

# CHLOROPLAST STRUCTURE OF THE CRYPTOPHYCEAE

## Evidence for Phycobiliproteins within Intrathylakoidal Spaces

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### ABSTRACT

Selective extraction and morphological evidence indicate that the phycobiliproteins in three Cryptophyceae algae (*Chroomonas*, *Rhodomonas*, and *Cryptomonas*) are contained within intrathylakoidal spaces and are not on the stromal side of the lamellae as in the red and blue-green algae. Furthermore, no discrete phycobilisome-type aggregates have thus far been observed in the Cryptophyceae. Structurally, although not necessarily functionally, this is a radical difference. The width of the intrathylakoidal spaces can vary but is generally about 200–300 Å. While the thylakoid membranes are usually closely aligned, grana-type fusions do not occur. In *Chroomonas* these membranes evidence an extensive periodic display with a spacing on the order of 140–160 Å. This periodicity is restricted to the membranes and has not been observed in the electron-opaque intrathylakoidal matrix.

### INTRODUCTION

The varied characteristics of the cryptomonads are responsible for their indefinite taxonomic position (17), but at the same time they enhance their status in evolutionary schemes (1, 4). Their chloroplast structure is distinct from that of every other group examined. In possessing chlorophylls *a* and *c*, they resemble the diatoms, brown algae, and dinoflagellates. However, in addition to chlorophyll *c* they also contain phycobiliproteins as photosynthetic accessory pigments. The present study was prompted by the presence of these pigments in *Rhodomonas lens*, *Chroomonas* sp., and *Cryptomonas ovata*. Their phycobiliproteins are different from those found in the red and blue-green algae (2), although they are functionally equivalent.

Many of the chloroplast features and the morphology of the cells have been described by Gibbs (8, 9) in her early extensive ultrastructural study of algal groups, and more recently by Dodge

(3), Greenwood *in* Kirk and Tilney-Bassett (10), and Lucas (13). The thylakoids have a tendency to be arranged in pairs, that is, for two of them to be closely associated with a 30–50 Å space between them. They do not fuse as in green plants and some other algae. Two sets of double membranes enclose the chloroplast; the outer set is the chloroplast endoplasmic reticulum, and the inner the chloroplast-limiting membranes which are characteristic of all chloroplasts.

Our study has confirmed most of the observations of the above mentioned authors, and in addition has yielded new data on chloroplast membrane structure and phycobiliprotein location in the cryptomonads.

### MATERIALS AND METHODS

#### *Growth Conditions*

Cultures of red *Rhodomonas lens* and blue-green *Chroomonas* sp. were grown in a liquid medium in

stationary Erlenmeyer flasks with cotton stoppers. Each liter of medium had the following ingredients: 18 g NaCl, 0.6 g KCl, 0.5 g NaNO<sub>3</sub>, 5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g CaCl<sub>2</sub>, 0.03 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g Na<sub>2</sub>SiO<sub>3</sub>·9H<sub>2</sub>O, 0.48 mg FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.003 mg vitamin B<sub>12</sub>, 0.1 mg thiamine HCl, 0.001 mg biotin, 1 g Tris, and 3 ml of Provasoli's PII metal mix plus distilled water to a total of 1 liter. The medium was adjusted to pH 7.6–8.0 and filtered. The cultures were given constant illumination with "cool white" fluorescent light (incident light intensity, 75–100 ft-c) at room temperature. Cultures of *Cryptomonas ovata* var. *palustris* (Indiana Culture Collection of Algae No. 358) were grown on proteose agar.

#### *Fixation for Electron Microscopy*

Numerous variations in the fixation procedure were carried out, but the best results were obtained by fixation in 2–4% glutaraldehyde, pH 6.8–7.6, in 0.1 M phosphate or cacodylate buffer, and postfixation in 1% OsO<sub>4</sub> in the appropriate buffer. Since *Rhodomonas* cells were too fragile (probably due to activation of trichocyst discharge) to be harvested by centrifugation, double-strength buffered glutaraldehyde was added directly to the liquid cultures in 1:1 proportions. Fixation was allowed to proceed for 1 hr at room temperature, then the material was rinsed and extracted by various procedures, or it was post-fixed in 1% OsO<sub>4</sub>, dehydrated, and embedded in Epon. Examination of sections stained with lead citrate, or uranyl acetate and lead citrate, were made with a Philips-200 or Siemens Elmiskop I electron microscope.

