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Events Surrounding the Early Development of *Euglena* Chloroplasts

V. CONTROL OF PARAMYLUM DEGRADATION[1]

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ABSTRACT

The degradation of the storage carbohydrate, paramylum, is induced by light in wild-type *Euglena gracilis* Klebs var. *bacillaris* Pringsheim and in a mutant, W$_3$BUL, which lacks detectable plastid DNA. Treatment of wild type with cycloheximide in the dark produces 60% as much paramylum breakdown as light, whereas treatment with levulinic acid in the dark yields a slightly greater response than light. Both cycloheximide and levulinic acid produce a greater paramylum breakdown in the light than they do in the dark. Treatment of W$_3$BUL with levulinic acid in darkness produces a larger paramylum degradation than light, with values similar to wild type in the light. Treatment of W$_3$BUL with cycloheximide induces paramylum degradation in darkness, and as with wild type, light is slightly stimulatory in the presence of both cycloheximide or levulinic acid. Streptomycin brings about only a very small amount of paramylum breakdown in the dark and only slightly inhibits breakdown in the light. Thus paramylum breakdown induced by light does not require the synthesis of proteins on cytoplasmic or plastid ribosomes. A model which explains these results postulates the existence of a protein which inhibits paramylum breakdown. When the synthesis of this protein is prevented either by light, cycloheximide, or by levulinic acid acting as a regulatory analog of delta amino levulinic acid, paramylum breakdown takes place. Because levulinic acid is a better inducer than light in W$_3$BUL, W$_3$BUL may not be able to form as much delta amino levulinic acid in light as wild type. The small amount of induction by streptomycin is viewed as a secondary regulatory effect attributable to interference with plastid protein synthesis which affects regulatory signals from the plastid to the rest of the cell.

Chloroplast development in *Euglena* requires the participation of both the chloroplast and nonchloroplast compartments of the cell (25, 29). The control of these compartments seems to involve separate photoreceptors, the plastid compartment being regulated by a red-blue photoreceptor similar or identical to PChl(ide) (13) whereas the nonchloroplast compartment(s) are regulated by a photoreceptor absorbing mainly in the blue region of the spectrum (12, 27). Among the processes which have been shown to be under light control and required in the nonchloroplast portion of the cell is the aerobic metabolism of paramylum which is necessary to supply energy and carbon for plastid development (9, 10, 14, 19, 25). In this paper we show that light can be replaced by CEX[4], a specific inhibitor of cytoplasmic protein synthesis (1, 18), or by LEV, a structural analog of ALA (2, 23), the precursor of tetrapyrroles such as Chl. These results suggest the participation of certain proteins and ALA in the regulation of the nonchloroplast contribution to chloroplast development.

MATERIALS AND METHODS

Euglena gracilis Klebs var. *bacillaris* Pringsheim maintained in the dark for many years and a mutant W$_3$BUL derived from this strain in which plastid DNA and most chloroplast structures are undetectable were used in the experiments to be described (11).

Cells were grown on a rotary shaker in the dark at 26 C on the pH-3.5 medium of Hutner (16) in 2 liter flasks containing 1 liter of medium. Resting cells were prepared by mixing 200 ml of a late log-phase culture with 500 ml of resting medium (33). In experiments with LEV, pH 5 resting medium was used (33) but in all other experiments pH 6.8 resting medium was used (17). After 3 days on the resting medium, cell division ceased (33) and the cultures were exposed to light (zero time in all experiments) for the initiation of plastid development. Cells maintained on resting medium for up to 30 days show little loss of viability (33). In all experiments, aseptic conditions were maintained and, up to the point of light exposure, the cells were kept in the dark and transferred under dim green safelights (24).

Optimal light for chloroplast development was supplied from a bank of alternating 20 w General Electric daylight and red fluorescent tubes with an apparent intensity of 150 ft-c measured normal to the surface of the cultures with a Weston Sunlight illumination meter (Model 756). The conditions for preillumination and continuous illumination are the same as described previously (32).

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[4] Abbreviation: CEX: cycloheximide; ALA: aminolevulinic acid; LEV: levulinic acid; SM: streptomycin.

