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

CARBON DIOXIDE FIXATION

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It was shown that cycloheximide inhibits CO_2 fixation in *Euglena* cells in the dark, but no effect of chloramphenicol was found. The light-dependent CO_2 fixation was inhibited by chloramphenicol and by large amounts of cycloheximide, but was stimulated by small amounts of cycloheximide. The presence of the stimulatory concentration of cycloheximide abolished the inhibition effect of high concentrations of chloramphenicol. The results indicate that the light-dependent CO_2 fixation is controlled by a repression-de-repression regulatory system, which seems to be independent of chlorophyll synthesis.

Photosynthetic CO_2 fixation becomes established after illumination of dark-grown *Euglena* cells for about 6 h (Brody *et al.*, 1965), although chlorophyll appears earlier. This indicates that the chlorophyll is not at first capable of participation in photosynthesis and leads to the belief that CO_2 fixation must await the independent synthesis of new enzyme proteins.

The synthesis of chlorophyll could be stimulated by cycloheximide even if protein synthesis was inhibited on the 80S ribosomal fraction, indicating that a repression system for chlorophyll synthesis was inhibited. The repressor has been shown to be inhibited by a protein synthesized on the 70S ribosomes (Perl, 1972).

The present paper deals with the question of whether or not the same repression-de-repression system affects the onset of CO_2 fixation in *Euglena* cells as well as with the relationship between CO_2 fixation and chlorophyll synthesis in the cell.

Materials and Methods

Euglena gracilis var. *bacillaris* cells were grown as previously described (Perl, 1971).

Measurement of CO_2 fixation

Batches (2ml) of cells in their normal growth medium were transferred into 12ml capped vials. For drug experiments cycloheximide or chloramphenicol, or both, were added and the vials were kept under dark or light conditions (Perl, 1971). After 12–16h, $^{14}\text{CO}_3^{2-}$ (10^5 c.p.m.; 33.3 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added and the vial was immediately capped and returned for the indicated time to normal growth conditions.

Fig. 1. Effect of chloramphenicol on CO_2 fixation

A batch (2ml) of dark-grown *Euglena* cells was incubated for 16–18h with different amounts of chloramphenicol, in the dark (○) or under normal conditions in the light (●), and $^{14}\text{CO}_3^{2-}$ (10^5 c.p.m.) was added and incubated for 2h at the same growth conditions. The free $^{14}\text{CO}_2$ was removed by ventilation and the cells were washed on a Millipore filter with water adjusted to pH 3.0 with HCl. The filter was dried at room temperature and counted for radioactivity.

Fig. 2. Effect of cycloheximide on CO_2 fixation

A batch (2ml) of dark-grown *Euglena* cells was incubated for 16–18 h in the presence of the indicated amounts of cycloheximide in the dark (○) or under normal conditions in the light (●). After the cell number had been measured, $^{14}\text{CO}_3^{2-}$ (10^5 c.p.m.) was added and reincubated for 2 h. Further treatments for radioactivity measurements were as described in Fig. 1.

The incorporation was stopped by opening the vials in a fume cupboard. The cells were immediately filtered on a Millipore filter ($5\mu\text{m}$ pores). The filter was washed three times with 3 ml of water adjusted to pH 3.0 with HCl, dried at room temperature and counted for radioactivity in toluene scintillation solution (Perl, 1972) in a Packard Tri-Carb liquid-scintillation counter.

Cell number was determined with a Coulter counter and by counting on a cytometer. Chlorophyll was determined by the method of Arnon (1949).

Results

The CO_2 fixation was examined by the methods described, at different incubation times and with different numbers of cell in the experimental vials. The amount of the CO_2 fixed was directly proportional to the number of cells and linear with time for up to 5 h.

Fig. 3. Effect of cycloheximide or chloramphenicol on 'light-dependent CO_2 fixation' in *Euglena* cells

The curves presented are the result of plotting the difference between CO_2 fixation in the light and in the dark from Fig. 1 (curve with chloramphenicol, ○) and from Fig. 2 (curve with cycloheximide, ●).

In preliminary experiments when dark-grown cells were used we noticed a relatively high CO_2 fixation in the dark-grown cells kept in darkness with $^{14}\text{CO}_2$. This 'dark CO_2 fixation' was lower in cells illuminated for 2 h. On the other hand, illuminated *Euglena* cells developed a 'light-dependent CO_2 fixation' which was inactive in the darkness. Therefore two kinds of measurements have been done for each sample, i.e. 'dark CO_2 fixation' and 'light CO_2 fixation'.

The presence of chloramphenicol in the medium as an inhibitor of the 70S ribosomal activity does not affect the 'dark fixation' of CO_2 even at relatively high concentrations, but the 'light fixation' is strongly inhibited (Fig. 1). Since chloramphenicol inhibits the 70S ribosomal protein synthesis, it seems that the 'light-fixation' system is synthesized on this ribosomal fraction.

When cycloheximide was used as an inhibitor for the 80S ribosomal activity, the 'dark fixation' was inhibited. This inhibition was dependent on the cycloheximide concentration in the medium (Fig. 2).

These results indicate that the effect of cycloheximide and chloramphenicol is on a specific protein system synthesized on the 70S or 80S ribosomes, rather than on a non-specific inhibitor for CO₂ fixation.

