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AN INVESTIGATION OF THE CHLOROPHYLLS OF SELECTED PRASINOPHYTE ALGAE

A Thesis

Presented to the

Department of Biology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

University of Nebraska at Omaha

by

Leslie Carlat Kwasnieski

May, 1986

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THESIS ACCEPTANCE

Accepted for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree Master of Arts, University of Nebraska at Omaha.

Thesis Committee

Name	Department
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Eric R. Manley	Chemistry

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21 April 1986
Date

ACKNOWLEDGEMENTS

I would like to thank Dr. Barbara Hayhome for providing this project; it has helped me to realize that I should go through life without so many preconceived expectations. Also, a sincere thank you for helping me realize that it was time to get along with my life by putting this thesis on paper.

A very special thanks to Dr. Suzanne Moshier who saw the positive side of everything and helped me see it too, even when I really didn't want to. Thanks also to Dr. Eric Manley for the patience that he has shown me when he had to explain it "just one more time". I have learned more chemistry from you than I would ever admit to knowing. I would also like to thank Dr. Bob Egan for his support in printing this thesis and for being such a good friend.

I want to thank the spaghetti-night bunch (Pete, John, Mark, Connie, Jeanette) for helping me keep everything in perspective, that must have been difficult for you, since we all know what a low-key person I am. Special thanks to Connie who has been through thick and thin with me over the past few years, I wouldn't have wanted to do it without you.

My deepest appreciation goes to my husband Mark for being there every time I needed him, even when he probably should have been doing something else (like work). I also want to thank you for our two beautiful little girls, their kisses and hugs helped me over the rough times when you were on the road.

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INTRODUCTION

Chlorophyll a is the prevalent light-collecting pigment in eukaryotic, photosynthetic organisms. Several different chlorophylls exist in nature, but all autotrophic plants contain chlorophyll a (Bogorad, 1976). The majority of these chlorophyll a molecules function to absorb light and channel the excitation energy to photochemical sites in reaction centers. Chlorophylls b, c, d, and e, carotenoid pigments and phycobiliproteins are organized into antenna systems which absorb light maximally at different wavelengths. This design permits the visible light spectrum to be exploited (Foyer, 1984) for energy by different photosynthetic organisms. Chlorophyll b is present in higher plants, Chlorophyta, Prasinophyceae, and Euglenophyta; chlorophyll c is found in some members of Cryptophyceae, Dinophyceae, Rhaphidophyceae, Chrysophyceae, Haptophyceae, Bacillariophyceae, Xanthophyceae, and Phaeophyceae; chlorophyll d is found in some Florideophyceae in the Rhodophyta; and chlorophyll e has been found in feral populations of two members of Xanthophyceae. Chlorophyll e is believed to be a breakdown product of chlorophyll c (Meeks, 1974). Additional forms of chlorophyll, the bacteriochlorophylls, are found in bacteria other than the cyanobacteria (Holt, 1965).

The biosynthesis of chlorophyll is a complex process and only after years of probing have the structures of intermediates been identified. The availability of the ^{14}C isotope and the

introduction of chromatographic techniques in the 1940's were instrumental in elucidation of the pathway. Much of the information collected has been from experiments conducted using animal tissues or enzymes, with the assumption that the biosynthetic pathway to the heme molecule is identical in plants and animals. Early tracer experiments using ^{14}C concluded that the α-carbon and nitrogen of glycine, and the citric acid cycle intermediate succinyl coenzyme A join to form the five carbon molecule δ-aminolevulinic acid (ALA). Two ALA molecules condense to form the precursor pyrrole porphobilinogen (PBG). Four PBG molecules are joined by sequential head-to-tail addition to form uroporphyrinogen I (Urogen I), a cyclic tetrapyrrole. Urogen I side chains are modified by decarboxylation of the acetate side chains and oxidative decarboxylation of two specific propionic side chains, resulting in formation of a molecule of protoporphyrinogen IX (Protogen IX), into which magnesium ions are incorporated. Further alterations of side chains resulting in formation of the chlorophyll molecule include esterifications, reductions, formation of a cyclopentanone ring, and reduction of one pyrrole ring (Bogorad, 1976).

Chlorophyll a and b differ only at the third carbon on the second ring where the methyl group of chlorophyll a is replaced by an aldehyde group in chlorophyll b. The synthetic pathway of chlorophyll b remains a mystery, although four possible pathways have been suggested by investigators (Jones, 1968):

1. Each pigment is formed by a separate pathway.
2. Chlorophyll a is synthesized from previously produced chlorophyll b.
3. Both pigments are formed from the same precursor molecule in the same pathway.
4. Chlorophyll b is synthesized from previously produced chlorophyll a.

The first two of these possibilities are improbable and are not given serious consideration by investigators. At present there is no evidence that two separate biosynthetic pathways exist for the biosynthesis of chlorophyll a and b. It is not probable that chlorophyll a is synthesized from chlorophyll b since many plants contain only chlorophyll a and no chlorophyll b is present from which to synthesize chlorophyll a. It is possible that chlorophylls a and b have a common precursor; although that branch point could lie anywhere from glycine to chlorophyllide a. In higher plants, it seems most probable that chlorophyll b is formed from previously present chlorophyll a by oxidation of the methyl group. The actual mechanism is unknown, however it is believed to be similar to the oxidation of methyl groups as it occurs in sterol biosynthesis. It has been suggested that chlorophyllide a (a chlorophyll precursor lacking the phytyl group) is converted to chlorophyllide b by oxidation of the methyl group, which in turn is converted into chlorophyll b by addition of phytol (Shylk, 1971). However, chlorophyllide b has been isolated in only one

system. In Chlamydomonas reinhardtii y-1, a mutant strain that requires light for chlorophyll synthesis, 1,7-phenanthroline stimulated the conversion of protochlorophyllide to chlorophyllide b in cells that were kept in the dark. Bednarik and Hoober (1985) suggested that chlorophyllide a, when present at threshold levels, acts as a positive effector for conversion of protochlorophyllide directly into chlorophyllide b. Since synthesis of chlorophyllide b occurred in the dark and the presence of light caused synthesis of chlorophyllide a, chlorophyllide a is not seen as an intermediate.