A stock solution of CEX containing 1 mg/ml was prepared in distilled H_2O and was sterilized by filtration. CEX was added to yield a final concentration of 12 μg/ml. The stock solution of SM was prepared as described previously (3) and was added to the cultures to yield a final concentration of 0.05% (w/v) SM-free base; cells were preincubated with SM for 12 hr before light exposure. A 3 M stock solution of LEV was prepared in pH 5 resting medium and was adjusted to a final pH of 4.8 with KOH. This solution was sterilized by filtration and was added to a final concentration of 30 mM.

Determination of Cellular Paramylum Content. Paramylum was purified by a modification of the technique of Freyssinet *et al.* (14). At appropriate times during development, approximately 10^7 cells were recovered by centrifugation and resuspended in approximately 1 ml of culture medium. The actual number of cells in the sample was determined with the aid of a Coulter counter as described previously (35). The resulting cell suspension was frozen in a Dry Ice-acetone bath and the frozen samples were stored at −20 C for the subsequent determination of paramylum.

Paramylum was purified by resuspending the frozen cells in a solution containing 1% (w/v) ethanol-recrystallized SDS and 5% (w/v) Na_2EDTA. This suspension was incubated for 30 min at 37 C and the paramylum granules were recovered by centrifugation for 10 min at 1000*g*. The SDS-Na_2EDTA treatment was repeated and the paramylum was washed twice with hot, 70 C, glass-distilled H_2O. After the second wash, the paramylum granules were dried overnight at 90 to 95 C.

The dried paramylum was resuspended in a known volume of 1 N NaOH and assayed colorimetrically using the anthrone reagent as described by Cook (6). A series of glucose standards were included in each assay and a correction factor of 0.88 was applied to the A_{630} of the paramylum solutions prior to determining the paramylum concentration from the glucose standard curve in order to correct for the increased color developed by paramylum (6). The results represent the average of duplicate samples which were purified and assayed separately.

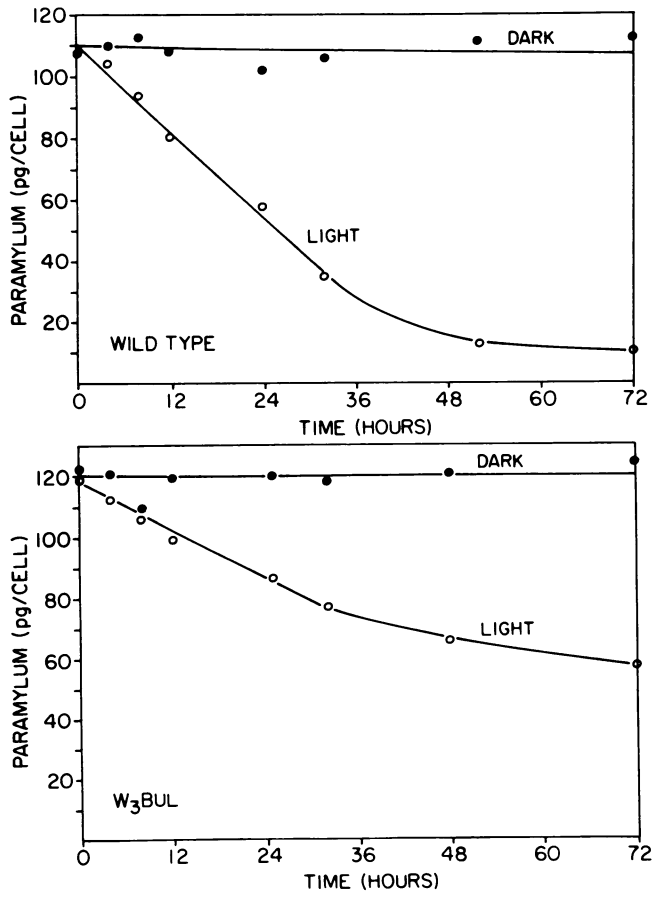

FIG. 1. Kinetics of light-induced paramylum degradation in wild type cells of *Euglena* and in the mutant, W_3BUL, which lacks detectable plastid DNA. At 0 time, dark-grown resting cells were exposed to continuous illumination or incubated in the dark and at appropriate times samples were withdrawn for the determination of paramylum.

RESULTS AND DISCUSSION

Kinetics of Light-induced Paramylum Degradation in Resting Wild-type *Euglena gracilis* var. *bacillaris* and Mutant W_3BUL. Figure 1 shows that paramylum breakdown in resting wild type cells is strictly light dependent and is linear for about 32 hr. These kinetics are somewhat different from those described by other workers using the Z strain and different experimental conditions, inasmuch as these workers found that paramylum degradation was virtually complete by about 24 hr of light exposure (9, 14).

