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Purification and identification of chlorophyll c_1 from the green alga *Mantoniella squamata*

Christian Wilhelm

Institut für Allgemeine Botanik, Johannes-Gutenberg-Universität, Mainz (F.R.G.)

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The prasinophycean alga *Mantoniella squamata* contains besides chlorophyll *a* and *b* a third chlorophyll *c*-like pigment in its light-harvesting antenna. This third chlorophyll was purified by reverse phase and polyethylene chromatography in order to identify its chemical structure. The absorption and fluorescence spectra were measured not only from the doubly purified pigment, but also from its Mg-free derivates. The spectra were compared with those of authentic chlorophyll *c* and of Mg-2,4-desethyl-2,4-divinylpheoporphyrin a_5 monomethyl ester which was isolated from *Rhodobacter capsulata*. The results show that the pigment from *Mantoniella* agrees best with chlorophyll c_1. In order to clarify the spectral data, chlorophyll c_1 and c_2, the pigment from *Mantoniella* and Mg-2,4-desethyl-2,4-divinylpheoporphyrin a_5 monomethyl ester were resolved by polyethylene chromatography. The chromatographic analysis clearly shows that the pigment from *Mantoniella* comigrates with chlorophyll c_1 and not with the bacterial pigment or chlorophyll c_2. Mantoniella is the first organism which has been demonstrated to contain chlorophyll *a*, *b* and *c*.

Introduction

In the most common view of eucaryotic algal taxonomy the early evolution of photosynthetic organisms has resulted in three different lines: the Chl *b*-containing green algae, the Chl *c*-containing heterocontic algae and the red algae with phycobilins [1]. This classic system is strongly based on the distribution of the accessory chlorophylls within the different taxa. More recently the classification of algal groups has been based on ultrastructural features [2,3]; most of this information confirmed the traditional system, but in some cases inconsistencies have become obvious.

One of the organisms which does not fit very well in the common held view is *Mantoniella*. It is a uniflagellate, very small alga (2 μm in diameter), which is known to contain Chl *a* and *b* and small amounts of a third Chl *c*-like pigment [4]. Ricketts reported this Chl to be Mg-DVPP, a precursor in the porphyrin pathway of bacteria [4]. This pigment differs from authentic Chl c_1 only by the fact that it possesses a propionic acid instead of an acrylic acid as side chain in position 7 of the ring IV.

Recently, Foss et al. identified the carotenoids from yellow colored prasinophytes [5]. They showed that the xanthophylls differ from all other green algae and named the main component 'prasinoxanthin'. It was demonstrated by Brown

Abbreviations: Chl, chlorophyll; Chl *c**, chlorophyll *c*-like pigment from *Mantoniella*; Mg-DVPP, Mg-2,4-desethyl-2,4-divinylpheoporphyrin a_5 monomethylester.

Correspondence: C. Wilhelm, Institut für Allgemeine Botanik, Johannes-Gutenberg-Universität, Saarstrasse 21, 6500 Mainz, F.R.G.

[6] and Wilhelm et al. [7] that the Chl *c*-like pigment, as well as the brown-colored prasinoxanthin act in photosynthesis.

This study reports the reidentification of Chl *c**. The aim of the experiments is focussed on the comparison between authentic Mg-DVPP, Chl *c* and the pigment isolated from *Mantoniella.*

Materials and Methods

Mantoniella squamata (Plymouth No LB 1965/5) was cultivated under conditions as previously described [7]. Cells of *Rhodobacter* (*capsulata*; strain Ala was a gift from Prof. Drews in Freiburg, F.R.G.) were cultivated according to Ref. 8.

