A Possible Ribosomal-Directed Regulatory System in *Euglena gracilis* CHLOROPHYLL SYNTHESIS

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Cycloheximide at concentrations of 0.1–100 μM stimulated chlorophyll synthesis when dark-grown cells of *Euglena* were illuminated. Chloramphenicol (1–4 mM) inhibited chlorophyll synthesis. The effect of cycloheximide on the incorporation of [^{14}C]leucine into material insoluble in trichloroacetic acid, and its failure to affect the incorporation of [^{32}P]orthophosphate into such material in short incubations, are interpreted as evidence that cycloheximide specifically inhibits protein synthesis by 80S ribosomes. Since the inhibitory effect of chloramphenicol on chlorophyll synthesis is counteracted by the presence of cycloheximide, it is suggested that chlorophyll synthesis is subject to control by a cytoplasmic repressor synthesized on 80S ribosomes, and to a de-repressor synthesized on 70S ribosomes.

Euglena gracilis cells grown in darkness lack chlorophyll and therefore grow organotrophically. Exposure to light causes 'greening', a process that involves the formation of chlorophyll. During illumination the cell develops chloroplasts and grows phototrophically.

Since the *Euglena* cell contains nuclear, chloroplastic and mitochondrial DNA (Lyman *et al.*, 1961; Edelman *et al.*, 1966), together with cytoplasmic 80S ribosomes and chloroplast 70S ribosomes (Brawerman, 1963; Rawson & Stutz, 1969), the origin of the genetic information and the site of synthesis of the proteins required for development of chlorophyll is still obscure. There is some evidence that chloroplast DNA directs RNA synthesis in chloroplast ribosomes (Scott & Smillie, 1967).

Several experiments have been reported that were designed to answer this question by determining the activities of the enzymes concerned with chloroplast synthesis in cells kept in the presence of antibiotics specifically inhibitory to protein synthesis (Ebbon & Tait, 1969; Smillie *et al.*, 1967; Kirk, 1968) either in the dark or in the light. The results indicate that inhibition of protein synthesis on either cytoplasmic ribosomes or chloroplast ribosomes decreases the chlorophyll synthesis that occurs on illumination of *Euglena* cells. Relatively large amounts of the drugs studied were used in these experiments.

There is evidence for a specific inhibitory effect of cycloheximide on 80S ribosomes and of chloramphenicol on 70S ribosomes (Clark & Chang, 1965; Siegel & Sisler, 1965; Godchaux *et al.*, 1967; Key *et al.*, 1967; but see also Ellis, 1969). By using these two antibiotics in low but effective concentrations we have shown that greening of dark-grown *Euglena* cells exposed to light is controlled by an 80S ribo-

somal product which acts as a repressor and which may be inactivated by a 70S ribosomal product.

Materials and Methods

Euglena gracilis var. *bacillaris* was grown in darkness as previously described (Perl, 1971).

For the drug-effect experiments, 50 ml of the cell suspension during the early exponential phase of growth was transferred to 250 ml flasks (2×10^4 – 4×10^4 cells/ml). The drug was added as indicated, and the cells were exposed to light. After various time-intervals samples (5 or 10 ml) of the cell suspension were centrifuged and the chlorophyll was extracted with aq. 80% (v/v) acetone.

The chlorophyll was determined by the method of Arnon (1949).

Cell numbers were determined with a Coulter counter.

Measurement of protein synthesis

To 10 ml of a suspension of green *Euglena* cells in 50 ml flasks the indicated amounts of the drug were added and the cells were allowed to grow in the light. After 18 h, L-[^{14}C]leucine (2.5×10^5 c.p.m.; 55 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added and the cells were kept continuously under normal growth conditions. At the indicated times 0.5 ml of cold 10% (w/v) trichloroacetic acid was added to 0.5 ml of cells from each experimental flask and the mixtures were kept in ice for 15–20 min. The suspension was filtered under vacuum on a 0.45 μm Millipore filter; the filter was washed three times with 2 ml of cold 5% trichloroacetic acid containing 100 μg of non-labelled DL-leucine, and then dried and counted for radioactivity.

in scintillation liquid containing 1 litre of toluene, 50mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 3g of 2,5-diphenyloxazole, by using a Packard Tri-Carb liquid-scintillation counter.

Incorporation of [32 P]orthophosphate

To each 10ml sample of green *Euglena* cell suspension (in 50ml flasks) 0, 10 or 100 μ g of cycloheximide was added, followed by [32 P]orthophosphate (10^6 c.p.m.; carrier-free; Nuclear Research Centre, Negev, Israel) and the flask was kept under normal growth conditions. After the indicated time-intervals 0.5ml samples of cells were transferred to 0.5ml of cold 10% trichloroacetic acid and kept in ice. After 30min the cell suspension was filtered on a 0.45 μ m Millipore filter under vacuum. The filter was washed three times with 5ml of cold 10% trichloroacetic acid and three times with 3ml of diethyl ether, and counted for radioactivity as described above.

