exposed to a final concentration of 0.2% deoxycholate for 10 min. The two samples were centrifuged again, and the supernatant fluid was discarded. Before fixation, the pellets were washed with buffer.

METHANOL-ACETONE EXTRACTION: Cells were treated with a mixture of 20% acetone and 80% methanol to extract the chlorophyll and many of the carotenoids. Phycobilins are not extracted with this solvent mixture. Extraction was continued until no further color was detectable visually or with a Cary 14 spectrophotometer. Glutaraldehyde fixation was carried out before methanol-acetone extraction.

NEGATIVE STAINING: Of the number of methods tried, two gave consistently good results: (*a*) Cells were rinsed twice in 0.1 M ammonium acetate (pH 6.8) and resuspended (1 g wet wt. per 10 ml). A 10 ml sample was transferred to a frozen metal beaker, and cells were broken with a Branson Sonifier (Branson Instruments Inc., Stamford, Connecticut) operating at a setting of 4 with a maximum acoustical output of 4 dc amps. After 45 sec of sonication, the material, which was kept at 4°C, was applied to carbon-coated Formvar grids and stained with a 1% solution of phosphotungstic acid in 0.1 M ammonium acetate (pH 6.8). (*b*) the cells were washed twice in distilled water. After resuspending them in distilled water (1 g wet wt per 10 ml), glutaraldehyde was added immediately prior to sonication to give a final concentration of 4%. The cells were sonicated for 90 sec at a setting of 4 with a maximum acoustical output of 4 dc amps. The material was then applied to carbon-coated Formvar grids and stained with 1% phosphotungstic acid in distilled water at pH 7.

A variety of plants were examined with the 4% glutaraldehyde–1% osmium tetroxide fixation, which was most successful in preserving the chloroplast granules of *P. cruentum*. The plants examined belong in either one of two categories: those that have phycobilins as accessory pigments, and those that do not. The groups which possess phycobilins consist of the red algae and the blue-green algae. Five red algae were examined: *Bangia fusco-purpurea*, *Antithamnion glanduliferum*, *Nemalion multifidum*, *Spermothamnion* sp. and *Porphyridium aerugineum*. All but the last are marine algae. The fresh-water, blue-green algae were: *Anacystis nidulans*, *Phormidium flaveolarum*, *Nostoc muscorum*, and *Tolypothrix tenuis*. One unidentified oscillatorialike marine blue-green was also examined.

The group examined which lacks phycobilins consisted of the algae: *Fucus* sp., *Ulva* sp., and *Euglena*

gracilis; and the vascular plants: *Matteuccia struthiopteris*, *Lemna*, *Wolffia*, *Nicotiana*, and *Haemanthus katherina*.

OBSERVATIONS

FIXATION: The small granules associated with the chloroplast lamellae of *P. cruentum* are very sensitive to the method of fixation. This is confirmed by the fact that this alga has been examined with the electron microscope by a number of people (4, 8, 18) who did not observe the presence of granules. We have tried a number of fixatives and various buffers. From the results shown in Table I, it can be concluded that the granules are best preserved with glutaraldehyde, hydroxyadipaldehyde, or formaldehyde. They are not preserved by fixation in 10% acrolein, $KMnO_4$, and 1% OsO_4 alone, and they are actually destroyed when exposed to 2% OsO_4 even when prefixed in glutaraldehyde. The three buffers tried gave essentially the same result.