For the isolation of Chl *c**, the cells of *Mantoniella* were harvested by centrifugation and homogenized in 80% acetone. The pigments were separated from cell debris by centrifugation. By adding two volumes of petrolether (boiling point, 140 °C) to 1 vol. of crude extract, most of the lipophilic pigments were removed. The pigments from the acetonic hypophase were transferred to diethylether, dried, evaporated and prepared for the injection in a HPLC separation system. The HPLC separation was run on a Knauer instrument with a 250 mm × 4.6 mm Hypersil ODS 3 μm column. The ultraviolet/VIS detector was adjusted to 440 nm. Runs were made at room temperature. A gradient was running from 70% B (acetonitrile/ methanol/ ethylacetate = 1 : 1 : 0.1, v/v) and 30% water at the beginning (flow rate, 1 ml/min) to 85% B after 10 min, to 100% B after 15 min (flow, 1.5 ml/min). At the end of 30 min all components were completely eluted from the column. This pigment was used for the synthesis of the pheoporphyrin *c* methyl esters and for polyethylene chromatography.

The Mg-DVPP was purified from *Rhodobacter.* The cell crude extract was prepared by the same procedure used for *Mantoniella.* Mg-DVPP was separated from other pigments by polyethylene chromatography. This chromatographic system was also used for the separation of Chl c_1 and c_2. A HPLC column (300 mm × 7.5 mm) was filled with polyethylene (RPX 1027 chromatographic grade Polyscience Inc., Warrington). The elution was carried out with 85% acetone/water at a flow rate of 3 ml/min. This system was used to purify all pigments from small amounts of alteration products in order to compare the chromatographic mobility and spectroscopical characteristics.

The Mg-free derivatives of the different pigments were prepared by the adition of 50 μl trichloroacetic acid into the cuvette. The pheoporphyrin *c* methyl esters were synthesized by the addition of 1 ml borontrifluoride-methanol complex (20%) to the dried pigment. This reaction mixture was kept overnight in the refrigerator at 4 °C. The pheoporphyrin *c* methyl esters were transferred to a diethylether solution, dried and separated using a HPLC system with a 250 mm × 4.6 mm Hypersil 5 μm column. The components were eluted from the column by a gradient running from 85% B (acetonitrile/ methanol/ ethylacetate = 1 : 1 : 0.1 (v/v) in 5 mM triethylamine) and 15% 5 mM triethylaminehydrochloride at the beginning to 90% B after 4 min. 10 min after injection all components were completely eluted from the column. The flow was adjusted at 1.5 ml/min. The wavelength of detection was 412 nm.

The absorption spectra were determined by a Shimadzu MPL 2000 spectrophotometer. The fluorescence spectra were recorded with a Hitachi F-3000 fluorometer at very low pigment concentrations (0.5 μg Chl/ml) in order to avoid self-aggregation. The excitation spectra were performed with a band pass of 1.5 nm for the excitation and 20 nm for the emission. The emission spectra were recorded with a band pass of 20 nm for the excitation and of 1.5 nm for the emission. The wavelengths of excitation and emission are given in the figure legends.

Results

The separation of the photosynthetic pigments from unialgal cultures of *Mantoniella* shows an unusual variety of chlorophylls as well as of xanthophylls. The most important carotenoids are the yellow-brown prasinoxanthin and its epoxide, both with a high chromatographic mobility (Fig. 1). Lutein, the most common xanthophyll in green plants, is lacking. The cells contain in addition to high amounts of Chl *b* a third green pigment which runs in front of all other components. Its