In experiments using the inhibitor of photosynthesis, DCMU, the onset of inhibition in the formation of Chl has been shown to occur at about 24 hr of development in the Z strain (9, 23) but at about 48 hr in *bacillaris* (26). This inhibition is probably attributable to the limited availability of reduced carbon. It is interesting that the onset of this inhibition correlates with the point at which paramylum degradation is complete. Light-induced respiration is required for chloroplast development (19). Thus, it seems likely that photosynthetic carbon reduction becomes developmentally important only after the paramylum reserves have been respired away.

Two hours of preillumination and a 12-hr dark period before exposure of dark-grown resting cells to continuous light eliminate the usual 12-hr lag in Chl synthesis (17). Paramylum degradation occurs during the 2-hr preillumination period and continues for only the first 2 hr of the dark period after which it ceases. After the dark period upon reexposure to light, the rate of paramylum degradation in preilluminated cells is similar to the rate in cells which have not been preilluminated (data not shown). Thus paramylum degradation is a light dependent process in *bacillaris* as it is in the Z strain (9, 14). The paramylum degraded during the preillumination period may provide the source of carbon and energy for the biosynthetic processes occurring during the dark period in the absence of respiration (19) and which are required for the development of the potential for rapid Chl synthesis (29).

Figure 1 shows that light induces paramylum breakdown in the mutant W_3BUL, which lacks detectable chloroplast DNA and PChl(ide) (11). The magnitude of the breakdown is not as great as in wild-type cells (Fig. 1) but the kinetics are similar. These results suggest that the breakdown of paramylum is under control of the nonplastid photoreceptor and that the events involved occur external to the plastid. Inasmuch as less paramylum is degraded in the mutant, the presence of a plastid may be necessary to provide regulatory interactions or a sink for paramylum degradation products to sustain a more complete breakdown of the polysaccharide. Experiments with levulinic acid, however, (see below) offer an alternative explanation.

Influence of Inhibitors of Protein Synthesis on Paramylum Degradation. Figure 2 shows that CEX, a specific inhibitor

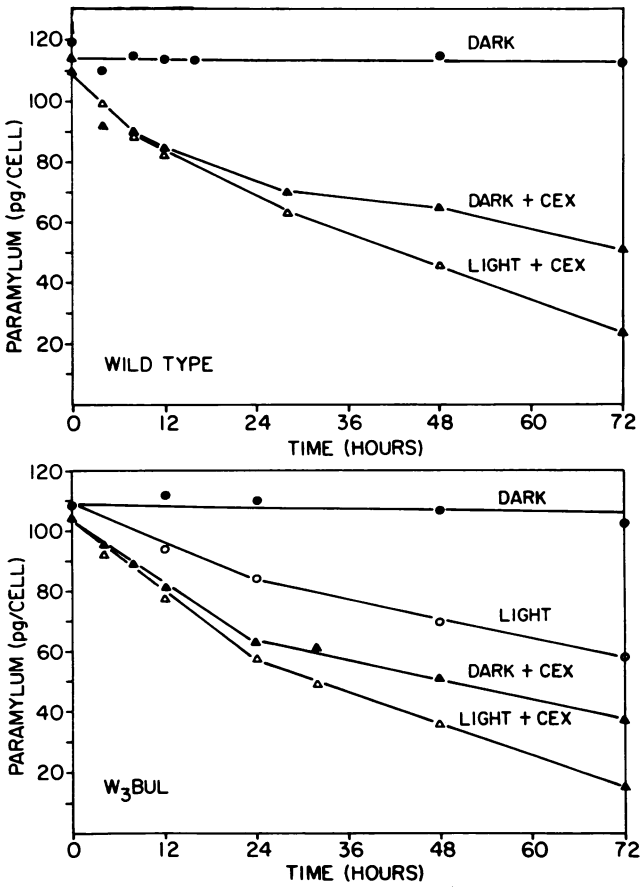

FIG. 2. Kinetics of cycloheximide-induced paramylum degradation in wild type cells, and in the plastidless mutant, W_3BUL. At 0 time, cycloheximide was added to the appropriate cultures of dark-grown resting *Euglena* to give a final concentration of 12 μg/ml. The cells were exposed to continuous illumination or incubated in the dark and at appropriate times samples were withdrawn for the determination of paramylum.

of protein synthesis on the 89S cytoplasmic ribosomes of *Euglena* (1, 18), does not block the breakdown of paramylum; indeed it causes the breakdown of the polysaccharide in the dark, replacing light as an inducer both in wild type and W_3BUL. The CEX-induced rate in W_3BUL is actually higher than the light-induced rate in untreated cells. After 12 to 24 hr of CEX treatment, the rate of paramylum degradation is higher in the light than in the dark.

