

Nutritional Regulation of Organelle Biogenesis in *Euglena*

REPRESSION OF CHLOROPHYLL AND NADP-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE SYNTHESIS¹

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ABSTRACT

Nitrogen deficiency and the presence of specific organic carbon sources prevent chloroplast development in *Euglena*. In exponentially growing cultures, chlorophyll levels were low and independent of the nitrogen content of the growth medium. Chlorophyll levels increased in stationary phase and the amount of chlorophyll formed was proportional to the initial nitrogen content of the growth medium; the greater the concentration of nitrogen, the greater the amount of chlorophyll synthesized during stationary phase. Washing experiments demonstrated that the major nutritional factor inhibiting chlorophyll synthesis in stationary phase cultures grown on medium containing a high carbon to nitrogen ratio was the absence of nitrogen rather than the presence of utilizable organic carbon.

The light-induced synthesis of chlorophyll and of NADP-glyceraldehyde-3-phosphate dehydrogenase was inhibited when acetate or ethanol was added at the time of exposure of dark-grown resting cells to light. Malate addition, however, stimulated chlorophyll and enzyme synthesis. Both cell number and total cell protein increased after ethanol, acetate, or malate addition, indicating that the resting cells were not nitrogen-deficient. Ethanol and acetate specifically repress light-induced chlorophyll synthesis. NADP-glyceraldehyde-3-phosphate dehydrogenase synthesis was inhibited at a time, the first 24 hours of light exposure, when chlorophyll synthesis was unaffected by carbon addition.

In addition to being photoregulated, chloroplast development is nutritionally regulated. Chl and chloroplast lipid synthesis in *Euglena* was repressed when cells were grown in the light on medium containing low amounts of utilizable N (18). When *Euglena* was grown in the dark on medium containing a high C/N², starved and exposed to light, Chl synthesis was repressed (1, 14, 25). Normal Chl synthesis was restored when a utilizable N source was added to the growth medium (14, 25). Since N-reversible repression was obtained with acetate, ethanol, succinate, malate, glucose, or fructose as the sole C source, Harris and Kirk (14) concluded that N-reversible repression is a function of the C/N of the growth medium rather than a specific metabolic effect of the C source.

Recent studies suggest that under appropriate conditions, specific C sources may repress Chl synthesis in *Euglena* independently of cellular N status. Ethanol (12, 25) and glucose (25) repressed Chl synthesis whereas succinate (12) had little effect. Ethanol repression was reversed by N addition; glucose repression was not (25). Other laboratories have failed to obtain repression with

glucose, and glucose-supplemented cultures have been routinely used in studies of chloroplast development (4, 26). Here, we identify the conditions required to obtain ethanol- and acetate-specific repression of the light-induced synthesis of Chl and of NADP-glyceraldehyde-3-P dehydrogenase. Ethanol is shown to inhibit the synthesis of NADP-glyceraldehyde-3-P dehydrogenase at a time, 0-24 h of development, when there is little inhibition of Chl synthesis. Cellular N status rather than C/N is also shown to be the critical factor regulating Chl synthesis in stationary phase cultures. A preliminary report of this work has appeared (23).

MATERIALS AND METHODS

Euglena gracilis Klebs var. *bacillaris* Cori maintained in the dark for many years (obtained from Dr. J. A. Schiff, Brandeis University) was used throughout this work. Cultures were routinely maintained in the light or the dark on Difco *Euglena* broth. Studies of the dependence of Chl synthesis on the C/N were performed with cells grown on pH 6.8 medium (7) using ethanol or Na-acetate as the sole source of C and NH₄Cl as the sole N source. Cells were rested at pH 6.8 (15). Conditions for cell growth and induction of chloroplast development (15, 27), the preparation of resting cells (15), the technique for washing cells (24), the determination of cell number (28), and the determination of Chl content (27) have been described. Total cell protein was determined by mixing an aliquot of cells with an equal volume of cold 10% trichloroacetic acid. After standing overnight, the precipitated proteins were recovered by centrifugation, the protein pellet was dissolved in a known volume of 0.1 M NaOH, and total protein was determined colorimetrically (17) using BSA as a standard. Activity of NADP-glyceraldehyde-3-P dehydrogenase was determined as described previously (9). Enzyme activity in crude extracts is stable for over 3 h (9). For all determinations, replicate samples differ by less than 10%. The data presented are the results from a typical experiment and each experiment has been repeated several times.

