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A GREEN DINOFLAGELLATE WITH CHLOROPHYLLS *A* AND *B*: MORPHOLOGY, FINE STRUCTURE OF THE CHLOROPLAST AND CHLOROPHYLL COMPOSITION[1]

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ABSTRACT

A green-colored marine unicell has been grown in unialgal culture and its morphology, chloroplast fine structure, and chlorophyll composition investigated. The organism is typical of dinoflagellates in its shape, flagellation, nucleus, mitochondria, and trichocysts. It is similar to Gymnodinium *but possesses fine body scales. Chloroplasts and two kinds of vesicles bounded by double membranes, but no organelles obviously identifiable as nuclei or mitochondria, are associated in ribosome-dense cytoplasm separated by a double membrane from the dinophycean cytoplasm. The chloroplasts are unlike any previously reported for dinoflagellates. Each is enclosed by an envelope consisting of a double membrane. Chloroplast lamellae consist of three appressed thylakoids. Interlamellar pyrenoids are present. Pigment analysis reveals chlorophylls* a *and* b *but not chlorophyll* c. *It seems likely that the organism is an undescribed dinoflagellate containing an endosymbiont with chlorophylls* a *and* b *and that the reduction of the endosymbiont nucleus and mitochondria has permitted a more initmate symbiosis.*

Key index words: chlorophylls a *and* b; *chloroplast; dinoflagellate; endosymbiont; fine structure; morphology*

Dinoflagellates are characterized by chlorophylls *a* and *c*, β-carotene and several xanthophylls, especially peridinin, which gives most organisms containing these pigments a brownish coloration. Relatively few dinoflagellates, however, have been described as being red, green, or blue-green. Recent reports of cryptomonad symbionts in blue-green gymnodinioids (Hu et al. 1980, Wilcox and Wedemayer 1984) focus attention on atypically colored dinoflagellates.

We have isolated and cultured a green dinoflagellate from a seawater sample collected on the northern coast of Japan. Research on the morphology and pigmentation of this dinoflagellate has been carried out in order to answer questions regarding its taxonomy and phylogeny. In the present paper we report our initial studies on the morphology, chloroplast fine structure, and chlorophyll composition of this organism.

MATERIALS AND METHODS

A unialgal clone (Y-100) of the green dinoflagellate was obtained from a seawater sample collected on August 23, 1985 from a depth of 0–20 m off Miyako (40°40′ N 141°55′ E), Iwate Prefecture, northern coast of Japan during a cruise of the Tansei Maru (KT 85-12) organized by the Laboratory of Oceanography, Faculty of Agriculture, Tohoku University.

The strain was maintained in a screw-capped test tube (18 × 150 mm) with 10 mL of ESM medium (cf. Watanabe and Kasai 1985) under illumination of ca. 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ with a photoperiod of 12:12 h LD from the daylight fluorescent lamps at 20 ± 1° C. These light and temperature conditions were used throughout the present studies.

For critical observation of the living cells under the light microscope, a drop of 20 mM $NiCl_2$ was added to 0.1 mL of the medium containing actively swimming cells on a glass slide just before observations were made. $NiCl_2$ inhibited flagellar movement without causing damage to the cell structure.

For transmission electron microscopy, cells were grown in 200 mL Erlenmeyer flasks with 100 mL of medium. Harvested cells were fixed for 1 h with 2.5% glutaraldehyde solution containing 0.2 M sodium cacodylate buffer (pH 7.2) and 0.2 M sorbitol, and post-fixed with 2% OsO_4 for 1 h. Specimens were then dehydrated through a 30–100% ethanol series and embedded in Spurr's resin. Serial sections were cut with a diamond knife on a Porter-Blum

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Mt-1 Ultramicrotome, stained with 2% uranyl acetate and lead citrate (Reynolds 1963), and examined with a JEOL JEM-100 CX Electron Microscope.

For chlorophyll analysis, cells were grown in two sets of 2 L Erlenmeyer flasks with 1 L of medium for about one month. The cells were harvested and washed with a 3% NaCl solution twice by centrifugation, followed by repeated extraction with acetone. The combined acetone solution was evaporated under reduced pressure at 25° C to dryness. When a small amount of water remained, the evaporation procedure was repeated after adding acetone. The dried residue was dissolved in acetone and used for pigment analysis. The ratio of chlorophyll *a* to chlorophyll *b* was determined spectrophotometrically in 80% acetone (Arnon 1949) and ether solutions (Smith and Benitez 1955). The same procedures were applied to spinach leaves to prepare chlorophyll *a* and *b* standards.