#### *Methanol Acetone Extraction*

Cells which had been previously fixed in glutaraldehyde and well rinsed in 0.1 M phosphate buffer were treated with a mixture of 20% acetone and 80% methanol for 1 hr at room temperature. This treatment extracted the chlorophyll, many carotenoids, and other lipid constituents, but not the biliproteins. This was continued until no further color was extractable.

#### *Pronase Extraction*

Fixed (in 4% glutaraldehyde for 1 hr, pH 6.8) and rinsed cells were suspended in 0.1 M phosphate buffer (pH 6.8) and incubated at 37°–40°C in pronase (2 mg/ml) (Calbiochem, Los Angeles, Calif.). 1 ml samples were removed just prior to adding pronase, and at 0, 5, 10, 60, 120, 180, and 240 min after addition of pronase. The 1 ml samples were put on individual millipore filters, and with vacuum filtration the cells were separated from the filtrate.

The filtrate was stored on ice until used for spectrophotometric readings. To stop the pronase digestion the cells were immediately suspended in very dilute HCl at pH 3.0. (There was no noticeable effect of dilute HCl on the ultrastructure of glutaraldehyde-fixed cells.) Postfixation in OsO<sub>4</sub> and embedding for electron microscopy followed.

## OBSERVATIONS

### *General Structure*

Each cell of the Cryptophyceae can be regarded as consisting of two compartments which are separated by membrane envelopes. The chloroplast and nucleus are enclosed by the chloroplast-endoplasmic reticulum and represent one compartment, while the rest of the cell with mitochondria, Golgi apparatus, and trichocysts (ejectosomes) represent the second compartment (Fig. 1). The cell pictured in Fig. 1 is typical with its asymmetric shape and the emergence of one of the mastigoneme-covered flagella through a gullet-like invagination. A well developed Golgi apparatus is seen to be surrounded by fibrous-filled vesicles, and large trichocysts are located in the cup-shaped area created by the chloroplast-nuclear complex. A periplast, whose features are to be discussed in another publication (*J. Phycol.*), surrounds the whole cell.

The shape of the chloroplast generally conforms to that of the cell. Newly divided cells possess a crescent-shaped chloroplast. In fully developed cells it usually has the form of a bilobed cup with a pyrenoid between the lobes. One of the lobes is usually more prominent and extends around and above the gullet-like region (particularly in *Rhodomonas* and *Chroomonas*). The chloroplast is surrounded by a double membrane envelope and, outside of it, by a sac of endoplasmic reticulum. Starch grains are formed near the pyrenoid, but outside of the chloroplast-limiting membrane (Figs. 1 and 4). An eyespot when present is within the stroma of the chloroplast and faces toward the nucleus, but is at some distance from the ciliary apparatus.

The stroma is continuous with the pyrenoid region. A few thylakoids traverse the pyrenoid in *Chroomonas* and in *Cryptomonas*, but not in *Rhodomonas*. In certain regions of the chloroplasts pictured in Figs. 1, 4, and 5 the tendency to form thylakoidal pairs is observable. However, any number from 2 to 25 (Fig. 4) or more can be part of a stack for

part of their length. In spite of the association of neighboring thylakoids the small space first noted by Gibbs (9) is always present and is about 40 Å wide, with our fixation conditions. The stainable portion of the thylakoid membranes has an average width of 50–55 Å.