RESULTS

Dependence of Cell Number and Chl Content on N Content of Growth Medium. The final cell yield and total Chl/cell in stationary phase cultures grown with ethanol as the sole C source were directly proportional to the initial N content of the growth media (Fig. 1). Below an initial N concentration of 14.8 mM NH₄Cl, growth was N-limited and ceased, depending on N concentration, 7-11 days after inoculation. Growth was C-limited (supplied as ethanol, 84 mM initial concentration) when the initial NH₄Cl concentration was greater than 14.8 mM (Fig. 1, data not shown). At the onset of stationary phase, 7 days after inoculation, total cellular Chl content was proportional to the initial N content of the growth medium (Fig. 1). Between 7 and 11 days after inoculation, cellular Chl content increased. By 11 days, C-limited cells contained similar amounts of Chl. Chl/cell was only dependent

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² Abbreviation: C/N: carbon to nitrogen ratio.

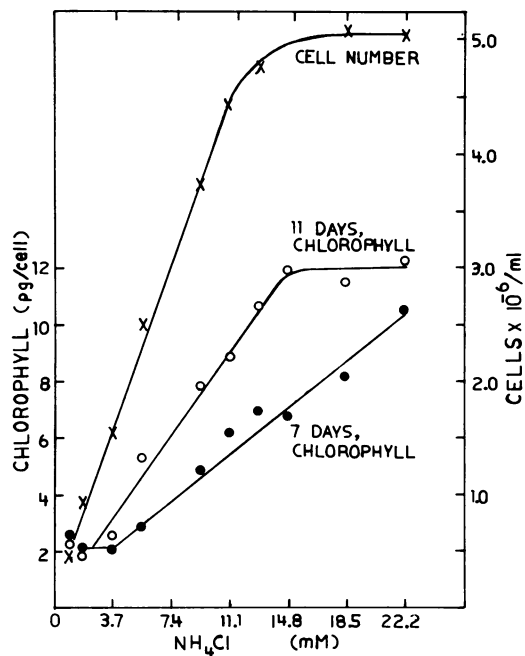


FIG. 1. Dependence of cell number and Chl content on the initial concentration of NH_4Cl . Cultures were inoculated to a final density of approximately 4×10^4 cells/ml into medium containing 84 mM ethanol and increasing amounts of NH_4Cl as the sole source of N. The cultures were incubated under constant illumination. Chl/cell was determined 7 days, or 11 days after inoculation. Cell number 11 days after inoculation is also shown.

on the initial N content of the growth medium when growth was limited by N (Fig. 1). Comparable results were obtained with acetate-butanol medium (data not shown).

Chl Synthesis during Growth on Medium Containing a High or Low C/N Ratio. Previous studies have concluded that the nutritional repression of Chl synthesis in *Euglena* is a function of the C/N of the growth medium (14). To determine whether cellular N status rather than the C/N of the growth medium is the factor regulating cellular Chl levels, the kinetics of Chl synthesis during unrestricted (exponential) growth (0–5 days) and stationary phase (after 5 days) was compared in cells growing on medium containing a high or low C/N with ethanol as the sole source of C. Growth on both media was unbalanced with respect to Chl synthesis; total cellular Chl increased and subsequently decreased during unrestricted growth (Fig. 2). The Chl content of the exponentially growing cells was relatively independent of the C/N (Fig. 2). The doubling time on both media was 12 h. In stationary phase, Chl content was dependent upon the initial C/N. In cells grown with a low C/N, Chl/cell increased 3-fold during stationary phase while remaining constant in cells grown with a high C/N (Fig. 2).

