STUDIES WITH CYANIDIUM CALDARIUM

I. The Fine Structure and Systematic Position of the Organism

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ABSTRACT

The fine structure of *Cyanidium caldarium*, as seen in thin sections of $KMnO_4$-fixed cells examined with the electron microscope, is described. This organism, whose taxonomic position among algae is undetermined, contains a single well defined chloroplast, a nucleus, and mitochondria. Studies, with the electron microscope, of *Chlorella pyrenoidosa* and *Nostoc* are also reported. Structural differences within cells of *Cyanidium*, *chlorella*, and *Nostoc* are discussed. It is concluded that if *Nostoc* can be taken as a *typical* Cyanophyte and *Chlorella* as a representative Chlorophyte and if the items of fine structure examined are diagnostic, then *Cyanidium* is certainly not a Cyanophyte and, while it has numerous features in common with *Chlorella*, is not a green alga similar to *Chlorella*. Comparisons are also made between *Cyanidium* and other algae whose fine structure has been described by others.

The systematic position of *Cyanidium caldarium* has been questioned for many years. It has been classified variously as a blue-green alga, a green alga, a red alga, a coccoid cryptomonad, or a symbiotic association between a blue-green alga and a colorless chlorophyte (1–6).

The purpose of the present report is to compare the fine structure of the normal wild-type strain with that of *Chlorella* and *Nostoc*, as determined by electron microscopy, and to provide information which may be useful in establishing the systematic position of this organism. A brief description of some aspects of the fine structure of *C. caldarium* was published recently by Rosen and Siegesmund (6).

All descriptions refer to wild-type cells of a strain kindly provided by M. B. Allen of the Kaiser Foundation, and a mutant III-D-2 which resembles the wild type but contains more chlorophyll and phycocyanin, some of which can be formed in darkness (7). The cells were grown in a liquid medium (5) with 1 per cent glucose at 43°C ± 2°C under fluorescent illumination of 150 to 500 ft-c. Growth was vigorous, and the cells were harvested after 4 or 5 days. For fixation the cells were centrifuged, and the pellet was resuspended in 2 per cent buffered $KMnO_4$ fixative pH 7.2 (veronal acetate, calcium and magnesium chloride 0.001 M, respectively). After 30 minutes the cells were washed briefly in water before being dehydrated in an ethanol series: 40, 70, 100 per cent. The cells were then embedded in methacrylate (75 per cent butyl, 25 per cent methyl, 0.05 per cent benzol peroxide, polymerized at 70°C). Sections were prepared with a Porter-Blum microtome using a diamond knife,

and examined in a Siemens Elmiskop I at 80 kv. Electron micrographs were taken at 10,000 to 40,000 magnifications. Osmium tetroxide fixation was attempted but was unsuccessful; most cells failed to embed after treatment with osmium tetroxide.

Phase contrast examination of the living material established the growth stages of *Cyanidium*. The cells are spherical, 3 to 5 μ in diameter, but little detail of internal structure can be detected. There is a single lobed chloroplast, which appears to lack a pyrenoid, filling much of the cell. An ill defined region with areas of different refractive densities occupy the rest of the cell, but a definite nucleus and other organelles cannot be distinguished.

Multiplication occurs by endospore formation. First, the chloroplast becomes vague. Then, four daughter cells appear within the mother cell; they are released after the mother cell wall ruptures. This description of the growth stages and cell structure corresponds with those reported by previous authors.

Several electron micrographs of sections of adult cells are shown in Figs. 1 to 5. In contrast to its appearance under the light microscope, the cell appears highly differentiated: a cell wall plasmalemma, ground matrix, endoplasmic reticulum, chloroplast, mitochondria, nucleus, and vacuoles may be identified.

The cell wall appears as a homogeneous dense layer, 0.1 to 0.05 μ in thickness in $KMnO_4$-fixed cells (Figs. 1 to 5); it is of low density in OsO_4. No evidence of stratification or layering has been observed, and it is not clear whether the wall has the fibrillar structure characteristic of plant cell walls in general.

The protoplast is bounded externally by an indistinct plasma-membrane approximately 100 A in thickness, which appears as a double structure of two dense lines separated by a light intermediate layer in favorable sections (Fig. 5). Although the membrane follows the general curvature of the protoplast, it is not always in close contact with the cell wall. It is frequently conspicuously indented and convoluted; the resulting space between the cell wall and the membrane contains a material of low density when fixed with $KMnO_4$.

Various organelles, vacuoles, and membranes are embedded in a granular ground cytoplasm of medium density which is not obviously different in texture from the matrix between the chloroplast lamellae or the mitochondrial matrix, as is to be expected in $KMnO_4$-fixed tissue.

Some of the organelles are obviously mitochondria. They have the typical mitochondrial structure consisting of a double membrane with cristae arising from the inner membrane, and contain a matrix of light density between the cristae (Fig. 4). From the shapes of the profiles, it appears that rods and filaments (0.2 μ in diameter $\times$ 0.4 to 1.5 μ in length) are the common forms of the mitochondria in *Cyanidium*. The cristae are not numerous. They are irregularly spaced and probably occur as infrequently branching tubes; circular and tubular profiles, occasionally forked, are common. No pattern of distribution of the mitochondria within the protoplast has been found. They appear to occur at random. As endospore formation commences the mitochondria become more elongate, up to 2 μ in length, the cristae become indistinct and the matrix much denser. Some of the dense rod-shaped bodies become too indefinite to be identified with certainty as mitochondria (Fig. 3).