### Localization of Phycobiliproteins

In all three species examined an electron-opaque material was found in the intrathylakoidal region (Figs. 1, 2, 4, and 5). Since the pyrenoid and stroma are continuous, one can ascertain that the dense material is indeed inside the thylakoids and not in the stroma (Figs. 1 and 4). Occurrence of electron-opaque material such as is present within the thylakoids is not a general feature in mature chloroplasts of other plants. The width of the intrathylakoidal lumina is variable, and ranges from about 100 Å to 500 Å or more, but it is usually 200–300 Å. It should be noted that the lumina widths are quite uniform within the same chloroplast, and that there are no observable connections of the thylakoids with the chloroplast-limiting membrane. Furthermore, in none of the three species studied was there any evidence of phycobilisome-type aggregates, either in the electron-opaque matrix, or on the stromal side of the membranes.

In order to obtain information on the nature of the intrathylakoidal material, fixed *Chroomonas* and *Rhodomonas* cells were treated with enzyme or with lipid solvents and monitored by electron microscopy and spectrophotometry. Cells treated with a methanol-acetone mixture lost their chlorophyll and carotenoids. All the stainable membrane components, eyespots, and "osmiophilic" bodies were removed, but the intrathylakoidal matrix remained unaffected (Fig. 6). Upon treatment with pronase, which was also monitored by spectrophotometry (Table I) and electron microscopy

(Figs. 7–10), the following results were obtained: Pronase extracted most of the cytoplasmic background, the chloroplast stroma, and the pyrenoid matrix in a few minutes. A comparison of Fig. 7 (0 min pronase) and Fig. 8 (5 min pronase) reveals that in as short a time as 5 min most of the chloroplast stroma was extracted, but the intrathylakoidal dense material was still present and relatively unaffected. At 10 min the stromal material had disappeared and, as can be seen in Table I, some phycoerythrin had begun to appear in the filtrate.

After 1 hr much of the electron-opaque intrathylakoidal matrix (Fig. 9) had disappeared, and there was a great increase in the phycoerythrin content outside the cells. As the enzyme extraction of the fixed cells progressed, discontinuities in the chloroplast lamellae became common. Immediately adjacent to these discontinuities small empty regions appeared in the electron-opaque matrix. This suggests that the membranes are more susceptible to pronase digestion in some areas than in others, and it also indicates that the intrathylakoidal matrix was more readily extractable at these points. After 2 hr (Fig. 10) the intrathylakoidal spaces were essentially free of electron-opaque material, and the chloroplasts were free of phycoerythrin as judged from the remaining green-colored pellet. The 2- and 3-hr pellets had the same ultrastructural appearance, but by 4 hr the membranes had become ragged and fragmented. Structures with a droplet appearance, similar to osmiophilic bodies, were noticeable after about 2 hr of extraction (Fig. 10). These are believed to be artifacts caused by the extraction because they increased as the pronase digestion progressed. They may contain chlorophyll and some other lipid-solvent extractable pigments (since the droplets disappeared on acetone extraction).

The reported phycoerythrin peak in *Rhodomonas* at 550 nm (15) is normally obtained by us in

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FIGURE 1 This micrograph is a cross-section of a typical *Chroomonas* cell. A periplast surrounds the cell. One of the two flagella is present in the gullet-like invagination at the upper left. Directly below are various sectional views of the large trichocysts (*T*). In close association with the Golgi apparatus (*G*) are numerous, large vesicles with a fibrous material. The chloroplast endoplasmic reticulum can be traced around the chloroplast nuclear complex. Chloroplast lamellae are aligned parallel to one another and are usually paired. Electron-opaque material characteristically fills the intrathylakoidal lumina. Starch (*S*) is present outside the chloroplast-limiting membrane and can be in the shape of plates, such as that near the pyrenoid (*P*), or irregularly shaped grains. An eyespot, sometimes present, is not shown in this section. *N*, nucleus.  $\times 29,000$ .





FIGURE 2 Various planes of section through and across the chloroplast lamellae are present in this chloroplast of *Chroomonas*. The electron-opaque material fills the intrathylakoidal lumina. Note the space between the closely aligned lamellae. Arrows indicate the regular periodic pattern evident all over the lamellae. The periodicity can always be traced back to a definite membrane face.  $\times 80,000$ .

buffer extracts. However, with pronase treatment of fixed cells the peak is at 530 nm during the first 2 hr of pronase exposure. At about 3 hr the peak changed to 525 nm, and to 523 nm at 4 hr. Since essentially all phycoerythrin had been extracted by the end of 2 hr, this is not significant to our results. It is probably due to increased turbidity and degradation of the phycoerythrin molecules.