TABLE I

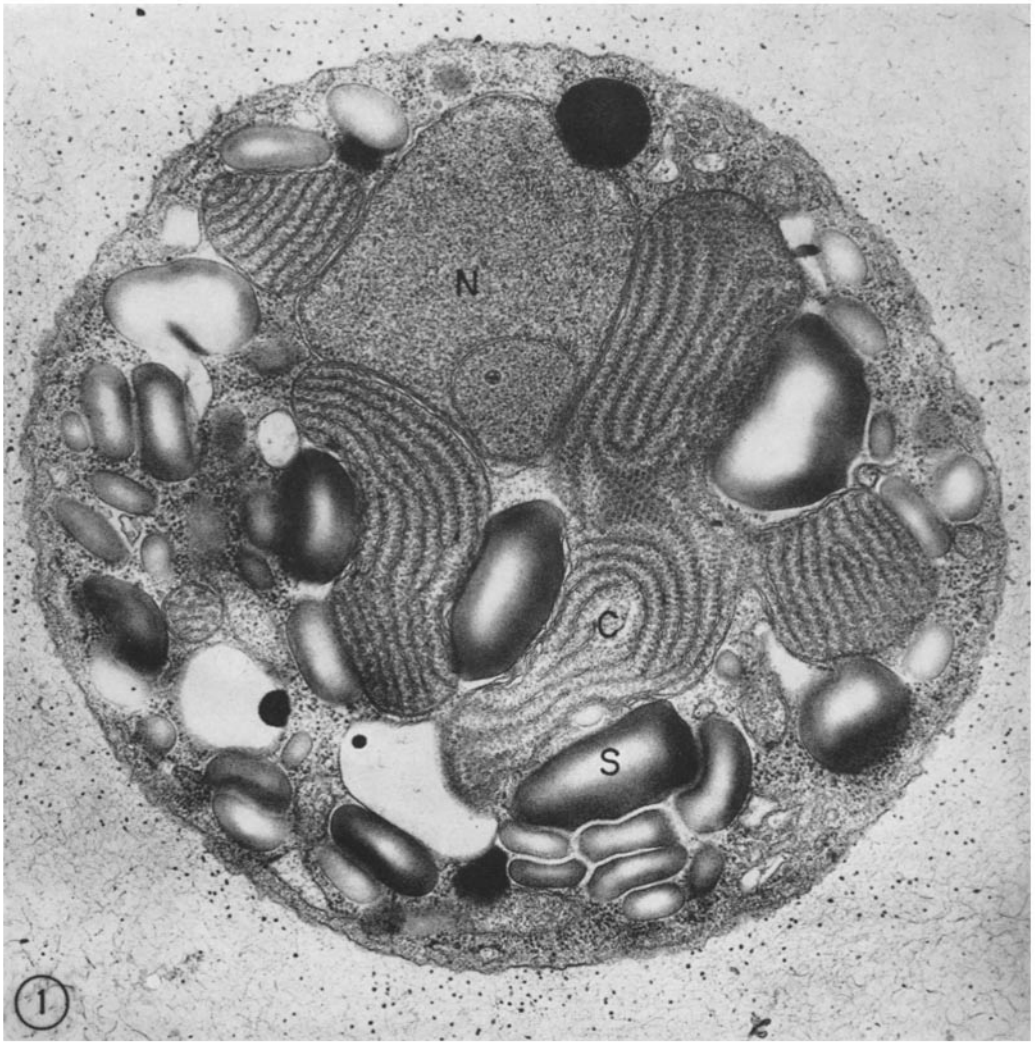
Preservation of Granules Associated with the Chloroplast Lamellae of Porphyridium cruentum

Fixatives	Buffer	Preservation of granules
4% glutaraldehyde (2 hr) + 1% OsO_4 (1 or 2 hr)	0.1 M phosphate, pH 6.8	++
4% glutaraldehyde (2 hr) + 1% OsO_4 (1 or 2 hr)	0.1 M Tris HCl, pH 7.6	++
4% glutaraldehyde (2 hr) + 1% OsO_4 (1 or 2 hr)	0.1 M cacodylate, pH 6.8	+
4% glutaraldehyde (2 hr) + 2% OsO_4 (2 or 4 hr)	0.1 M phosphate, pH 6.8	−
4% hydroxyadipaldehyde (2 hr) + 1% OsO_4 (1 hr)	0.1 M phosphate, pH 6.8	+
4% formaldehyde (2 hr) + 1% OsO_4 (1 hr)	0.1 M phosphate, pH 6.8	+
12% formaldehyde (2 hr) + 1% OsO_4 (1 hr)	0.1 M phosphate, pH 6.8	doubtful
10% acrolein (2 hr) + 1% OsO_4 (1 or 2 hr)	0.1 M phosphate, pH 6.8	−
1% OsO_4 (1 or 2 hr)	0.1 M phosphate, pH 6.8	−
2% OsO_4 (1 or 2 hr)	0.1 M phosphate, pH 6.8	−
2% $KMnO_4$ (1 hr)	aqueous	−

MORPHOLOGY AND ARRANGEMENT: In our previous paper (6) we reported that the granules had a diameter of about 320 A; this measurement should be considered merely as the approximate size, for sometimes granules are less than 320 A, while at other times their diameter approaches 400 A (Fig. 3). The culture conditions, the age of the culture, and the method of preservation influence the variation in size. Granules in cells from a stationary phase culture are conspicuously larger than those from early log phase. Although no critical quantitative determinations have been made, we know that, within limits, the concentration of phycobilins also increases as a culture ages. Grazing sections over chloroplast lamellae show that the granules are arranged in repeating rows with a center-to-center granule distance of 400 to 500 A (Fig. 1). The granules of adjacent rows can often be observed to interdigitate. Interdigitation of alternate granules attached to neighboring lamellae can also be seen in regions where the lamellae are closely spaced (Fig. 3). Whether or not the granules have this regular spacing in all areas of the lamellae is not certain. However, when one examines sections such as those shown in Fig. 2, one is impressed by the great regularity of the granule spacing in the entire chloroplast, and strongly suspects that it occurs on all the chloroplast lamellae of this alga.

