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The Effect of Light on the Synthesis of Mitochondrial Enzymes in Division-synchronized *Euglena* Cultures

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ABSTRACT

The development of the mitochondrial enzymes fumarase and succinate dehydrogenase has been followed in *Euglena* cultures division-synchronized by 14-hour light periods alternating with 12-hour dark periods. The activity of both enzymes was unaltered over the light phase, doubled in early dark phase, and thereafter remained constant over the rest of the cycle. The increase in enzyme activity in early dark phase probably represented *de novo* enzyme synthesis because it was prevented by the addition of cycloheximide at a concentration known to inhibit protein synthesis on *Euglena* cytoplasmic ribosomes.

When division-synchronized cultures were darkened in early light phase, a doubling of both fumarase and succinate dehydrogenase activity resulted, showing that light was repressing enzyme synthesis. The addition of acetate did not have a similar effect to darkening cultures: enzyme activity being unaltered over the light phase of the cycle. Enzyme expression was also unaffected by the addition of 3-(3,4 dichlorophenyl)-1,1-dimethylurea, a potent inhibitor of photosynthetic carbon dioxide fixation. The addition of 6-methylpurine (an inhibitor of transcription) at the beginning of the light phase inhibited enzyme increase in early dark phase, but when added at a later stage of the light phase (hour 8), increase in enzyme activity in early dark phase was unaffected. We concluded that transcription for these enzymes occurs in early light phase but light exerts a post-transcriptional control so that enzyme synthesis does not result until cells enter the dark phase of the cell cycle.

There exists in photosynthetic eukaryotes a subtle balance between the relative numbers per cell of the two types of organelles capable of generating ATP for use in cell metabolism. In algae the relative numbers of mitochondria and chloroplasts per cell is greatly influenced by environmental conditions. In particular, the provision of an organic carbon source as opposed to dependence on carbon compounds resulting from the photosynthetic fixation of carbon dioxide is a major factor in the regulation of organelle development. Thus, cells of *Chlorella protothecoides* when incubated in a medium containing a high concentration of glucose or acetate are bleached with degeneration of chloroplasts and decrease in photosynthetic capacity (11). Similarly, *Euglena* cells are bleached when grown on carbon sources in the dark but regain their chloroplasts and full photosynthetic capacity on reillumination (19). While the effects of light and carbon nutrition on chloroplast development have received some attention, their possible effects on mitochondrial development have not, although Smillie (20) reported that malate dehydrogenase activity in heterotrophically grown *Euglena* was about twice that for phototrophically grown cells. While investigating the biogenesis of glyoxysomes in *Euglena* we observed that the transfer of cells from autotrophic growth conditions to heterotrophic growth on acetate resulted in a great increase in the number of mitochondria per cell. In the present investigation, the effect of light and carbon nutrition on mitochondrial development has been determined using division-synchronized cultures of *Euglena* in the hope that the analysis of results would be facilitated by having a cell population at a uniform stage of development. The enzymes succinate dehydrogenase and fumarase have been used as mitochondrial markers because they are specifically mitochondrial enzymes in contrast to malate dehydrogenase where specific isoenzymes are present in *Euglena* outside the mitochondrion (6, 17).

MATERIALS AND METHODS

Growth, Synchronization Regime and Sampling of Culture. Division-synchronized cultures of *E. gracilis* Klebs. strain Z were obtained exactly as described previously (4). As before, samples were removed at t_8, t_{12}, t_{17}, and t_{23}; this refers to the hour of sampling after commencement of the 24-hr cycle in which the culture was used. Cell counts were made with a Coulter counter Model T. Antibiotics and DCMU were added to cultures at the beginning of the light phase. DCMU was dissolved in 7 ml of 95% (v/v) ethanol and added to 1493 ml of culture to give a final concentration of 10 μM. Seven ml of 95% (v/v) ethanol were added to a control culture. Chloramphenicol was dissolved in 100 ml of medium by heating to 49 C accompanied by continuous stirring; when added to 1400 ml of culture, the final concentration was 1 mg/ml. Cycloheximide was dissolved in 30 ml of growth medium and added to 1470 ml of culture to give a final concentration of 15 μg/ml. In control cultures, identical amounts of the culture were removed and replaced by fresh growth medium. All solutions were sterilized by Seitz filtration before addition to the culture.

