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A Yellow Marine *Chlamydomonas*: Morphology and Pigment Composition

Tsutomu Sasa[1], Shoichiro Suda[1,3], Makoto M. Watanabe[1,4] and Shinichi Takaichi[2]

[1] *National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki, 305 Japan*
[2] *Biological Laboratory, Nippon Medical School, Kosugi-cho 2, Nakahara-ku, Kawasaki, Kanagawa, 211 Japan*

A unicellular yellow marine microorganism was isolated from water samples collected in Hachinohe Harbor, on the northern coast of Japan, and Off Tsushima Island, on the western coast of Japan, and its structure and pigment composition were investigated. Light and electron microscopy indicated that the alga belongs to the genus *Chlamydomonas* and it is identified as *C. parkeae*.

Pigment analysis by high-performance liquid chromatography revealed the presence of 2,4-divinylprotochlorophyllide (DVP) as a third chlorophyll in addition to chlorophylls *a* and *b*. Such a pigment composition has been reported previously only for some prasinophytes among autotrophically grown algae. With respect to carotenoids, the alga contains, in addition to the carotenoids of higher plants (neoxanthin, violaxanthin, zeaxanthin, lutein, β-carotene), siphonaxanthin and siphonein (siphonaxanthin dodecenoate); the latter have been detected previously only in marine benthic ulvophycean algae and in some prasinophytes. However, the coexistence of DVP, siphonein and siphonaxanthin in a single species has never been reported for either ulvophycean or prasinophycean algae. In addition to siphonaxanthin dodecenoate, the alga was found to contain two "siphoneins", siphonaxanthin decenate and siphonaxanthin octanoate.

Key words: Carotenoids — *Chlamydomonas* — *Chlamydomonas parkeae* — Chlorophyta, ultrastructure — 2,4-Divinylprotochlorophyllide (DVP) — Flagellar apparatus — Siphonaxanthin — Siphonaxanthin decenoate — Siphonaxanthin dodecenoate — Siphonaxanthin octenoate.

The unicellular, volvocalean biflagellate, *Chlamydomonas*, of which there are hundreds of species have been described, is widely distributed in the natural world and has been studied from various perspectives in the laboratory as well as in the field. It contains both chlorophylls *a* and *b* and is a member of the class Chlorophyceae (Mattox and Stewart 1984, Melkonian 1989) in the Chlorophyta. Among species of *Chlamydomonas*, wild and mutant strains of *C. reinhardtii* have been used predominantly in studies of carotenoids and their relation to photosynthesis (Sager and Zalokar 1958, Krinsky and Levine 1964, Francis et al. 1975). The authors cited, as well as Fawley and Lee (1990), reported the existence of the following major carotenoids in wild strains of *Chlamydomonas*: β-carotene, loroxanthin, lutein, neoxanthin and violaxanthin.

During a survey of pigment patterns in microalgae maintained in our facility, our attention was drawn to a set of cultured strains of yellow *Chlamydomonas* isolated from waters off the northern and western coasts of Japan. Our preliminary experiments showed that the yellow alga is morphologically typical of *C. parkeae* but is quite unusual in terms of its pigment composition.

The present report describes our studies of the structure and pigment pattern of this alga.

Materials and Methods

Two strains of yellow *Chlamydomonas* were isolated from surface water samples collected in Hachinohe Harbor, Aomori Prefecture, in January 1985 (NIES-441;

Abbreviations: DVP, 2,4-divinylprotochlorophyllide, magnesium 2,4-divinylphaeoporphyrin a5; MVP, monovinylprotochlorophyllide; ODS, octadecylsilane-bonded silica; GLC, gas-liquid chromatography.

[3] Present address: Nippon Roche Research Center, Kajiwara, Kamakura, Kanagawa, 247 Japan.

[4] Address for reprint requests.

Watanabe and Satake 1991) and in Izumi Bay, Tsushima Island, Nagasaki Prefecture, in March 1986 (NIES-440; Watanabe and Satake 1991), respectively. Each strain was maintained in a screw-capped test tube (18 mm i.d. × 150 mm) with 10 ml of f/2 medium (cf. Watanabe and Satake 1991) under illumination of about 100 μE m^{-2} s^{-1} with a photoperiod of 12 h of light and 12 h of darkness from daylight fluorescent lamps at 20°C. *C. reinhardtii* was grown in C medium (cf. Watanabe and Satake 1991) instead of f/2 medium.