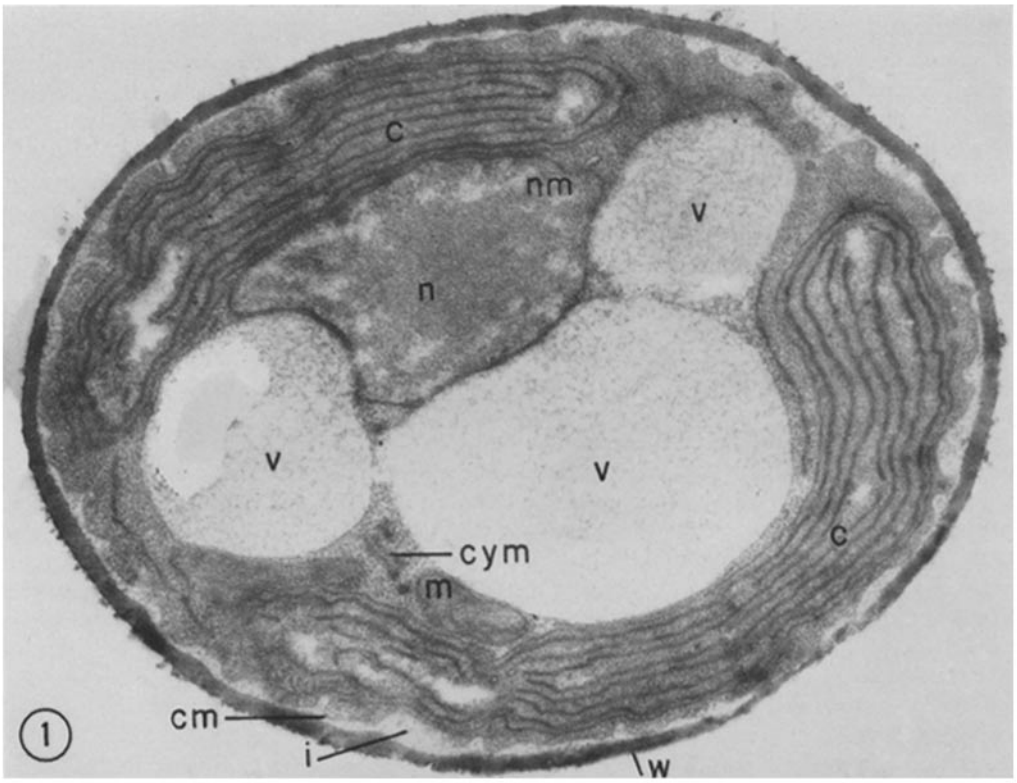
The mature cell contains a single, much lobed chloroplast which occupies a large part of the

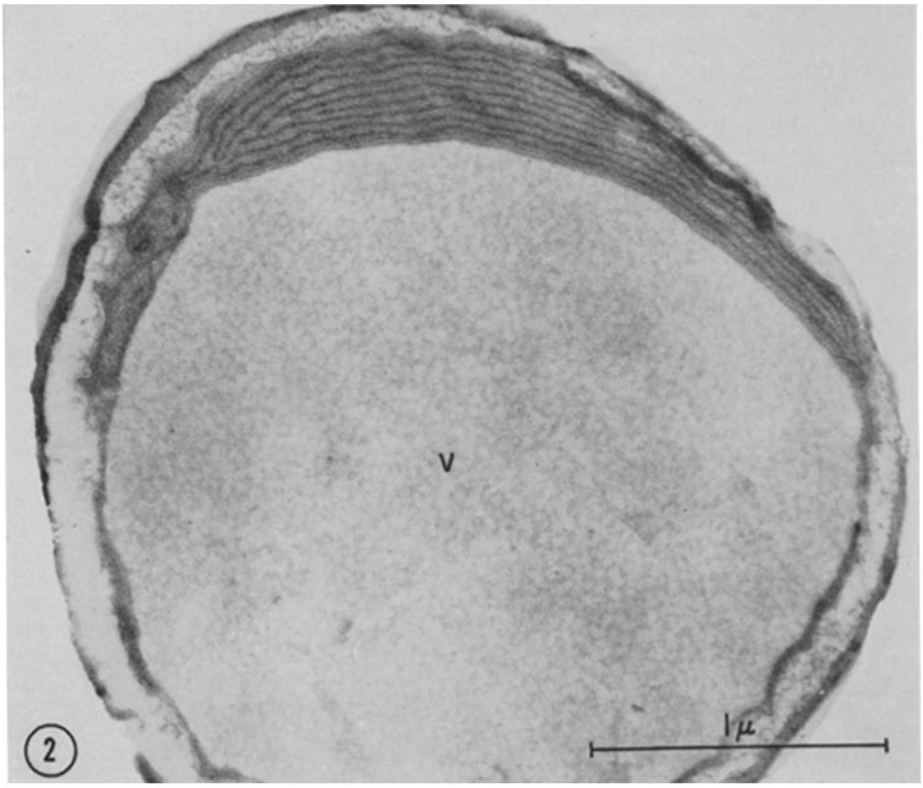
FIGURE 1

Section of adult cell of *Cyanidium caldarium* showing the dense cell wall (*w*), several chloroplast profiles (*c*) with bands of lamellae, enclosed by a membrane, the nucleus (*n*), and nuclear membrane (*nm*), vacuoles (*v*), mitochondria (*m*), cytoplasmic membranes (*cym*), and the cell membrane (*cm*) with numerous invaginations (*i*). $\times$ 40,000.