### Membrane Structure

The chloroplasts of *Chroomonas* evidence an extensive periodic display particularly when membranes (Figs. 2 and 3) are sectioned at an angle (tangential). While this display is commonly observed, it is not found in every section. The periodicity is due to a parallel array where each repeating band or cord width is on the order of 140–160 Å. In regions such as seen in Fig. 3 the plane of section is believed to be passing parallel just over a membrane, or to be passing through it (and separating it as in opening a sandwich), thus revealing the linear banding. Such a view suggests that the membrane is entirely composed of cords which are about three times wider (140–160 Å) than thick (50 Å). The cobblestone appearance seen in some regions (Figs. 2 and 3) is probably due to the cords being sectioned tangentially and being viewed more or less head on. It could also be explained by imagining a linear array of closely packed subunits. The periodicity is believed to be only in the membrane because it can always be traced back to membrane faces. Occasionally there are indications of a membrane periodicity in the chloroplasts of *Cryptomonas* and *Rhodomonas*, but it is not so clear as in *Chroomonas* and, therefore, is still equivocal.

### DISCUSSION

The structure of the chloroplasts of the three cryptophycean algae studied is very similar. The significant structural features are: (a) the pairing tendency of the thylakoids, (b) the absence of phycobilisome-type aggregates, and (c) the presence of the electron-opaque intrathylakoidal matrix which is believed to contain the phycobiliproteins. Differences such as the pyrenoid shape, variations in the width of the intrathylakoidal space, and whether or not lamellae pass through the pyrenoid are not considered particularly significant. Pairing of the lamellae had first been noted by Gibbs (8, 9) and has since been confirmed

in every photosynthetic cryptophyte examined (3, 10, 13). While pairing is perhaps the most striking in *Cryptomonas*, it occurs in all, although sometimes it is obscured when the thylakoid number is great. The intrathylakoidal matrix was not observed in this genus by Greenwood *in* Kirk and Tilney-Bassett (10) or Lucas (13). This is somewhat puzzling, particularly since we found it in our preparations which were fixed by a fixation procedure very similar to that employed by Lucas. Dodge (3), who also used glutaraldehyde fixation, showed the dense material in *Chroomonas* very clearly, and logically suggested that it may contain phycobiliproteins.

The evidence presented here for the location of the phycobiliproteins in the intrathylakoidal spaces can be taken with a good degree of confidence even though it is not unequivocal proof. As seen in the results, there is a positive correlation between the stepwise extraction of the cells, the removal of the electron-opaque intrathylakoidal material (Figs. 7–10), and the appearance of the phycoerythrin in the aqueous medium surrounding the fixed cells (Table I). While the cytological approach employed here has its limitations, it is one of the few possible approaches that can be used when one is dealing with the localization of a water-soluble pigment.

In the past we had expected that phycobilisomes would be found in all algae which possessed phycobiliproteins as accessory pigments. The presence of phycobilisomes on the stromal side of the photosynthetic membranes of red and blue-green algae is well established (see references cited in 5, 6, and 7). Whereas it is true that phycobilisomes have not yet been found in every species of red and blue-green algae, they are believed to exist in all photosynthetic species of these two groups. From our experience we know that phycobilisomes are observable when the growth conditions and fixation are good, and that they are absent when the growth conditions and fixation are poor (as is often the case with blue-green algae). These two factors are not responsible for the lack of observable phycobilisomes in the cryptophytes. Therefore, we are presently assuming that this algal group does not have phycobilisomes, and that the biliproteins are contained in the intrathylakoidal space where they exist in solution or in a semi-crystalline state.

