EVIDENCE FOR THE SYNTHESIS IN VIVO OF PROTEINS OF THE CALVIN CYCLE AND OF THE PHOTOSYNTHETIC ELECTRON-TRANSFER PATHWAY ON CHLOROPLAST RIBOSOMES

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One function of chloroplast DNA appears to be the formation of a protein-synthesizing system within the chloroplasts. Specifically, chloroplast DNA codes for the RNA of chloroplast ribosomes (Scott and Smillie, 1967). However, the exact role of the chloroplast protein-synthesizing system during the differentiation of chloroplasts and, in particular, the identity of the proteins synthesized on chloroplast ribosomes is not known. Evidence that the synthesis of both stromal and lamellar-bound enzymes of photosynthesis occurs within chloroplasts and on the chloroplast ribosomes has been obtained by comparing the effects of two inhibitors of protein synthesis, chloramphenicol and cycloheximide, on cells containing differentiating chloroplasts.

While protein synthesis in most non-bacterial systems is only marginally affected by chloramphenicol (Gale, 1963), chloroplasts constitute a notable exception. Thus the incorporation of amino acids into protein by ribosomes isolated from chloroplasts is sensitive to chloramphenicol (Spencer 1965), and the development of chloroplasts in Euglena gracilis is prevented by chloramphenicol at concentrations which have little effect on essential

cytoplasmic protein synthesis (Smillie et al., 1963). These effects have been attributed to the greater capacity of chloroplast ribosomes, as compared with cytoplasmic ribosomes, to bind chloramphenicol (Anderson and Smillie 1966). Thus a study of the action of chloramphenicol on the synthesis in vivo of individual chloroplast proteins may reveal the identity of those proteins whose synthesis involves the chloroplast ribosomes. The results of such a study are reported in this paper together with parallel studies carried out with cycloheximide which, in contrast to chloramphenicol, inhibits protein synthesis by ribosomes isolated from higher organisms, including yeast (Siegel and Sisler, 1965) and mammalian cells (Ennis and Lubin, 1964), but not from bacteria (Ennis and Lubin, 1964).

MATERIALS AND METHODS

Euglena gracilis, strain Z, was cultivated in continuous darkness at 25°C in the growth medium of Hutner et al. (1956). To study chloroplast formation, cells in the exponential phase of growth were transferred to fresh medium from which all carbon compounds, other than thiamine and vitamin B_{12}, had been omitted. The cells were shaken in the dark for 48 hours, D-glucose (22.5 g/l) was then added, and the cells were immediately exposed to continuous white light (125 to 150 ft-candles). Inhibitors were added with the glucose.

Cell-free extracts were obtained by passing a suspension of cells in 0.1 M Tris-HCl buffer, pH 7.8, through a French pressure cell. Isolated lamellae were assayed for bound ferredoxin-NADP-reductase by the reduction of 2,6-dichlorophenol indophenol in the presence of $NADPH_2$, and cytochrome-561 was determined spectrophotometrically after extracting the lamellae with cold 80% acetone. Particle-free extracts were assayed for cytochrome-552 spectrophotometrically, for Fraction I protein after separation by electrophoresis at pH 8.3 on polyacrylamide gel, and for ribulose-1,5-bisphosphate carboxylase and NADP-glyceraldehyde-3-phosphate dehydrogenase by procedures based on those described by Smillie (1962) and Gibbs (1955), respectively.

RESULTS

Fig. 1 shows the effects of chloramphenicol and cycloheximide on chlorophyll synthesis and cell division. At 1 mg/ml, chloramphenicol inhibited