Cell Disruption Techniques. Total enzyme activity per cell was determined by harvesting the cells by centrifugation, washing once, resuspending in 0.1 M potassium phosphate buffer, pH 7.0, and disrupting by treatment with three 15-sec bursts of ultrasonic waves (M.S.E. ultrasonic disintegrator 1.5 amp). After centrifugation at 150*g* for 10 min, the supernatant was used in enzyme assays.

Enzyme Assays. Succinate dehydrogenase (EC.1.3.99.1) was assayed by a modification of the procedure of Ells (7),

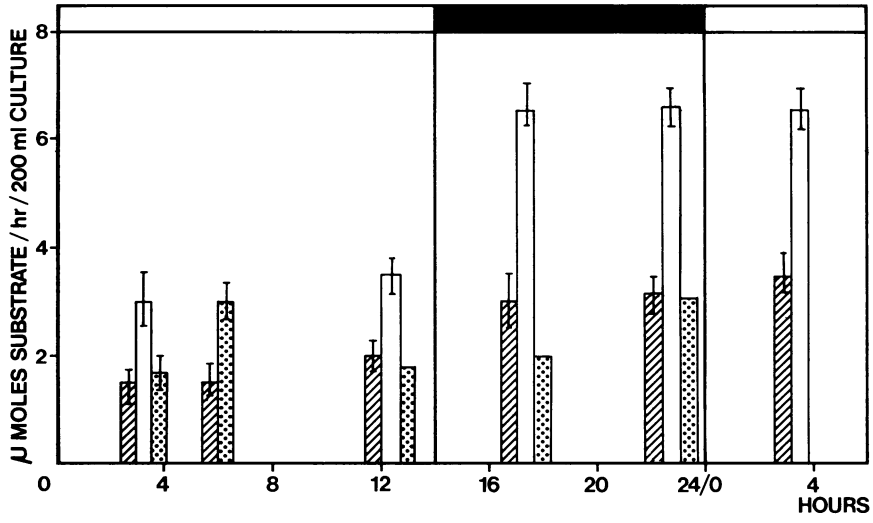

FIG. 1. Succinate dehydrogenase and fumarase activities over the cell cycle in division-synchronized *Euglena* cultures. Fumarase activity (□); succinate dehydrogenase activity (▨); succinate dehydrogenase activity with culture kept in light at dark phase of cycle (⊡). Bar denotes spread of determinations.

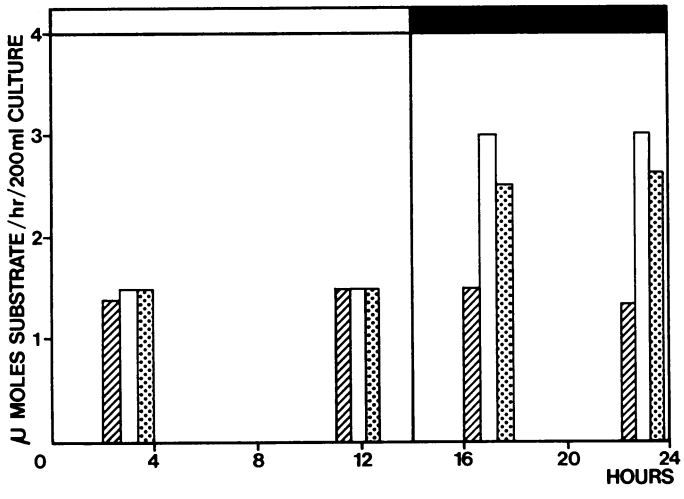

FIG. 2. Effect of chloramphenicol and cycloheximide on succinate dehydrogenase activity over the cell cycle in division-synchronized *Euglena* cultures. Control culture (□); cycloheximide added at t_0 stage (▨); chloramphenicol added at t_0 stage (⊡).

which uses phenazine methosulfate to couple electron transfer between succinate and 2,6-dichlorophenol indophenol. The reaction mixture contained in a final volume of 3.0 ml, 33 mM potassium phosphate buffer, pH 7.6, 0.1 mM 2,6-dichlorophenolindophenol, 3.3 mM KCN, 6.6 mM sodium succinate, and enzyme. The reaction was started by the addition of 0.1 ml of 1% (w/v) phenazine methosulfate and was measured by following the decrease in extinction at 600 nm.