Nutritional Limitation of Chl Synthesis During Stationary Phase. The nutritional factor limiting Chl synthesis in stationary phase cultures grown with a high C/N was identified by washing and resuspending stationary phase high C/N cultures in fresh resting medium and determining the effect of the addition of ethanol, NH_4Cl , or ethanol and NH_4Cl , on Chl synthesis. Cells receiving no addition or 84 mM ethanol synthesized little Chl during the subsequent 144 h of incubation in the light (Fig. 3). NH_4Cl addition, however, resulted in rapid Chl synthesis (Fig. 3) and by 144 h, total cellular Chl had increased 4-fold. From 12 to 144 h after nutrient addition, cells supplemented with both ethanol and NH_4Cl synthesized 30% less Chl than cells supplemented only with NH_4Cl . Thus, ethanol partially inhibits the N-stimulated synthesis of Chl. Cell number remained constant throughout the experimental period indicating that a nutrient other than C or N

limited cell division but not Chl synthesis. The amount of Chl/cell was therefore an accurate indicator of the Chl-synthetic capacity of the culture.

Ethanol- and Acetate-specific Repression of Light-induced Chl Synthesis. Studies with cells grown under continuous illumination indicated that both the absence of a utilizable N source as well as the presence of ethanol can repress Chl synthesis. Repression due to N deficiency cannot be readily distinguished from metabolic

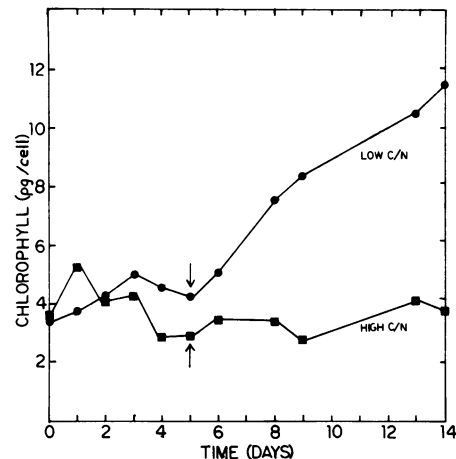


FIG. 2. Chl synthesis during growth in the light on medium containing a high or low C/N. Stationary phase cells grown under continuous illumination on media containing a high C/N (84 mM ethanol, 1.9 mM NH_4Cl) were inoculated to a final density of approximately 4×10^4 cells/ml into fresh growth medium containing a high C/N or a low C/N (84 mM ethanol, 13 mM NH_4Cl). Cells were incubated under continuous illumination and at various times, samples were removed for the determination of total cell number and Chl. Arrows indicate the beginning of stationary phase.

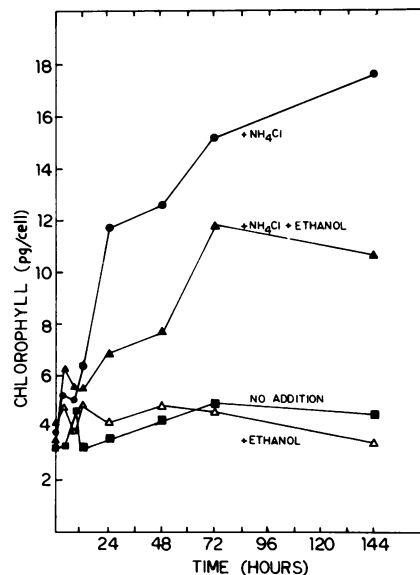


FIG. 3. Nutritional requirements for Chl synthesis in stationary phase cells grown in the light on medium containing a high C/N. Cells were grown to stationary phase under continuous illumination on medium containing a high C/N (84 mM ethanol, 1.9 mM NH_4Cl). Eleven days after inoculation, the cultures were washed and resuspended in resting medium containing no addition; 84 mM ethanol; 13 mM NH_4Cl ; or 84 mM ethanol and 13 mM NH_4Cl . The cultures were incubated under continuous illumination and at various times, samples were removed for the determination of cell number and total Chl. Zero time is the time of resuspension in fresh resting medium.

effects of the C source. We have, therefore, turned to the dark-grown resting cell system (20, 27) to characterize metabolic (ethanol) repression of chloroplast development. In resting cells, intracellular reserves are mobilized to provide the carbon and energy required by the developing chloroplast (8, 11, 22); development does not require nutrients supplied by the resting medium (24).