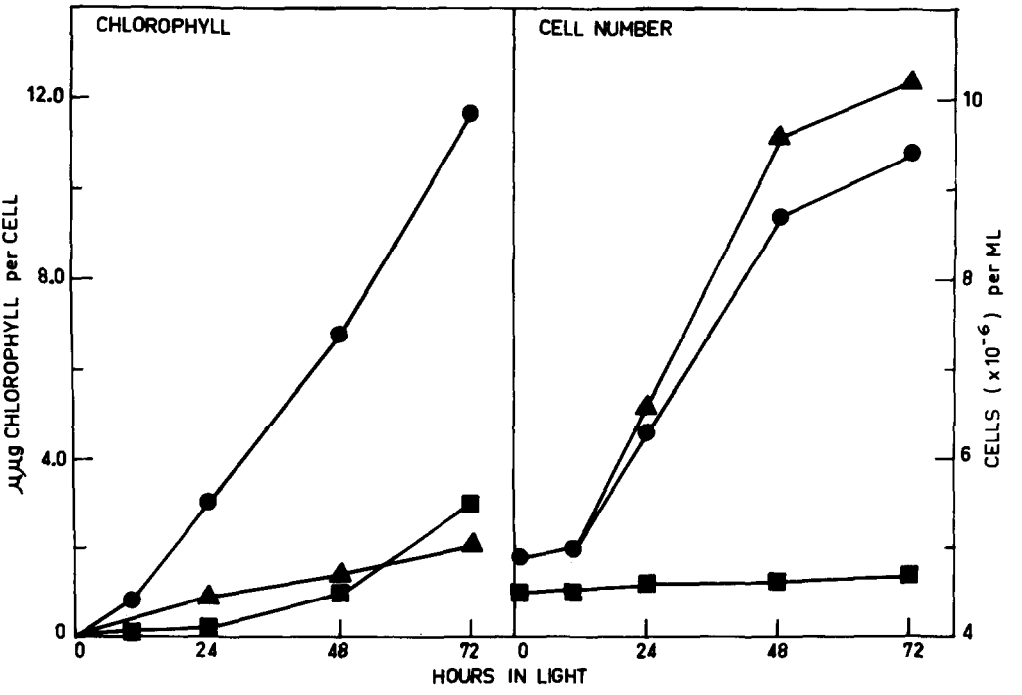

Fig. 1. The effect of chloramphenicol and cycloheximide on chlorophyll synthesis and cell division. ● , control; ▲ , chloramphenicol (1 mg/ml); ■ , cycloheximide (15 μg/ml).

chlorophyll formation by 82% after 72 hours of illumination, but there was no inhibition of cell division; indeed the rate of cell division was slightly increased by chloramphenicol. Cycloheximide at 15 μg/ml inhibited chlorophyll synthesis by 75% after 72 hours and completely stopped cell division.

Table I shows the effect of chloramphenicol and cycloheximide on the formation of chloroplast proteins. Both inhibited the synthesis of ferredoxin-NADP-reductase, cytochrome-552 (a c-type cytochrome), and

cytochrome-561 (a b-type cytochrome), all proteins of the photosynthetic electron transfer pathway. Chloramphenicol also severely inhibited the production of two enzymes of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase and NADP-glyceraldehyde-3-phosphate dehydrogenase. In contrast, cycloheximide did not inhibit the formation of these enzymes, even though both cell division and the synthesis of chloroplast lamellar proteins were inhibited.

Table I

Effect of chloramphenicol (CAP) and cycloheximide on the synthesis of chloroplast proteins

	No Inhibitor	CAP	Cycloheximide
Ferredoxin-NADP-reductase	8.55	1.58	3.06
Cytochrome-552	46.4	7.7	11.4
Cytochrome-561	44	0.9	13.3
Ribulose-1,5-bisphosphate carboxylase	0.62	0.07	1.34
NADP-glyceraldehyde-3-phosphate dehydrogenase	13.0	-0.6	15.3

Values shown are the increases in components of cells during illumination for 72 hr. Units: activities, μmoles substrate per min per 10^9 cells; cytochromes, mμmoles per 10^9 cells.

Fraction I protein, the major soluble protein of chloroplasts and the site of activity of ribulose-1,5-bisphosphate carboxylase, was likewise synthesized in cycloheximide-treated cells (Fig. 2), although the level attained was not as high as in the control cells. Fraction I protein was barely detectable in cells grown in the presence of chloramphenicol.

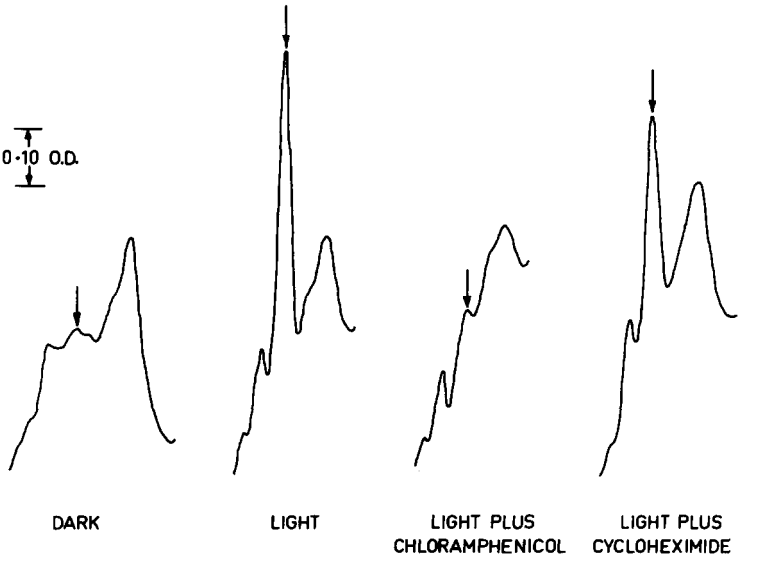

Fig. 2. Densitometer traces of Euglena proteins separated on polyacrylamide gel in the region occupied by Fraction I protein. The proteins were stained with amido black. Arrows point to the peaks attributed to Fraction I protein. Inhibitor concentrations were as in Fig. I.