Results

The effect of cycloheximide on protein synthesis is shown in Fig. 1. Cycloheximide very strongly inhibits the incorporation of [14 C]leucine into the fraction

Fig. 1. Inhibition of protein synthesis by cycloheximide in *Euglena* cells

Green *Euglena* cells were incubated in the light under normal growth conditions in the presence of different amounts of cycloheximide. After 18h the cells were labelled for the indicated times with [14 C]leucine; the cell number and radioactivity in the fraction insoluble in cold trichloroacetic acid were measured. Cycloheximide concentrations: $\bullet$, 0; $\circ$, 0.1 μ M; $\blacktriangle$, 10 μ M; $\square$, 1 mM.

insoluble in trichloroacetic acid. Possible influences of this drug on the coupling between electron transport and respiratory chain (MacDonald & Ellis, 1969; Ellis & MacDonald, 1970; Ellis & Hartley, 1971) were excluded by examination of its effects on *Euglena* cell respiration in the dark and on incorporation of [32 P]orthophosphate into the fraction of the cells that was insoluble in cold trichloroacetic acid. The respiration curve obtained in the presence and in the absence of cycloheximide shows that no changes

Fig. 2. Effect of cycloheximide on incorporation of [32 P]orthophosphate

Cycloheximide ($\bullet$, 0; $\circ$, 1 μ g/ml; $\blacktriangle$, 10 μ g/ml) was added to 10ml of green *Euglena* cells in normal growth medium in the presence of [32 P]orthophosphate (10^6 c.p.m.). At the indicated times 0.5ml batches of cells were transferred to cold trichloroacetic acid, washed and counted for radioactivity as described in the Materials and Methods section.

occur during the first 2 h. Similar results have been reported by Kirk (1970). The incorporation curve of [32 P]orthophosphate (Fig. 2) shows that this activity decreased only 2 h after cycloheximide was added. The rapid effect of cycloheximide seems therefore to be primarily on protein synthesis, and the later inhibition phenomena are probably a consequence of this effect.

Chloramphenicol inhibited the incorporation of leucine by only 10–15%, i.e. much less than cycloheximide (Fig. 3). This seems to correlate with the ratio of the 70S ribosomes to the 80S ribosomal fraction in the cell. The ability of cells that had been kept in the presence of cycloheximide (20 μ g/ml) for 18 h to incorporate [14 C]leucine was restored after 4 h of growth in its absence. These results indicate that cycloheximide is not lethal to the *Euglena* cell under the conditions used; its effect is fully reversible and operates only while it is present in the cells.

When different amounts of cycloheximide were added to a dark-grown cell population just before they were permitted to grow in normal light (Fig. 4a), cell division was partly blocked at a concentration of 0.1 μ M, although growth was completely inhibited only at 0.1 mM. The greening of the cells, as measured by chlorophyll content, was inhibited at 1 mM, but between 0.1 mM and 0.1 μ M greening was markedly stimulated.

Fig. 3. Effect of chloramphenicol on protein synthesis in *Euglena* cells

Euglena cells were labelled with [14 C]leucine for the indicated times after they had been incubated for 18 h with 2 mM-chloramphenicol. The cell number and radioactivity in the fraction insoluble in cold trichloroacetic acid were measured. Chloramphenicol concentrations: ●, 0; ○, 2 mM.

Since cycloheximide inhibits 80S ribosomal activity, the results suggest that the cell contains a cytoplasmic repressor for greening, which is synthesized by the 80S ribosomes. Synthesis of this repressor is inhibited by cycloheximide, thus causing stimulation of the greening process. When 70S ribosomal activity was inhibited by different concentrations of chloramphenicol, growth and cell division of the *Euglena* cells did not seem to be affected even by relatively large amounts of the drug (up to 3 mM) (Fig. 4b), but

Fig. 4. Effects on chlorophyll synthesis and cell division of different concentrations of cycloheximide and chloramphenicol

A sample (50 ml) of dark-grown *Euglena* cells was exposed to light in the presence of the indicated concentrations of cycloheximide (a) and chloramphenicol (b). After 48 h of illumination chlorophyll content (●) and cell number (○) were determined as described in the Materials and Methods section.

Table 1. Chlorophyll synthesis effected by the presence of cycloheximide and chloramphenicol in the culture medium

The indicated antibiotics were added to 50 ml of dark-grown *Euglena* cells. After 24 h of illumination chlorophyll and cell number were determined as described in the Materials and Methods section.

Antibiotic added	Chlorophyll (mg/24h per 10 ⁶ cells)
None	9.0
Cycloheximide (1 μ M)	19.2
Chloramphenicol (2 mM)	2.6
Cycloheximide (1 μ M) and chloramphenicol (2 mM)	10.5

the greening of the cell was completely inhibited at this concentration.

This phenomenon may be explained by supposing either that a protein synthesized by 70S ribosomes was required for chlorophyll synthesis or that such a protein de-repressed chlorophyll synthesis that had been inhibited by a repressor synthesized on the 80S ribosomes. This question was examined by using both antibiotics on the same *Euglena* culture.