Location of biliproteins in the intrathylakoidal space raises some interesting points. It implies that the thylakoid membranes are “inverted” when

compared with those of the red and blue-green algae (where the biliproteins are on the stromal side of the lamellae). Since cryptophytes also possess chlorophyll *c*, which also functions as an accessory pigment, the question can be raised whether or not the "inversion" is related to the location of this chlorophyll. The lack of perceptible aggregates and the smaller size (1, 14) of cryptophyte biliproteins suggest that they do not aggregate as readily as the biliproteins of the red and blue-green algae (2, 12). It seems reasonable to assume that a pigment in solution or in a semi-crystalline state would function more efficiently in transferring energy if the pigment were concentrated in a membrane sac.

It is interesting to note here that Berns (2) has shown that the cryptophyte biliproteins are immunologically distinct from those of the red and blue-green algae. The cryptophyte algae have long been regarded as an anomalous group (17). By their chloroplast composition and structure alone, they cannot be grouped with any other line. Our observations on their biliprotein location underline this difference even more.

To our knowledge, the periodicity in the thylakoidal membranes has not been described before in sectioned material. While the periodicity of the chloroplast membranes is clear only in *Chroomonas*, we believe that it is not peculiar to this species alone, since we have observed suggestions of it in the other two species. The dimension of the periodic spacing (140–160 Å) described here is within the same range as those observed by other methods. Data from low angle X-ray determinations made on chloroplasts of higher plants (11) and data from shadowed replicas have yielded similar spacings. Kreutz's "crystallite" has a square side of about 150 Å, and the particle which Park and Biggins had called a "quantasome" (16)

TABLE I  
Optical Density Changes of Filtrate from Pronase-Extracted *Rhodomonas* Cells (2 mg/ml), pH 6.8, 37°–40°C

Time of digestion	OD, 530nm	ΔOD, 530nm
<i>min</i>		
0	0.0	—
5	0.15	+0.15
10	0.24	+0.09
60	1.94	+1.70
120	2.37	+0.43
180	2.49*	+0.12
240	2.28‡	–0.21

\* Peak at 525nm.

‡ Peak at 523nm.

is about 185 Å long, 155 Å wide, and 100 Å thick. It is not our intention to equate these structures with either of the above, but merely to emphasize that this spacing is not uncommon in chloroplast membranes. Neither the structural arrangement nor the possible functional significance of the membrane periodicity is understood at this time. Work in progress hopefully will lead to a more thorough understanding of the structure of these membranes.

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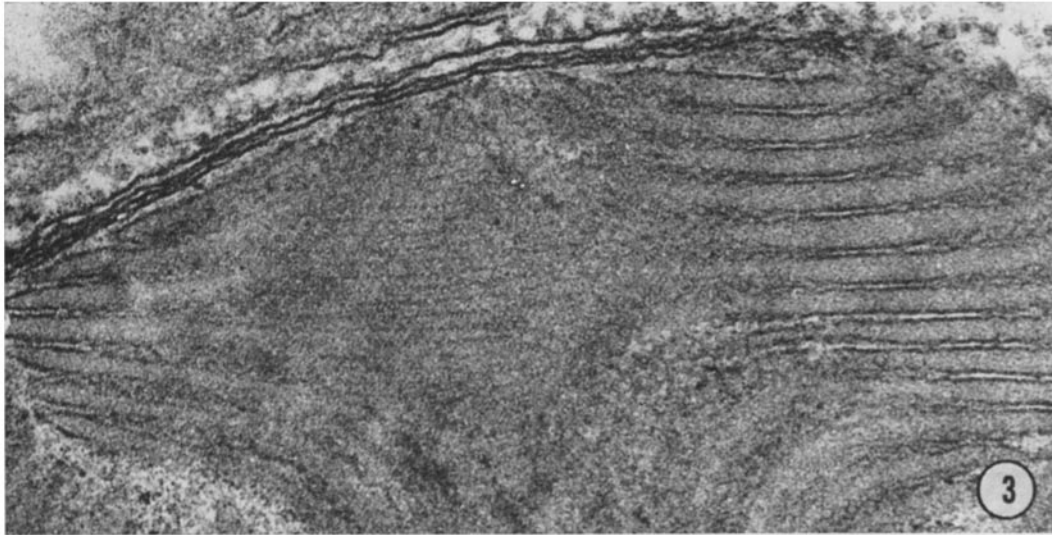
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FIGURE 3 Chloroplast section of *Chroomonas* at higher magnification in which the periodic spacing (140–160 Å) is illustrated. The area with the exposed linear structure is believed to belong within a thylakoid membrane. Structures reminiscent of cobblestones can be seen in regions where the membranes are sectioned tangentially (right side of figure). Compare with similar regions in Fig. 2. Of the four membrane layers at the chloroplast periphery on the left, two belong to the chloroplast envelope, while the other two belong to the chloroplast endoplasmic reticulum. × 119,000.