That the granules are definitely attached to the lamellae is evident not only from stereo-electron microscopy but also from the fact that they adhere to the membranes in chloroplast fragments (Fig. 5) and to the lamellae of isolated chloroplasts (Figs. 8 and 9 in reference 6) when fixed soon after cell breakage. However, with most methods of disruption in an aqueous medium, the granules detach readily from the membranes. Granules attached to or free from membranes begin to dissolve in an aqueous solution. The shape of the granules seen in sections, as well as in negatively stained material, is spherical to oblong. At high magnification, as seen in Fig. 3, the granules have several small, rounded projections, a fact which suggests that they may be composed of subunits.

Negatively stained material, which is prepared in a manner completely different from the sectioned material, confirms and extends the observations made from chemically fixed preparations.



Figs. 1 through 7 are of *Porphyridium cruentum.*

FIGURE 1 This micrograph represents a noncentral section of a cell. The nucleus (*N*) is surrounded by several distinct sections which belong to one chloroplast (*C*). Small granules are seen to be attached to the chloroplast lamellae. Their highly regular two-dimensional spacing is particularly evident in the region of the cell slightly to the right of center. *S*, starch. Glutaraldehyde–osmium tetroxide fixation. × 25,000.

Fig. 7 shows the same close spacing of the granules. It also gives an indication that the membrane pairs have a tendency to stick together even after fragmentation of the chloroplast. The granules are still attached to the sides facing away from its membrane pair. The granules are also clearly composed of subunits. The subunits, now under investigation, can be seen at very high magnification to have a ringlike appearance.

RIBONUCLEASE TREATMENT: We carried out the ribonuclease treatment to ascertain whether or not the granules are ribosomes. We already knew (6) that the granules are significantly different from ribosomes in that they are less electron-opaque than ribosomes, they have a greater diameter than ribosomes, and they exhibit a two-dimensional type of crystalline array which has not been seen in membrane-attached ribosomes. The