For microscopic observations, the cells were cultured in 200-ml Erlenmeyer flasks in 100 ml of medium for about 10 days. Fixation of the cells was carried out as follows. A 5% solution of glutaraldehyde, prepared in 0.2 M sodium cacodylate buffer (pH 7.2), was added to an equal volume of the suspension of cells. After fixation for 1 h at room temperature, part of the mixture was put aside for examination under a Nikon Optiphot microscope equipped with differential interference-contrast optics.

The cells in the rest of the mixture were rinsed several times in 0.2 M sodium cacodylate buffer (pH 7.2) and post-fixed in the same buffer supplemented with 2% osmium tetroxide for 2 h. After dehydration in a graded ethanol series, which was followed by a graded transfer to propylene oxide, the cells were embedded in Spurr's resin (Spurr 1969). Ultrathin sections were cut with a diamond knife, collected on slot grids and double-stained with uranyl acetate and lead citrate. Observations were carried out with a JEOL JEM 100CX-II electron microscope.

For the analysis of pigments, the cells were grown in several Erlenmeyer flasks with 100 or 1,000 ml medium. After about ten days, the cells were harvested on filter paper (Whatman GF/C) and washed with distilled water, and then they were extracted repeatedly with 90% acetone. The combined acetone extract was used for the analysis. If necessary, the extract was further subjected to column chromatography on DEAE-Toyopearl CL-6B (Tosoh, Japan), by method of Omata and Murata (1980), as modified by Araki et al. (1984). The column was eluted successively with acetone to obtain the "carotenoid fraction", with an acetone-methanol mixture to remove chlorophylls, and finally with 80% acetone that contained 1% ammonium acetate to obtain a "nonesterified chlorophyll fraction" that contained chlorophyll *c* and protochlorophyllide species.

Pigments from spinach leaves and thalli of *Codium divaricatum* were employed as standards of known composition (Konishi et al. 1968, Kleining 1969, Yokohama 1981).

For a preliminary survey of photosynthetic pigments, high-performance liquid chromatography (HPLC) was performed with a Shimadzu system equipped with an SPD-M6A photodiode array, a UV-VIS spectrophotometric detector, and an octadecylsilane-bonded silica (ODS) column (Shimpack LC-ODS, 4.6 mm i.d. × 150 mm; Shimadzu, Japan). The system was operated as described by Mantoura and Llewellyn (1983) and modified by Kohata and Watanabe (1988). The acetone extract was mixed at a ratio of 10 : 3 (v/v) with an ion-pairing solution of tetrabutylammonium (10 ml of 0.5 M solution) and ammonium acetate (7.7 g), which had been made up to 100 ml with water, with the pH adjusted to 7.1 with acetic acid. Then 100 μl of the mixture were injected into the system. Elution was started with a linear gradient of solvent A (ion-pairing solution, water and methanol, 5 : 5 : 90, v/v) to solvent B (ethyl acetate) over a 20-min period, which was followed by isocratic elution with ethyl acetate for 5 min at a flow rate of 1.0 ml min^{-1}. Conditions were reversed for 5 min and the column was held under the initial conditions for at least 10 min prior to the next injection.

In order to idetify nonesterified chlorophylls (Chls), the "nonesterified Chl fraction" was analyzed by chromatography on a column packed with polyethylene powder by the method of Shioi and Beale (1987). The column was eluted with 65% acetone at a flow rate of 0.2 ml min^{-1}.

When the Shimadzu HPLC system was used, the column eluent was monitored by the sum of the absorbance between 400–440 nm and that between 600–670 nm. Furthermore, at the peaks of the separated pigments, the entire spectrum between 400–670 nm was recorded by the photodiode-array detector.

The chlorophyll *a*/*b* ratio was determined spectrometrically with the 80% acetone extract by the method of Arnon (1949). Absorption spectra of the intact cells were measured by the opal glass method (Shibata et al. 1954) with a spectrophotometer (model 557; Hitachi, Japan).