The kinetics of Chl synthesis in ethanol-supplemented, acetate-supplemented, malate-supplemented, and unsupplemented dark-grown resting cells exposed to light is shown in Figure 4. Acetate and ethanol repressed Chl synthesis whereas malate had little effect. Ethanol was a more effective repressor than acetate. It should be noted that the lag period of Chl synthesis (0–12 h) was relatively unaffected by any of the compounds tested. This insensitivity does not appear to be due to the failure of ethanol to enter immediately and be metabolized by the cells since repression was not significantly enhanced by incubation with ethanol prior to light exposure even though a significant synthesis of paramylum, storage carbohydrate, occurred during the preincubation period (data not shown).

The addition of acetate, ethanol, or malate to dark-grown resting cells promotes cell division (Table I). To assess the effects of specific C sources on Chl synthesis independently of the effects of these C sources on cell division, we determined the amount of Chl synthesized per ml culture in unsupplemented and C-supplemented resting cells (Fig. 5). The addition of malate to the resting medium stimulated the synthesis of Chl while ethanol and acetate

repressed Chl synthesis (Fig. 5). Ethanol was a more effective repressor than acetate. The decreased Chl content of ethanol- and acetate-supplemented cultures represents a true metabolic repression of Chl synthesis rather than an artifact caused by a stimulation of cell division and thus, a decrease in the amount of Chl/cell as compared to a nondividing population.

The stimulation of Chl synthesis by malate supplementation suggested that the dark-grown resting cells were not N-deficient. To verify this, we determined the effect of C supplementation on two N-dependent culture parameters, cell number and total cell protein, after 72 h in the light or the dark (Table I). Cell number, protein/ml and protein/cell, remained relatively constant in the nondividing unsupplemented resting cells (Table I). Addition of acetate, ethanol, or malate to the resting medium induced cell division and a net increase in protein/ml culture. There was, however, an appreciable decrease in the amount of protein/cell indicating that growth of C-supplemented resting cells was unbalanced with respect to protein synthesis. Based on g atoms of C added, ethanol was more effective than acetate or malate in stimulating cell division. The nature of the C source rather than N availability appears to be the nutritional factor limiting cell division and the net synthesis of proteins in resting cultures.

Ethanol- and Acetate-specific Inhibition of Light-induced NADP-Glyceraldehyde-3-P Dehydrogenase Synthesis. Ethanol specifically inhibited the light-induced synthesis of NADP-glyceraldehyde-3-P dehydrogenase at a time when Chl synthesis was unaffected by ethanol (Fig. 6). Exposure of dark-grown resting *Euglena* to light produced in the presence or absence of malate a

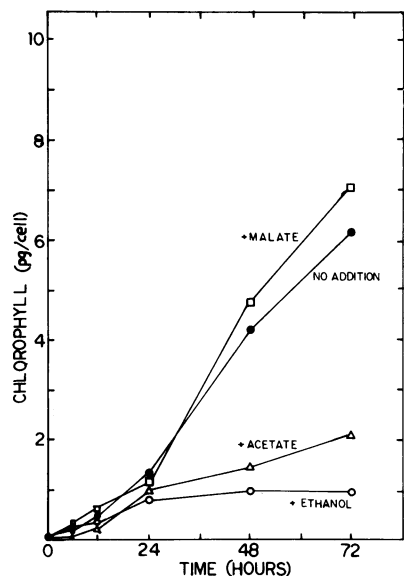


FIG. 4. Ethanol repression of Chl synthesis in dark-grown resting *Euglena* exposed to light. At the time of exposure to light (zero time) the following compounds were added to the resting medium: no addition; 60 mM malate; 84 mM acetate; 84 mM ethanol; samples were removed at appropriate times for the determination of Chl/cell.