FIGURE 4 The chloroplast of *Rhodomonas lens* is also composed of linearly arranged thylakoids filled with an electron-opaque matrix surrounded by stromal material (*St*). The pyrenoid (*P*) is generally not traversed by lamellae in this species. The chloroplast-limiting membranes are not obvious in this section, but they also separate the starch plates from the pyrenoid. *N*, nucleus; *S*, starch. × 35,000.





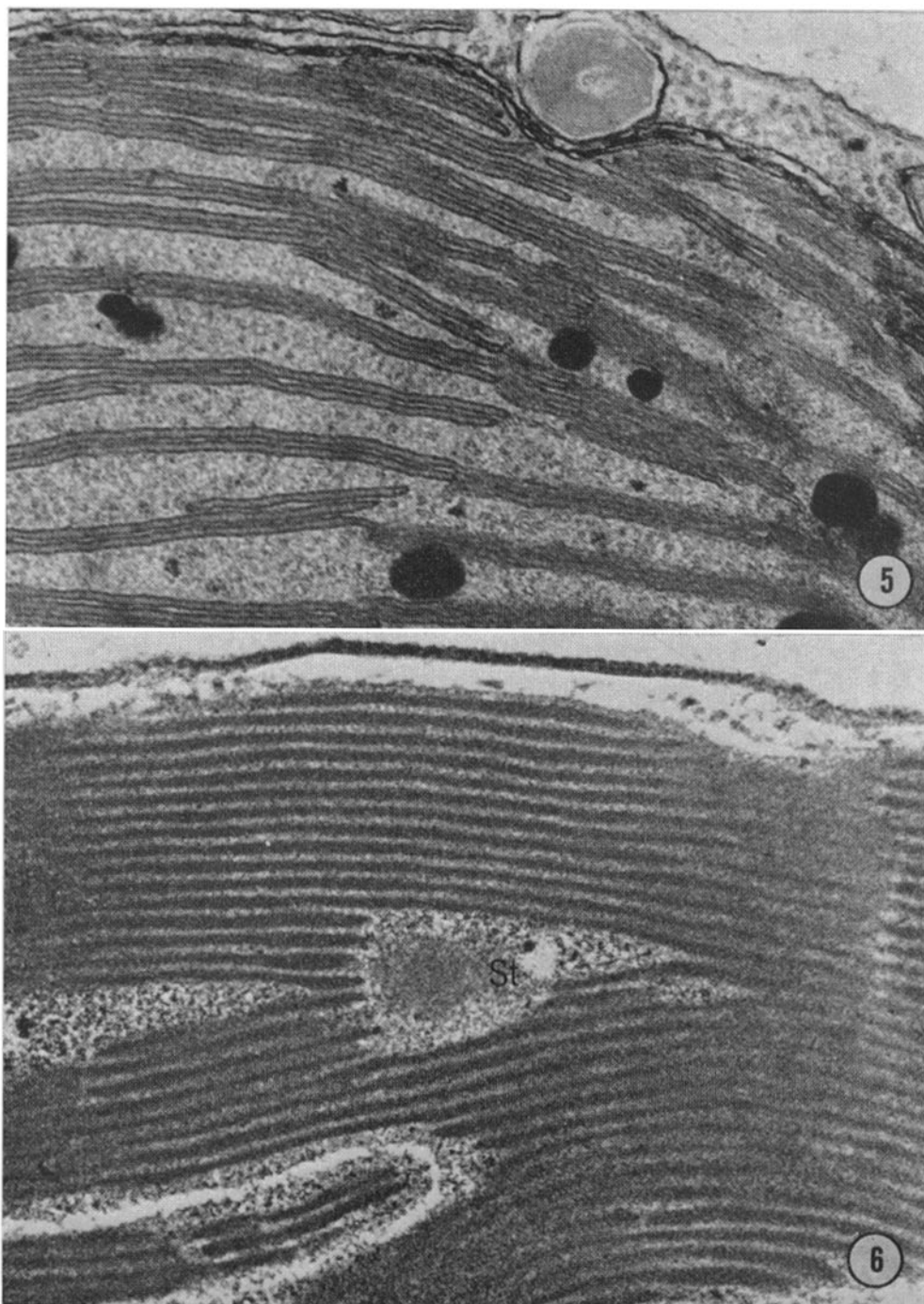
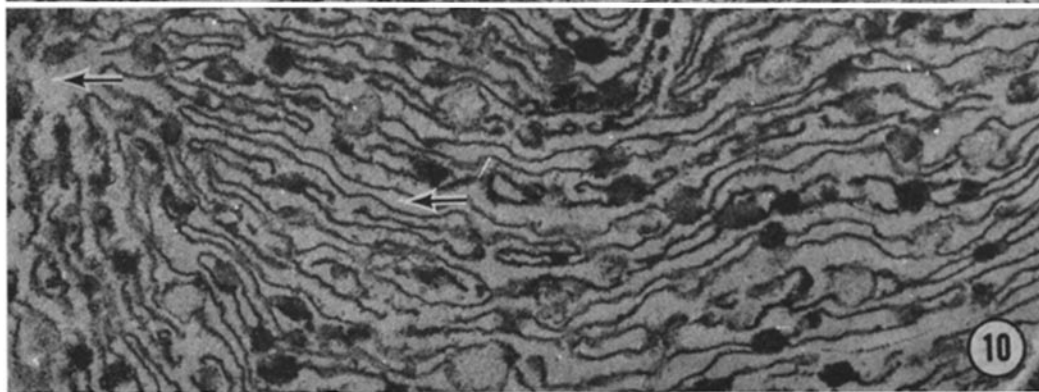
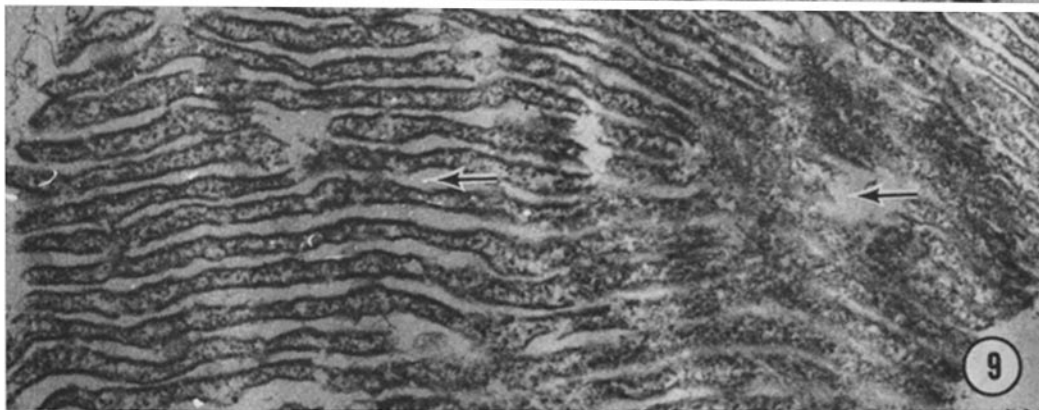
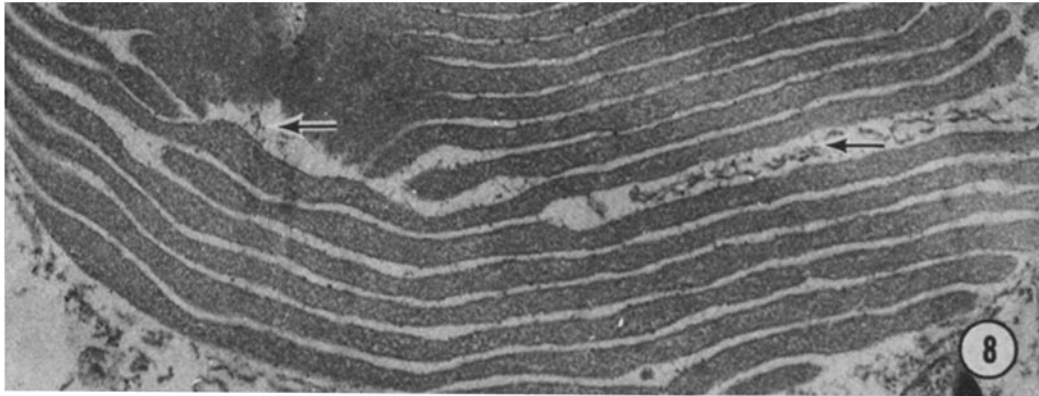
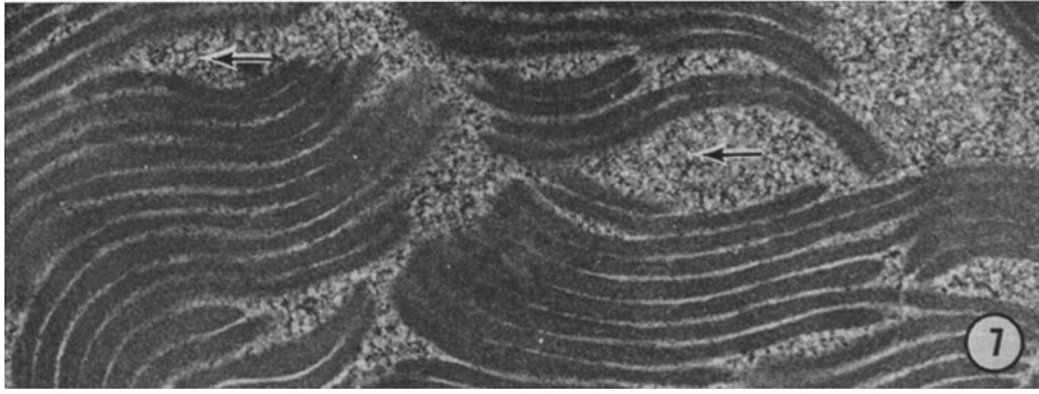