Authentic 2,4-divinylprotochlorophyllide (DVP) [i.e., magnesium 2,4-divinylphaeoporphyrin a5 (Mg 2,4-D)] was extracted and partially purified from the growth medium of *Rhodobacter sphaeroides* that had been cultured in nicotinamide-enriched medium by the method of Shioi et al. (1988). Authentic monovinylprotochlorophyllide (MVP) was partially purified from the acetone extract of dark-grown cells of the YG6 mutant of *Chlorella regularis* (Shioi and Sasa 1984).

To identify individual carotenoids, the "carotenoid fraction" was fractioned by reverse-phase thin-layer chromatography (TLC; KC18; Whatman, U.K.) with methanol as the developing solvent. Further purification, when necessary, was performed by silica gel high-performance thin-layer chromatography (HP-TLC; Merck, U.S.A.) on plates developed with a mixture of dichloromethane and ethyl acetate (3 : 1, v/v), and/or by an ODS-HPLC system (Waters, U.S.A.) which included a prepacked column that consisted of a Radial-PAK μ Bondapak C_{18} cartridge (8 mm i.d. × 100 mm). The absorption spectrum was measured by a photodiode array detector, MCPD-350 PC System II (Otsuka Electronics, Japan, 230–800 nm, 1.4 nm

resolution, 1-s interval) that was part of the Waters system (Takaichi et al. 1990, Takaichi and Shimada 1992).

The molecular weight of each carotenoid was analyzed by mass spectrometry in the field desorption mode (FD-MS; Takaichi and Ishidsu 1992).

Acidic butanolysis of siphonaxanthin esters was performed in a 5% HCl–*n*-butanol solution (GL Science) at 70°C for 30 min. After evaporation, the fatty acid butyl esters were extracted with *n*-hexane. The esters were further purified by silica gel HP-TLC (Takaichi and Ishidsu 1992), and then analyzed with a gas-liquid chromatograph (GLC; model GC-6A; Shimadzu), fitted with an HR-Thermon 3000A capillary column (0.25 mm i.d. × 25 m). The temperature of the column was increased from 150° to 200°C at a rate of 3°C min^{-1} (Sato and Murata 1988). *n*-Butyl esters of *n*-octanoic, *n*-decanoic, *n*-dodecanoic and *n*-tetradecanoic acids (Tokyo Kasei) were used as standards.

Results

Morphological observations—The algal cultures appeared brown, but each cell was yellow or yellowish-green under the light microscope. Thus, superficially, the alga would seem to be a prasinophyte or chrysophyte. However, a detailed examination under the light microscope showed that the features and coloration of the cells corresponded closely to those described for the marine species *Chlamydomonas parkeae* Ettl (Ettl 1967), as shown below. The cells are broadly ovoid or ellipsoid, 4.0–6.5 μm long and 4.0–5.5 μm wide. A ridge-like papilla is located at the anterior end of the cell and appears to be of type "cc" (Ettl 1976) (Fig. 1). The two long flagella, which are three to four times longer than the cell body, arise from the edges of the papilla when viewed from the cell's broad side. The chloroplast is cup-shaped and a single pyrenoid is situated at the bottom of the chloroplast (Figs. 1 and 4). There is a single, oval eyespot located in the median to subanterior peripheral portion of the chloroplast (Fig. 4). Asexual reproduction was observed only during the dark period because the culture was partially synchronized by a 12 : 12 LD cycle. One or two successive cell divisions occur within the wall of the mother cell, after detachment of flagella, prior to propagation (Figs. 2 and 3).

The wall is thick, having several layers of distinct electron-density. In favorable sections, three thin outer layers and a thick relatively electron-lucent layer can be observed as the wall components (Figs. 5 and 6). The innermost layer seems to contain fibrous materials which often protrude inward, with resultant formation of many papillate protuberances to which the plasmalemma is aligned in parallel (Fig. 6). The chloroplast is yellowish-green and usually consists of thylakoid lamellae (Fig. 4). The multilayered thylakoid lamellae were also observed to be differentiating into grana-like and stromal lamellae (Fig. 7). The single pyrenoid is surrounded by several starch bodies (two to six in single sections), and the matrix is traversed by several, single-layered thylakoids from various directions with no obvious or regular pattern (Fig. 8).