Paper chromatography was performed with a solvent system of toluene-ethanol (200:1) according to Chiba and Noguchi (1954). Absorption spectra were recorded on a Model 228 spectrophotometer (Hitachi, Tokyo).

Fluroescence measurements were made at 20° C with a Hitachi MPF-4 fluorospectrophotometer, the slit width being fixed at 10 nm for both emission and excitation.

RESULTS

Light Microscopy (Figs. 1–6)

Cells of strain Y-100, which are most commonly 20–30 μm diam., are unarmored, with no indication of thecal plates. They are green, subglobular, and separated by a cingulum into a hypocone and a slightly smaller epicone (Fig. 1). The two ends of the cingulum, which encircles the cell and houses the transverse flagellum, are offset vertically by the width of the cingulum. The sulcus, which houses the longitudinal flagellum, extends from the antapex to the apex, becoming very narrow in the epicone to form the apical groove (Figs. 2, 3). A short peduncle-like projection is situated between the sites of emergence of the two flagella (Figs. 4, 5). A large nucleus is located centrally. Variable numbers of irregularly shaped, greenish chloroplasts are located peripherally. These morphological characteristics are diagrammed in Figure 6.

Electron Microscopy (Figs. 7–12)

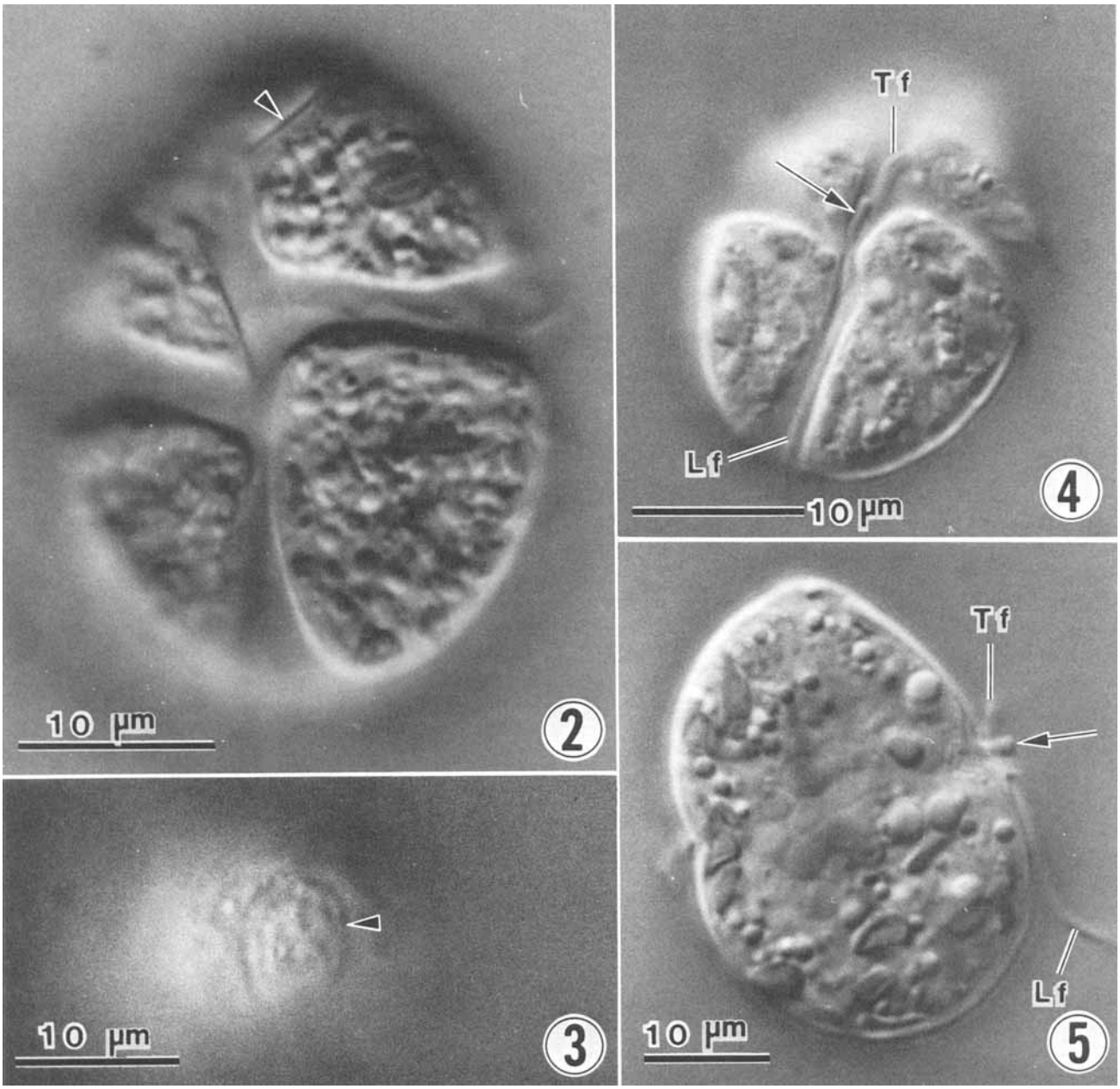
The fine structure of most of the organelles of strain Y-100 is characteristic of dinoflagellates. The nucleus is large, centrally located, and contains numerous long, condensed chromosomes dispersed in ribosome-filled nucleoplasm (Fig. 7). The nuclear envelope is pierced by numerous pores. Mitochondria, which are randomly distributed and variably shaped, possess tubular cristae and are surrounded by a double membrane (Figs. 7, 8). Starch granules only occur in the dinoflagellate cytoplasm (Fig. 8). Trichocysts and mucocysts are located peripherally and fine scales cover the cell surface (Fig. 7).