FIGURE 2

Old cell of *Cyanidium* in section. Most of cell is occupied by a large vacuole (*v*) and the remainder of the protoplast forms a thin lining to the cell. $\times$ 40,000.


c
nm
v
n
v
v
cym
m
c
cm
i
w
1


v
1μ
2

volume of the cell. Frequently several distinct profiles are seen in a section (Fig. 1). Serial sections show these to be part of the multi-lobed chloroplast, and not separate chloroplasts. The shape and number of profiles is determined by the plane of the section through the cell. In the mature cell the chloroplast is enclosed by a membrane, possibly several membranes, (Fig. 1), consisting of two dense parallel layers, *ca.* 30 A in thickness separated by a space *ca.* 25 A in thickness. But the chloroplast membrane is not always distinguishable from the outermost lamellae and the membranes in the cytoplasm (Fig. 5). The chloroplast lacks a pyrenoid but contains from several to about twelve dense bands embedded in a matrix of granular material. It is assumed, for comparison with *Chlorella* and *Nostoc*, that a lamella, as seen in the electron micrographs, consists of a single dense line *ca.* 25 to 30 A wide, a light space also *ca.* 25 to 30 A wide, and another dense line *ca.* 25 to 30 A wide; the over-all width is about 75 to 90 A. On this basis each band in *Cyanidium* contains usually two, or occasionally three, lamellae. Starch grains have not been identified, nor have localized distortions of the lamellae which are associated with starch grains in other species of chloroplast been detected.

In cells approaching endospore formation the chloroplast disintegrates, but only the general pattern of breakdown is known. The chloroplast membrane along with much of the lamellar system breaks down, and many of the lamellar membranes become dispersed in the cytoplasmic matrix. How the chloroplast becomes organized after the formation of the endospores has not been determined (Fig. 3).

A well defined nucleus is present. It is usually most conspicuous in cells forming endospores and is most commonly located in the concavity of the chloroplast. The nucleus is variable in shape, usually much lobed, and up to 0.5 × 2.0 μ in size. The nuclear membrane is double, and the outer member of the double membrane is continuous with membranes in the cytoplasm (Fig. 1; Fig. 3).

Cytoplasmic membranes are common both in the cytoplasm in the interior of the cell and in the cytoplasmic layer between the cell wall and the chloroplast. The membranes are usually paired but do not appear to form conspicuous closed vesicles or cisternae. Presumably these membranes correspond to the endoplasmic reticulum of other cells. Golgi bodies have not been identified, although occasionally clusters of membranes which may correspond to Golgi bodies are present (Fig. 1; Fig. 5).

Vacuoles are invariably present. In mature cells their number varies from one to three or four (Fig. 1). In old cells, probably those no longer capable of undergoing endospore formation, the smaller vacuoles have fused to form a large vacuole which fills most of the volume of the cell (Fig. 2). Also, as the cells age the contents of the vacuole change from a granular material to a