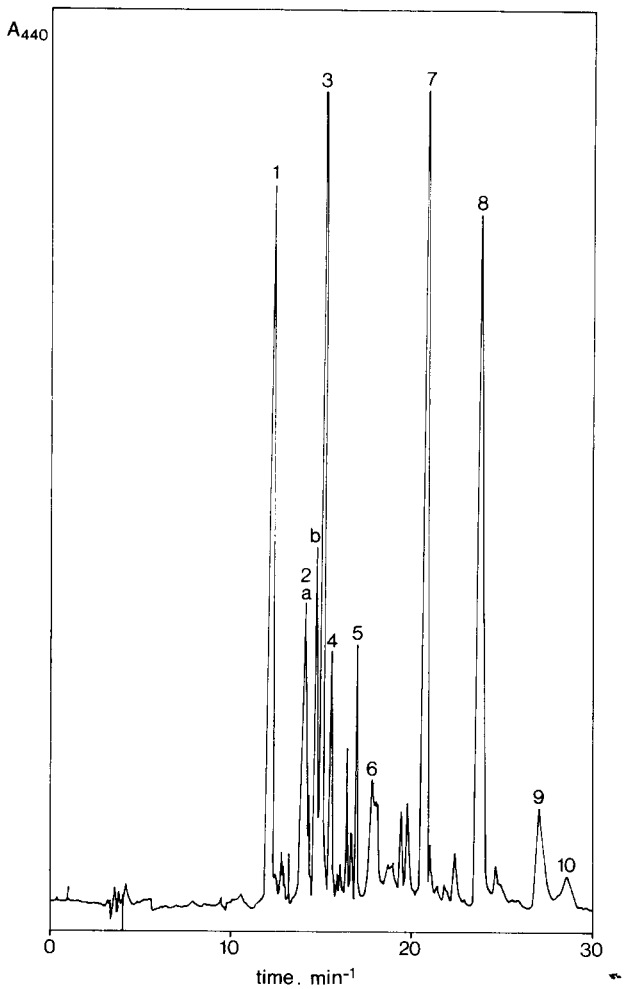

Fig. 1. HPLC-scan of the photosynthetic pigments from *Mantoniella* (for separation conditions, see Materials and Methods). The identification of the different fractions is as follows: 1, Chl *c**; 2a, neoxanthin; 2b, violaxanthin; 3, prasinoxanthin; 4, prasinoxanthin-epoxide; 5, lateoxantin; 6, not identified; 7, Chl *b*; 8, Chl *a*; 9 and 10, carotene.

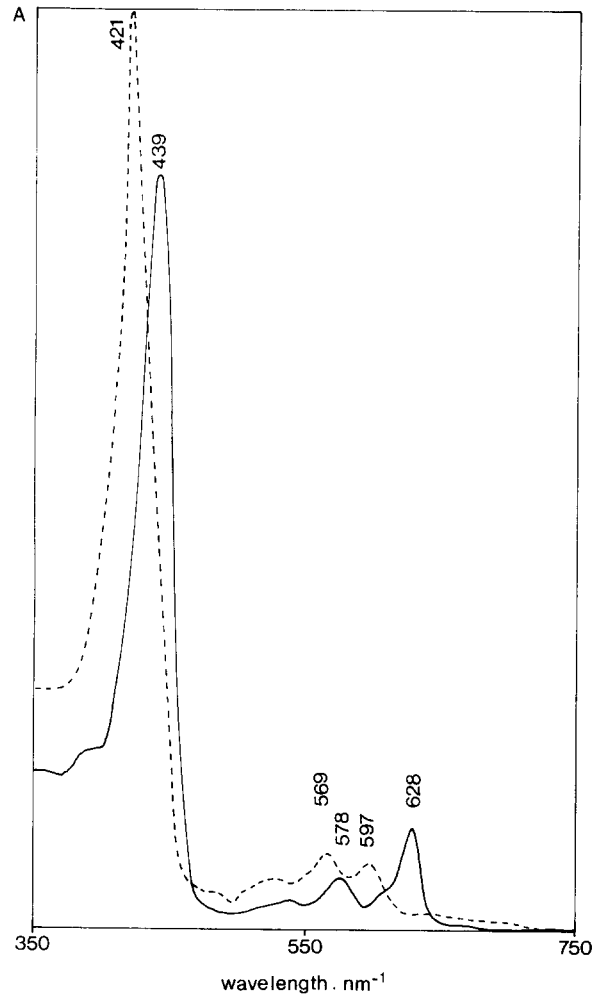

Fig. 2. Absorption spectra of Chl *c** (solid line) and of pheoporphyrin *c** (dotted line) in 90% acetone.