Because CEX does not inhibit respiration in *Euglena* (18), these results suggest that a protein(s) is ordinarily synthesized which inhibits the breakdown of paramylum in the dark. Light prevents the production or action of this protein while cycloheximide prevents its synthesis. The result is that either light or cycloheximide induce the degradation process. This protein could act directly upon the enzymes of the paramylum degradation pathway.

Figure 3 shows that treatment with SM, a specific inhibitor of translation of 68S plastid ribosomes in *Euglena* (3, 30) produces only a very small amount of paramylum breakdown in the dark and very little inhibition of the light-induced degradation. The lack of inhibition is consistent with the nonchloroplast localization of the degradation process implied by the results of the experiments with mutant W_3BUL. The SM induction of paramylum breakdown in the dark is probably a secondary effect of SM treatment. The plastid seems to control paramylum degradation indirectly as is also the case for cytoplasmic RNA turnover (5, 36) and the turnover of cytoplasmic proteins (15, 28). Inhibition of plastid protein synthesis by SM may interfere with this regulatory system resulting in an increased rate of paramylum breakdown in the dark. The 32-hr lag period for SM-induced paramylum breakdown in the dark (Fig. 3) and the failure of mutant W_3BUL (which lacks many plastid localized functions) to degrade paramylum in the dark are consistent with the view that proteins synthesized on plastid ribosomes do not directly control paramylum degradation.

Induction of Paramylum Degradation by Levulinic Acid. LEV, a competitive inhibitor of ALA dehydratase in *Euglena* (23), can also replace light as an inducer of paramylum degradation (Fig. 4). Wild type cells treated with LEV in the dark or the light degrade approximately the same amount of paramylum during the first 12 hr of treatment (Fig. 4). During this time, cells exposed to light degrade less paramylum than LEV-treated cells. After 12 hr of treatment, the LEV-induced rate of paramylum degradation is higher in the light than in the dark.

LEV also induces paramylum breakdown in mutant W_3BUL (Fig. 4). The LEV-induced rates of paramylum breakdown are the same during the first 12 hr of treatment in the light or the dark. After this time, cells treated with LEV in the light degrade paramylum faster than cells treated with LEV in the dark. The rate of paramylum degradation in LEV-treated cells is, however, higher than the light-induced rate (Fig. 4). The ability of LEV to restore the rate and the extent of paramylum degradation in W_3BUL to the wild-type light-induced rate and extent (*cf.* Figs. 1 and 4) indicates that LEV is a more effective inducer of paramylum breakdown than light.

The induction of paramylum breakdown in the plastidless mutant W_3BUL indicates that paramylum breakdown may be regulated outside of the plastid, although nuclear-coded, cytoplasmically translated chloroplast enzymes may be present at levels comparable to dark-grown wild type (25). Because ALA dehydratase is a plastid-localized enzyme (4), it is unlikely that LEV induces paramylum breakdown by inhibiting this enzyme although it is possible that there is enough de-