FIGURE 3

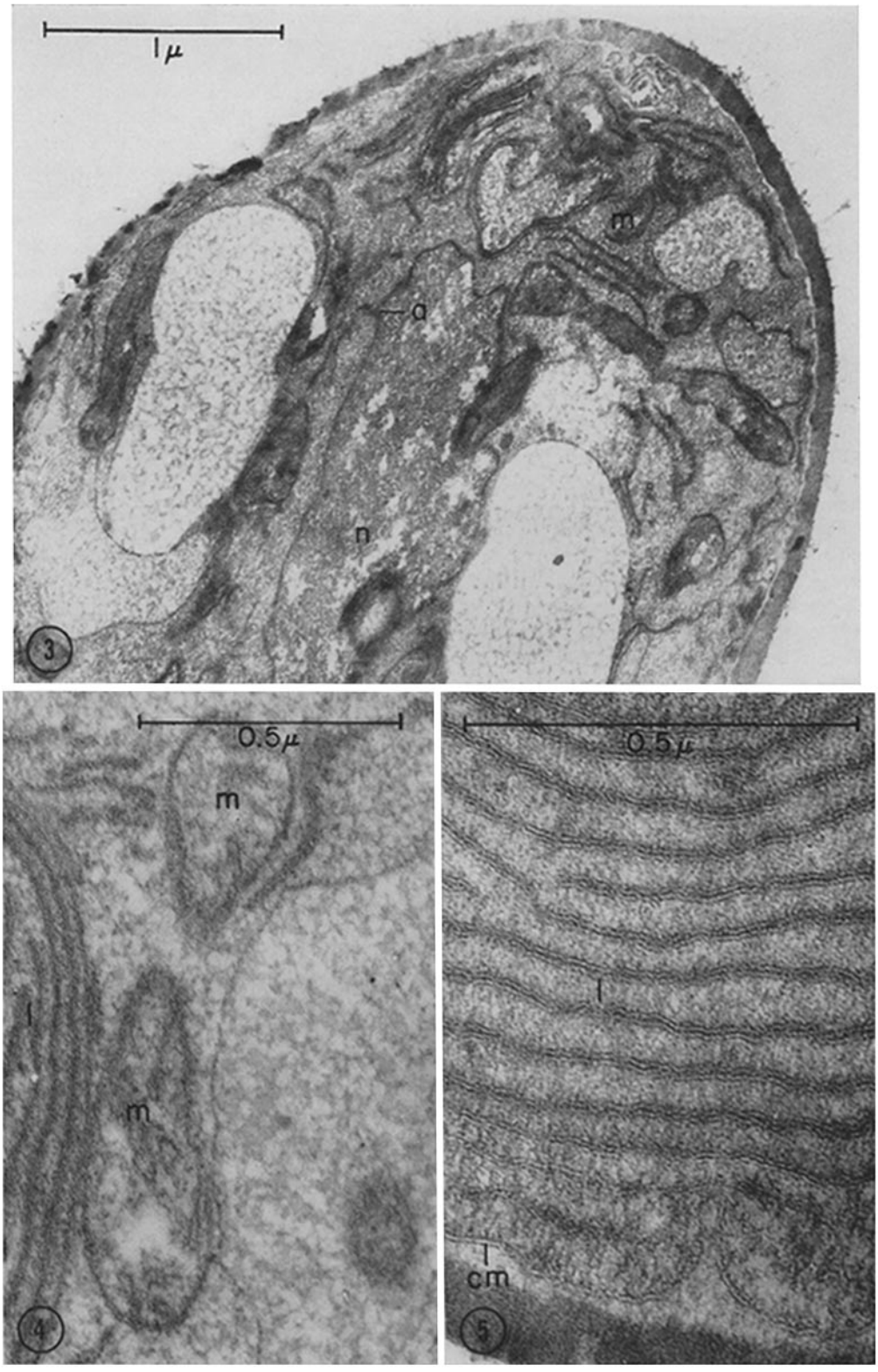
A section of a *Cyanidium* cell mutant III D-2 at endospore formation. The chloroplast has broken down and numerous cytoplasmic membranes are dispersed in the cytoplasm. Some of these may be derived from the chloroplast. Numerous bodies, some mitochondria (*m*), but others unidentified, are present. The lobed nucleus (*n*) is elongated and at (*a*) the nuclear membrane is continuous with a cytoplasmic membrane. × 36,000.

FIGURE 4

Portion of *Cyanidium* protoplast showing mitochondria (*m*) and chloroplast lamellae (*l*). × 80,000.

FIGURE 5

Median section of portion of chloroplast with bands of lamellae (*l*). The bands consist of two lamellae, *i.e.* one disc. The chloroplast membrane cannot be distinguished from cytoplasmic membranes, but the cell membrane (*cm*) is seen as two dense layers enclosing a light space. × 120,000.


1μ
m
a
n
3
0.5μ
m
l
m
4
0.5μ
l
l
cm
5

material having a reticular structure after $KMnO_4$ fixation (Fig. 2).

Chlorella

The basic structure of the adult cell of *C. pyrenoidosa* after $KMnO_4$ fixation is shown in Figs. 6 to 8. The cell is highly organized and a cell wall, plasmalemma, endoplasmic reticulum, Golgi bodies, nucleus, mitochondria, chloroplast, and pyrenoid may be identified.

The cell wall appears as a homogeneous layer, enclosed within two thin dense layers. There is an external cell membrane, the plasmalemma, which has a double structure of two dense members separated by a less dense zone. In some sections the inner member appears continuous with the cytoplasmic membranes. The plasmalemma closely follows the curvature of the wall.

Numerous organelles, circular, oval, and elongate in section are scattered through the cytoplasm. All are enclosed by a double membrane, and contain a matrix of medium density. A few, the more elongated ones, have well developed cristae and can be identified as mitochondria. The majority have a few poorly developed cristae, and, therefore, are probably mitochondria, while others consist of only an external membrane and matrix. Whether the latter are to be described as mitochondria is debatable, and may depend upon obtaining biochemical data about their function. If the presence of cristae is to be accepted as the diagnostic criterion of a mitochondrion, then such crista-less organelles should not be classified as mitochondria.

A single flask-shaped chloroplast occupies a large part of the cell. The chloroplast membrane is conspicuous; it is seen as two dense lines, each *ca.* 25 A in thickness, separated by a zone of low density, *ca.* 25 A wide. The lamellar system is highly differentiated, consisting of distinct bands of well orientated lamellae. The bands are separated by a homogeneous matrix or stroma of varying thickness. In suitable sections, a lamella is seen as a dense line *ca.* 25 to 30 A wide, a light space *ca.* 25 to 30 A wide, and a dense line *ca.* 25 to 30 A wide, but usually such detail is not resolved and the lamella appears as a single dense line *ca.* 75 to 90 A wide. Assuming that a single lamella corresponds to a 75 to 90 A line, the number of lamellae per band ranges from six (common) to fourteen. Owing to the close packing of the lamellae, adjacent lamellae are usually not resolved as two units, but appear as a dense single line *ca.* 150 to 180 A wide. Frequently at the edges of the bands adjacent lamellae are continuous to form closed structures similar to the disc-structure proposed by Sager and Palade (8) for the chloroplast of *Chlamydomonas*, and as elaborated for chloroplasts in general by Gibbs (9). If the disc-structure is real, and not an artifact due to the breaking and coalescing of lamellae during fixation, a band of, for example, six lamellae consists of three discs, and each band is made up of three to seven discs.