The 'light fixation' was stimulated by the drug at 1–100 μ M. The decrease of the CO₂ fixation at higher concentrations of cycloheximide seems to reflect in part the decrease in the 'dark-fixation' system which is included in the 'light fixation'. At high concentrations of cycloheximide there is a decrease in CO₂ fixation; nevertheless the 'light fixation' is always higher than the dark activity (Figs. 2 and 3). The differences between 'light CO₂ fixation' and 'dark CO₂ fixation' plotted against the concentration of cycloheximide (Fig. 3) illustrate the existence of a 'repression curve', i.e. there is a range of cycloheximide concentration that stimulates the 'light-dependent CO₂ fixation'. This curve is similar to the curve demonstrated for chlorophyll synthesis (Perl, 1972).

The decrease in 'light-dependent fixation' of CO₂ may have been due to inhibition of chlorophyll syn-

thesis by chloramphenicol or to inhibition of the synthesis of the enzymes required for CO₂ fixation. Perl (1972) has shown that the presence of cycloheximide at a low concentration in the medium restores the chlorophyll synthesis inhibited by a relatively high amount of chloramphenicol. Hence by the use of both inhibitors, cycloheximide at its stimulatory concentration in the medium and chloramphenicol at different concentrations, it was shown the chlorophyll synthesis was practically unaffected (Fig. 4). The CO₂ fixation was still inhibited by high concentration of chloramphenicol, but the inhibition caused by chloramphenicol was much less in the presence of cycloheximide than in its absence. After the cells were grown for 48 h in the presence of both drugs, the inhibition of 'light fixation' by chloramphenicol was abolished (Fig. 5).

Discussion

The pathways of photosynthetic CO₂ fixation in *Euglena* appear to be typical of those found in many higher plants (Lynch & Calvin, 1953; Norris *et al.*,

Fig. 4. Effect of chloramphenicol on chlorophyll synthesis in dark-grown cells during illumination in the presence and in the absence of stimulatory amounts of cycloheximide

Dark-grown cells were grown in the absence and in the presence of 3 μ M-cycloheximide and in the presence of the indicated amounts of chloramphenicol. After 24 h (●, + cycloheximide) and after 48 h (○, + cycloheximide; ▲, no cycloheximide) of illumination, cell number and chlorophyll content were measured. (A) and (B) represent no chloramphenicol and no cycloheximide respectively.

Fig. 5. Effect of chloramphenicol on CO_2 fixation in the presence of stimulatory amounts of cycloheximide

Dark-grown cells were grown for 24h (a) or 48h (b) in darkness (○) or in light conditions in the absence (□) and in the presence (●) of $3 \mu\text{M}$ -cycloheximide and in the presence of the indicated amounts of chloramphenicol. Then 2ml of cells was incubated in screw-top vials with $^{14}\text{CO}_3^{2-}$ (10^5 c.p.m.) in the dark or in light conditions for 2h. The labelled cells were washed and radioactivity was measured as described in Fig. 1. (A) and (B) represent no chloramphenicol and no cycloheximide respectively.

1955). By the use of chloramphenicol it has been shown that at least one enzyme involved in this process, the non-metal aldolase (EC 4.1.2.13), was present in lower amounts in cells treated with the drug than in untreated cells (Smillie, 1968). In the preceding paper (Perl, 1972) we have obtained evidence that chlorophyll synthesis is under a regulatory system in which a product of the 80S ribosomes represses chlorophyll synthesis, whereas a product synthesized by the 70S ribosomes de-represses. The results in the present paper show that the *Euglena* cells have a 'dark- CO_2 -fixation' and a 'light- CO_2 -fixation' system. By using different amounts of cycloheximide or chloramphenicol in the growth medium, we have shown that cycloheximide inhibits the synthesis of the enzymes of the 'dark CO_2 fixation' whereas that of 'light CO_2 fixation' is stimulated by this drug at low concentration. This indicates that the enzymes of photosynthetic fixation of CO_2 are inhibited by a product of the 80S ribosomes.

Chloramphenicol inhibits only the synthesis of enzymes of the 'light-fixation' system but does not affect the synthesis of enzymes of the 'dark-fixation' system. The inhibition of CO_2 fixation may be explained either by an inhibition of synthesis of the enzymes essential for this process of the 70S ribosomes, or by inhibition of production of a de-repressor by this ribosomal fraction.

By the use of the stimulatory concentration of cycloheximide together with chloramphenicol in the growth medium of *Euglena*, it was shown that the inhibitory effect of chloramphenicol was abolished by the addition of cycloheximide. This indicates that the process of the 'light-dependent CO_2 fixation' is activated by proteins synthesized on the 80S ribosomal fraction, but that it is regulated by a repression system synthesized on the same ribosomal fraction. This repression is apparently de-repressed by a product of the activity of the 70S ribosomes.

Comparing the results obtained for chlorophyll synthesis in the preceding paper (Perl, 1972) with those for CO_2 fixation, it is suggested that synthesis of enzymes of the 'light- CO_2 -fixation' system is dependent either on the chlorophyll content or on the energy-producing ability of the cell.

Comparing the results obtained for chlorophyll synthesis in the presence of both antibiotics with those obtained for CO_2 fixation under the same conditions, it seems that there is no direct correlation between these two processes: at the same cell content of chlorophyll, the CO_2 fixation/unit weight of chlorophyll is diminished as the concentration of chloramphenicol is raised. It therefore seems that development of the ability to produce the enzymes needed to fix CO_2 during illumination is independent of the

development of the enzyme apparatus for chlorophyll synthesis.

References

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