Fumarase (EC.4.2.1.2) was assayed by the method of Massey (10). The reaction mixture contained in a final volume of 3.0 ml, 50 mM potassium phosphate buffer, pH 7.3, 50 mM L-malate, and 0.1 ml of cell extract. The reaction was started by the addition of malate and followed by measuring fumarate formation at 300 nm. Protein was determined by the method of Lowry *et al.* (9).

Respiration. Measurements of respiration were made at intervals over the cell cycle by removing 200 ml aliquots from the culture, centrifuging at 1000*g* for 4 min and carefully resuspending the cells in 2 ml of fresh growth media, or growth media containing cycloheximide or chloramphenicol. Oxygen uptake was measured using a Rank oxygen electrode in a continuously stirred Perspex vessel around which was circulated water from a thermostatically controlled water bath at 25 C. Results were recorded on a Rikandenki Model B two-pen recorder. The electrode was calibrated as described by Cockburn *et al.* (3).

RESULTS

Fumarase and Succinate Dehydrogenase Activity over the Cell Cycle. The activities of fumarase and succinate dehydrogenase were followed by removing aliquots from the culture, preparing cell extracts and assaying at intervals over the cell cycle. Both enzymes showed similar patterns of activity, remaining constant over the light phase, except for a slight increase at the t_{12} stage of the cycle, followed by a rapid doubling of activity in early dark phase, after which activity remained constant throughout the rest of the dark phase (Fig. 1). This pattern of activity was shown only by division-synchronized cultures. Fumarase activity increased steadily over the light phase and early dark phase in random cultures subjected to the same light/dark periods. Similar results to those for fumarase were obtained for succinate dehydrogenase activity. When division synchronized cultures were kept in continuous light, the doublings of enzyme activity that occurs in early dark phase of a normal light/dark cycle did not result, the increase in enzyme activity being delayed until much later in the cycle (Fig. 1).

Effect of Specific Inhibitors of Protein Synthesis upon Enzyme Synthesis. Although the changes in succinate dehydrogenase and fumarase activities that result on transfer from light to dark occur only in division synchronized cultures of *Euglena*, the possibility of a direct effect of light on enzyme activity cannot be excluded. The use of a light/dark regime to impose cell synchrony raises the possibility of changes in enzyme activity resulting from a light activation or inactivation of specific enzymes. To investigate this possibility specific inhibitors of protein synthesis were added to cultures at the beginning of the light phase and enzyme activity followed throughout the cycle. The increase in succinate dehydrogenase activity in the early dark phase of the cycle was effectively inhibited by cycloheximide (Fig. 2). The addition of chloram-

phenicol inhibited only slightly the increase in succinate dehydrogenase (Fig. 2). Similar results were obtained for the effect of inhibitors on fumarase activity over the cell cycle. Thus the increase in succinate dehydrogenase and fumarase activities at the beginning of the dark phase of the cycle apparently represents *de novo* enzyme synthesis rather than activation of latent enzyme activity.

Effect of Light on Enzyme Synthesis. The characteristic pattern of activity for the mitochondrial markers fumarase and succinate dehydrogenase resulting from the transfer of cells from light to dark was shown only by division-synchronized cultures. Although the primary effect of a light/dark regime is to impose cell synchrony the possibility of a secondary effect of light in division-synchronized cultures cannot be excluded. The rapid increase in enzyme activity in early dark phase could result from the removal of a temporal restriction on the expression of mitochondrial enzymes imposed by light in division synchronized cultures. This possibility was investigated by darkening division synchronized cultures of *Euglena* at different stages of the cell cycle and determining the effect on fumarase and succinate dehydrogenase activity. When cells were darkened 3 hr after commencement of the light phase, both fumarase and succinate dehydrogenase had nearly doubled by the t_6 stage of the cell cycle (Figs. 3 and 4) and did not show appreciable further increase over the rest of the cycle. This increase in enzyme activity was not observed if cycloheximide was added to the culture at the beginning of the light phase, showing that the increase in activity resulting from premature darkening of the cells was probably the result of enzyme synthesis.