Dark-grown *Euglena* cells were exposed to light in the presence of both chloramphenicol and cycloheximide. Under these conditions the chlorophyll synthesis was restored to a normal value (Table 1) (see also Fig. 4 of Wolfvitch & Perl, 1972). Since the addition of both cycloheximide and chloramphenicol results in a normal greening activity, cytoplasmic ribosomes are evidently involved in a repressor synthesis, and the 70S (chloroplast?) ribosomes are synthesizing a de-repressor which inactivates the repressor synthesized in the cytoplasm.

To examine whether this repression-de-repression system is present in dark-grown cells or whether it appears only after illumination, cycloheximide and chloramphenicol were added separately to a dark-grown culture at different time-intervals as indicated before the cells were exposed to light (zero time on the abscissa; Fig. 5). After 24 and 48 h of illumination chlorophyll content was determined in all cultures. Early addition of cycloheximide in the dark greatly stimulated chlorophyll synthesis (Fig. 5). This stimulation was less when the drug was added either after or just before the start of the illumination that resulted in the development of chlorophyll. On the other hand, early addition of chloramphenicol to the culture caused greater inhibition of greening. The effect of early addition of cycloheximide or chloramphenicol indicated that the cytoplasmic repressor and the de-repressor synthesized on 70S ribosomes are present in dark-grown *Euglena* cells.

The effect of the early addition of chloramphenicol can be seen only up to 24 h of illumination: this indi-

Fig. 5. Effects on chlorophyll synthesis of pretreatment of dark-grown cells by cycloheximide and chloramphenicol

Chloramphenicol (1 mM; $\circ$) or cycloheximide (1 μ M; $\bullet$) was added to dark-grown *Euglena* cells, and illumination for 24 h (a) or 48 h (b) was started at various times after this addition. At the end of the illumination the chlorophyll content and cell number were determined. ($\square$, Cells illuminated without drug.)

cates that the de-repressor is not very stable and that its effectiveness is limited to this relatively short time. The possibility that cycloheximide causes a feedback inhibition of amino acid synthesis (Fletcher & Beevers, 1971), which could thus increase the amount of material available for chlorophyll synthesis, has been examined by adding different amounts of amino acids (0.1–10 mM, individually or together), but no stimulation of chlorophyll synthesis was observed. Some inhibition was noted at relatively high concentrations (1 mM) of proline and hydroxyproline.

Discussion

The synthesis of chloroplast proteins and chlorophyll is readily affected by a variety of environmental factors that have no appreciable effect on cell growth.

Chloroplast formation in *Euglena gracilis* requires the continued presence of light. In the dark, the synthesis of structural proteins and soluble photosynthetic enzymes associated with the chloroplasts ceases. Since cycloheximide affects cytoplasmic 80S ribosomal activity, the stimulation of chlorophyll synthesis by this drug at a low concentration may be explained by the existence of a repressor synthesized on the 80S ribosomal fraction that inhibits chlorophyll formation. Horowitz *et al.* (1970) demonstrated a similar inhibition by cycloheximide of the repression of tyrosinase synthesis. On the other hand, the inhibitory effect of chloramphenicol indicates that chlorophyll synthesis also depends on the 70S ribosomal fraction (chloroplast?) activity.

The fact that the amount of chlorophyll per ml of cell culture increased at the stimulatory concentration of cycloheximide (the increase is not only per cell, as can be calculated from Fig. 4a) excludes the possibility that the stimulatory effect may result from the greater inhibition by cycloheximide of cell division than of chlorophyll synthesis.

The results obtained on chlorophyll synthesis in the presence of both cycloheximide and chloramphenicol indicate that the decrease evoked by chloramphenicol can be reversed by cycloheximide, presumably because the synthesis of repressor is prevented.

Schwartz *et al.* (1965) have indicated that the chloroplast ribosomes do not possess any coding specificity, but they may play a role in the selective regulatory process concerned with chloroplast protein synthesis. With this in mind, our results may be interpreted as follows.

Scheme 1. Scheme proposed for controlling chlorophyll synthesis in *Euglena*

The arrows show the influence of internal or external materials (or products) on chlorophyll synthesis. The broken lines indicate repression activity.

The enzymes that make chlorophyll are formed on the 80S ribosomal fraction, and the same cytoplasmic ribosomes produce a substance that represses chlorophyll synthesis.

A protein synthesized by the 70S ribosomes de-represses the inhibitory factor.

In the presence of both cycloheximide and chloramphenicol the greening of the cell is not inhibited. The cycloheximide inhibits synthesis of the repressor and therefore no de-repression is needed for chlorophyll synthesis; chloramphenicol thus has no effect on greening if cycloheximide is present.

The effect of light on the chloroplast-ribosomal activity seems to be through the synthesis of the de-repressor in the light by this ribosomal fraction. The results in Fig. 3 show that the synthesis of the repressor during the dark period of growth proceeds more rapidly than the synthesis of the de-repressor by the 70S ribosomes. This indicates that the synthesis of the de-repressor is intensified in the light.

The repression effect could explain the findings published by Richards *et al.* (1971) that cycloheximide causes a relatively higher incorporation of [32 P]-orthophosphate into the chloroplast DNA than into cytoplasmic DNA.

Our results confirm the existence of an 80S ribosome product that may act on the chloroplast DNA as a repressor (see Scheme 1).

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