The fine structure of the chloroplast, however, is quite different from that of dinoflagellate chloroplasts. Several chloroplasts appressed to one another are enclosed by a double membrane (Figs. 8, 9). Cytoplasm associated with the chloroplasts within the double membrane possesses numerous ribosome-like particles (Figs. 8, 10) and two kinds of vesicles bounded by double membranes. One kind of vesicle has amorphous, electron-dense contents and pores in the enveloping membranes (Fig. 10); the other has neither electron-dense contents nor pores (Fig. 9). Serial sectioning shows both kinds of vesicles to be subglobular. Although it appears from a single section that several units consisting of chloroplasts, cytoplasm, and vesicles are present (Fig. 7), serial sectioning suggests that there is only one ramifying or reticulate structure per cell (not shown). Each chloroplast is enclosed by an envelope consisting of a double membrane (Fig. 9). An interlamellar pyrenoid bounded by a single membrane and having a paracrystalline matrix (Figs. 11, 12) was found in almost all chloroplasts sectioned (Figs. 7, 8). Chloroplast lamellae consist of three appressed thylakoids (Fig. 12) and lie more or less parallel to one another and to the long axis of the chloroplast. A girdle lamella is not present.

FIG. 1. A green dinoflagellate, strain Y-100, isolated from the northern coast of Japan.

Pigment Analysis (Figs. 13, 14)

Paper chromatography of the acetone extract showed one blue-green spot and one green spot, the Rf values of which (0.45 and 0.28 respectively) coincided exactly with those of chlorophyll *a* and *b*



FIGS. 2–5. Light micrographs of strain Y-100. FIG. 2. Ventral view of cell. The ends of the cingulum are offset by the width of the cingulum and the sulcus becomes very narrow in the epicone to form the apical groove (arrowhead). FIG. 3. Apical view of cell, showing apical groove (arrowhead) encircling the greater part of the apex. FIGS. 4, 5. Oblique and lateral views of cell. The peduncle-like projection (arrows) is located at the sites of emergence of the longitudinal flagellum (Lf) and transverse flagellum(Tf).

standards prepared from spinach leaves (Fig. 13). The absorption spectra prepared from the acetone extract of the cells and that of spinach leaves are shown in Figure 14. A shoulder at around 645 nm is much more obvious in the cell extract than in the spinach leaf extract, a result suggesting that the chlorophyll a/b ratios are much lower in the cells than in spinach. Chlorophyll a/b ratios determined spectrophotometrically from cells from cultures of various ages ranged from 0.8 to 1.4.

An attempt to confirm the chlorophyll composition was made by separating and purifying the pigment according to the method of Omata and Murata (1980) as modified by Araki et al. (1984). The absorption spectra of the chlorophyll *a* and *b* fractions obtained from the cells were identical to those obtained from spinach with respect to the position of the two peaks for each chlorophyll and the absorption ratio of the two peaks. For chlorophyll *a* there were peaks at 430 nm and 663 nm with the absorption ratio of 430 nm to 663 nm being 1.24. For chlorophyll *b* there were peaks at 455 nm and 645

nm with the absorption ratio of 455 nm to 645 nm being 2.84. In a quantitative purification procedure starting from 1 g fresh weight of cells, 62 nmol of chlorophyll *a* and 76 nmol of chlorophyll *b* (*a*/*b* ratio = 0.8) were prepared.