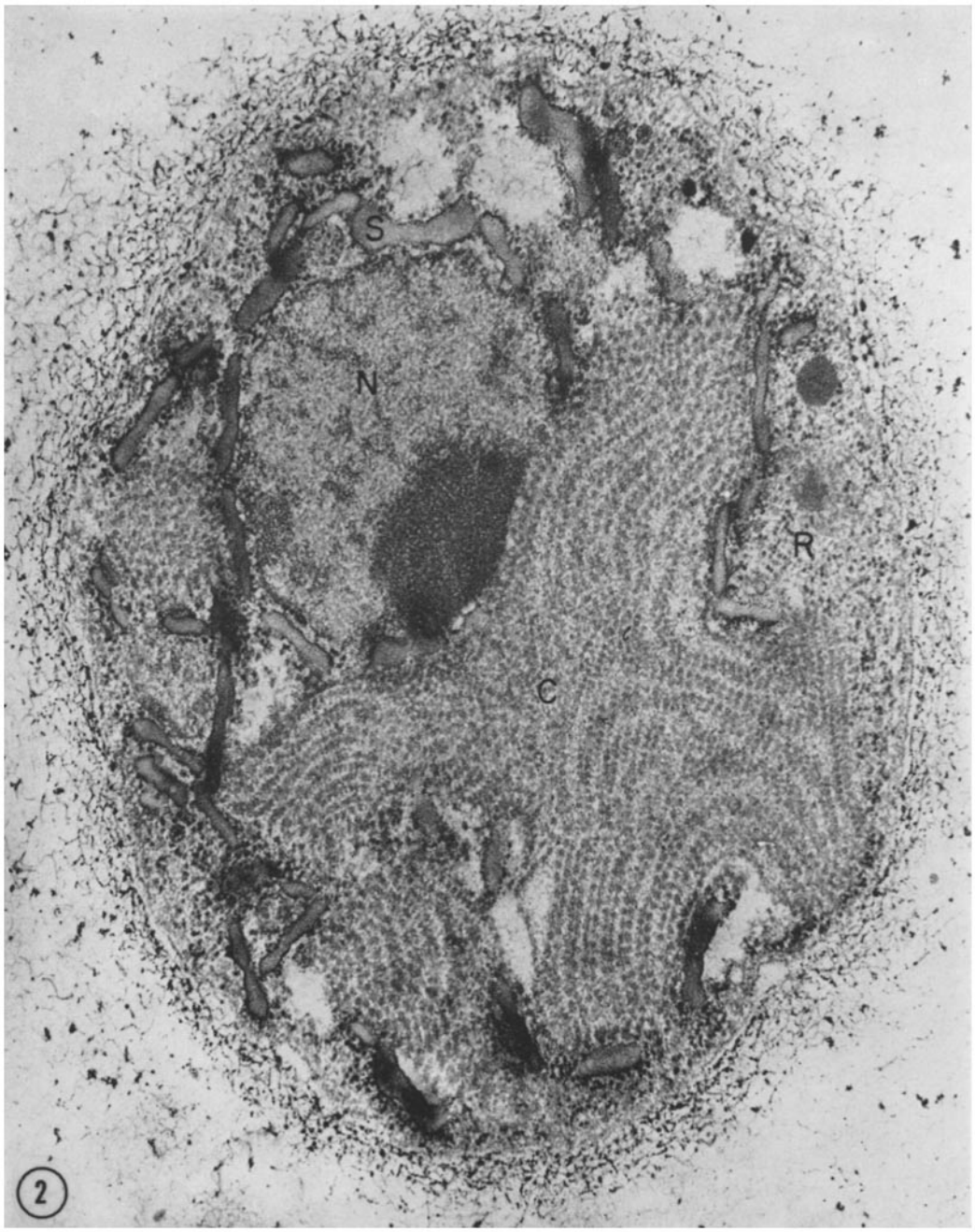


FIGURE 2 Section of a cell prefixed with glutaraldehyde and extracted with methanol-acetone prior to postfixation with osmium tetroxide. Although the cell as a whole shrank, owing to dehydration by methanol-acetone extraction, the usual components of the cell, such as a nucleus (*N*), chloroplast (*C*), ribosomes (*R*), and starch (*S*), can still be recognized. We have removed all the stainable membrane components, a large part of which consisted of chlorophyll. The granules are still present and exhibit the highly ordered spacing over the whole chloroplast. A cell thus treated has a purple-pink color, whereas prior to methanol-acetone extraction it is brick-red in color. × 37,000.

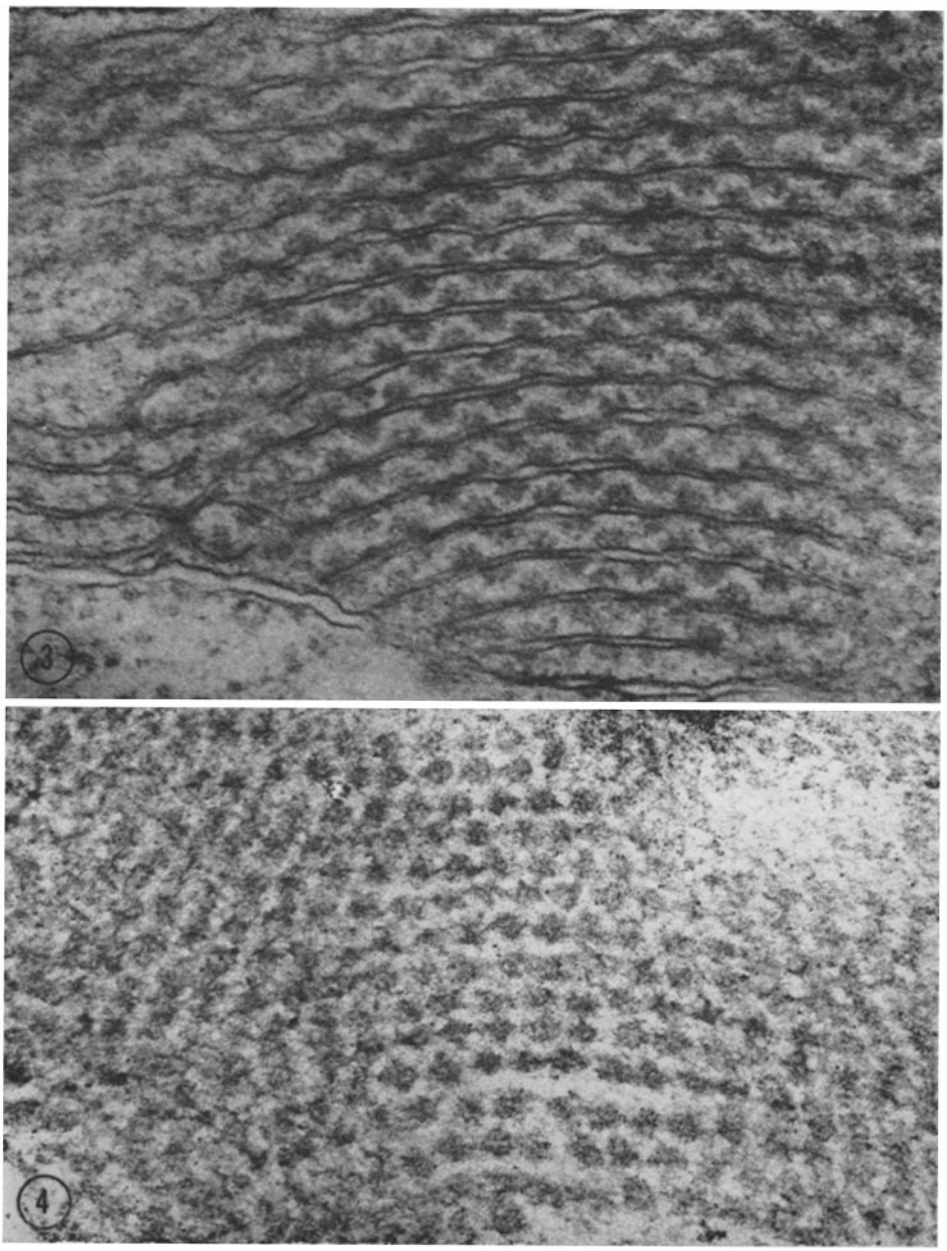
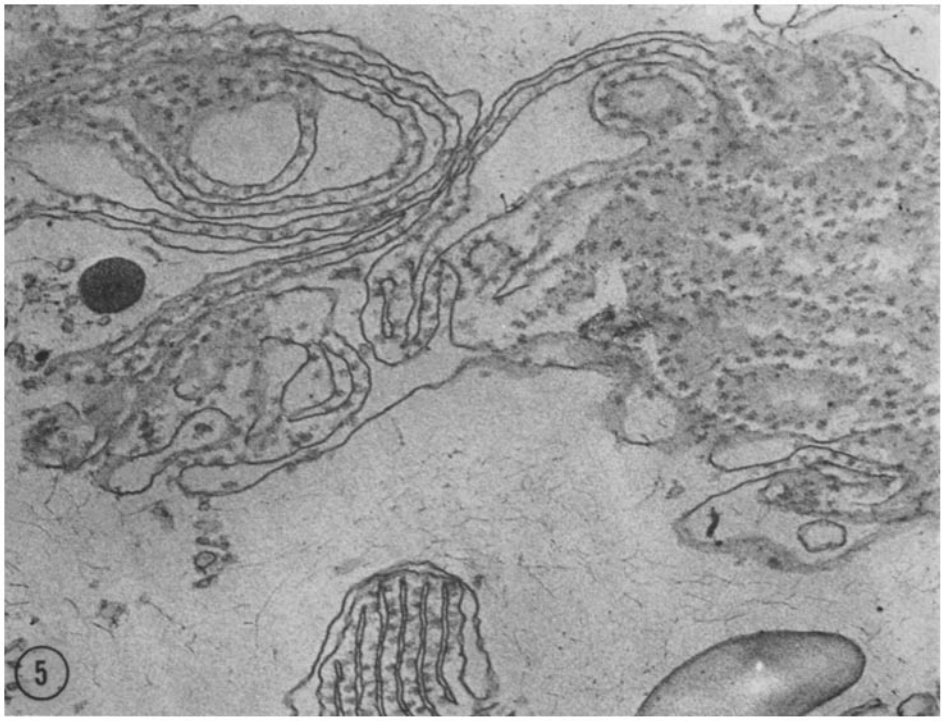