mobility is comparable with chlorophyllides. The absorption spectrum of this pigment, termed Chl *c**, is very similar to Chl *c*: it exhibits a red absorption maximum at 628 nm and an 8-times higher Soret band at 439 nm (Fig. 2). In addition, the spectrum of pheoporphyrin *c** is similarily shifted compared to the pheoporphyrin from authentic Chl *c* [9]. Since Mg-DVPP, Chl *c** and Chl c_1/c_2 have similar absorption spectra, the different pigments were isolated and compared by absorption and fluorescence spectroscopy. Fig. 3 gives the excitation and emission fluorescence spectra of Chl *c**. The fluorescence maxima and the ratios of the different bands were found to be identical with those of Chl c_1. In order to compare all three chlorophylls under identical conditions, the absorption and fluorescence spectra were recorded from chlorophylls that were isolated in parallel. The results are listed in Table I. In all spectra, Mg-DVPP from *Rhodobacter* exhibits maxima at shorter wavelength in the red region as well as in the blue. No differences can be observed between Chl *c** and Chl c_1. Chl c_2 is shifted to longer wavelengths, especially in the blue absorption and excitation maxima (Table I). Although

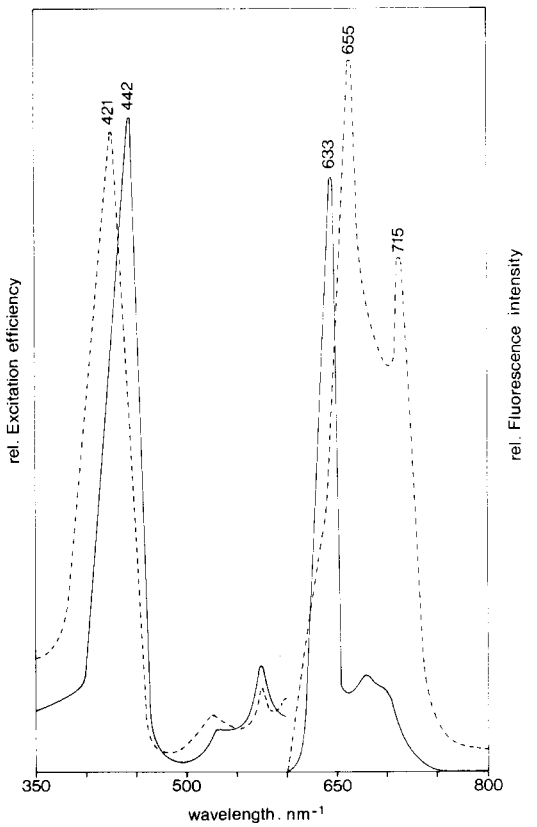

Fig. 3. Fluroescence excitation ad emission spectra of Chl c^* (solid line) and of pheoporphyrin c^* (dotted line) in acetone. In excitation spectra the emission wavelength was adjusted at 630 nm for Chl c^* and at 655 nm for pheoporphyrin c^*. In emission spectra the excitation wavelength was 440 nm for Chl c^* and 420 nm for pheoporphyrin c^*.