Organisms that contain both chlorophylls a and b usually contain a much higher amount of chlorophyll a. The proportion of chlorophyll a and b is represented as an a:b ratio; the chlorophyll a:b ratio generally ranges from 2.1 to 3.1 in higher plants and freshwater green algae (Meeks, 1974). Marine macrophyte a:b ratios have been reported to be significantly lower, ranging from 1.3 to 2.2. Wood (1979) reported a:b ratios for 27 species of Prasinophyceae and Chlorophyceae. More than 60% of the a:b ratios were less than 2.0. It has been suggested that low a:b ratios are a common trend in all chlorophyll b-containing marine algae (Nakamura, et al., 1976). One Prasinophyte, Platymonas stipitata Rey 2 has been reported to contain 55% of its total chlorophyll (a+b) as chlorophyll b (Hayhome, et al., 1979).

The Prasinophyceae are a group of single-celled algae with a varied taxonomic history. However, most investigators place them in a separate class in the division Chlorophyta (Norris, 1980).

Their characteristics have led some investigators to suggest that a primitive scaly green flagellate belonging to this class may be ancestral to three major lines of evolution, the Chlorophyceae, Charophyceae, and Ulvaphyceae (Norris, 1980). Prasinophytes are flagellated, scale-covered cells that lack cellulose. The heart-shaped cells usually have four flagella that arise from an anterior pit and are covered with three layers of scales. Platymonas and Prasinocladus cells are surrounded by a theca that is made from a polysaccharide containing galactose and uronic acid subunits, other species may be scale-covered (Lee, 1980). Seen with a light microscope, the cells contain a single bowl-shaped chloroplast where thylakoids are stacked in lamellae in groups from two to five. This arrangement is seen in other members of the Chlorophyta, unlike higher plants which have lamellae stacked into grana. Prasinophyte cells have one nucleus near the flagellar base, an eyespot and pyrenoid located within the chloroplast, and starch granules. Unlike other green algae that store soluble photosynthates as sucrose, fructose, glycerol or glucose, the principle storage product in the Prasinophyceae is mannitol (Norris, 1980).

The purposes of my research were to extend the study of chlorophyll a:b ratios to previously uncharacterized prasinophytes and to examine the effects of certain chlorophyll biosynthesis inhibitors on one isolate of particular interest- Platymonas stipitata Rey 2. Previous work has shown this isolate to have the

lowest reported chlorophyll a:b ratio and, in cultural studies, to exhibit bleaching in the presence of glycerol (Hayhome, 1980).

MATERIALS AND METHODS

ISOLATES AND CULTURE METHODS

The following axenic prasinophyte clonal isolates were obtained from Dr. R. R. L. Guillard at the Bigelow Laboratory for Ocean Sciences in West Boothbay Harbor, Maine.

Platymonas stipitata Rey 2

Prasinophyte sp. unknown SL 48-23

Unidentified BT-5

Unidentified 1326-1

Platymonas svecica PS-305

Tetraselmis svecica UW 483

Tetraselmis sp. unknown UW 498

All isolates were grown in batch culture in diluted sterile natural sea water (0.357 Osm per ml) with F/2 enrichment (Guillard, 1975) at 21 C under a photoperiod of 18 hours of light and 6 hours of dark at *ca.* 50 $\mu E\ m^{-2}\ s^{-1}$ light intensity. Under this regime, cultures started by a 200 fold dilution of late log phase cells reached late log phase in 21 days (7.2×10^6 cells/ml). Cell numbers in cultures were determined by hemacytometer counts (mean of 8 counts for each sample) following immobilization by dilution into Transeau's solution (Tiffany, 1938).

PIGMENT EXTRACTION

Late log phase cells of all isolates were collected by centrifugation (1000 X g, 10 min) and stored at -20 C. For extraction the cells were thawed, covered with reagent grade methanol (Riemann, 1978) and placed in the dark at 4 C overnight (Hansmann, 1978). Pigments were extracted into reagent grade petroleum ether (30/60) and dried with a rotary evaporator. A sample of Platymonas stipitata Rey 2 was saponified (Davies, 1965) with 5% KOH to remove any chlorophylls, extracted and stored for analysis by high-pressure liquid chromatography. Due to the photosensitivity of chlorophyll pigments, all extractions were completed in a darkened room in the presence of a safelight with a Kodak # 7 green filter. All pigment extracts were stored under nitrogen in tightly sealed flasks, wrapped in foil, and kept at -20 C.

HIGH-PRESSURE LIQUID CHROMATOGRAPHY ANALYSIS OF CHLOROPHYLLS

High-pressure liquid chromatography (HPLC) was chosen over thin layer chromatography for separation of pigments and their analysis. HPLC cleanly separates the chlorophylls from each other and provides easily quantifiable results. The high-pressure liquid chromatography system used was a Beckman 112 solvent delivery system, Beckman 340 injector, Beckman 160 UV-visible detector with interchangeable filters, an Ultrasil 25 cm X 4.6 mm ODS C-18 Reverse Phase Column with a 75 mm X 7.5 mm guard column and a Hewlett Packard 3390A integrator. All HPLC work was done

with a 436 nm filter and a flow rate of 1.0 ml per minute. The mobile phase used by other researchers to separate chlorophylls and their degradation products are methanol:water (97:3) (Bessriere & Montiel, 1982), methanol:water (90:10, followed by 98:2) (Falkwoski & Sucher, 1981), methanol:acetone:water (75:22:3) (Goeyens, et al., 1982), and methanol:water (95:5) (Burke & Aronoff, 1979). Several concentrations of methanol, from 100% methanol to 98% methanol and 2% water were tested in an attempt to find the mobile phase that gave the best peak resolution and the shortest run time. The mobile phase chosen was 98% methanol and 2% water. All solvents were filtered and degassed through a millipore system with a 0.45 µm filter.

For all samples, small amounts of extracted pigments were taken up in 98% methanol and filtered through a 0.22 µm Durapore filter. Samples were diluted with solvent until they read approximately 0.6 absorbance at 436 nm on a spectrophotometer in a 1 cm cuvette. A spectrum was run for each sample from 350 to 700 nm. A 20 µl sample was injected onto the column. All samples were detected at 436 nm using a sensitivity of 0.005 absorbance units full scale. Chlorophyll a and b standards from spinach (Sigma Chemical Co., St. Louis, Mo.) were injected separately onto the column to identify the location of the chlorophyll a and b peaks. The saponified pigments of Platymonas stipitata Rey 2 were run then compared to a non-saponified sample of pigments from the same alga to verify that the chlorophylls were the only pigments being

detected at 18 and 32 minutes. For comparison, fresh spinach leaves were extracted and stored for later analysis by HPLC.