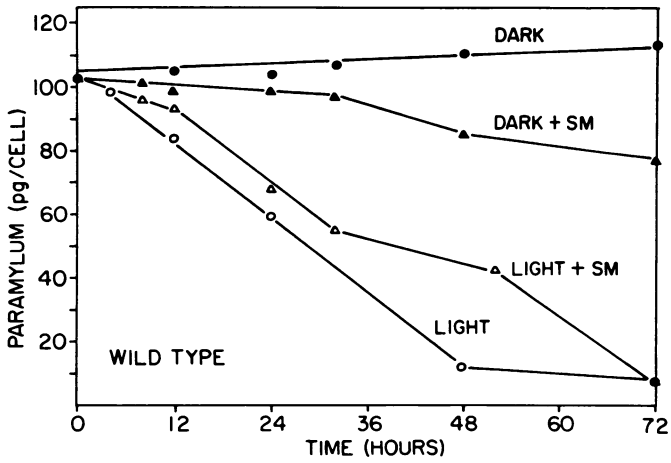

FIG. 3. Kinetics of light-induced paramylum degradation in wild type *Euglena* treated with streptomycin. Twelve hours prior to light exposure, streptomycin was added to the one culture of darkgrown resting *Euglena* to give a final concentration of 0.05% (w/v). At 0 time, both treated and untreated cells were exposed to continuous illumination or were incubated in the dark and at appropriate times samples were withdrawn for the determination of paramylum.

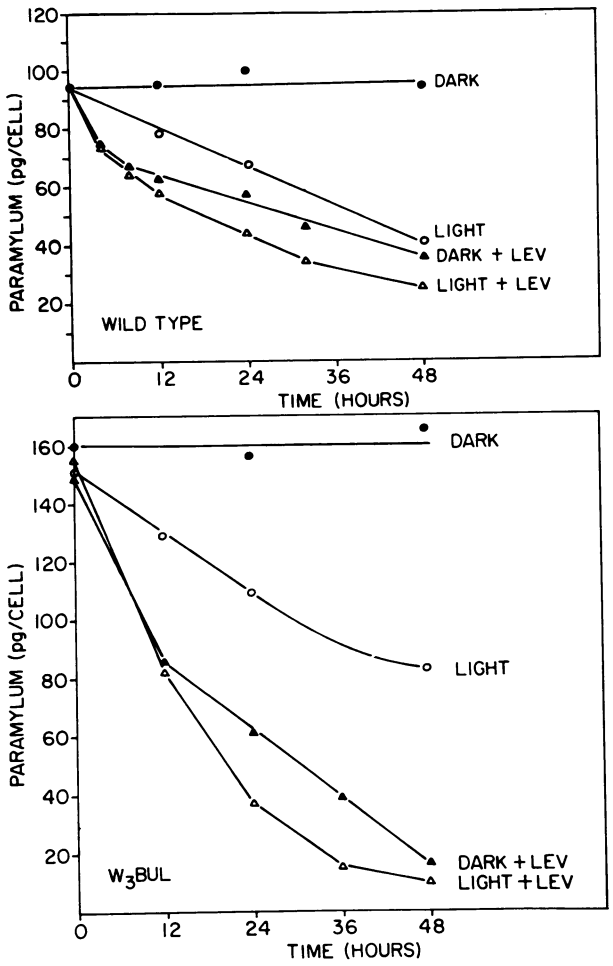

FIG. 4. Kinetics of levulinic acid-induced paramylum degradation in wild type *Euglena* and in the plastidless mutant, W_3BUL. At 0 time, levulinic acid was added to one culture of dark-grown resting *Euglena* to give a final concentration of 30 mM and both treated and untreated cultures were exposed to continuous illumination or were incubated in the dark. At appropriate times samples were withdrawn for the determination of paramylum.

hydratase in W_3BUL to drain off ALA, which may be the regulatory molecule involved. LEV might induce by blocking the dehydratase, thereby increasing the ALA pool. Another possibility is that LEV exerts its action on paramylum breakdown by acting as a structural analog of ALA. Thus it is suggested that light induces the synthesis of ALA which serves as a corepressor of the synthesis of certain proteins which inhibit paramylum breakdown. In this case LEV would serve the same function by interacting with the same site. LEV-treated cells, therefore, would have a higher intracellular concentration of induced and a higher rate of paramylum degradation than light-induced cells.

CONCLUSIONS

Blue light regulates many aspects of plant metabolism (31, 34 and references cited therein). In *Euglena*, a nonchloroplast, blue-absorbing photoreceptor controls cytoplasmic ribosomal RNA synthesis (5, 36), carotenoid synthesis (8), the synthesis of a number of proteins (27), and the events required for the elimination of the lag period in Chl synthesis (12, 17). Cell division (7, 20), and protein synthesis and turnover (15, 28) are also regulated by a nonchloroplast photoreceptor. Many of these processes are also coregulated by a chloroplast photoreceptor (5, 12, 15, 17, 27, 28). The photoinduction of paramylum degradation in the plastidless mutant, W_3BUL, indicates that carbohydrate metabolism is also controlled by a nonchloroplast photoreceptor. Thus, the nonchloroplast photoreceptor of *Euglena* is functionally analogous to the blue-absorbing photoreceptor found in other plant systems (31, 34 and references cited therein).