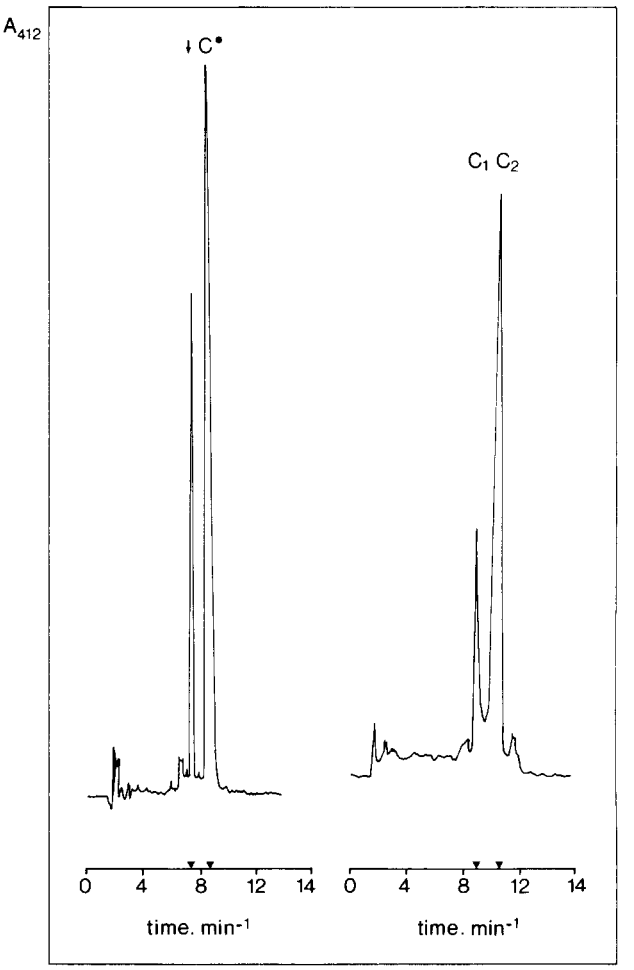

Fig. 4. HPLC scan of the pheoporphyrin c_1/c_2 methyl esters of the pheoporphyrin c^* methyl methyl ester. (For separation conditions see Materials and Methods.)

the differences between the pigments are not very drastic, the given data clearly refute the idea that the pigment from *Mantoniella* is the bacterial Mg-DVPP. The most important difference becomes obvious in the emission spectrum of the pheoporphyrins. Chl c_1 and Chl c^* emit at the same wavelength, but 10 nm longer than Mg-DVPP.

In order to verify the hypothesis that the Chl *c*-like pigment in *Mantoniella* is Chl c_1 and not Mg-DVPP, the pheoporphyrin *c* methyl esters were synthesized. Fig. 4 shows the chromatography of the pheoporphyrin c_1/c_2 methyl esters and from the respective pigment derived from *Mantoniella*. The retention times are indicated by arrows. The first fraction from Chl c^* is either an alteration product or an impurity, which was not separated by the reverse phase chromatography, or a true component of Chl c^*. Its amount varies from preparation to preparation, even from the same batch of cells. The second band comigrates with the methyl ester from Chl c_1. Their absorption spectra are identical.

Because no alga is known to contain only Chl c_1 without Chl c_2, a chromatographic system had to be developed to separate these two components. The reverse-phase systems do not have enough resolution when free acids need to be

TABLE I

THE ABSORPTION AND FLUORESCENCE MAXIMA OF Chl c_1, Chl c_2, Chl c* and Mg-DVPP AND THEIR Mg-FREE DERIVATES IN DIFFERENT SPECTROSCOPIC SYSTEMS

Pigments	Absorption (nm)												Fluorescence in ether (nm)	
	in 90% acetone			band ratios			in ether			band ratios			emission	excitation
	III	II	I	III	II	I	III	II	I	III	II	I		
Chl c_1	442	580	629	7.3	0.5	1	438	577	625	8.7	0.5	1	632	443
Chl c_2	450	584	629	8.8	0.8	1	445	577	628	9.3	0.9	1	632	446
Chl c*	439	578	628	7.4	0.5	1	438	574	625	9.5	0.6	1	631	441
Mg-DVPP	433	572	623	8.3	0.4	1	431	570	622	14	0.3	1	628	436
Pheoporphyrin c_1							421	568		11.3	1		655 718	422
Pheoporphyrin c_2							432	572		12.3	1		655 718	432
Pheoporphyrin c*							421	567		11.4	1		654 717	421
Pheoporphyrin Mg-DVPP							416	564		16.1	1		645 711	419