Published extinction coefficients for chlorophylls a and b are available for either acetone or ether (Svec, 1978), but not for 98% methanol. To be able to accurately calculate the chlorophyll amounts from the HPLC separation required the determination of extinction coefficients for a 98% methanol solution at 436 nm. Approximately 0.03 mg chlorophyll a standard was placed in a cuvette and diluted with 3.0 ml of peroxide-free ethyl ether (peroxides were removed by washing ether in water, then drying over calcium chloride). The absorbance was measured at 428nm, the absorption maximum for chlorophyll a in ether (Strain, 1963). The exact amount of chlorophyll a was then calculated using the published extinction coefficient of 11.2×10^4 M^{-1} cm^{-1} (Strain, 1963). The sample was evaporated under a stream of nitrogen. The chlorophyll was redissolved in 3.0 ml of 98% methanol. The absorbance was read at 436 nm, the wavelength of the detector filter. While 436 nm does not correspond to the absorption maximum for either chlorophyll a or b, it is a commonly available wavelength for a variety of filter selectable detectors. There is sufficient absorbance by both compounds at this wavelength that reasonable sensitivity is obtained. The extinction coefficient of chlorophyll a in 98% methanol was calculated to be 5.7×10^4 M^{-1} cm^{-1} at 436 nm.

This basic procedure was repeated for chlorophyll b. The samples were read at 452 nm (Strain, 1963) and the exact amount

was calculated using the published extinction coefficient of 15.9 X 10^4 M^{-1} cm^{-1} (Strain, 1963). The extinction coefficient for chlorophyll b in 98% methanol was calculated to be 4.2 X 10^4 M^{-1} cm^{-1} at 436 nm.

Pigment extracts from each of the seven prasinophyte species were loaded onto the column individually and the amount of chlorophyll a and b in each clonal isolate was calculated using the extinction coefficients from 98% methanol at 436 nm and the area percent figures from the integrator. The chlorophyll a:b ratio was calculated for each species. Five samples from one pigment extract of Platymonas stipitata Rey 2 were run separately on the column to verify reproducibility.

INHIBITION OF CHLOROPHYLL BIOSYNTHESIS

One isolate, Platymonas stipitata Rey 2, was selected for an inhibition study of chlorophyll biosynthesis because of its unusual chlorophyll a to chlorophyll b ratio. Five possible chlorophyll inhibitors were chosen from the literature for initial survey experiments. These included l-cysteine, thiophenol, p-chloromercuribenzoate (PCMB), α,α'-dipyridyl (dipyridyl), and glycerol. Thiophenol was used by Rao and Sane (1981) to inhibit chlorophyll formation in two places. Concentrations of 5.0 mM completely inhibited the formation of porphobilinogen and the conversion of chlorophyllide to chlorophyll in leaves of Vigna radiata. Rao et al. (1981) used 1.0 mM concentrations of dipyridyl to inhibit conversion of chlorophyllide to chlorophyll

in leaves of Arachis hypogaea. Gibson et al. (1958) used 0.3 μM PCMB to inhibit formation of δ-aminolevulenic acid in chicken erythrocytes. 0.035 M concentrations of l-cysteine (Granick, 1958) inhibit δ-aminolevulenic acid formation in chicken erythrocytes. Hayhome (1980) reported that 0.025 M concentrations of glycerol inhibited chlorophyll formation in Platymonas stipitata Rey 2 by unknown methods.

Each inhibitor was tested at 0.1 mM, 0.5 mM and 1.0 mM concentrations. The inhibitor was weighed and mixed with 3 ml of solvent (l-cysteine and glycerol in water, dipyridyl and thiophenol in 100% ethanol, and PCMB in dimethylsulfoxide). Inhibitors were then filtered through a 0.45 μm cellulose acetate filter and enough sterile medium was added to bring the volume up to 10 ml. Serial dilutions were made to achieve the above concentrations in 100 ml of sterile medium. Each dilution was inoculated with 10 ml of late log phase Platymonas stipitata Rey 2 and grown to late log phase. The number of cells/ml and the condition of the cells was determined every seven days by hemacytometer counts (mean of 8 counts for each sample). All inhibitors tested except l-cysteine suppressed the growth rate of the cells when compared to the control; l-cysteine apparently stimulated photorespiration causing the cells to have a shorter generation time than the control. From these results thiophenol, PCMB, dipyridyl and glycerol were chosen for further study as they supressed growth but did not adversely affect culture viability.

New concentrations for each of the four remaining inhibitors were chosen after analysis of the survey experiment results. Thiophenol and PCMB were tested the second time at 0.0001 mM, 0.001 mM, 0.01 mM and 0.1 mM; glycerol was tested at 0.0025 M, 0.025 M, and 0.25 M; and dipyridyl was tested at 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, and 1.0 mM. The number of cells/ml and the condition of the cells were recorded every seven days for three weeks. In addition, a 5.0 ml sample of each culture was pelleted (1000 X g, 10 min) and frozen. These samples were extracted with methanol and chlorophyll a and b amounts were estimated by measuring absorbance at 650 nm and 665 nm (Holden, 1976). After analysis of these results 0.25 M glycerol and 0.05 mM dipyridyl were chosen for further study of inhibition of chlorophyll b biosynthesis in Platymonas stipitata.

In the final inhibitor study, recovery from exposure to 0.25 M glycerol and 0.05 mM dipyridyl was monitored. Three liters of Platymonas stipitata Rey 2 was grown to late log phase and harvested by centrifugation (4000 X g, 10 min). These cells were divided into three aliquots and cultured for three weeks in 1000 ml of fresh F/2 medium alone, or media supplemented with either 0.25 M glycerol or 0.05 mM dipyridyl. After three weeks' incubation, the cells of each liter were harvested by centrifugation (4000 X g, 10 min). The batches of cells were each resuspended in 1000 ml of fresh medium not containing inhibitor and observed for cell morphology as they greened up over an 18 day period.