FIGURE 3 This chloroplast section shows that the granules are attached to the lamellae and alternate with one another. The granules have small rounded projections, a fact which suggests they are composed of smaller subunits. Glutaraldehyde–osmium tetroxide fixation. × 115,000.

FIGURE 4 Enlarged section from a chloroplast of a cell extracted with methanol-acetone. No parts of the chloroplast lamellae are visible in this cross-section. The granules retain their morphological structure and display the interdigitation of alternate granules. × 115,000.



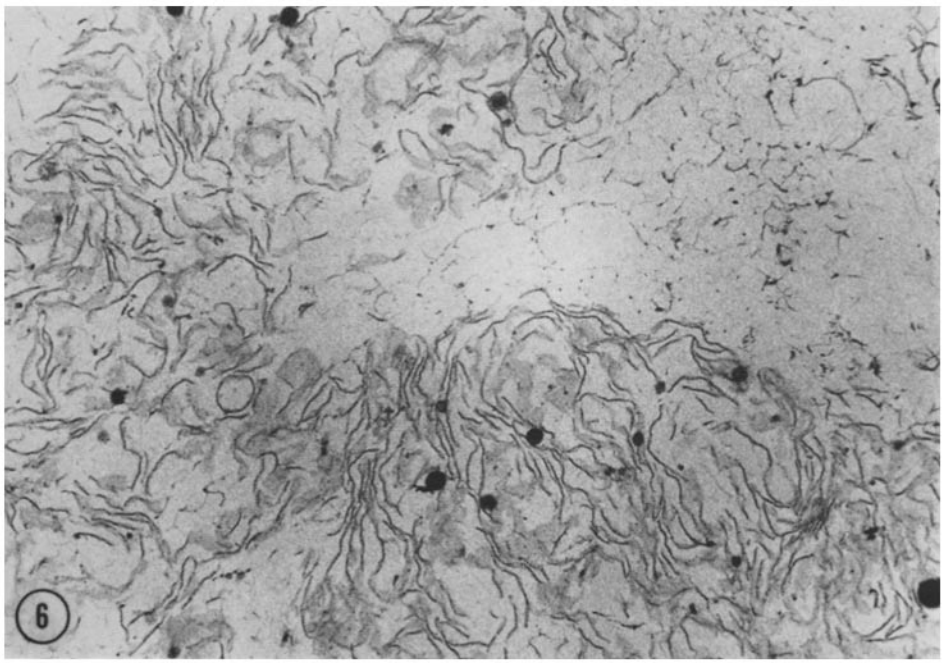


FIGURE 5 Section of a chloroplast fragment from a pellet of disrupted cells. This pellet was brick-red in color; the granules are still attached to the chloroplast lamellae. Fixation in glutaraldehyde–osmium tetroxide after cell disruption. × 43,000.