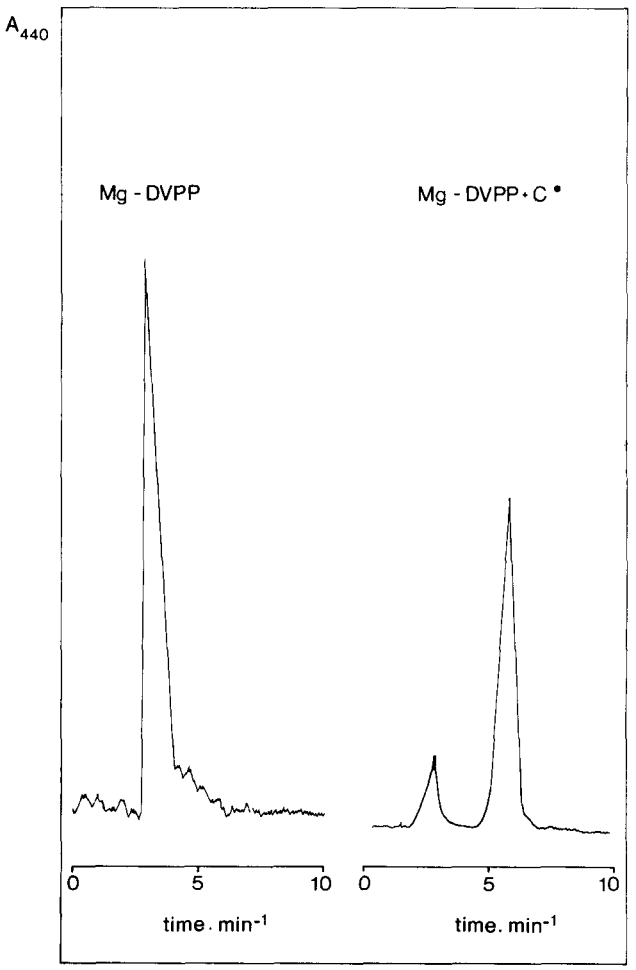

Fig. 5. HPLC scans of the cochromatography of Mg-DVPP and Chl c* in the RPX-system (see Materials and Methods).

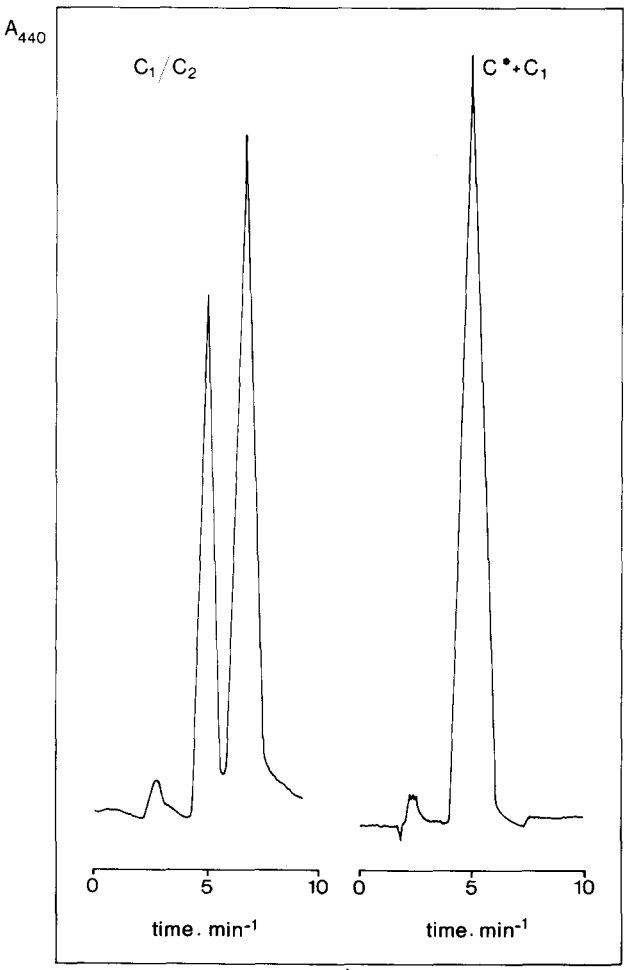

Fig. 6. HPLC scans of Chl c_1/c_2 and the cochromatography of Chl c* and Chl c_1 in the RPX-system (see Materials and Methods).

chromatographed. Therefore, polyethylene according to Jeffrey [9] was packed in a stainless-steel column and used as HPLC stationary phase. This system yields sufficient resolution and reproducibility to separate Chl c_1 and c_2 in sufficient amounts within 10 min. Chromatograms of mixtures of Chl c*/Chl c and Chl c*/Mg-DVPP were used to identify the different pigments by their retention times. Fig. 5 demonstrates that Mg-DVPP is clearly distinguished from Chl c*. The polyethylene chromatography resolves Chl c_1 and Chl c_2 as two distinct fractions, whereas Chl c_1 and Chl c* are eluted in a single symmetric band (Fig. 6). In accordance with all other results, Chl c* comigrates with Chl c_1, whereas Mg-DVPP is chromatographically different from the *Mantoniella* pigment.