RESULTS

PIGMENT ANALYSIS BY HPLC

Based on earlier work by other investigators, I began using methanol:water (95:5) as the mobile phase for HPLC of pigment extracts from *Platymonas stipitata* Rey 2. Although this gave good peak resolution, each run took in excess of 60 minutes. In an attempt to shorten run time, I tried methanol with 0.0%, 0.25%, 0.5%, 0.75% and 2.0% water as the mobile phase. As table I shows, with 100% methanol, run time was shortened to 20 minutes. This system was inadequate, however, because resolution of the chlorophyll b peak at 11 minutes and an unknown peak at 12 minutes was not sufficient to allow reliable quantification by the integrator. Addition of 0.25%, 0.5%, or 0.75% water shortened run time but the resolution between the two peaks did not increase significantly. Two percent water in methanol gave a run time of 42 minutes and increased the resolution between the peaks sufficiently to allow reliable quantification by the integrator. All high-pressure liquid chromatography was completed subsequently using 2% water in methanol.

HPLC of the chlorophyll a standards from spinach produced a peak with a shoulder at approximately 33 minutes. Chlorophyll b standards from spinach produced a peak with a shoulder at approximately 18 minutes. As shown in table II, the chlorophyll a and b peaks from the standards correspond to peaks seen in

chromatograms from pigment extracts of the Prasinophytes examined. When standards were allowed to sit in ether for a period of time, degradation products were produced. When separated by HPLC, chlorophyll a breakdown products appeared at 24 and 37 minutes and chlorophyll b breakdown products appeared at 17 and 20 minutes. The specific identity of these degradation products is unknown.

Purification of chlorophyll was attempted using a method that involves precipitation of chlorophylls, leaving the carotenoid pigments in solution. Pigment extracts were mixed with reagent grade dioxane, then precipitated by drop-wise addition of water (Iriyama, et al., 1974). When precipitated chlorophyll was dissolved in 98% methanol and injected onto the column, several chlorophyll degradation products were found. In addition to the chlorophyll a and b peaks, eight unidentified degradation product peaks were seen. Due to the presence of so many impurities, this method was not used to purify chlorophyll extracts.

Figure 1 is a representative chromatogram of pigments from Platymonas stipitata Rey 2. Five runs of the same Platymonas stipitata Rey 2 pigment extract were run sequentially to verify reproducibility. In table III the variability between these five runs is shown. The chlorophyll b peak varied from 18.31 minutes to 18.64 minutes (0.33 min) and the percent of chlorophyll b as calculated from the area percent varied from 45.1% to 47.7% (2.6%). The chlorophyll a peak varied from 33.57 minutes to 34.40 minutes (0.83 min) and the percent of chlorophyll a varied from 52.3% to 54.9% (2.6%). Although there is a small difference

between these percentages, when they are used to calculate the a:b ratio, that difference is intensified. The average a:b ratio for Platymonas stipitata Rey 2 is 1.15, but the values range from 1.10 to 1.22. The chlorophyll a:b ratios (and percentages) for both Platymonas stipitata Rey 2 and the other Prasinophyte isolates are summarized in table IV.

For comparison, chlorophyll a:b ratios (and percentages) of the Prasinophytes were computed using spectral readings. Pigments were extracted into 100% methanol and readings were taken at 650 nm and 665 nm. These results are given in table V.

The chlorophyll a:b ratios (and percentages) derived by these two methods appear to differ. A comparison of the two sets of data in tables 4 and 5 reveals substantial differences for some specimens. Platymonas stipitata Rey 2 has a lower chlorophyll a:b ratio when the pigments were analyzed on the HPLC, more chlorophyll b was reported by that method than by spectral analysis. The unidentified Prasinophyte, BT-5 has similar chlorophyll a:b ratios as measured by both methods. For the five remaining algae, more chlorophyll b was reported by spectral measurements than by HPLC. Two Prasinophytes, SL 48-23 and the unidentified species 1326-1 have ratios that are comparable for both methods. The remaining three Prasinophyte species Platymonas svecica PS-305, Tetraselmis svecica UW 483, and Tetraselmis sp. UW 498 all had substantially higher chlorophyll a:b ratios as measured by HPLC. Because of these differences, pigment extracts from one selected isolate, Platymonas stipitata Rey 2 were

subjected to saponification. The rationale for this step was to degrade the chlorophylls present in extracts, leaving carotenoids intact to assess if there were carotenoid contributions to the HPLC peaks for chlorophyll a and b.

A chromatogram from the saponification of Platymonas stipitata Rey 2 pigments was compared to a chromatogram from non-saponified pigments of the same alga. Saponification removes all chlorophyll pigments from the extract by removing the phytyl tail that allows the chlorophyll to be sufficiently non-polar to dissolve in petroleum ether. Carotenoid pigments are non-saponifiable and thus remain soluble in petroleum ether. When saponified pigments were analyzed by HPLC, a peak at 18 minutes remained. This is the same location that the chlorophyll b peak occupies in the chromatogram from non-saponified extracts. This finding suggests the presence of a non-saponifiable pigment at 18 minutes, the same location as chlorophyll b. This pigment could be a carotenoid pigment or a unique form of chlorophyll b that is non-saponifiable.

To better characterize this unknown pigment a large amount of Platymonas stipitata Rey 2 pigment was injected onto the HPLC column. The complex peak at 18 minutes was collected and a spectrum was made from 350 to 700 nm. The spectrum was characteristic of both chlorophyll and carotenoid pigments. There was a single peak at 650 nm as in chlorophyll and a triple peak with maxima at 420, 440, and 475 nm as in a carotenoid pigment. Fractionation and spectral analysis of the components of this peak

at 18 minutes revealed a chlorophyll b peak, a phaeophytin b peak and a peak that might be a carotenoid. Separation of this carotenoid was not possible using 2% water in methanol as it was located directly under the chlorophyll b peak.