The insertion of the two flagella is such that they pass through the papilla cylinders and are linked proximally with the basal bodies. The basal bodies are arranged in a V-shape, at 90–95 degrees to one another, and they do not overlap (Fig. 9). They are interconnected by one distal and two laterally arranged proximal fibers (Fig. 10). Each basal body has two microtubular rootlets, a right and a left one (Fig. 11). When the basal bodies are viewed from the anterior side of the cell, they show a clockwise orientation, and the four microtubular rootlets have a cruciate profile (Fig. 11). The two accessory basal bodies are always present at the vegetative stage (Fig. 12).

The nucleus, containing a conspicuous nucleolus, is situated just beneath the basal bodies, and no specific structures are present between them (Fig. 4). There is no contractile vacuole in the anterior region of the cell, a feature that is not unusual in marine cells. There are two dictyosomes posterior to the nucleus and one or two large vacuoles are often observed between the dictyosome and the chloroplast.

Pigment analysis—Figure 13 shows the elution profiles of acetone extracts prepared from spinach leaves, *Chlamydomonas parkeae* and *Codium* thalli after HPLC. The system gave adequate separation of chlorophyll *a*, chlorophyll *b* and most of the carotenoids. The pigments of spinach and *Codium* thalli were identified tentatively from their retention times and absorption spectra as follows: peaks 2, 4, 5, 6, 8, 9, 10, 11 and 12 corresponded to siphonaxanthin, neoxanthin, violaxanthin, antheraxanthin, lutein, siphonein, chlorophyll *b*, chlorophyll *a* and β-carotene, respectively.

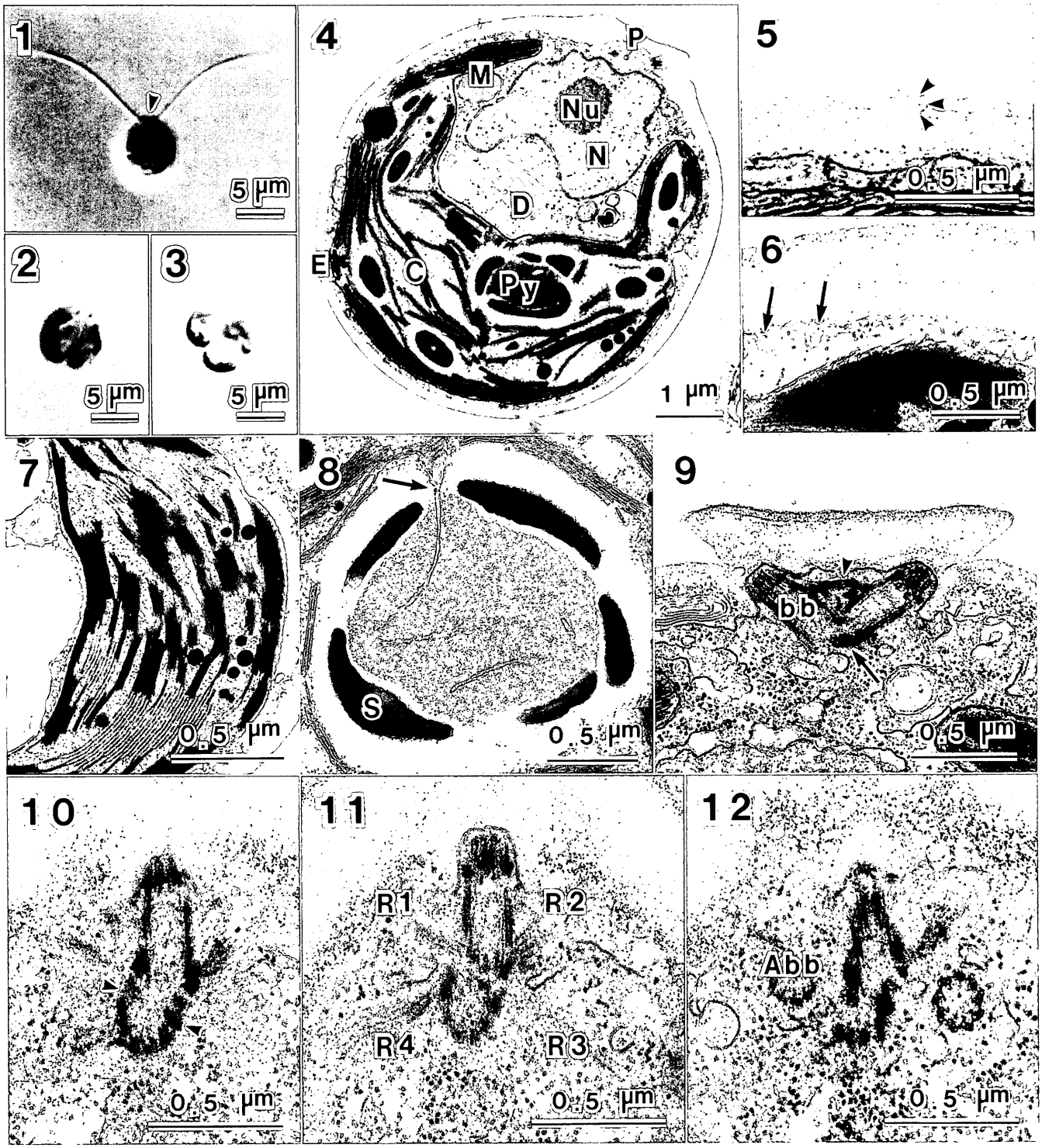
Twelve pigments were separated from the extract of *C. parkeae* in less than 20 min. Because the retention times and absorption spectra of the peaks from *C. parkeae* were in complete agreement with those from spinach and *Codium*, peaks 2, 4, 5, 6, 9, 10, 11 and 12 from *C. parkeae* were identified tentatively as siphonaxanthin, neoxanthin, violaxanthin, antheraxanthin, siphonein, chlorophyll *b*, chlorophyll *a* and β-carotene, respectively.