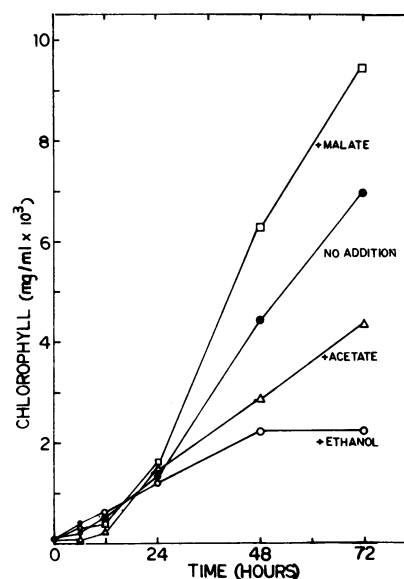


FIG. 5. Ethanol repression of Chl synthesis in dark-grown resting *Euglena*. Experimental conditions are identical to Figure 4. Results are expressed as Chl/ml culture. At the time of exposure to light, all cultures had the same cell density.

Table I. Cell Number and Protein Content of Carbon-supplemented Resting *Euglena* Exposed to Light

Additions to Media	Number			Protein/ml Culture			Protein/per Cell		
	0 h	72 h L ^a	72 h D ^b	0 h	72 h L	72 h D	0 h	72 h L	72 h D
	cells/ml × 10 ⁻⁵			mg/ml			ng/cell		
None	2.86	3.11	3.19	0.10	0.11	0.11	0.36	0.34	0.33
84 mM acetate	2.82	6.94	5.94	0.09	0.17	0.18	0.33	0.24	0.30
84 mM ethanol	2.88	9.19	9.24	0.09	0.16	0.18	0.32	0.17	0.19
40 mM malate	2.84	5.39	6.59	0.09	0.14	0.15	0.31	0.27	0.23

^a L: light.

^b D: dark.

7-fold increase in the specific activity of NADP-dependent glyceraldehyde-3-P dehydrogenase (Fig. 6 and Table II). In all cultures maintained in the dark, the specific activity of NADP-dependent glyceraldehyde-3-P dehydrogenase was unaltered over a 72-h period (Fig. 6 and Table II).

The addition of ethanol to the resting medium inhibited the light-induced increase in the specific activity of glyceraldehyde-3-P dehydrogenase (Fig. 6 and Table II). After 72 h of light exposure, there was less than a 2-fold increase in specific activity in ethanol-supplemented cultures (Fig. 6 and Table II). Ethanol inhibition of the light-induced increase in enzyme activity was apparent at a time 0–24 h after light exposure (Fig. 6) when little inhibition of Chl synthesis was observed (Fig. 5). Mixing experiments using extracts obtained from ethanol-supplemented and unsupplemented cultures which had been exposed to light for 48 h indicated that this decreased enzyme activity was not due to the presence in the cell-free extracts of enzyme inhibitors or inactivators.

The basal, dark, synthesis of NADP-glyceraldehyde-3-P dehydrogenase was relatively unaffected by ethanol and acetate (Table II). Although ethanol, acetate, and malate supplementation induced cell division and a net synthesis of protein, the specific enzyme activity remained relatively constant in the dark over a 72-h period (Fig. 6 and Table II). The amount of enzyme/cell was also relatively unchanged by carbon supplementation (Table II). The amount of enzyme/ml culture was, however, increased by carbon supplementation (Table II). The uninduced or basal synthesis of the enzyme in the dark was proportional to the synthesis of total cell protein and the extent of cell division, resulting in the maintenance of a relatively fixed amount of enzyme/cell.

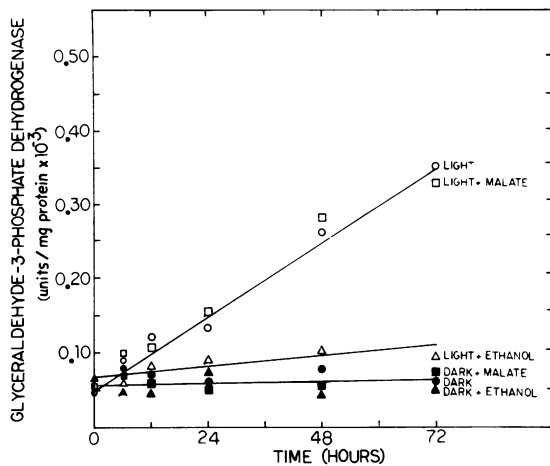


FIG. 6. Ethanol repression of light-induced synthesis of NADP-glyceraldehyde-3-P dehydrogenase. At zero time, the following compounds were added to dark-grown resting cells and the cultures were incubated in the light or the dark: no addition; 84 mM malate; 84 mM ethanol. Samples were removed at appropriate times and the specific activity of NADP-glyceraldehyde-3-P dehydrogenase was determined.