INHIBITION OF CHLOROPHYLL BIOSYNTHESIS

In the initial survey experiment, inhibitor concentrations of 0.1 mM, 0.5 mM, and 1.0 mM were chosen to test for chlorophyll biosynthesis inhibition. Each inhibitor was added to medium at these concentrations; cell numbers and condition were monitored for 14 days. As seen in table VI the addition of l-cysteine stimulated photorespiration, causing the cultures to grow at a rate faster than the control. Since l-cysteine did not have an inhibitory effect on the organism, it was discarded as a possible chlorophyll inhibitor. At the concentrations chosen thiophenol, p-chloromercuribenzoate (PCMB) and α,α'-dipyridyl (dipyridyl) were toxic and monitoring was discontinued at 7 days. Cultures containing glycerol grew at the same rate as the control with cells in all concentrations being motile, green and healthy.

These results were used to determine new concentrations for the second set of inhibitor studies with thiophenol, PCMB, dipyridyl and glycerol. The concentrations for glycerol were increased to 0.0025 M, 0.025 M, and 0.25 M; concentrations for thiophenol and PCMB were decreased to 0.0001 mM, 0.001 mM, 0.01

mM, and 0.1 mM; and concentrations for dipyridyl were decreased to 0.01 mM, 0.05 mM, 0.1 mM., 0.5 mM, and 1.0 mM.

Table VII shows that as the glycerol concentration increased, the rate of cell division decreased. At 0.25 M there was no increase in cell numbers. At a concentration of 0.0025 M the cells were green and motile, at 0.025 M the cells were a pale golden-green color and only a few cells were still motile. At 0.25 M concentrations of glycerol, all the cells were non-motile and golden in color. Cells appeared enlarged after 21 days.

Results from the PCMB inhibition experiment appear in table VIII. Concentrations of 0.0001 mM did not affect growth rate of Platymonas stipitata Rey 2 until the third week, when the growth rate dropped. Growth rates in the culture containing 0.001 mM PCMB were lower than the control. At both concentrations, the cells were green and motile at 14 days, but had acquired a green-brown color at 21 days. Concentrations of 0.01 mM and 0.1 mM were toxic, no living cells were seen at 7 days and the cells were discarded after 14 days. No bleaching of cells was seen with PCMB at these concentrations.

Results of the thiophenol inhibition experiment are given in table IX. Concentrations of 0.0001 mM did not affect the growth rate of Platymonas stipitata Rey 2 and the cells were green and motile. Concentrations of 0.001 mM and 0.01 mM slowed the growth rate of the alga by day 21, but the cells were still motile and green. Some clumping of cells was seen at 21 days at 0.01 mM concentrations. 0.1 mM concentrations of thiophenol were toxic,

all cells were dead by 7 days and the cultures were discarded after 14 days. No bleaching of cells was seen with thiophenol at these concentrations.

Results from the dipyridyl inhibition experiment are shown in table X. Concentrations of 0.01 mM did not inhibit the growth rate of Platymonas stipitata Rey 2; cells were green and motile. Cultures with a concentration of 0.05 mM dipyridyl did not show a decrease in growth rate or motility but by day 21 at this concentration, the cells were a golden-green color. Concentrations of 0.1 mM, 0.5 mM and 1.0 mM were toxic to the cells and were discarded after 14 days.

The total amount of chlorophyll per cell was estimated every seven days for each inhibitor concentration using spectral methods. These results are shown in tables XI-XIV. As seen in table XI, 0.025 M and 0.25 M concentrations of glycerol caused the level of chlorophyll in the cells to decrease significantly at 7 days. As shown in tables XII and XIII, PCMB and thiophenol did not decrease the amount of chlorophyll per cell. At 0.01 mM concentrations, as seen in table XIV, dipyridyl inhibited the amount of chlorophyll per cell at 21 days and at 0.05 mM concentrations the amount of chlorophyll was inhibited at 14 days. These results confirm visual observations that 0.25 M glycerol and 0.05 mM dipyridyl inhibit chlorophyll biosynthesis in Platymonas stipitata Rey 2.

In the final inhibition experiment, identical aliquots of cells from cultures of Platymonas stipitata Rey 2 were placed in

cultures containing either 0.25 M glycerol or 0.05 mM dipyridyl. These cells were recovered after 21 days and placed in fresh medium where their greening up was monitored for an 18 day period. The cells that had been in 0.25 M glycerol for three weeks were not motile when placed in fresh media; many of the cells were dead and those that were not were a golden color. There was no change in the cells until day 7 when a green band was noted around the top of the culture. By day 11 the cells had entered a stage of exponential growth that continued until day 18. The greened cells were enlarged. The cells that had been placed in 0.05 mM dipyridyl were a golden-green color, half of the cells were motile. Evidence of greening up was visible by day 3 when the cells entered a period of rapid growth that lasted until day 18.

DISCUSSION

Pigment analysis by high-pressure liquid chromatography was used to calculate chlorophyll a:b ratios in Platymonas stipitata Rey 2 and six other Prasinophytes. When variations were seen between ratios calculated by HPLC and spectral methods, pigments of Platymonas stipitata Rey 2 were saponified for comparison with a non-saponified extract. These comparisons indicated the presence of a non-saponifiable peak in the same location as chlorophyll b. The mobile phase used (98% methanol) did not separate chlorophyll b and the unknown pigment sufficiently to allow identification of the unknown pigment. Future analysis by this HPLC method should include a comparison of saponified and non-saponified pigment samples to detect the presence and amount of unknown pigments. In order to be precise, chlorophyll b levels must be calculated by subtraction of such pigments from the total chlorophyll b peak area.

When chlorophyll a:b ratios of the seven isolates calculated from HPLC and spectral measurements were compared, the ratios differed in some cases. In multiple runs from the same pigment sample of Platymonas stipitata, it was shown that chlorophyll a:b ratios vary as much as 0.2 within the same extract. Considering this variation, a difference in a:b ratios of similar magnitude between two completely different methods could not be viewed as extreme. Under this premise, Platymonas stipitata Rey 2, an

unknown Prasinophyte SL 48-23, and two unidentified isolates BT-5 and 1326-1 were shown by the two methods to contain similar amounts of chlorophyll a and b. The remaining three isolates: Platymonas svecica PS-305, Tetraselmis svecica UW 483, and an unidentified Tetraselmis species UW 498 were shown to have a substantially higher a:b ratio as measured by HPLC. It is interesting to note that the two genera Platymonas and Tetraselmis are considered by some investigators to be the same genus (Guillard, personal communication).

