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Short Communication

Sterols and Chloroplast Structure of *Cyanidium caldarium*

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A brief survey on the occurrence of algal sterols shows that green algae (Chlorophyta) are characterized by the presence of sitosterol (12) and a complex mixture of sterols as occurs in higher plants. However, among few exceptions to that, *Chlorella*, a "typical" green unicellular alga, possesses ergosterol as the major constituent (13). Recent investigations of Alcaide *et al.* (1) with marine red algae reveal the presence of C_{27}-C_{29} sterols, the C_{28} predominant. This algal group (Rhodophyta) contains primarily cholesterol (12, 13) and may possess sitosterol (13), methylene-24 cholesterol, and campesterol (1).

Recently, reports have shown that the cyanophytes (blue-green algae) contain cholesterol and other 24-alkyl sterols (4). In fact, cholesterol seems to be very widely distributed among all groups of plants tested, since it occupies a key position in the biosynthesis of other steroids. Further information on algal sterols was reviewed by Klein and Cronquist (10) and Patterson (13).

The unicellular thermophilic alga *Cyanidium caldarium* has not been classified to a fixed taxonomic position. At one time or another it has been considered as a cyanophyte (10), rhodophyte (3, 8), chlorophyte (10), or as harboring an endosymbiote (14) because of special features of this fascinating alga which combines characteristics of other algal groups and which possesses specialties of its own. The principal features of this monotypic organism can be summarized as follows: morphologically it resembles *Chlorella* in size (15) and by dividing into 4 autospores (Fig. 1); it possesses a red alga type of chloroplast (Figs. 1 and 2), a nucleus and mitochondrion; as photosynthetic pigments it contains only chlorophyll *a*, a blue-green algal type of phycocyanin, β-carotene and zeaxanthin. The storage sugar and enzyme pattern of *Cyanidium* are similar both to the general distribution of blue-green and red algae (5). Ecologically, *Cyanidium* is found in hot springs (50–60 C) throughout the world and grows in acidic medium (pH about 2) and even under pure CO_2 (15).

Klein (11) and Fredrick (5) re-examined the taxonomic questions and were tempted to consider *Cyanidium* as a transition or "bridge form" between the primitive blue-green and the more advanced red algae.

Since lipids comprise a substantial portion of cellular membranes, we undertook this study of *C. caldarium* to examine sterol composition and the ultrastructure of the chloroplast lamellae. We attempted to elucidate the controversial taxonomy of *C. caldarium* by using these two criteria as phylogenetic markers. Experimental evidence and observation have been obtained which support the proposal that this alga is a rhodophyte (red alga).

Algal cells were grown in a mineral acid medium (pH 2–3) as previously described (15). Cells were cultured in 4-liter flasks; the suspension was agitated with magnetically driven stirring bars and kept at 45 to 50 C. Continuous illumination was provided by fluorescent tubes supplying an intensity of about 450 ft-c. Cultures were aerated with either 5% CO_2 in air or with pure CO_2 (gases prefiltered and humidified) in order to eliminate environmental atmospheric influence. After a few weeks of growth, the cells were harvested by centrifugation, and pellets were washed three times with distilled water and lyophilized. Dry cells were extracted with hot chloroform-methanol (2:1) for 6 hr. The filtrate was concentrated under reduced pressure leaving dark green viscous residue. This mixture was hydrolyzed with methanolic 7% KOH for 6 hr under reflux. Methanol was removed (*in vacuo*) and replaced by water, and the solution was extracted with chloroform. Filtration and evaporation left the unsaponifiable fraction which was chromatographed on a silica gel column. Each of the collected fractions was subjected to thin layer chromatography on Silica Gel (GF-254) plates and developed with petroleum ether (boiling point 40–60 C)-ethyl ether-acetic acid (20:4:1). Spraying with H_2SO_4 (50%) and charring (at 180 C) revealed the presence of sterols at R_F 0.43. The fractions containing sterols were concentrated and subjected to preparative thin layer chromatography, and, after a double development, the sterol zones were scraped off and extracted with chloroform-ether (1:1).

A sample of the sterols was chromatographed on Silica Gel G plates impregnated with $AgNO_3$ (9) with chloroform serving as developing solvent. Observations of the developed plate under UV light revealed the presence of two fluorescent spots, one corresponding to ergosterol (R_F 0.15) and the other spot with an R_F value of 0.17. The band corresponding to ergosterol had the following UV absorption peaks (in ethanol): 264, 273, 283, and 295 nm, characteristic of ergosterol (16). This conclusion was supported also by the typical blue color formed in the Liebermann-Burchard test. Analysis of the sterol fraction by gas-liquid chromatography (3% XE-60 on Gas Chrom Q, at 245 C) revealed the presence of cholesterol (0.3%), ergosterol and 5,6-dihydroergosterol (55.0%), campesterol (5.6%), β-sitosterol (38.9%), and 7-dehydrositosterol (0.2%); these compounds correspond to the following molecular ions found in the mass spectra: 386, 396, 398, 400, 414, 412. The predominant compounds were ergosterol, β-sitosterol, and campesterol. One should keep in mind that there is still a possibility that some of these compounds may exist as a mixture of 24α- and β-isomers.