The chlorophyll *a*/*b* ratio of *C. parkeae* was approximately 1.8, the value generally obtained from marine algae (Wood 1979, Yokohama and Misonou 1980).

The presence of peak 1 suggests the existence of protochlorophyllide or chlorophyll *c*, but the ODS-HPLC (Shimadzu system) did not allow complete separation of these pigments. The "nonesterified Chl fraction" was dried in vacuo, dissolved in ethyl ether, and its absorption spectrum was recorded. The absorption maxima at 437, 573 and 624 nm coincided well with those obtained from DVP from *Micromonas pusilla* CS-86 (Jeffrey and Wright 1987)

and *Rhodobacter sphaeroides* (Dr. Y. Shioi, personal communication). Furthermore, when the fraction was subjected to polyethylene-HPLC (Shimadzu system) and its retention time was compared with that of DVP prepared from a culture of *R. sphaeroides* and with that of MVP from *Chlorella regularis*, complete agreement was observed in terms of retention times and absorption spectra between DVP from *Rhodobacter* and the pigment from *C. parkeae*. These data were further evidence that the pigment was DVP.

After the materials in peaks 2, 4, 5, and 12 had been purified, they were identified as siphonaxanthin, neoxanthin, violaxanthin and β-carotene, respectively, from their absorption spectra, which were recorded with the Waters



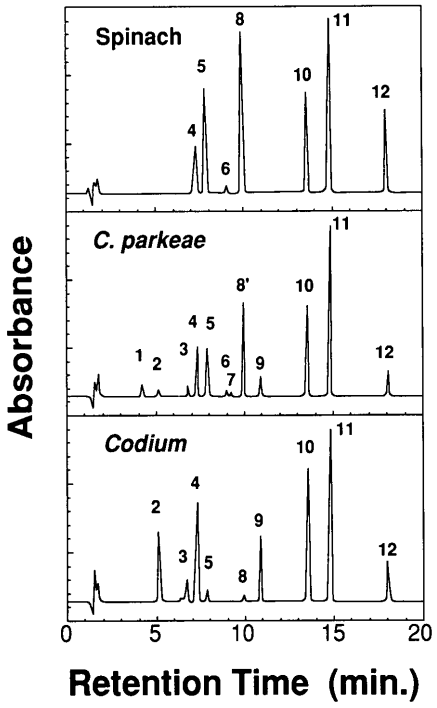


Fig. 13 Elution profiles of acetone extracts prepared from spinach leaves, *Chlamydomonas parkeae* cells and *Codium* thalli after ODS-HPLC (Shimadzu system). Peaks were identified as follows: 1, DVP; 2, siphonaxanthin; 3, unknown; 4, neoxanthin; 5, violaxanthin; 6, antheraxanthin; 7, siphonaxanthin octenoate; 8, lutein; 8′, lutein+zeaxanthin+siphonaxanthin decenoate; 9, siphonein (siphonaxanthin dodecenoate); 10, Chl. *b*; 11, Chl. *a*; and 12, *β*-carotene.