Five of the isolates had more chlorophyll b according to spectral measurement. It is widely accepted that chlorophyll a can be over-estimated by dichromatic measurements when degradation products are present in the pigment extracts (Goeyens, et al., 1982). These degradation products absorb at the same wavelengths as chlorophyll a and increase the apparent amount of chlorophyll a detected. It is possible that the presence of degradation products from chlorophyll b increased the amount of chlorophyll b detected spectrophotometrically in these five isolates. With the mobile phase chosen, the HPLC effectively separated chlorophyll degradation products from the chlorophylls, but separation of the carotenoid pigments presented some difficulty.

The need to prevent formation of chlorophyll degradation products cannot be overemphasized. Although the presence of some of these products is normal, steps must be taken not to increase the amounts of these products during processing of samples. These steps include protecting the extracts from light, oxygen and

changes in temperature. Despite efforts to reduce chlorophyll degradation, in extracts that were exposed to one or all of these factors the amount of degradation of the chlorophylls increased significantly; in some samples no chlorophyll b remained intact. Pigments that had been extracted, then frozen for later HPLC analysis showed a marked increase in production of degradation products when compared to pigments that were extracted and immediately injected onto the HPLC. Substances such as $CaCO_3$, $MgCO_3$, $NaHCO_3$ and dimethylaniline can be added to prevent formation of pheophytin during the extraction process (Holden, 1976). Acidification techniques used by investigators to account for the degradation products of chlorophyll a do not function similarly for chlorophyll b and should not be used (Lorenzen, 1981).

Degradation products can also be formed while the sample is on the column. For this reason, it is critical that the amount of time that the sample is on the column be minimized. In separations for chlorophyll a measurements, run times as short as 14 minutes have been achieved by changing the mobile phase from methanol:water (95:5) to methanol:acetone:water (75:20:5) and increasing the flow rate (Brown, et al., 1981). Further investigation into other mobile phase systems is necessary to achieve better resolution of the chlorophyll b peak. Perhaps the use of methanol:water (95:5) at a higher flow rate will increase resolution without sacrificing run time.

Inhibition of chlorophyll biosynthesis in Platymonas stipitata Rey 2 was achieved with 0.25 M glycerol and 0.05 mM α,α'-dipyridyl (dipyridyl). Thiophenol and p-chloromercuribenzoate (PCMB) did not inhibit chlorophyll biosynthesis in Platymonas stipitata Rey 2 at the concentrations surveyed. It is possible that these inhibitors are toxic to the organism at the level that is necessary to achieve inhibition of chlorophyll biosynthesis, and for this reason we did not see inhibition. L-cysteine apparently stimulated photorespiration in Platymonas stipitata Rey 2 at concentrations surveyed; perhaps higher levels would inhibit chlorophyll biosynthesis.

Inhibition of chlorophyll biosynthesis with dipyridyl has been shown during greening of etiolated Arachis hypogaea leaves (Rao, et al., 1981). The site of inhibition was found to be at the level of conversion of chlorophyllide a to chlorophyll a. The activity of dipyridyl, a metal ion chelator, was partially reversed by addition of cations. This inhibitor could be used to answer some questions about chlorophyll b biosynthesis in Platymonas stipitata Rey 2. Future studies on the inhibition of chlorophyll biosynthesis by dipyridyl in cells from Platymonas stipitata Rey 2 should include the monitoring of the cells to see when chlorophyll b is produced during the greening process. If chlorophyll b is produced first, this might indicate synthesis by an alternate pathway, possibly directly from protochlorophyllide a or from chlorophyllide a via chlorophyllide b. If chlorophyll a

is produced first, this would be consistent with synthesis of chlorophyll b from chlorophyll a.

High concentrations of glycerol have been shown to uncouple the energy transfer chain from phycoerythrin to chlorophyll a in Anacystis (Williams, et al., 1981). Thus, the inhibitory effect of glycerol on chlorophyll formation has been explained for organisms that contain phycobiliproteins. Such uncoupling may have the effect of inhibition of chlorophyll synthesis in Platymonas stipitata Rey 2. Although 0.25 M concentrations of glycerol do inhibit chlorophyll formation in Platymonas stipitata Rey 2 cells, whether that inhibition involves uncoupling is unknown at this time. Because the actual mechanism of chlorophyll biosynthesis inhibition by glycerol is potentially at a level well removed from the terminal synthetic steps, this inhibitor is not the best choice for biosynthesis studies. However, further study of this glycerol-induced chlorophyll inhibition would be interesting in Platymonas stipitata Rey 2 at another time.

Study of the effect of 1,7-phenanthroline on cells of Platymonas stipitata Rey 2 may prove to be worthwhile. In etiolated cells of the mutant Chlamydomonas reinhardtii y-1 it was established that 1,7-phenanthroline stimulated production of chlorophyllide b from protochlorophyllide in the dark without the formation of new chlorophyllide a (Bednarik & Hoober, 1985). A similar investigation in Platymonas stipitata Rey 2 could provide a clue to the biosynthetic relationship of chlorophyllide b to chlorophyllide a.

SUMMARY

Although chlorophyll a biosynthesis has been elucidated, the terminal steps for chlorophyll b biosynthesis are unknown. Some Prasinophyceae are reported to have high amounts of chlorophyll b (Wood, 1979) and the prasinophyte Platymonas stipitata Rey 2 is reported to have 55% of its chlorophyll as chlorophyll b, (Hayhome, 1979).

Pigment extracts from Platymonas stipitata Rey 2 and six other prasinophytes were analyzed by high-pressure liquid chromatography (HPLC) for their chlorophyll a:b ratios. These results were compared to chlorophyll a and b amounts calculated from dichromatic measurements taken spectrally (Holden, 1976). The chlorophyll a:b ratios from the two methods differ in some cases, possibly due to interference by chlorophyll degradation products or carotenoids. Saponification of pigments from Platymonas stipitata Rey 2 reveals the presence of an unidentifed pigment peak at the same location as chlorophyll b. With the mobile phase chosen HPLC effectively separates chlorophylls a and b, but separation of other pigments from chlorophylls a and b was not achieved.

Chlorophyll biosynthesis inhibitor studies with one selected isolate, Platymonas stipitata Rey 2, show that dipyridyl and glycerol are potentially useful as inhibitors of chlorophyll biosynthesis in prasinophytes.