Little structure can be resolved in the pyrenoid

FIGURE 6

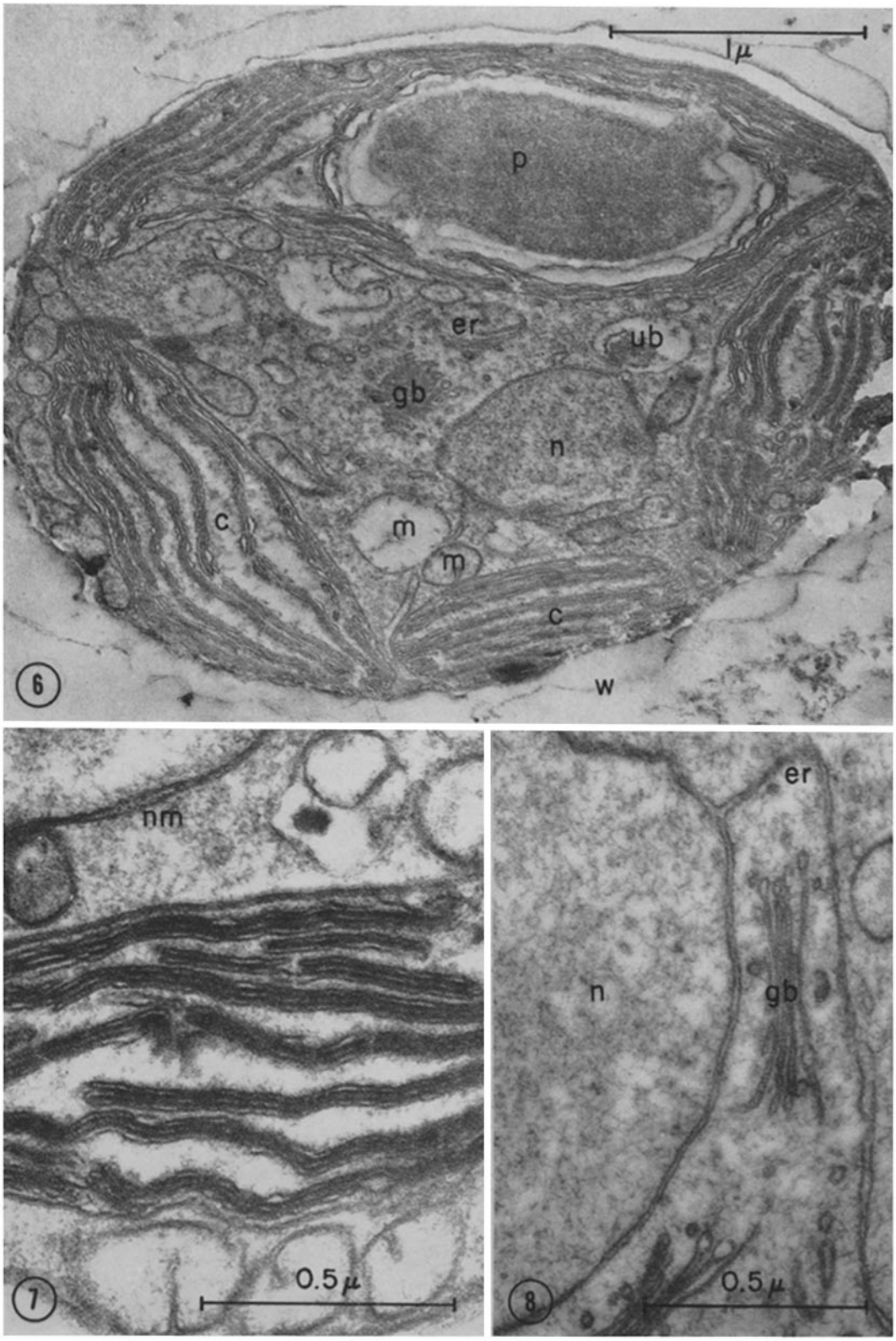
Mature cell of *Chlorella pyrenoidosa* in section showing cell wall (*w*) of low density, nucleus (*n*), mitochondria (*m*), unidentified cytoplasmic bodies (*ub*), Golgi bodies (*gb*), chloroplast (*c*) with pyrenoid (*p*) and enclosed by a conspicuous membrane, and endoplasmic reticulum (*er*). × 40,000.

FIGURE 7

Median section of portion of *Chlorella* chloroplast. The bands of lamellae contain three or four discs, and each disc appears as two dense lines separated by a region of low density. Nuclear membrane, *nm*. × 80,000.

FIGURE 8

Portion of nucleus of *Chlorella* (*n*) with double membrane which is continuous with the endoplasmic reticulum (*er*), and possibly with the Golgi bodies (*gb*). × 60,000.


1μ
p
er
ub
gb
n
c
m
m
c
w
6
nm
0.5μ
7
er
n
gb
0.5μ
8

of $KMnO_4$-fixed cells. It is surrounded by the lamellar system to which it is attached by extensions of material similar in appearance to the body of the pyrenoid, and appears to be enclosed by the membrane which encloses the starch sheath. Starch is present in the starch sheath between the body of the pyrenoid and the lamellae, and between the bands of lamellae.