ODS-HPLC system (Takaichi and Shimada 1992), and their molecular weight, as determined by FD-MS. Peak 9 gave a carbonyl-type spectrum and its molecular ion (M^+), m/z 780, which is indicative of siphonein (siphonaxanthin dodecenoate), was observed in the FD-MS spectrum. Siphonein from *Eutreptiella gymnastica* had the same M^+ (Fisksdahl et al. 1984) as that from the present strain. Furthermore, the *n*-butyl ester of dodecenoic acid was detected by GLC.

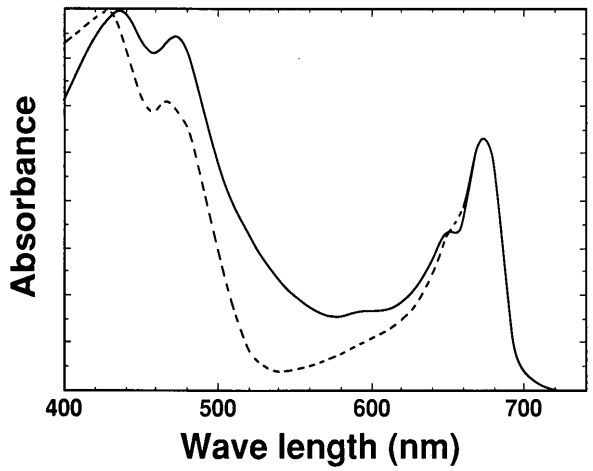


Fig. 14 Absorption spectra recorded from cells of *Chlamydomonas parkeae* (solid line) and *C. reinhardtii* (dotted line).

The retention time of peak 8′ was consistent with that of peak 8 from spinach (lutein), but the two absorption spectra did not coincide. The material in peak 8′ was separated into three carotenoids by HP-TLC on silica gel. Two of the components were identified as lutein and zeaxanthin by their absorption spectra (Takaichi and Shimada 1992) and mass patterns. The third gave a carbonyl-type spectrum and m/z 752 (M^+). The absorption spectrum of its reduced form indicated that it was a nonconjugated *β*-ring with eight conjugated double bonds (Takaichi and Shimada 1992). It was, therefore, identified as siphonaxanthin decenoate. In addition, the material in peak 7, the retention time of which was quite close to that of antheraxanthin on the Shimadzu ODS-HPLC system, was identified as a new third type of siphonein, siphonaxanthin octenoate, and its M^+ was detected at m/z 724. The *n*-butyl esters of decenoic and octenoic acids were also detected by GLC.

The absorption spectrum of the material in peak 3 was that of a carbonyl-type compound, with the largest peak at m/z 614 (M^+). However, it remains to be identified.

Figure 14 shows the absorption spectra recorded from

Figs. 1–3 Light micrographs of *Chlamydomonas parkeae.* Fig. 1. Vegetative cell. Arrowhead indicates papilla. Figs. 2 and 3. Asexual reproduction.

Figs. 4–12 Transmission electron micrographs of *Chlamydomonas parkeae.* Fig. 4. Median to longitudinal section of a vegetative cell. Almost all organelles can be seen: chloroplast (C), dictyosome (D), eyespot (E), mitochondria (M), nucleus (N), nucleolus (Nu), papilla (P) and pyrenoid (Py). Fig. 5. Higher-magnification view of the cell wall, in which three distinct thin layers (arrowheads) and a thick layer can be seen. Fig. 6. The innermost layer of the cell wall has many papillate protuberances (arrows). Fig. 7. Multi-thylakoid lamellae observed in a chloroplast. Fig. 8. Section of a pyrenoid surrounded by five starch bodies (S). The matrix is traversed by several single-layered thylakoids. Arrow indicates the connection between chloroplast thylakoid and pyrenoidal matrix thylakoid. Fig. 9. Median to longitudinal section of the flagellar apparatus. Two basal bodies (bb) are connected by a distal fiber (arrowhead) and a proximal fiber (arrow). Figs. 10–12 Anterior side view of serial sections of the flagellar apparatus. Fig. 10. Basal bodies are connected by two striated proximal fibers (arrowheads). Fig. 11. Basal bodies have a clockwise orientation, and four microtubular rootlets (R1-R4) have a cruciate profile. Fig. 12. Accessory basal bodies are evident (Abb).