Parallel experiments have been carried out with non-dividing cells (in these experiments the addition of glucose to the medium just prior to exposing the cells to light was omitted), but the results for the synthesis of chlorophyll and chloroplast proteins were substantially the same as in the dividing system.

DISCUSSION

The data shown in Fig. 1 and Table I are clearly consistent with the hypothesis that the light-dependent synthesis of ribulose-1,5-bisphosphate carboxylase, NADP-glyceraldehyde-3-phosphate dehydrogenase, and Fraction I protein occurs on the chloramphenicol-sensitive ribosomes of chloroplasts.

Their synthesis was inhibited by concentrations of chloramphenicol which gave no inhibition of cell division. Cycloheximide inhibited both pigment synthesis and cell division in Euglena, confirming Kirk and Allen's (1965) earlier observations; but, more significantly, increases in the activities of two of the Calvin cycle enzymes and a net synthesis of Fraction I protein continued.

Chloramphenicol also inhibited the synthesis of the three electron transfer proteins that were assayed, and in addition, it is known to inhibit chlorophyll synthesis and the light-dependent incorporation of Mn into lamellae (Smillie et al., 1963). These results implicate chloroplast ribosomes as sites of synthesis of proteins of the photosynthetic electron transfer pathway, but the question arises as to why their synthesis, in contrast to that of proteins in the stroma, is at the same time inhibited by cycloheximide. One possible explanation is that the inhibitory effect of cycloheximide is an indirect one resulting from a block in the synthesis of certain structural elements essential for the synthesis of the electron transfer proteins and their incorporation into lamellae.

Our results for chloroplasts contrast sharply with recent studies on the function of the protein-synthesizing system of mitochondria. Certain of the soluble enzymes catalyzing the cyclic oxidation of carbon compounds to CO_2 do not appear to be synthesized in the mitochondrion (Roodyn et al., 1962), nor is their synthesis in vivo inhibited by chloramphenicol (Clark-Walker and Linnane, 1967). In contrast, it is the chloroplast rather than the cytoplasm that appears to be the site of synthesis of enzymes catalyzing the cyclic reduction of CO_2 in photosynthesis. Similarly, the synthesis of at least one mitochondrial electron transfer protein, cytochrome c, is extra-mitochondrial (Beattie et al., 1966) and is insensitive to chloramphenicol (Huang et al., 1966); whereas the synthesis of the easily extractable c-type cytochrome of Euglena chloroplasts, cytochrome-552, as well as of the other electron transfer proteins investigated, is inhibited by chloramphenicol and appears to involve organelle-localized ribosomes. These results suggest that chloroplast

ribosomes are involved in the synthesis of a wider range of organelle proteins than is the case with the protein-synthesizing system of mitochondria.

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REFERENCES

Anderson, L.A. and Smillie, R.M., Biochem. Biophys. Res. Commun. 23, 535 (1966).
Beattie, D.S., Basford, R.E. and Koritz, S.B., Biochemistry 5, 926 (1966).
Clark-Walker, G.D. and Linnane, A.W., J. Cell Biol. 33, in press (1967).
Ennis, H.L. and Lubin, M., Science 146, 1474 (1964).
Gale, E.F., Pharmocol. Rev. 15, 481 (1963).
Gibbs, M. in S.P. Colowick and N.O. Kaplan (Eds.) Methods in Enzymology, Vol. I, Academic Press, Inc., New York, 1955, p.411.
Huang, M., Biggs, D.R., Clark-Walker, G.D. and Linnane, A.W., Biochim. Biophys. Acta 114, 434 (1966).
Hutner, S.H., Bach, M.K. and Ross, G.I., J. Protozool. 3, 101 (1956).
Kirk, J.T.O., and Allen, R.L., Biochem. Biophys. Res. Commun. 21, 523 (1965).
Roodyn, D.B., Suttie, J.W. and Work, T.S., Biochem. J. 83, 29 (1962).
Scott, N.S. and Smillie, R.M., Biochem. Biophys. Res. Commun. 28,598 (1967).
Siegel, M.R. and Sisler, H.D., Biochim. Biophys. Acta 103, 558 (1965).
Smillie, R.M., Plant Physiol. 37, 716 (1962).
Smillie, R.M., Evans, W.R. and Lyman, H., Brookhaven Symp. Biol. 16, 89 (1963).
Spencer, D., Arch. Biochem. Biophys. 111, 381 (1965).