FIGURE 6 Section from a pellet of chloroplast fragments treated with deoxycholate after disruption. This pellet was green after the deoxycholate treatment. The granules are not associated with the lamellae, a fact which correlates with loss of red color. (The red pigments were present in the supernatant.) Fixation in glutaraldehyde–osmium tetroxide after cell disruption. × 43,000.

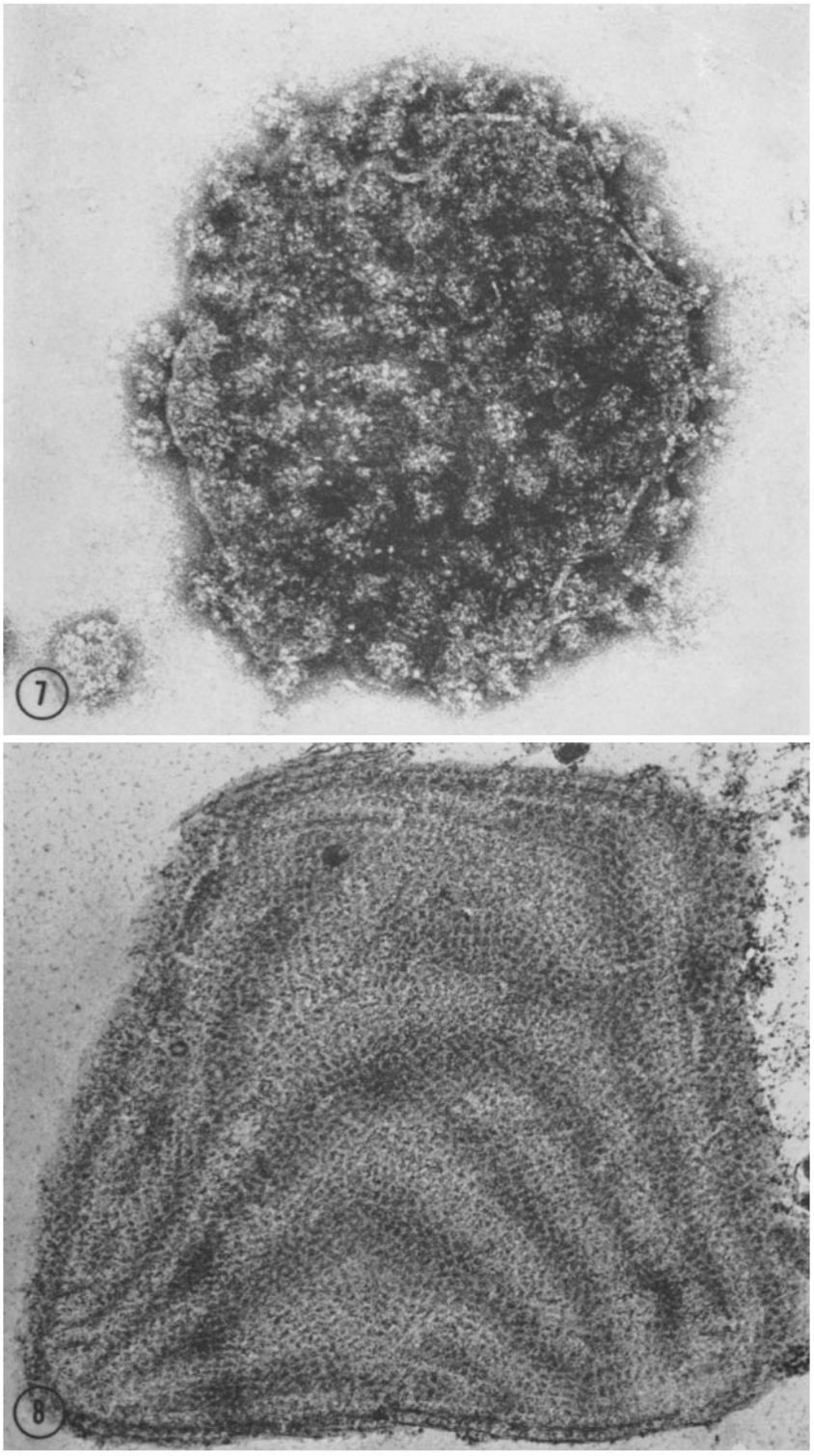


FIGURE 7 The granules on this chloroplast fragment are composed of small, distinct subunits. The two membranes comprising a chloroplast lamella can be seen at the lower right. Stained with 1% phosphotungstic acid. × 164,000.