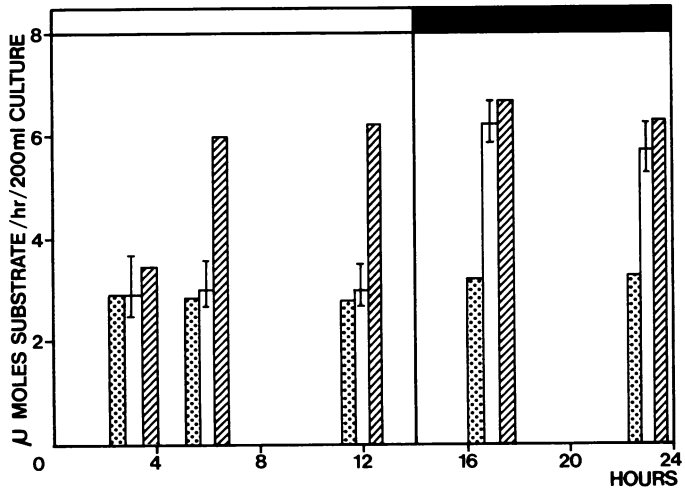

FIG. 3. Effect of light on the expression of fumarase activity over the cell cycle in division-synchronized *Euglena* cultures. Control culture, normal light/dark cycle (□); culture darkened at t_3 stage of the cell cycle (▨); cycloheximide added at t_0 and culture darkened at t_3 (⊡). Bar denotes spread of determinations.

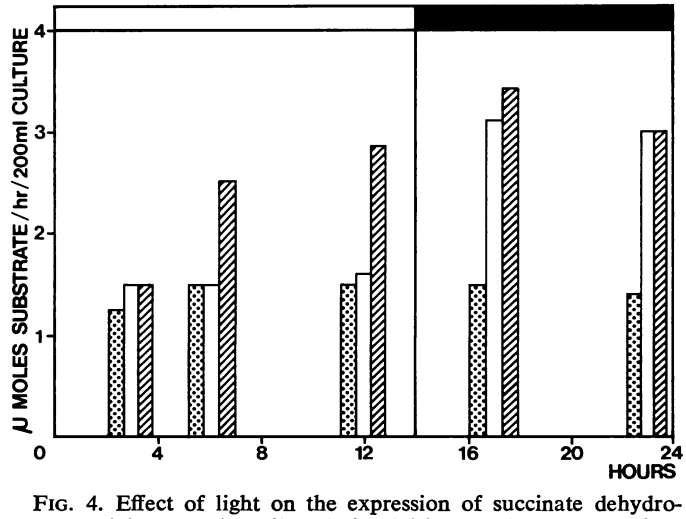

FIG. 4. Effect of light on the expression of succinate dehydrogenase activity over the cell cycle in division-synchronized *Euglena* cultures. Control culture, normal light/dark cycle (□); culture darkened at t_3 stage of the cell cycle (▨); cycloheximide added at t_0 and culture darkened at t_3 (⊡).

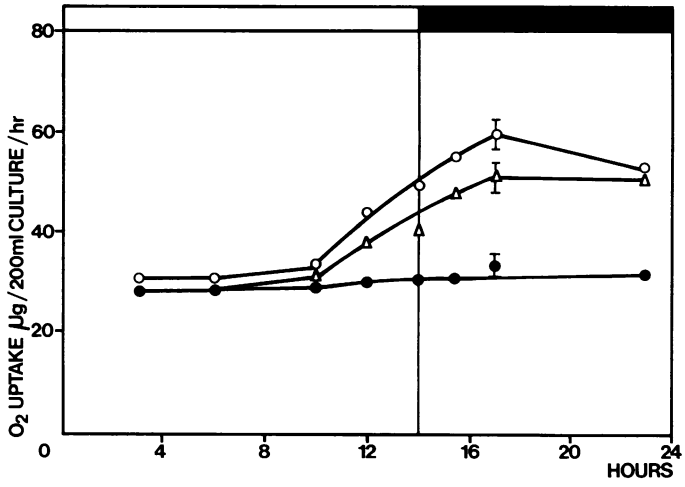

FIG. 5. Respiration over the cell cycle in division-synchronized *Euglena* cultures. Control culture (○); chloramphenicol added at t_0 (△); cycloheximide added at t_0 (●).

Respiration over the Cell Cycle. Respiration was followed by removing aliquots from the culture at intervals over the cell cycle. Respiration rate remained constant over the first 10 hr of the light cycle, from then until t_{17} respiration increased progressively with time until by t_{17} the respiration rate had doubled per unit of culture (Fig. 5). In the presence of cycloheximide the respiration rate over the cell cycle was constant, presumably reflecting the inhibition of the synthesis of mitochondrial enzymes in the presence of cycloheximide. In the presence of chloramphenicol, respiration showed the same pattern as control cultures but the increase in respiration over the cell cycle was a third less than that of the control (Fig. 5).