The composition of algal sterols has been discussed as a guide in classification and for phylogenetic questions (10, 12, 13). The sterol composition of *C. caldarium* appears generally to agree with the earlier proposals that this paradoxical alga is transitional form between the procaryotic blue-green and the higher algae. However, it resembles closer that of the Rhodophyta by possessing campesterol, the major sterol of some rhodophytes (1, 13) and (even though in small amount)

cholesterol—the widely distributed sterol of red algae. Although *Cyanidium* also contains sitosterol and ergosterol which were reported to be common in green algae, some red algae possess the former one (12). This wide range of *Caldarium* sterol content agrees with Patterson's (13) statement which pointed out the confusion concerning the sterols of the red algae.

New ultrastructural aspects of *Cyanidium* chloroplast are shown (Figs. 1 and 2). They are virtually identical to those of many rhodophytes described by others (2, 6, 7). *Cyanidium* chloroplast shows the following features: single, nongranal, concentric thylakoids (Figs. 1 and 2); the outermost lamella (girdle band) encircles the rim of the chloroplast (Fig. 1). Phycobilisome-like granules are attached to the lamellae (Fig. 2); storage grains are absent from the plastid and are embedded in cytoplasm (Fig. 1). Similar characteristics to these were described for many red algal chloroplasts (2, 6, 7, 10) and also for the procaryotic blue-green algal *cells* (7, 10, 14).

Based on older data, Hirose (8) and Chapman *et al.* (3) proposed that *Cyanidium caldarium* is a red alga, called it *Rhodococcus caldarius* (8) and placed it with the primitive members of Rhodophyta (3). From our study and observation