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Figure 1. Chromatogram of methanol extracted pigments from Platymonas stipitata Rey 2 on an Ultrasil-ODS column (4.6 mm X 250 mm) using methanol:water (98:2).

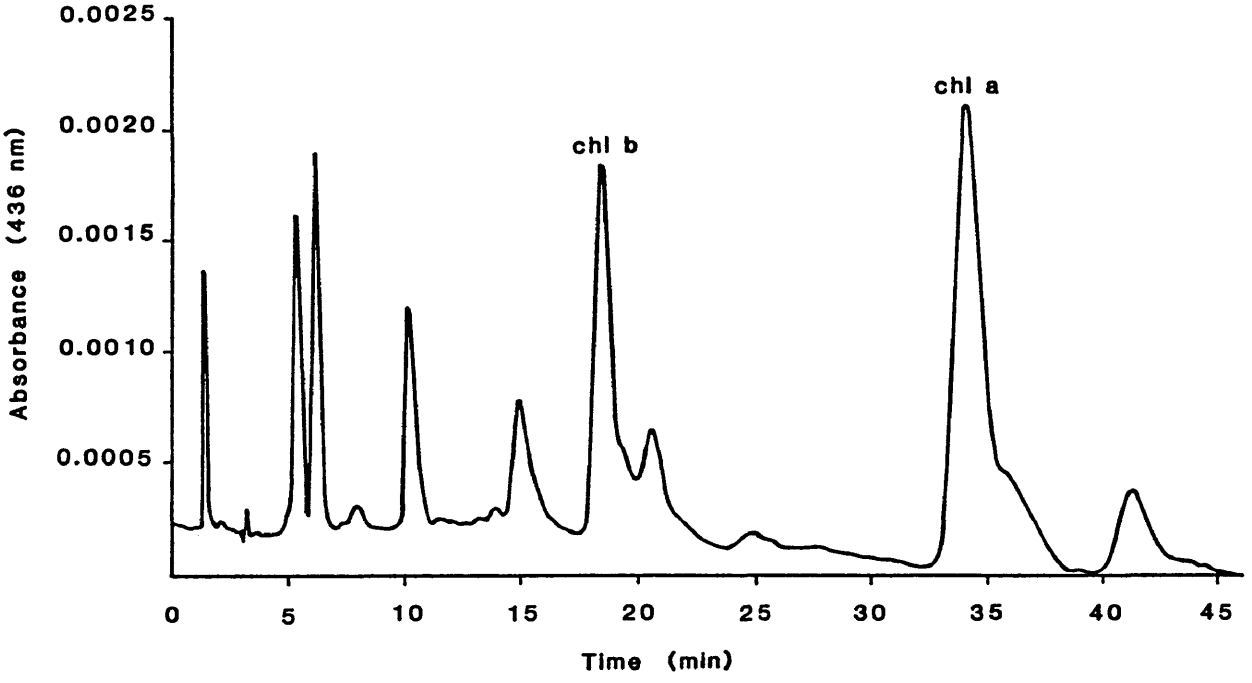

Table I. A comparison of solvent, run time and location of chlorophyll a and b peaks from high-pressure liquid chromatographic separation of Platymonas stipitata Rey 2 pigments.

SOLVENT SYSTEM	TOTAL RUN TIME	LOCATION OF CHL. A PEAK	LOCATION OF CHL. B PEAK
100% methanol	20 min	18.5 min	11.4 min *
0.25% water in methanol	27 min	20.7 min	12.5 min *
0.5% water in methanol	30 min	21.7 min	12.9 min *
0.75% water in methanol	32 min	23.1 min	13.6 min *
2.0% water in methanol	42 min	34.3 min	18.6 min

* These peaks were not resolved sufficiently from adjoining peaks to allow reliable quantification by the integrator.

Table II. A comparison of the chlorophyll a and b peak locations in chlorophyll a and b standards and in select Prasinophytes.

PRASINOPHYTE ISOLATE	CHL. A LOCATION (min)	CHL. B LOCATION (min)
Standards from spinach	33.6	18.4
Platymonas stipitata Rey 2	33.9	18.4
Prasinophyte sp. 48-23	33.9	18.5
Unidentified BT-5	34.0	18.5
Unidentified 1326-1	33.1	18.2
Platymonas svecica PS-305	33.1	18.1
Tetraselmis svecica UW 483	34.7	18.9
Tetraselmis sp. UW 498	33.1	18.1

Table III. A comparison of the location of chlorophyll a and b peaks, chlorophyll a:b ratios and % chlorophyll a and b in five sequential runs from the same pigment sample of Platymonas stipitata Rey 2.

RUN #	CHL. A LOCATION (min)	CHL. B LOCATION (min)	%A	%B	A:B RATIO
1.	34.40	18.64	54.1	45.9	1.18
2.	33.57	18.33	54.9	45.1	1.22
3.	34.09	18.53	54.0	46.0	1.17
4.	33.58	18.34	52.3	47.7	1.10
5.	33.59	18.31	52.8	47.2	1.11

Table IV. Chlorophyll a:b ratios and % chlorophyll a and b of several Prasinophytes as calculated by high-pressure liquid chromatography.

ORGANISM	CLONE	A:B	%A	%B
Platymonas stipitata	Rey 2	1.16	53.6	46.4
Prasinophyte sp.	SL 48-23	1.22	55.4	44.6
Unidentified	BT-5	1.63	61.5	38.5
Unidentified	1326-1	1.50	59.7	40.3
Platymonas svecica	PS-305	2.57	72.2	27.8
Tetraselmis svecica	UW 483	1.86	64.7	35.3
Tetraselmis sp.	UW 498	2.57	72.2	27.8

Table V. Chlorophyll a:b ratios and % chlorophyll a and b of several Prasinophytes as calculated by spectral measurements at 650 and 665 nm. in 100% methanol.

ORGANISM	CLONE	A:B	%A	%B
Platymonas stipitata	Rey 2	1.52	60.4	39.6
Prasinophyte sp.	SL 48-23	0.96	49.1	50.9
Unidentified	BT-5	1.70	62.9	37.1
Unidentified	1326-1	1.16	53.7	46.3
Platymonas svecica	PS-305	1.68	62.6	37.4
Tetraselmis svecica	UW 483	1.16	53.8	46.2
Tetraselmis sp.	UW 498	1.50	60.0	40.0

Table VI. Average cells/ml of initial inhibitor survey experiment with Platymonas stipitata Rey 2.