Effect of DCMU on Fumarase and Succinate Dehydrogenase Activity. Consideration was given to the possibility that in the light some product (or products) of the photosynthetic carbon reduction cycle functioned as a repressor for the synthesis of mitochondrial enzymes. To investigate this possibility DCMU, an inhibitor of photosynthetic carbon dioxide fixation, was added at the beginning of the light phase and enzyme activity followed throughout the cycle. The pattern of fumarase activity over the cell cycle was unaffected by the addition of DCMU (Fig. 6). Apparently, the effect of light on enzyme activity does not result from some product of photosynthesis repressing mitochondrial enzyme formation.

Effect of Acetate on Enzyme Activity. When cells growing autotrophically are transferred to heterotrophic growth conditions, in addition to the absence of light, the other major change is the provision of an organic carbon source. The present work has shown that light has a major effect on the temporal expression of mitochondrial enzymes, but the possibility cannot be excluded that the presence of an organic carbon source may also affect enzyme development during the transi-

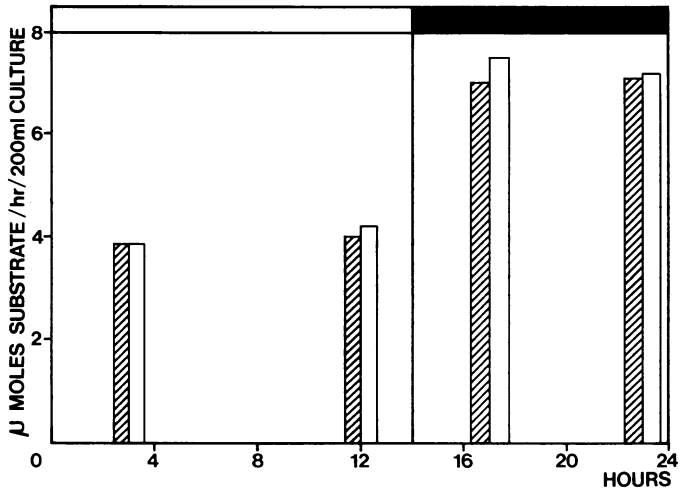

FIG. 6. Effect of DCMU on fumarase activity over the cell cycle in division-synchronized *Euglena* cultures. Control culture (□); DCMU added at t_0 to given 10 μM final concentration (▨).

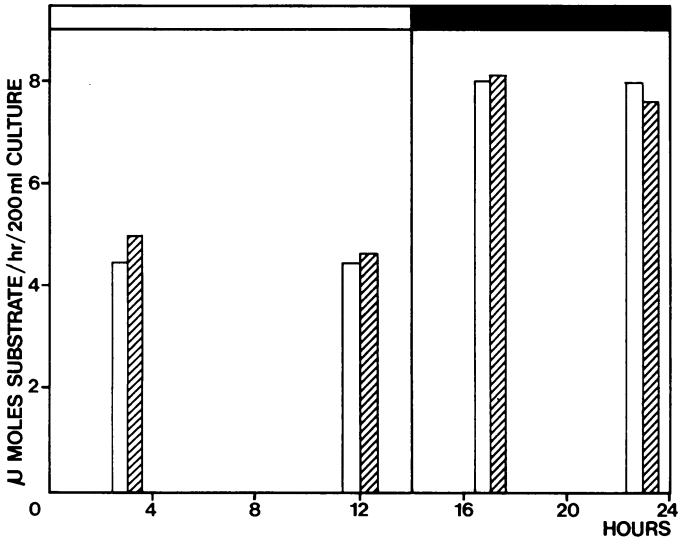

FIG. 7. Fumarase activity over the cell cycle in division-synchronized *Euglena* cultures in the presence of acetate. Control culture (□); acetate added at t_8 stage (▨).

tion from autotrophic to heterotrophic growth. When sodium acetate (final concentration 0.2% w/v) was added at the t_3 stage of the cell cycle, fumarase showed the same pattern of activity as in autotrophic division-synchronized cultures (Fig. 7). The addition of acetate did not produce the same doubling of fumarase activity that resulted from the darkening of early light phase cultures. A similar result was obtained for succinate dehydrogenase activity; when acetate was added at the t_3 stage of the cycle, an immediate effect upon enzyme activity was not observed and the usual increase in enzyme activity occurred in early dark phase. The major difference was a continuing increase in succinate dehydrogenase activity over the dark phase so that by the end of the dark phase, activity was nearly double that of the control culture (Fig. 8).