In the light, the enzyme/cell and enzyme/ml culture as well as the specific enzyme activity increased approximately 7-fold above the dark basal level in malate-supplemented and unsupplemented cultures (Table II). Exposure of ethanol-supplemented cultures to light for 72 h resulted in less than a 2-fold increase in enzyme as calculated from enzyme/cell, enzyme/ml, or specific activity (Table II). Acetate also inhibited enzyme synthesis but as found for Chl synthesis, was a less effective inhibitor than ethanol (Table II). Thus, ethanol and acetate specifically inhibit the light-induced synthesis of glyceraldehyde-3-P dehydrogenase.

DISCUSSION

This study has shown that N deficiency and the metabolism of specific C sources can independently repress light-induced Chl synthesis in *Euglena*. Cells growing exponentially on medium containing a high or low C/N contained comparable amounts of Chl throughout exponential growth. When cell division ceased, Chl synthesis continued at a rate which was dependent upon N availability; the greater the amount of N available, the greater the amount of Chl formed during stationary phase. Late stationary phase cells thus contained Chl levels ranging from the minimum level found in N-deficient cells to the maximum level found in N-sufficient cells.

Stationary phase high C/N cells washed and resuspended in N-containing C-free medium synthesized Chl while Chl levels were unchanged in cells resuspended in C-containing N-free medium. The absence of N rather than the presence of residual C limits the synthesis of Chl. Repression of Chl synthesis by growth on medium containing a high C/N, preadaptation, apparently results from the inability of N-deficient cells to synthesize Chl during stationary phase, the time at which previous studies with preadapted cells were performed (1, 14, 25). With preadapted cells, effects of specific carbon sources on the synthesis of Chl are masked by the effect of N deficiency on Chl synthesis.

Dark-grown resting cells exposed to light develop chloroplasts by mobilizing intracellular reserves to supply the molecules required for the synthesis of chloroplast components (5, 8, 11, 20, 22, 24). The addition of ethanol or acetate to the resting medium at the time of light exposure repressed Chl synthesis. The addition of malate, however, stimulated Chl synthesis. Malate, ethanol, or acetate addition caused an increase in cell number and total cellular protein—culture parameters whose increase is dependent upon N availability indicating that C-supplemented resting cells are not N-deficient. The inhibition of Chl synthesis by ethanol and acetate is a specific metabolic effect due to the presence of the C source rather than the absence of N. The ability of ethanol addition to inhibit Chl synthesis in exponentially growing, N-sufficient cells exposed to light (unpublished results) is a further indication that ethanol repression of Chl synthesis is unrelated to the inhibition of Chl synthesis produced by N deficiency.

The stimulation of Chl synthesis by malate disagrees with studies which found that malate repressed Chl synthesis (1, 14). This discrepancy may stem from the failure of previous studies to

Table II. Activity of NADP-Glyceraldehyde-3-P Dehydrogenase in Carbon-supplemented Resting *Euglena* Exposed to Light

Additions to Media	nunits ^a /Cell			munits/ml Culture			Specific Activity (munits/mg protein)		
	0 h	72 h L ^b	72 h D ^c	0 h	72 h L	72 h D	0 h	72 h L	72 h D
None	4.7	36.6	6.3	5.8	50.1	8.3	0.04	0.28	0.06
84 mM acetate	5.8	14.9	6.5	8.6	36.4	17.9	0.04	0.14	0.06
84 mM ethanol	11.7	3.0	6.1	9.0	7.5	17.6	0.08	0.07	0.03
60 mM malate	6.3	43.9	6.6	9.6	70.7	17.2	0.05	0.33	0.07

^a One unit = 1 μ mol NADPH oxidized/min.