The induction of paramylum breakdown by CEX and LEV in the dark and the restoration by LEV of the levels in W_3BUL to those observed with wild type cells in the light provides insight into the mechanism by which the nonchloroplast photoreceptor controls carbohydrate metabolism and, presumably, other biosynthetic processes in *Euglena*. We might speculate that a protein which turns over rapidly or a metabolite produced by this protein prevents paramylum degradation. Upon exposure of dark-grown cells to light, ALA would be synthesized (23, 29) and the newly synthesized ALA would induce paramylum degradation by inhibiting the synthesis of this protein. ALA may in fact be a corepressor of many of the enzymes such as malic enzyme (21), malate dehydrogenase (22), and malate synthetase (20) whose synthesis seems to be inhibited by light. Both the light inhibition of protein synthesis (15, 28) and the light induction of paramylum degradation may be the results of an increase in the intracellular level of ALA. CEX would induce paramylum degradation in the dark by directly preventing the resynthesis of the rapidly turning over inhibitory protein while LEV would induce paramylum degradation in the dark by acting as a structural analogue of ALA, or as an inhibitor of ALA-utilizing enzymes resulting in higher ALA levels, and so would repress the synthesis of this inhibitory protein. Thus LEV would restore the rate and extent of paramylum degradation in W_3BUL to the wild type level by increasing the intracellular concentration of the inducer.

Although the ability to degrade paramylum seems to be controlled by a rapidly degraded protein, the rate of paramylum degradation is apparently controlled by the rate at which the degradation products are metabolized. The rates of paramylum degradation in cells treated with CEX or LEV (Figs. 2 and 4) are similar in the light or the dark during the first 12 to 24 hr of light exposure. After this time, the rate of degradation is higher in the light than in the dark. Although SM does not prevent degradation (Fig. 3), the light-induced rate of degradation is lower in SM-treated cells than in untreated cells. Normally, the developing plastid utilizes both the carbon and energy produced by paramylum degradation for the synthesis of plastid constituents (10). When processes required for normal plastid development are inhibited by antibiotics, it is conceivable that energy, ATP, as well as carbon skeletons produced by carbohydrate degradation would accumulate within the cell, resulting in the feedback inhibition of the paramylum degrading enzymes. Because of this feedback inhibition, cells treated with SM, CEX, or LEV would have decreased final rates of paramylum degradation when compared with untreated cells in the light. The inhibitor-induced rates in the dark are also lower than the rates in the light since in the light, noninhibited light-induced processes utilize the metabolites produced by carbohydrate degradation. Consequently, the concentration of feedback inhibitors and thus the degree of feedback inhibition in the light is less than in the dark. Cells exposed to light in the presence of glucose fail to use their paramylum reserves (9) in agreement with the idea that paramylum degradation is inhibited by products produced by its degradation.

The complex photo and metabolic control of paramylum utilization insures that even under conditions of severe nutritional limitation sufficient carbon and energy remain available to form the photosynthetic apparatus. The amount of Chl synthesized is related to the amount of paramylum degraded during development (14), and tracer studies have shown that the carbon derived from paramylum degradation is incorporated into chloroplast lipids (10).

Euglena is a facultative phototroph. In the presence of sufficient nutrients and in the absence of light, the carbon and energy required to synthesize a fully developed plastid can be more efficiently used for cell growth. When light must serve as the major source of energy and reduced carbon, however, there must be sufficient carbon and energy available to build the enzymic machinery required for the formation of the photosynthetic apparatus. The photoregulation of paramylum degradation insures that sufficient carbon and energy will be available for plastid development when the cells are exposed to light. In addition to mobilizing paramylum, a mechanism must also exist to insure that the carbon and energy produced by paramylum degradation will be used for plastid development rather than cell growth. The nonchloroplast photoreceptor which promotes paramylum degradation is thus probably the same receptor which inhibits cell division in *Euglena* (7, 20). In this way, the limited amounts of nutrients available to the cell are used preferentially for plastid development. When the cell is photosynthetically competent, it can synthesize the additional carbon and energy required for cell division.

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