Effect of 6-Methylpurine on Enzyme Expression over the Cell Cycle. The base analog 6-methylpurine was selected as an inhibitor of RNA synthesis to determine whether the observed effect of light on the synthesis of mitochondrial enzymes occurs at the stage of transcription or some later stage of protein synthesis. Both fumarase and succinate dehydrogenase activities double in early dark phase so presumably transcription occurs before this stage of the cell cycle. This being so, the effect of adding 6-methylpurine (final concentration 1 mM) at either the t_0 or t_8 stage of the cell cycle was determined. The addition of 6-methylpurine at the t_8 stage of the cell cycle had little effect on the expression of fumarase over the cell cycle (Fig. 9). When 6-methylpurine was added at the t_0 stage, however, there was a complete inhibition of any increase in fumarase activity over the cell cycle (Fig. 9). Similar results were obtained for the effect of 6-methylpurine on the expression of succinate dehydrogenase over the cell cycle.

DISCUSSION

Although the expression of enzymes concerned with photosynthesis has recently been investigated (4, 5, 24), with the exception of malate dehydrogenase (6), the development over the cell cycle of enzymes catalyzing specific reactions of respiration has not been investigated in *Euglena*. Succinate dehydrogenase and fumarase revealed similar patterns of activity over the cell cycle, enzyme activity doubling over the first part of the dark phase and remaining nearly constant over the rest of the cell cycle. These results contrast with those obtained for malate dehydrogenase where activity increased mainly over the light phase of the cycle (6). However, unlike fumarase and

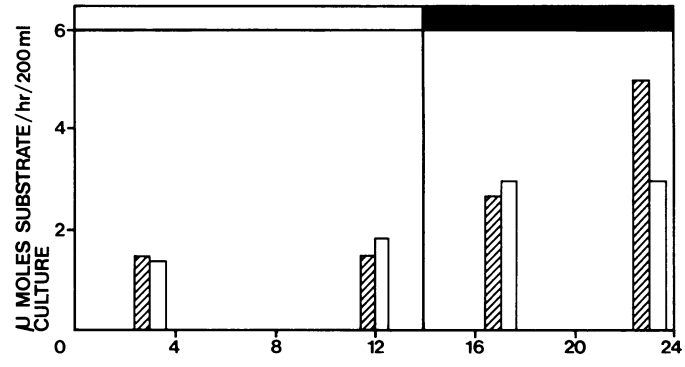

FIG. 8. Effect of acetate on succinate dehydrogenase activity in division-synchronized *Euglena* cultures. Control culture (□); acetate added at t_3 (▨).

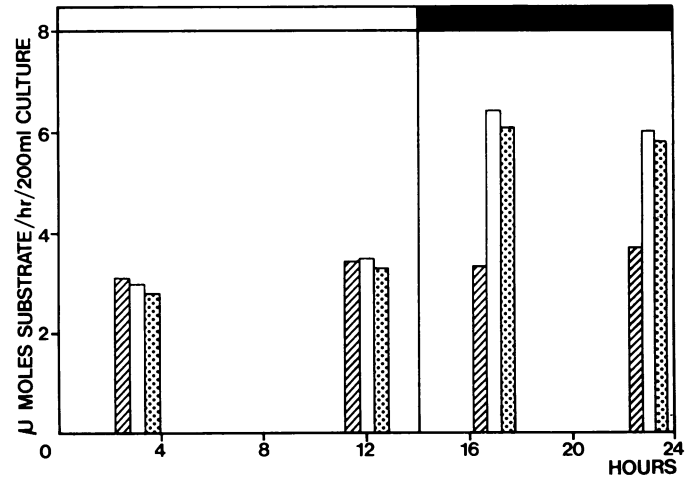

FIG. 9. Effect of 6-methylpurine on fumarase activity over the cell cycle in division-synchronized *Euglena* cultures. Control culture (□); 6-methylpurine added at t_0 (▨), and at t_8 (⊡).

Table I. *Effect of Various Treatments on Cell Synchrony in Euglena*

The data were normalized to 5×10^4 cells at beginning of the cell cycle.