Discussion

At the time Ricketts published his findings, that Chl c* from *Micromonas pusilla* was identical with Mg-DVPP, it was not necessary to differentiate the prasinophytes from green algae [4]. The Chl c-like pigment could be considered as an accumulated precursor of the catabolic porphyrin pathway. In the last few years it has been shown, however, that Chl c* participates in the energy transfer within the light-harvesting antenna in *Mantoniella* [6,710]. The pigment analysis of the isolated light-harvesting complex showed that this pigment is really protein-bound and must be considered as an integral component of the *Mantoniella* light-harvesting apparatus. In these studies, there was also clear evidence that this alga contains authentic Chl *a* and Chl *b* given by spectroscopical and chromatographical data [7].

Modern techniques of pigment separation reveal that Chl c is very sensitive to chemical degradation [9]. The altered products have very similar absorption spectra, but they can be differentiated by the infrared NMR fluorescence spectroscopy [11,12] and polyethylene chromatography [9]. Our results clearly show that doubly purified Chl c* from *Mantoniella* differs from Mg-DVPP chromatographically and spectrally (fluorescence), but that it is identical to Chl c_1 according to all criteria used. These differences and similarities are also observed in comparison of pheoporphyrin c methyl esters. There remains some doubt about the question if only Chl c_1 is present or if there is a mixture of at least two components in the Chl c* fraction running from reverse-phase chromatography. The chromatography of the pheoporphyrin c methyl esters reveals two components, but the identity of the second one is unclear. The preliminary results from NMR spectroscopy suggests two components, too. It can be suggested that the pigment which is purified only by reverse-phase chromatography is slightly contaminated. There is no doubt, however, based on its spectra that Chl c* has an acrylic side chain and contains authentic Chl c-1 (Wilhelm, C., unpublished results). Mg-DVPP differs chemically from Chl c_1 by the fact that it contains a propionic acid instead of an acrylic acid as side chain in position 7 of the ring IV, which can readily distinguished by NMR spectroscopy [13].

One could argue that the culture of *Mantoniella* used in this experiments was contaminated by chrysophytes or another yellow alga. However, the cultures used for the experiments were analyzed by electron microscopy to confirm their unialgality. Besides some bacteria no other species were found. In addition, the presence of prasinoxanthin is a good taxonomic marker for prasinophytes. This pigment would not be found if the culture were contaminated by green algae. If the culture were a mixture of green and yellow algae, lutein must be found, which was always absent. Finally, if a prasinophycean culture would be contaminated by a yellow or yellow-green alga, the appropriate xanthophylls like fucoxanthin had to be found.

The differences between these data and the first analysis by Ricketts cannot be explained by the different organisms [4]. In previous experiments we have analyzed the pigments from *Micromonas pusilla.* There are no differences in the pigment pattern between *Mantoniella* and *Micromonas* (data not shown). The only explanation is that modern techniques permit milder preparation and more sophisticated identification of the pigment studied.

If one accepts the evidences as stated here, a new view of algal systematics must be developed. The strong correlation between chloroplast ultrastructure and pigment pattern is no longer valid.

Hibberd and Norris [14] reported a new class of green alga which did not contain starch as storage product and exhibit more than two membranes of the chloroplast-surrounding envelope. This indicates also the independence of pigment composition and ultrastructure. *Mantoniella* is the first case of a Chl *c*-containing alga whose chloroplast is not surrounded by the endoplasmatic reticulum. In addition, the fact that Chl *b* and Chl *c* coexist in one chloroplast points out a new aspect in the hypothesis of endosymbiosis. In light of our results it is no longer necessary to demand a procaryotic Chl *c*-containing photosynthetic organism. It can be speculated that Chl *c*, or near derivatives like Mg-DVPP, are not only precursors in the biosynthesis of chlorophyll *b* [15], but also came first in evolution [16].

The present data reinforce the current tendency in algal systematics to integrate ultrastructural and biochemical features in defining the taxa. As a consequence, new combinations of fatures lead to the multiplication of taxonomic orders. This reflects the main problem of phycology: although our knowledge about many species has increased, our understanding of the true evolutionary path in algal development is still unresolved.

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