In the course of separation and purification of the chlorophylls, an unesterified chlorophyll fraction was obtained and it was necessary to determine whether this was chlorophyll *c* or chlorophyllides *a* and *b*. Since there was not enough of the substance for spectrophotometric measurement, fluorescence analysis was applied to the fraction. Two components with excitation-emission wavelength maxima at 430–670 nm and 450–650 nm were detected. This pattern corresponds with the excitation-emission maxima of chlorophyllides *a* and *b*, respectively (French et al. 1956). These chlorophyllides are presumably formed from chlorophylls *a* and *b* by a chlorophyllase reaction during preparation and/or cultivation. Excitation and emission maxima at 450–453 nm and 633–635 nm, which are characteristic of chlorophyll *c*, could not be detected.

In a preliminary analysis of carotenoids in strain Y-100 it was determined that beta carotene predominated. Neither peridinin nor fucoxanthin was detected.

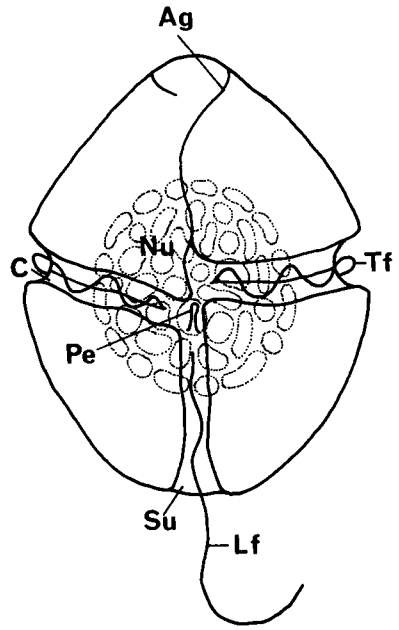


FIG. 6. Diagrammatic illustration of strain Y-100: apical groove (Ag), cingulum (C), peduncle-like projection (Pe), longitudinal flagellum (Lf), nucleus (Nu), sulcus (Su), and transverse flagellum (Tf).

DISCUSSION

It is apparent from this study that Y-100 is a colorless dinoflagellate that contains a pigmented endosymbiotic alga, or more properly, the phylogenetic vestiges of such an alga. Although dinoflagellates are known to host a variety of phylogenetically vestigial endosymbionts containing chlorophyll *a* and *c* (Dodge 1971a, Tomas and Cox 1973, Jeffrey and Vesk 1976, Wilcox and Wedemayer 1984), this report provides the first evidence for a phylogenetically vestigial endosymbiont containing both chlorophylls *a* and *b*. It should be noted that *Noctiluca miliaris* is known to host the endosymbiotic prasinophyte *Pedinomonas noctilucae* (Sweeney 1976), but the endosymbiont in that relationship is unreduced, swimming actively in the vacuole of the host.

Strain Y-100 is superficially similar to *Gymnodinium* but possesses body scales. This characteristic, which is considered highly valuable in dinoflagellate taxonomy (Morrill and Loeblich 1981), has never been found in *Gymnodinium*. A taxonomic treatment of strain Y-100 will be published in a subsequent paper.

Although the ultrastructure of strain Y-100 is for the most part characteristic of dinoflagellates, the ultrastructure of the vestigial endosymbiont is unlike anything that has been found in dinoflagellates heretofore. In other endosymbioses a single membrane separates the dinoflagellate cytoplasm from that of the endosymbiont (Dodge 1971a, Tomas and Cox 1973, Jeffrey and Vesk 1976, Wilcox and Wedemayer 1984), while in strain Y-100 the membrane is double. Tomas and Cox (1973) speculated that the single membrane facilitates a mutual interchange between the host and endosymbiont, but strain Y-100 demonstrates that reduction of the membrane from double to single is not a requirement of endosymbioses. The presence of starch in the dinoflagellate cytoplasm implies that metabolite exchange is occurring.

In previously studied endosymbioses both a nucleus and mitochondria are found in the cytoplasm of the phylogenetically vestigial endosymbiont. In the endosymbiont of strain Y-100 neither nucleus nor mitochondrion occurs, although it is possible that the subglobular, double membrane bounded vesicles represent vestiges of these organelles. The fact that one kind of vesicle has pores and electron dense contents is consistent with its being considered a vestigial nucleus. The fact that several such pored vesicles are present in each endosymbiont is less consistent. A definitive conclusion must await analysis of the contents.