C. parkeae and *C. reinhardtii* cells. The former gave much higher absorbance in the blue-green region than the latter.

Discussion

Light microscopy revealed that the present alga is assignable clearly to *Chlamydomonas parkeae*. Under the electron microscope, when the flagellar basal bodies of *C. parkeae* are viewed from the anterior side of the cell, they show a clockwise orientation and the four flagellar rootlets exhibit a cruciate profile, characteristic features of Chlorophyceae (Mattox and Stewart 1984, Melkonian, 1984). By contrast, in both Prasinophyceae and Ulvophyceae, the flagellar basal-body configuration exhibits a counter-clockwise orientation under the same conditions (Melkonian 1984, O'Kelly and Floyd 1984). However, pigment analysis indicated that the pigment pattern of *C. parkeae* is quite unusual for a chlorophycean alga. The presence of DVP, siphonein or siphonaxanthin, is more typical of Ulvophyceae and Prasinophyceae, as discussed below.

DVP is an intermediate in the chlorophyll biosynthetic pathway (Castelfranco and Beale 1983). The pigment is produced in cultures of photosynthetic bacteria that contain inhibitors of the chlorophyll biosynthesis (Jones 1963, Wong 1978, Shioi et al. 1988). Furthermore, its accumulation has been reported in dark-grown plants (Belanger and Rebeiz 1980, Kotzabasis and Senger 1986). However, Ricketts (1966, 1967, 1970, 1971) found that, in some marine flagellates (Prasinophyceae) grown under normal photoautotrophic conditions without the addition of any inhibitors, DVP was present in the cells as a stable component in addition to Chls *a* and *b*. With respect to the role of the pigment, Brown (1985) postulated that the Soret band near 450 nm in vivo may increase the light-harvesting capacity of algae that live in deep waters.

Since the first identification of DVP by Ricketts (1966), several reports of its presence in microorganisms have been published (Jeffrey and Wright 1987, Jeffrey 1989, Ricketts 1970). To date, information has been available only for primitive green algae, the prasinophytes, and so the present report provides the first evidence for the presence of DVP in Chlorophyceae. It is clear that DVP has only rather limited value as a phylogenetic indicator, as suggested by Moestrup (1991).

Siphonaxanthin and siphonein have been detected only in the eusiphonian algae (part of Ulvophyceae; Kleining 1969, Yokohama 1981, O'Kelly 1982) and in some prasinophytes (Ricketts 1970, 1971, Moestrup 1991) and they have not previously been reported in any species of *Chlamydomonas* (cf. Goodwin 1980, Harris 1989).

As cited above, the "main" siphonein contained in *C. parkeae* was found to be siphonaxanthin dodecenoate, as in the case of the euglenoid *Eutreptiella gymnastica* (Fiksdahl et al. 1984). In addition, a second and third siphonein were isolated and they were identified as siphonaxanthin decenoate and siphonaxanthin octanoate, respectively. In various siphonous green algae, such as *Codium latum, Bryopsis maxima, Caulerpa sertularioides* and *C. serrulata*, only one siphonein species, siphonaxanthin dodecenoate, was detected. The presence of such "multiple" siphoneins in individual algae other than *C. parkeae* must be the focus of future research. At present, it is suggested that the name "siphonein" should be changed to "siphonaxanthin ester"; for example, "siphonein esterified with dodecenoate" should be described as "siphonaxanthin dodecenoate".

As already mentioned, DVP has been detected only in some prasinophycean algae, and siphonaxanthin and/or its ester only in some prasinophycean algae and in marine, benthic, ulvophycean algae. The presence of the above three pigments in one algal species has never previously been reported.

As shown in Figure 14, *C. parkeae* has much higher absorbance in the green region than *C. reinhardtii*; in other words, the former absorbs green light more effectively than the latter. The pattern of absorbance by *C. parkeae* is very similar to that of some deep-water seaweeds, which carry out efficient photosynthesis under green light as a result of the presence of siphonaxanthin, its ester, and a higher concentration of chlorophyll *b* (Kageyama et al. 1977, Yokohama 1981, Yokohama and Misonou 1980) and DVP (Brown 1985). Thus, *C. parkeae* seems also to be able to live in deep waters, even though our samples were collected from surface waters.

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