Cytoplasmic membranes are conspicuous; they form an extensive endoplasmic reticulum with Golgi bodies. The reticulum is continuous with the nuclear membrane and possibly with the membranes of the Golgi bodies. Only the external member of the nuclear membrane is continuous with the endoplasmic reticulum (Fig. 8). The reticulum is not elaborate, and flattened vesicles are rarely observed. Usually it consists of only the double membranes which ramify through the cytoplasm (Fig. 8). An interesting feature of the Golgi body is the presence of small membrane-enclosed regions, circular ellipses in profile, which appear to be part of or formed from the Golgi body. The nucleus is conspicuous and is usually present in the cavity of the cup-shaped chloroplast. The nuclear membrane is double and has pores, but each dense member can be resolved into two thin dense lines separated by a less dense space, all *ca.* 25 to 30 A wide. Thus, the double membrane is seen as four dense lines *ca.* 25 to 30 A wide, two less dense lines *ca.* 25 to 30 A wide, and a less dense space of variable size up to 250 A wide, and is continuous with the membrane of the reticulum.

One or more vacuoles occur. The tonoplast is not conspicuous. Usually, the vacuole contains a material which appears granular after $KMnO_4$ fixation.

Nostoc

The structure of adult cells after $KMnO_4$ fixation is shown in Figs. 9 and 10. The cell is differentiated into two ill defined regions, a peripheral region and a central region. These probably correspond with the chromatoplasm and centroplasm regions as identified from light microscope studies.

A complex system of paired unit membranes is scattered throughout the peripheral zone. For comparison with the other species a unit membrane is assumed to be a lamella. In the electron micrographs each lamella (Fig. 10) is seen as a dense line, a less dense space, and another dense line, all *ca.* 25 to 30 A wide. Adjacent lamellae are frequently closely approximated; thus the two inner dense lines appear as a single unit *ca.* 50 to 60 A wide. A pair of lamellae may be considered to form a band.

A number of spherical-elliptical, membrane-enclosed spaces, possibly vacuoles, are a feature of the peripheral zone (Fig. 10).

The matrix of the peripheral zone is apparently continuous with that of the central region. There is no clear demarcation between the two zones. A number of dense bodies of granular texture and frequently of angular shape are embedded in the matrix of the central region.

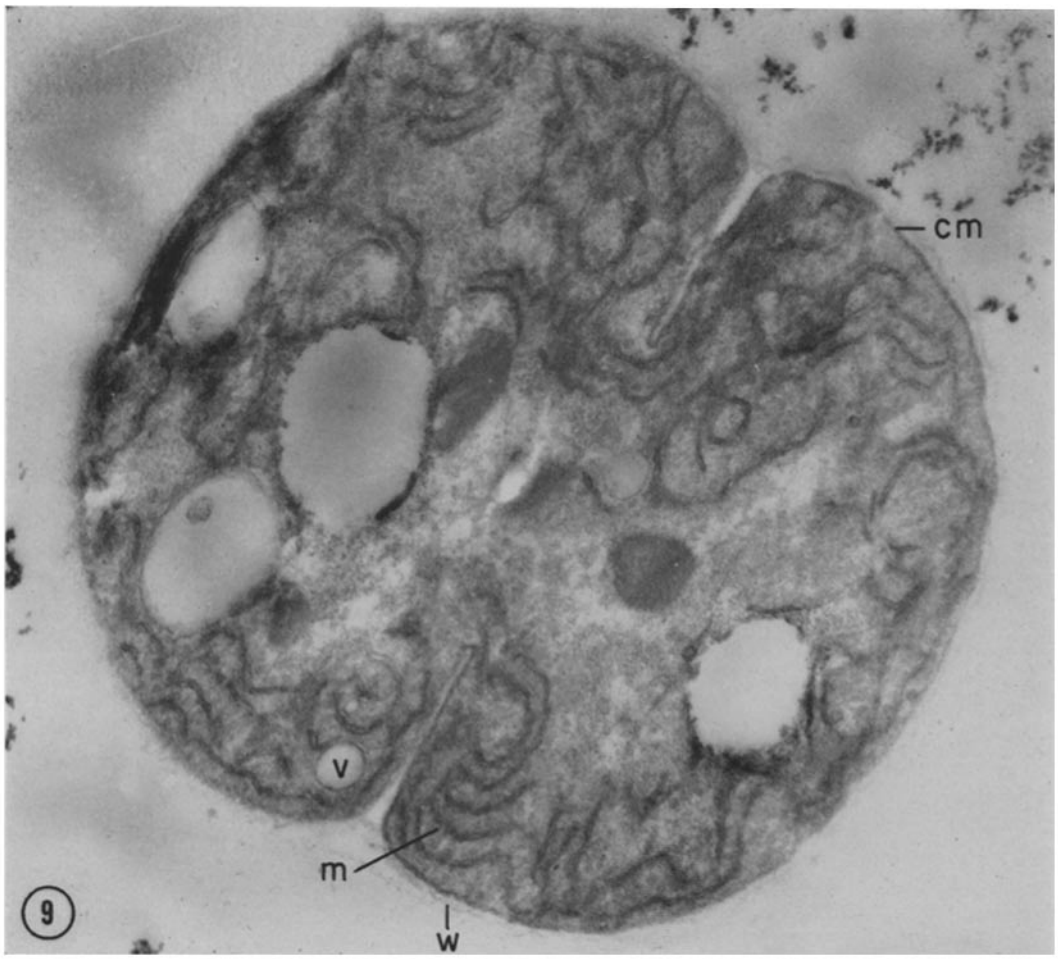
The cell wall is not conspicuous and apparently is not reactive with $KMnO_4$. It appears to consist of two layers, presumably corresponding to the inner investment and the cell sheath. These