FIGURE 8 Small granules, similar in appearance to those seen in *Porphyridium cruentum*, can be observed to cover the obliquely sectioned chloroplast lamellae of the red alga *Spermothamnion*. × 31,000.

various ribonuclease treatments on whole cells, chloroplast fragments, and on fixed and unfixed cells clearly showed that the granules are completely resistant to ribonuclease. Sections of ribonuclease-treated material looked exactly like that pictured in Fig. 5.

METHANOL ACETONE EXTRACTION: After establishing that the granules were not ribosomes, we tried an experiment which consisted of extracting whole cells with a mixture of 80% methanol and 20% acetone. This treatment removed chlorophyll and many carotenoids, but none of the phycobilins. The cells and the extracts were checked by spectrophotometry. Prior to extraction the cells have a brick-red color, but after extraction their color is purplish-pink with a broad absorption peak coinciding with that of phycoerythrin of whole cells (Fig. 9). Glutaraldehyde fixation before extraction keeps the cell components intact and does not affect the methanol-acetone extraction. Figs. 2 and 4 represent sections of cells after such treatment. All the stainable membrane components have been removed, the nucleus, chloroplast, ribosomes, and the cell as a whole are still intact, and the granules are present. The regularity of their arrangement over the entire chloroplast seems even more evident than in unextracted cells. They have not decreased in staining intensity, and they are still distinct and coherent.

DEOXYCHOLATE TREATMENT: Once we knew that the phycobilins remained in the cell after lipid solvent extraction, and that the granules are present when the purple-pink color is present, we attempted to remove the granules and leave the lamellae intact. After disrupting the cells in a French pressure cell, we collected a pellet, brick-red in color, by centrifugation. We discarded the supernatant fraction and divided the pellet. Half of the pellet was treated with a final concentration of 0.2% deoxycholate, and the other half was set aside as a control. Both halves were then fixed with glutaraldehyde and centrifuged. The control pellet was brick-red in color and, as Fig. 5 shows, the granules are still attached to the lamellae of the chloroplast fragment. However, the pellet treated with deoxycholate was green in color and, as Fig. 6 illustrates, granules were absent from the lamellae. The red color and the granules disappeared at the same time. The red color is due primarily to phy-

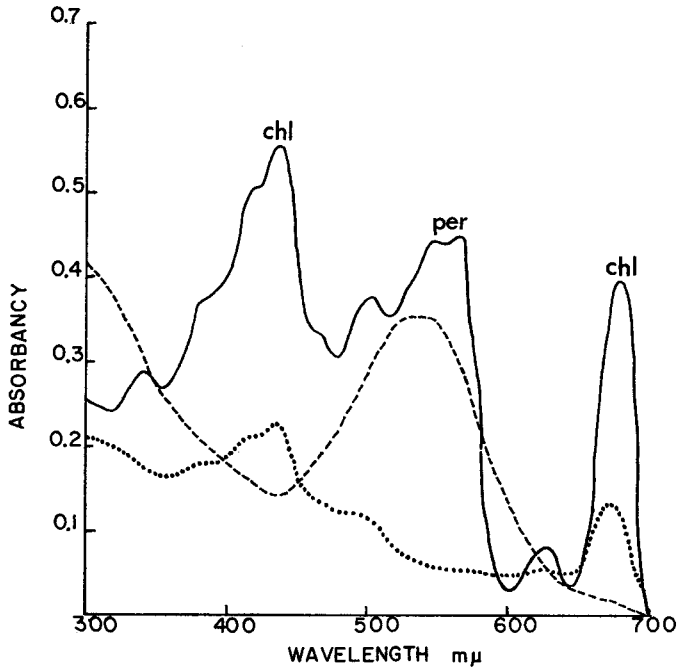


FIGURE 9 Absorption spectra of whole cells (———), cells extracted with a methanol-acetone mixture (- - - - - -), and of the green pellet of disrupted cells after deoxycholate treatment (· · · · · ·). *chl*, chlorophyll; *per*, phycoerythrin.

coerythrin, which is not destroyed by deoxycholate, but can be recovered from the supernatant fraction. Fig. 9 shows the absorption spectrum of the green pellet. The red pellet has an absorption spectrum like that of whole cells.