	Cell No. $\times 10^{-4}$			
	T_3	T_{12}	T_{17}	T_{23}
Normal light/dark cycle	5.0	4.9	9.9	9.9
Acetate added at t_3	5.0	4.9	6.4	7.0
Culture darkened at t_3	5.0	5.1	5.1	5.1
Cycloheximide added at t_0	5.0	5.0	5.0	5.0
Chloramphenicol added at t_0	5.0	5.0	9.8	9.8

succinate dehydrogenase, malate dehydrogenase is not located exclusively in the mitochondria; characteristic isoenzymes are also present in the peroxisomes (6) and the soluble fraction of the cell (6, 17). The increase in fumarase and succinate dehydrogenase over the early dark phase probably results from the synthesis of new enzyme because it was effectively prevented by cycloheximide added to the cultures at concentrations known to inhibit protein synthesis by *Euglena* on cytoplasmic ribosomes although not affecting chloroplast ribosomes (1, 19). Although over the usual light/dark cycle cell division and increase in fumarase and succinate dehydrogenase activities occur at more or less the same stage of the cell cycle, this increase in enzyme activity is not dependent on cell division. When cells were darkened at the t_3 stage, fumarase and succinate dehydrogenase activities had doubled by the t_6 stage, but cells darkened so early in the cycle failed to undergo cell division (Table I).

In previous experiments a direct effect of light on malate dehydrogenase activity over the cell cycle could not be detected (6), whereas light regulates the synthesis of succinate dehydrogenase and fumarase. When cells enter the dark phase of the cell cycle, enzyme activity increases, and the absence of light is a key factor because darkening cells at the t_3 stage of the cycle also results in a doubling of fumarase and succinate dehydrogenase activities. In both instances, increase in enzyme activity is inhibited by cycloheximide suggesting *de novo* enzyme synthesis rather than removal of a light inactivation of the enzyme. Similarly, when division-synchronized cultures are kept in continuous light over the dark phase of the cycle, the increase in enzyme activity normally observed in early dark phase is delayed until much later in the cycle. One possible mechanism by which light could repress the synthesis of mitochondrial enzymes would be by catabolite repression (15), acting either on existing mRNA, as occurs during the repression of isocitrate lyase by glucose in *Chlorella* (13), or at the level of transcription, as occurs with the lac operon of *E. coli* (23). DCMU, at the concentration used in the present experiments, effectively inhibits photosynthetic CO_2 fixation in *Euglena* without affecting chloroplast development (18), but when DCMU was added at the beginning of the cell cycle, fumarase activity was unaffected demonstrating the absence of catabolite repression.

The hypotheses of Tauro *et al.* (22) and Mitchison (14), concerning the mechanism by which temporal restriction is imposed on the synthesis of enzymes during the cell cycle, both propose that enzyme synthesis occurs during a period of concurrent gene transcription. If this were correct in all instances, then the present results would suggest a direct effect of light on transcription in *Euglena*. 6-Methylpurine has been shown to be an effective inhibitor of the synthesis of RNA in barley aleurone tissue (2), sugarcane stem tissue (8), and in *Chlorella* (12), while preliminary experiments have shown it to be equally effective in *Euglena*. The formation of fumarase and succinate dehydrogenase by cells either upon darkening at the t_3 stage or at the beginning of the dark phase of the cell cycle could result from the removal of light initiating transcription or transcription already having occurred and light exerting a post-transcriptional control. The addition of 6-methylpurine at the beginning of the cell cycle effectively inhibited increase in fumarase and succinate dehydrogenase activities, but when added at the t_8 stage of the cell cycle, there was only a marginal effect on increase in enzyme activity (Fig. 9). As enzyme synthesis is unaffected by the addition of 6-methylpurine at the t_8 stage of the cell cycle, transcription has occurred before this stage. However, as enzyme synthesis does not occur until the cells enter the dark phase of the cycle the regulation of enzyme synthesis occurs at a post-transcriptional level. It has been suggested by Sussman (21) that enzyme synthesis during morphogenesis in *Dictyostelium* is also regulated by controls operating at the post-transcriptional level. Soluble protein factors are important in the translational control of protein synthesis in eukaryotic animal cells (16) and the results obtained with division-synchronized cultures of *Euglena* suggest the possibility of this type of control operating in *Euglena*.

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