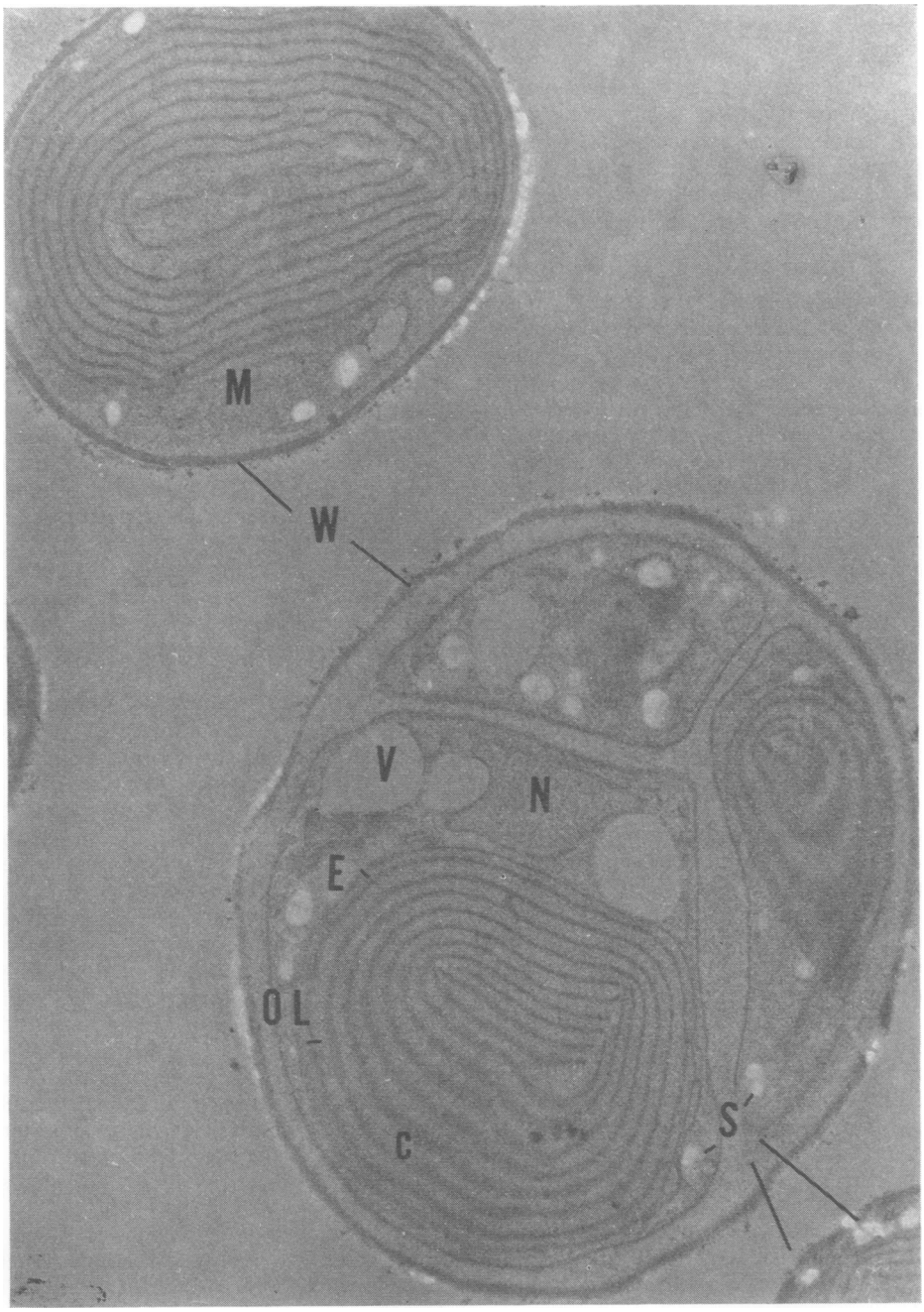

FIG. 1. This micrograph is a cross section of two *Cyanidium* cells cultured under pure CO_2 and fixed at 25 C with 2% $KMnO_4$. One cell divided and three autospores are visible within the maternal cell wall (W). The chloroplast (C) enclosed by an envelope (E) shows outermost lamella (OL) and single cicular or parallel thylakoids. The cell possesses a definite nucleus (N), mitochondrion (M), small vacuoles and storage grains (S). Unstained × 38,000.

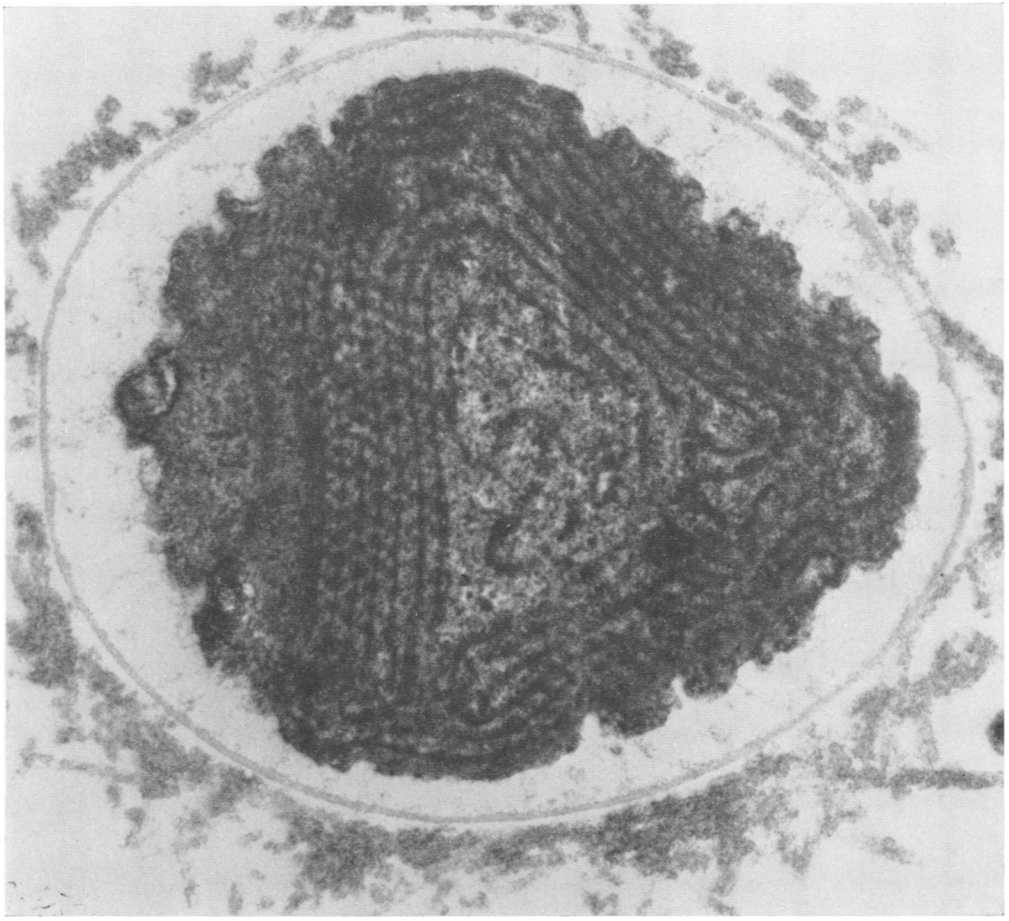

FIG. 2. A triangular shaped chloroplast of air-grown (0.03% CO_2) *Cyanidium* cell. The photosynthetic lamellae contain phycobiliprotein granules (phycobilisomes) arranged in parallel rows on the thylakoids. Fixed with 2.5% $KMnO_4$ at 4 C, poststained with lead citrate. × 51,500.

it seems reasonable to conclude that *C. caldarium* is a blue-green colored rhodophyte closely related to the unicellular red algae.

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