INHIBITOR		CONCENTRATION	#CELL/ML
control*	day 7		6.60×10^6
	day 14		3.90×10^6
l-cysteine	day 7	0.1 mM	1.48×10^6
		0.5 mM	1.08×10^6
		1.0 mM	2.08×10^6
	day 14	0.1 mM	4.58×10^6
		0.5 mM	5.50×10^6
		1.0 mM	3.40×10^6
thiophenol	day 7	0.1 mM	2.30×10^5
		0.5 mM	3.10×10^5
		1.0 mM	3.00×10^5
PCMB	day 7	0.1 mM	2.10×10^5
		0.5 mM	1.80×10^5
		1.0 mM	2.40×10^5
α,α'-dipyridyl	day 7	0.1 mM	1.50×10^5
		0.5 mM	2.80×10^5
		1.0 mM	2.80×10^5
glycerol	day 7	0.1 mM	6.50×10^5
		0.5 mM	8.40×10^5
		1.0 mM	1.40×10^5
	day 14	0.1 mM	4.60×10^5
		0.5 mM	4.00×10^5
		1.0 mM	2.60×10^5

* All cultures contained 2.37×10^4 cells/ml at inoculation.

Table VII. Average cells/ ml from glycerol-inhibited cultures of Platymonas stipitata Rey 2.

CONCENTRATION	DAY 7	DAY 14 (all values X 10^6)	DAY 21
control[#]	1.18 (0.82-1.44)*	5.25 (4.52-5.96)	4.65 (3.72-5.28)
0.0025 M	1.31 (1.04-1.56)	2.58 (1.68-3.40)	3.65 (2.68-4.18)
0.025 M	0.30 (0.22-0.34)	0.43 (0.36-0.53)	0.63 (0.26-0.40)
0.25 M	0.24 (0.16-0.27)	0.23 (0.16-0.29)	0.24 (0.12-0.35)

[#] All cultures contained 4.96 X 10^5 cells/ml at inoculation.
* Range

Table VIII. Average cells/ml of p-chloromercuribenzoate-inhibited cultures of Platymonas stipitata Rey 2.

CONCENTRATION	DAY 7	DAY 14 (all values X 10^6)	DAY 21
control[#]	3.69 (3.44-4.16)*	4.07 (2.92-4.58)	6.17 (4.32-8.08)
0.0001 mM	3.89 (3.10-4.22)	4.66 (3.84-5.48)	4.38 (2.50-5.14)
0.001 mM	2.15 (1.76-2.70)	3.81 (3.12-5.00)	2.22 (1.76-3.14)
0.01 mM	0.44 (0.33-0.51)	0.34 (0.22-0.47)	
0.1 mM	0.32 (0.20-0.35)	0.28 (0.18-0.38)	

[#] All cultures contained 4.24 X 10^5 cells/ml at inoculation.
* Range

Table IX. Average cells/ml of thiophenol-inhibited cultures of Platymonas stipitata Rey 2.

CONCENTRATION	DAY 7	DAY 14 (all values X 10^6)	DAY 21
control[#]	3.69 (3.44-4.16)[*]	4.07 (2.92-4.58)	6.17 (4.32-8.08)
0.0001 mM	1.42 (1.40-2.02)	4.15 (3.30-5.22)	5.83 (5.16-6.36)
0.001 mM	2.35 (1.94-2.82)	4.41 (3.56-4.94)	5.06 (3.60-5.80)
0.01 mM	2.23 (1.46-3.28)	2.85 (2.02-3.60)	4.28 (3.68-4.90)
0.1 mM	0.20 (0.17-0.36)	0.15 (0.11-0.19)	

[#] All cultures contained 4.24 X 10^5 cells/ml at inoculation.
[*] Range

Table X. Average cells/ml from α,α'-dipyridyl-inhibited cultures of Platymonas stipitata Rey 2.

CONCENTRATION	DAY 7	DAY 14 (all values X 10^6)	DAY 21
control[#]	2.11 (2.02-2.30)*	5.23 (3.52-6.38)	4.20 (3.16-5.00)
0.01 mM	2.83 (2.30-3.14)	5.30 (4.94-5.90)	6.70 (5.44-8.08)
0.05 mM	2.38 (2.24-2.48)	4.70 (3.22-6.12)	5.21 (4.36-6.08)
0.1 mM	0.18 (0.11-0.18)		
0.5 mM	0.30 (0.16-0.43)		
1.0 mM	0.42 (0.38-0.48)		

[#] All cultures contained 4.0 X 10^5 cells/ml at inoculation.
* Range

Table XI. The total amount of chlorophyll per cell in glycerol-inhibited Platymonas stipitata Rey 2 cells.

CONCENTRATION	DAY	pg CHL/CELL
control	7	1.15
	14	0.92
	21	1.28
0.0025 M	7	2.00
	14	1.67
	21	0.99
0.025 M	7	0.16
	14	0.08
	21	0.19
0.25 M	7	0.15
	14	0.08
	21	0.19

Table XII. The total amount of chlorophyll per cell in p-chloromercuribenzoate-inhibited Platymonas stipitata Rey 2 cells.

CONCENTRATION	DAY	pg CHL/CELL
control	7	0.68
	14	0.95
	21	0.53
0.0001 mM	7	0.58
	14	0.77
	21	0.67
0.001 mM	7	0.82
	14	0.92
	21	1.32

Table XIII. The total amount of chlorophyll per cell in thiophenol-inhibited Platymonas stipitata Rey 2 cells.

CONCENTRATION	DAY	pg CHL/CELL
control	7	0.68
	14	0.95
	21	0.53
0.0001 mM	7	0.16
	14	0.77
	21	0.54
0.001 mM	7	0.95
	14	0.75
	21	0.70
0.01 mM	7	0.94
	14	1.10
	21	0.65

Table XIV. The total amount of chlorophyll per cell in α,α'-dipyridyl-inhibited Platymonas stipitata Rey 2 cells.

CONCENTRATION	DAY	pg CHL/CELL
control	7	1.35
	14	1.11
	21	0.63
0.01 mM	7	1.10
	14	0.72
	21	0.32
0.05 mM	7	2.00
	14	0.68
	21	0.80