^b L: light.

^c D: dark.

distinguish between repression due to N deficiency and repression due to the metabolism of the organic C source. Malate as well as glucose and succinate were found to inhibit Chl synthesis in cells preadapted to the C source (1, 14, 25). Inhibition was reversed by the addition of a utilizable N source (14, 25) suggesting that inhibition was due to N deficiency rather than the metabolism of a specific organic C source. As we have found with malate, glucose and succinate did not inhibit Chl synthesis when they were added to resting cells at the time of light exposure (8, 12). Dark-grown resting cells supplemented with ethanol at the time of light exposure are thus the system of choice for studies of metabolic (ethanol) repression of Chl synthesis. Cells preadapted by growth on the repressing C source can not be used since repression due to N deficiency cannot be distinguished from repression due to the metabolism of the C source.

NADP-glyceraldehyde-3-P dehydrogenase is a light-induced, nuclear-coded, cytoplasmically synthesized, chloroplast-localized enzyme (2, 3, 20). The addition of acetate or ethanol to resting cells at the time of light exposure inhibited enzyme induction. Malate addition had no effect on the light-induced increase in the specific activity of this enzyme. Detailed action spectra indicate that the conversion of Pchl(ide) to Chl(ide) is the major photochemical event regulating enzyme induction (9, 10). Recent studies with mutants, however, suggest that NADP-glyceraldehyde-3-P dehydrogenase synthesis is jointly regulated by Pchl(ide) and the blue absorbing nonchloroplast photoreceptor of *Euglena* (19, 21). The photoconversion of either receptor appears to be sufficient for at least partial enzyme induction (19, 21). Since ethanol almost totally inhibited the light-induced synthesis of NADP-glyceraldehyde-3-P dehydrogenase at a time 0–24 h after light exposure, when at least one of the photochemical reactions, Pchl(ide) photoconversion (9, 10), as well as the net synthesis of Chl, was unaffected by ethanol treatment, it appears that ethanol inhibits enzyme induction at a site other than the photochemical reactions regulating enzyme induction. The induction by ethanol of malate synthetase and fumarase (unpublished results) and the dark synthesis of NADP glyceraldehyde-3-P dehydrogenase by ethanol-supplemented cultures indicates that ethanol is specifically inhibiting light-induced enzyme synthesis; ethanol addition does not result in a general nonspecific inhibition of cellular protein synthesis.

Euglena assimilates ethanol by converting it to acetate which is then converted to succinate and malate through the operation of the glyoxylate cycle (13). Previous studies have shown that *Euglena* can assimilate ethanol more effectively than acetate (6, 16). The molar growth yield on ethanol is almost twice that found with acetate (16). More ethanol C is assimilated than acetate C and the addition of ethanol can increase the amount of acetate C assimilated (6). We found that 84 mM ethanol is a more effective metabolic repressor of chloroplast development and inducer of cell division and protein synthesis than 84 mM acetate. With higher levels of acetate, greater repression was obtained (data not shown). Taken together, these results suggest that the actual repressor of chloroplast development is a metabolite produced during ethanol and acetate assimilation; the decreased repression obtained with acetate resulting from decreased intracellular levels of the actual repressor. Since succinate (12) and malate do not repress Chl development, the actual repressor is most likely either a primary metabolite produced during the conversion of acetate to succinate, probably glyoxylate, or a molecule whose intracellular level is influenced by glyoxylate levels. Since malate, as well as acetate and ethanol can be assimilated to paramylum (6, 12) and since light induces paramylum degradation (8, 22) we do not believe that the repressor is a glycolytic intermediate although this possibility is not completely eliminated by our results. Although the actual repressor of chloroplast biogenesis must still be identi-

fied, we have shown that ethanol repression of chloroplast biogenesis may be a useful tool for studying the reactions linking the chloroplast photoreceptor and the biosynthetic events controlled by that photoreceptor.

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