We examined a number of other algae and chloroplasts of some vascular plants, using exactly the same fixation procedure that gave the best preservation of the granules in *P. cruentum*. No granules, nor any suggestion of them, were found on the chloroplast lamellae of vascular plants or on any of the algae which do not have phycobilins as accessory pigments. Granules were observed on the chloroplast lamellae of all the marine Rhodophytes examined (*Spermothamnion*, *Bangia fusco-purpurea*, *Nemalion multifidum*, and *Antithamnion glanduliferum*). The granules in the chloroplast of most of these red algae are not generally as conspicuous as those in *P. cruentum*, but they are clearly present as Fig. 8 shows. The fresh-water red alga *Porphyridium aerugineum*, which has only phycocyanin as its major accessory pigment, was also found to have granules. Our results with all fresh-water, blue-green algae are inconclusive at this point, because the method of fixation used is not ideal for these algae. However, we have observed small granules attached to the lamellae of an unidentified oscillatorialike marine blue-green.

DISCUSSION

From our examination of the chloroplast lamellae of various plant groups we may conclude that only members of the red algae and some members of the blue-green algae have granules attached to the lamellae. We are quite certain of the lack of granules in the photosynthetic organisms which do not have phycobilins as accessory pigments that we examined, since we always had excellent preservation but no hint of granules. Fixation of blue-greens with our method was not as good as can be expected. It is clear that the granules are very sensitive to the method of fixation, as the results in Table I indicate. What the ideal conditions are for the preservation of the granules is not known at this point. It can be assumed, however, that mild fixatives, especially aldehydes, which are known to preserve proteins, are the most satisfactory. The ionic composition within the cell at the time of fixation may be one of the most critical factors in preserving the granules. This composition might be one explanation for the observation that granules are clearly present in all the marine Rhodophytes examined. Also, the only indication we have found that granules are present in the blue-green algae is in an oscillatorialike marine blue-green.

It is of great interest to us that Lefort (12) has shown that granules of the same type and arrangement are present in two Cyanophycean endosymbionts, *Glaucocystis nostochinearum* and *Cyanophora paradoxa*. It is significant not only that she showed the granules in blue-green algae, but also that they were preserved by osmium tetroxide fixation. However, she was unable to show granules in free living blue-greens with the same fixation.

A great amount of work has been done on phycobilins, as the literature cited in some of the latest review articles indicates (15, 17). Most of the work on phycobilins has been concerned with their chemical and physical properties both in vivo and in vitro. Very few people have worked on the localization of these pigments within the cell in either the red or the blue-green algae. With only a few exceptions, these two groups of algae have phycobilins as accessory pigments. In *Cyanidium caldarium*, an alga of doubtful taxonomic affinity, Bogorad et al. (2, 3) found that there were no observable differences in the thickness of the lamellae in mutants which either possessed or lacked phycocyanin and allophycocyanin; they assumed therefore that the phycobilins are not in the lamellae. Bergeron (1) considers it likely that phycocyanin in *Anacystis nidulans* is located between the lamellae, while Fuhs (5), who also worked on blue-greens, concluded that the phycocyanin is part of the lamellae. Working with *P. cruentum*, Brody and Vatter (4) assumed that the phycobilins are in the lamellae, while Giraud (8, 9) thinks that they are in the stroma between the lamellae. In our opinion, Giraud's most cogent arguments are that calculations indicate that there is not enough room on the lamellae to accommodate the phycobilins as a single layer over the chlorophyll, and that the colored pigments are readily released from broken cells when the lamellae are relatively intact.

Both Brody and Vatter's and Giraud's assumptions can be reconciled with the possibility that the granules are major sites of phycobilins. They are attached to the lamellae, and are thus within the 50 A distance necessary for effective energy transfer. It appears that they are not a structural part of the lamellae, for they are easily removed by cell disruption and treatment with deoxycholate. Furthermore, any increase in phycobilin con-

centration without a concomitant lamellar growth can be accommodated by an increase in the size of the granules.

To support our assumption that the granules may be sites of phycobilin aggregation, we have shown that when the granules disappear, the red color also disappears. Also, when we removed the chlorophyll and many carotenoids by methanol-acetone extraction, the stainable membrane constituents were removed but the purple-pink color (the color of the phycobilins after removal of the green chlorophyll) and the granules are left. If the phycobilins were in the form of a layer in or on the lamellae, we should have some indications of such a layer, and we do not.

The results given in this paper are, of course, only indications that the granules may be sites of phycobilins. We also have some other corroborating evidence. For instance, when cells are grown in a nitrate-deficient medium, they divide regularly, but turn a mustard color, and the granules either become greatly decreased or absent. When the nitrate is replaced, the red color and the granules appear together. The most convincing evidence we have is from preliminary results of isolated granules. Under certain conditions we can recover the granules from the red supernatant fluid of the deoxycholate-treated cell fragments. The recovered granules are morphologically identical with the granules on the lamellae in either sectioned or negatively stained material, and they have the spectrophotometric pattern of isolated phycobilins of *P. cruentum*.

At this time, the spatial arrangement of the subunits and the chemical and physical characteristics of the granules are being studied.

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