The fine structure of the chloroplast shows dissimilarities with that of chloroplasts of other phylogenetically vestigial endosymbionts. The chloroplast envelope is a double membrane, as in green algae, whereas it is composed of two double membranes in all other known vestigial endosymbionts. Moreover, the combination of the parallel type of lamellar arrangement (Dodge 1971b, 1975) and the simple interlamellar pyrenoid has been found only in Prymnesiophyceae (Dodge 1973, 1979), a group in which chlorophyll *b* is absent. The presence of chlorophyll *b* in strain Y-100 was established by pa-

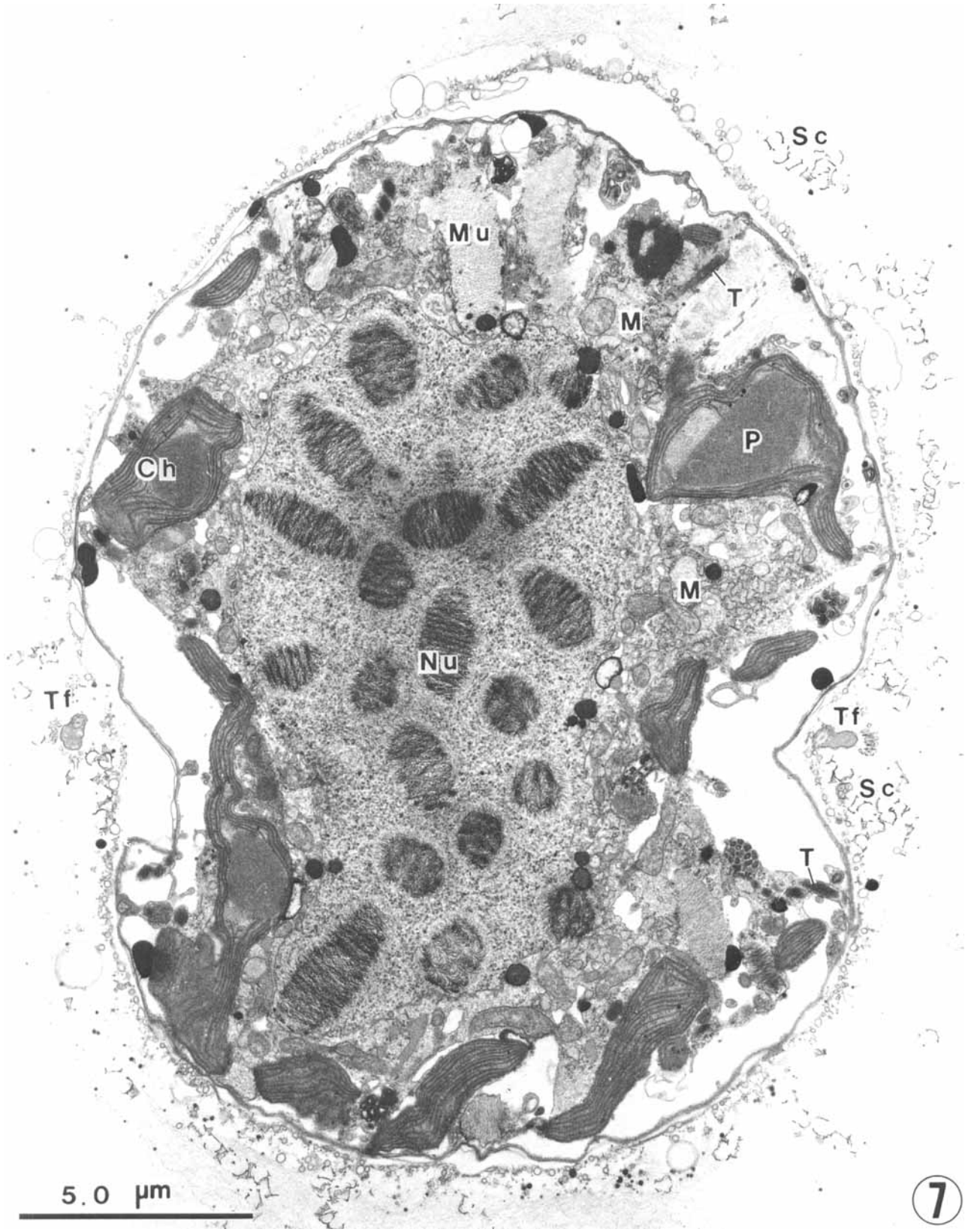
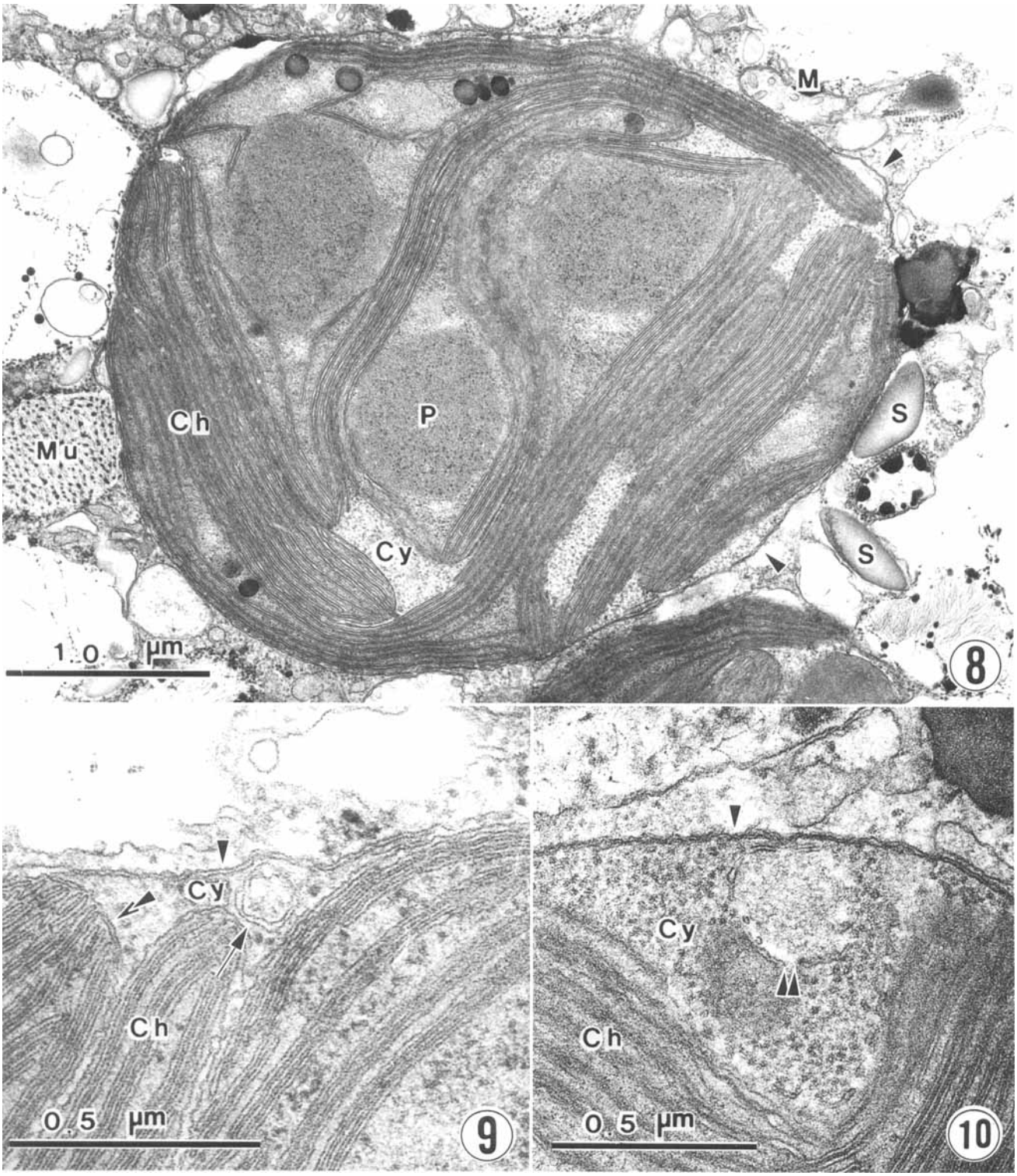
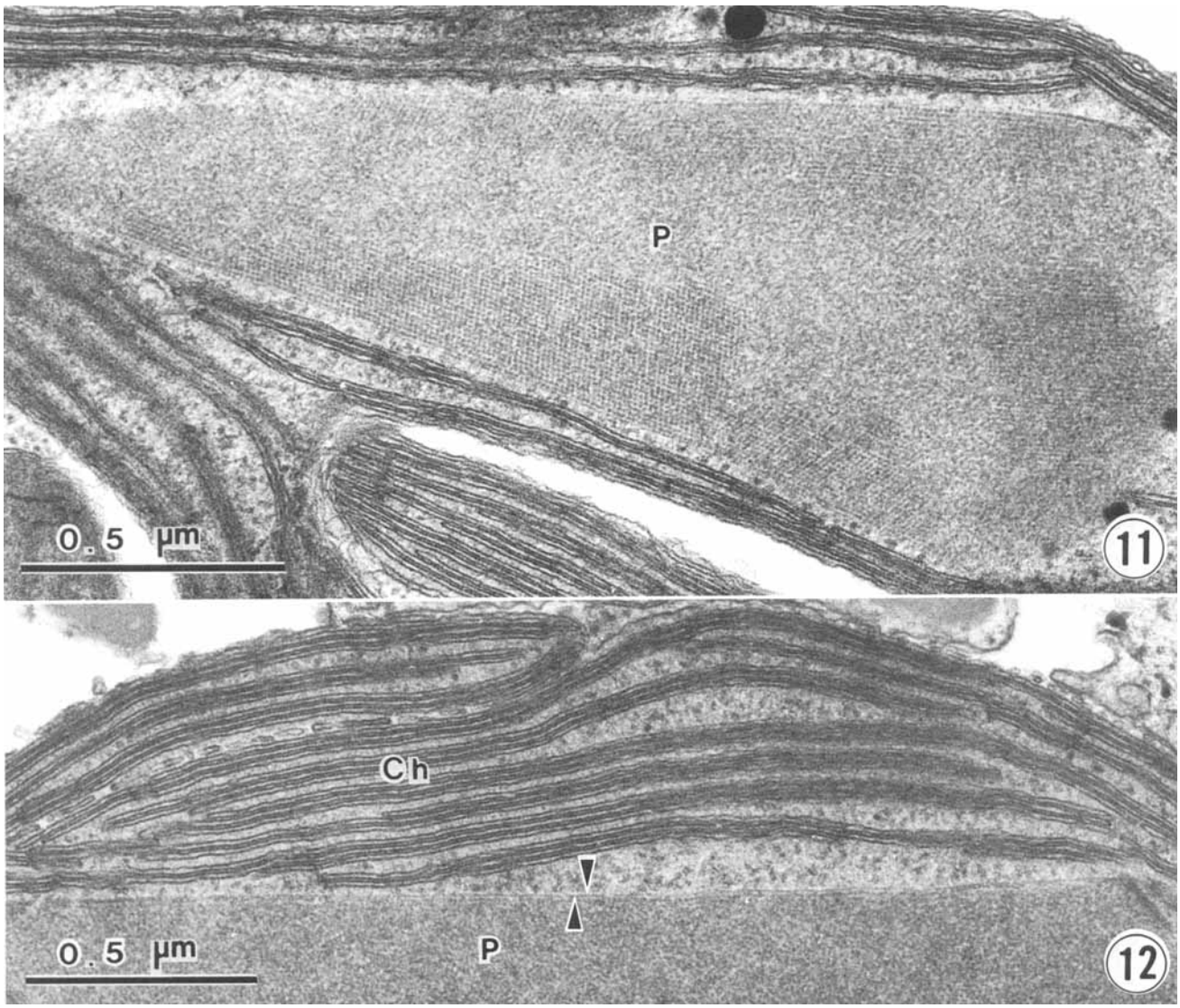