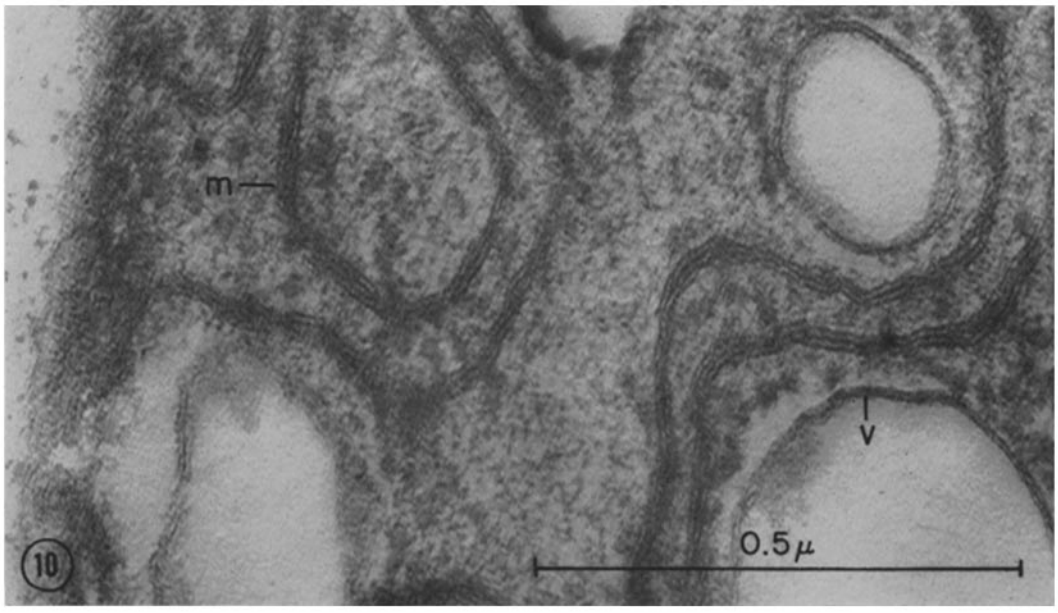
FIGURE 9

Two cells in filament of *Nostoc*. The protoplast is enclosed by a cell membrane (*cm*) and is differentiated into an outer region and an inner region. Numerous membranes (*m*) are scattered throughout the outer region which also contains a number of membrane-enclosed vacuoles (*v*). Several granular bodies are seen in the central region. The wall structure (*w*) is not easily distinguishable but it may consist of two layers of intermediate density enclosing a less dense region. × 40,000.

FIGURE 10

Portion of cell of *Nostoc* showing the double structure of the membranes (*m*) of outer region of the protoplast, and the membranes of the vacuoles (*v*). The double structure of the membranes shows a striking resemblance to the lamellae in the chloroplast of *Cyanidium*. × 130,000.


cm
v
m
w
9


m
v
0.5μ
10

details are more obvious after staining with lead. A detailed account of the structure of *Nostoc* and other blue-green algae is to be given in a later paper from this laboratory.

DISCUSSION

A comparison of the fine structure of *Cyanidium*, *Chlorella*, and *Nostoc* shows that the protoplast of *Chlorella* is more differentiated than that of *Cyanidium* which, in turn, is more differentiated than that of *Nostoc*. The protoplast of the Cyanophyte, which is relatively undifferentiated, is characterized by a lack of organelles, such as mitochondria, chloroplasts, Golgi bodies, and nucleus. The highly differentiated protoplasts of *Cyanidium* and *Chlorella* demonstrate that these organisms should not be placed in the same taxonomic group as *Nostoc*. The presence of organelles, including a nucleus, shows that *Cyanidium* cannot be a member of the Cyanophyta nor a symbiotic association of a blue-green alga and a colorless chlorophyte. Whether it should be classified with *Chlorella* in the Chlorophyta is not so obvious.

The protoplast of *Chlorella* differs from that of *Cyanidium* in many structural features. In general, *Chlorella* is more highly differentiated and the organelles are more definite structures. The cytoplasmic membrane system is relatively poorly developed in *Cyanidium*. As far as is known, Golgi bodies do not occur in *Cyanidium*, and membranes corresponding to the endoplasmic reticulum are not so conspicuous nor so extensive as those in *Chlorella*. But in both species the cytoplasmic membrane system is continuous with the nuclear membrane. One may argue that an endoplasmic reticulum with Golgi bodies cannot evolve in the absence of the double nuclear-membrane of a nucleus, which would mean that the membranes of the protoplast of the blue-green alga should not be identified as endoplasmic reticulum. Rather, if the membranes of the protoplast of the blue-green alga are the sites of photosynthetic activity, they may be related to the lamellae-structures of the chloroplasts of other plants.