FIGURE 5 This section from *Cryptomonas ovata* also displays the tendency for two lamellae to be aligned parallel with one another. Although the lamellae lumina are less extensive, they also contain the electron-opaque matrix characteristic of this group of algae.  $\times 97,000$ .

FIGURE 6 After extraction with methanol-acetone the chloroplast lamellae of *Chroomonas* are not visible. The electron-opaque matrix is still present. Due to an over-all shrinkage the chloroplast components are more compact. *St*, stroma.  $\times 97,000$ .



FIGURES 7-10. See p. 290 for legends.

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FIGURES 7-10 Sections of *Rhodomonas* chloroplasts from cells exposed to pronase and subsequently to dilute acid at pH 3.0 to stop digestion. Stromal regions marked with arrowheads. Figs. 7-10,  $\times 61,000$ .

FIGURE 7 Pronase treatment at 0 min. The chloroplast components are unchanged. To be compared with Fig. 4 of a control cell.

FIGURE 8 Pronase treatment at 5 min. Note that most of the stromal material has been extracted as has the cytoplasm in the lower part of the figure.

FIGURE 9 Pronase treatment at 60 min. At this point the electron-opaque intrathylakoidal matrix shows considerable extraction, concomitant with an increase of phycocerythrin in the supernatant.

FIGURE 10 Pronase treatment at 120 min. The biliproteins have been extracted at this point, and the intrathylakoidal matrix has disappeared. Some membranes have connected with neighboring ones, or have rounded up to form vesicles of various sizes. The darkly staining droplet-like areas were formed during the later stages of pronase extraction and are methanol-acetone soluble.