FIG. 7. Transmission electron micrograph of strain Y-100. Longitudinal section of cell. Major cell components are seen: chloroplast (Ch), mitochondria (M), mucocyst (Mu), nucleus (Nu), pyrenoid (P), body scales (Sc), transverse flagellum (Tf), and trichocyst (T).



FIGS. 8–10. Transmission electron micrographs of strain Y-100. FIG. 8. Several chloroplasts (Ch) enclosed by a common double membrane (arrowhead). Associated cytoplasm (Cy) and pyrenoids (P) are also found within the double membrane Mucocyst (Mu), mitochondria (M) and starch granules (S) are seen in dinophycean cytoplasm. FIG. 9. Double membrane (arrowhead) separating chloroplast, associated cytoplasm (Cy), and vesicle from dinophycean cytoplasm, and double membrane envelope of chloroplast (double arrowhead). The vesicle is enclosed by a double membrane without pore (arrow). FIG. 10. Vesicle enclosed by a double membrane with numerous pores (double arrowhead), involving electron-dense materials. The ribosome-rich cytoplasm (Cy) and chloroplast (Ch) are seen. The arrowhead shows the double membrane separating these two structures from the dinophycean cytoplasm.



FIGS. 11, 12. Transmission electron micrographs of strain Y-100. FIG. 11. Pyrenoid (P), showing latticed, paracrystalline structure. FIG. 12. Chloroplast (Ch) lamellae, composed of three appressed thylakoids and a single membrane (arrowheads) enclosing pyrenoid (P).

per chromatography, absorption spectroscopy, and direct purification. The ratios of chlorophyll *a* to chlorophyll *b* that were obtained are much lower than those in higher plants and freshwater green algae, which are usually approximately 3 (Boardman and Anderson 1964, Halldal 1970, Porra and Grimme 1974, Rabinowitch 1945). Similarly low ratios have been calculated, however, for more than ten species of marine planktonic Prasinophyceae and Chlorophyceae (Wood 1979).

We are unable without further analysis of carotenoids and additional detailed ultrastructural investigation to draw definitive conclusions as to the phylogenetic affinities of the endosymbiont. The entire chromophyte line, in which chlorophyll *b* is absent, can be ruled out by the pigment composition. Of the chlorophyll *b*-containing algae—Chlorophyceae, Prasinophyceae, and Euglenophyceae—a prasinophyte would seem to be the likeliest candidate for a progenitor. To our knowledge, however, no free-living organisms with chlorophylls *a* and *b* are known to have such a simple chloroplast structure.

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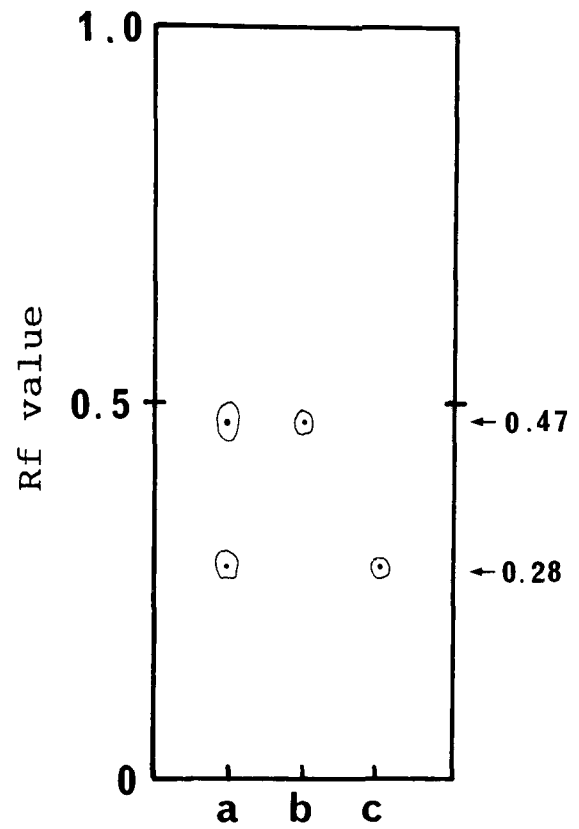

FIG. 13. Paper chromatogram of acetone extract of strain Y-100 (a), standard chlorophyll *a* (b); standard chlorophyll *b* (c). The two spots obtained from strain Y-100 coincide with those of the standards.

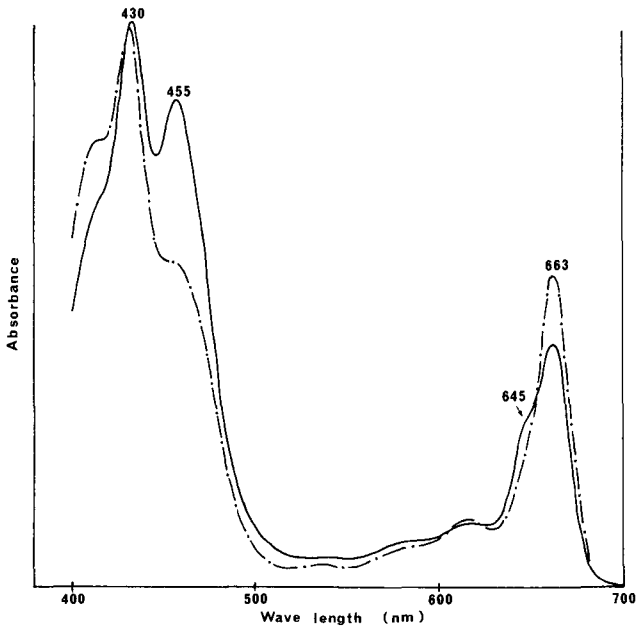

FIG. 14. Absorption spectra of acetone extracts of strain Y-100 (solid line) and spinach leaves (dotted line). A shoulder at 645 nm is clearly seen in strain Y-100, indicating the presence of chlorophyll *b*.

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