Similarly, the chloroplast system of *Cyanidium* is not so elaborate or distinct as that in *Chlorella*. Although the lamellae are aggregated into a definite organelle in *Cyanidium* the chloroplast membrane is frequently indistinguishable from the lamellae and adjacent cytoplasmic membranes. In contrast, in *Chlorella* the chloroplast membrane is always distinct from the chloroplast lmaellae and the chloroplast is always sharply differentiated from the rest of the protoplast. There is also a striking difference in the arrangement of the lamellae. The bands of lamellae in *Cyanidium* consist usually of two lamellae or one disc, whereas those in *Chlorella* consist usually of three discs, although four or five discs are also found. Recently Gibbs (9) showed that chloroplast bands, each composed of two to five closely appressed discs, are widespread in the algae. She also pointed out that the disc-structure has been observed in members of the dinoflagellates, chrysophytes, diatoms, and the red, brown, and green algae. Since the disc may be a universal structure it may be of little taxonomic value unless the number of discs per band is found to be unique for a particular group. Thus, the observation that the bands in *Cyanidium* contain mostly one disc, whereas those of *Chlorella* mostly include three, may or may not indicate that the two organisms are only distantly related.

The chloroplast of *Chlorella* contains a pyrenoid. Starch grains are found in a starch sheath and between the bands of lamellae. In contrast, the chloroplast of *Cyanidium* contains neither a pyrenoid nor starch grains; no carbohydrate storage product has been identified in its chloroplast.

Finally, the cell wall of *Cyanidium* appears to be different in composition from that of *Chlorella*. With fixation times from 1 to 12 hours, the former reacts strongly with $KMnO_4$ while the latter reacts poorly.

Thus, *Cyanidium* differs from *Chlorella* in a number of structural features: (*a*) the absence of a pyrenoid, (*b*) the absence of starch grains, (*c*) the indistinctness of the chloroplast membrane, (*d*) the fewer discs in the lamellae-bands, (*e*) the absence of Golgi bodies, (*f*) the poor development of the endoplasmic reticulum, and (*g*) the composition of the cell wall.

In the absence of information about the fine structure of a wide range of micro-organisms, it is difficult to decide how far fine structure can be used as an aid to classification. Obviously, it is of value in some instances; for example, differences in structure between *Nostoc* and *Cyanidium* and *Chlorella* demonstrate that the latter two are not blue-green algae, but does the absence of a pyrenoid constitute evidence against a close relationship between these two organisms? Possibly not, since the pyrenoid is known to be a plastic

structure. Some members of the Chlorophyta do not contain pyrenoids (10, p. 65). But since the two organisms differ in at least seven structural features, it seems reasonable to conclude that they are not closely related. Furthermore, since the basic structure of *Chlorella* is similar to that of *Chlamydomonas*, as reported by Sager and Palade (8), *Cyanidium* and *Chlamydomonas* are probably not closely related. *Chlorella* and *Chlamydomonas*, which have a similar fine structure, are classified in different orders of the Chlorophyceae, namely the Chlorococcales and the Volvocales, respectively. Thus, if two organisms which are grouped, on microscopic features, among different orders of green algae have a similar fine structure, it seems questionable whether *Cyanidium*, which differs from *Chlorella* and *Chlamydomonas* in so many structural features, should be included in either the Chlorococcales or the Volvocales. Whether or not *Cyanidium* should even be regarded as a member of the Chlorophyceae requires that more information be available about common features of fine structure among members of the group.

Unfortunately, through lack of material, it was not possible in the present study to make a comparison among *Cyanidium*, the unicellular red alga *Porphyridium*, and a coccoid cryptomonad. The description of an osmium-fixed unicellular red alga *Porphyridium cruentum* given by Brody and Vatter (11) suggests that *Cyanidium* and *Porphyridium* are not closely related. *Cyanidium* differs from *Porphyridium* in several features: (*a*) the absence of a pyrenoid, (*b*) the absence of a gelatinous sheath, (*c*) the absence of floridean starch in the cytoplasm, (*d*) the probable absence of well developed dictyosomes (Golgi bodies), (*e*) the indistinctness of the chloroplast membrane, and (*f*) the presence of mitochondria, although Brody and Vatter's observation that *Porphyridium* does not contain mitochondria seems improbable. Because the two organisms differ in a number of structural features, it is concluded that *Cyanidium* is not a red alga similar to *Porphyridium*, as suggested recently by Hirose (4).

Despite the obvious weaknesses of the technique used in the present study, the findings do exclude several of the alternative classifications proposed for *Cyanidium*. *Cyanidium* is not a blue-green alga, not a symbiotic association, not a red alga similar to *Porphyridium*, and not a green alga similar to *Chlorella* or *Chlamydomonas*. It is less differentiated than *Chlorella* and may be more primitive. On the basis of the structure of its chloroplast, *Cyanidium* resembles both *Nostoc* and *Chlorella*. The fine structure of the lamellae is similar to that of the photosynthetic membranes in *Nostoc* (Fig. 5 and Fig. 10), while in the arrangement of lamellae into a definite organelle *Cyanidium* more closely resembles *Chlorella* (Figs. 1 and 2, and Fig. 6). Whether it should be included in the Chlorophyceae remains a matter of conjecture.

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