Events Surrounding the Early Development of *Euglena* Chloroplasts: Cellular Origins of Chloroplast Enzymes in *Euglena**

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SUMMARY

During chloroplast development, the large increases in ribulose diphosphate carboxylase (RUDPCase) activity and cytochrome 552 concentration follow the pattern of chlorophyll synthesis, in that the formation of these two enzymes is inhibited by streptomycin (Sm) and by chloramphenicol (Cm) beyond 12 h of development. Neither enzyme can be detected in *w₃BUL*, a mutant of *Euglena* in which chloroplasts and chloroplast DNA are undetectable. In contrast, the NADP-linked triose phosphate dehydrogenase (NADP-TPDase), another plastid-localized enzyme, increases in activity without the 12 h lag normally observed for chlorophyll synthesis; this increase in activity is not inhibited by Sm and Cm, but is inhibited by cycloheximide, an antibiotic which acts on 80S cytoplasmic ribosomes. NADP-TPDase activity is present at the same level in *w₃BUL* as in the dark-grown wild-type organisms. These data are interpreted to mean that NADP-TPDase is coded in the nuclear DNA, and is translated on 80S cytoplasmic ribosomes. The sensitivity of the increase in cytochrome 552 and RUDPCase activities to Sm and Cm indicates that they are translated, at least in part, on the 68S ribosomes of the chloroplast. Thus, chloroplast differentiation in *Euglena* is dependent upon information and synthetic machinery from both the plastid and the rest of the cell. Since total cellular protein does not change significantly during chloroplast development in resting cells, we conclude that protein turnover probably occurs.

INTRODUCTION

The finding that many constituents required for chloroplast development may come from outside the plastid (Schiff, Zeldin & Rubman, 1967; Schiff, 1971; Schiff & Zeldin, 1968; Smillie, 1968) suggests that certain chloroplast molecules might be supplied through synthetic activities and informational contributions from outside the chloroplast. Certain antibiotics, such as chloramphenicol and streptomycin, selectively inhibit protein synthesis on 68S plastid ribosomes, but have little or no effect on cell division or viability in *Euglena*, implying that they do not inhibit protein synthesis on the 80S cytoplasmic ribosomes (Smillie *et al.* 1967; Provasoli, Hutner & Schatz, 1948; Avadhani & Buetow, 1972*b*; Schwartzbach, Freyssinet & Schiff, 1973; Bovarnick, Chang, Schiff & Schwartzbach, 1974). In addition, mutant strains of *Euglena* are available in which plastid DNA is undetectable (Edelman, Schiff & Epstein, 1965) providing a means of determining whether chloroplast DNA is involved in coding or controlling the formation of these plastid proteins. From the enzymes which have been localized in the plastids of *Euglena* (Smillie, Evans & Lyman, 1963; Smillie, 1968), we have chosen ribulose diphosphate carboxylase (Fuller & Gibbs, 1959; Latzko & Gibbs, 1968) the NADP-linked triose phosphate dehydrogenase

* No. 4 of a series.

(Heber, Pon & Heber, 1963; Pupillo, 1972), both enzymes of the carbon dioxide fixation cycle, and cytochrome 552 (Perini, Schiff & Kamen, 1964; Nishimura, 1959; Wolken & Gross, 1963; Katoh & San Pietro, 1967), a member of the photosynthetic electron transport chain. A preliminary account of this work has been presented (Bovarnick, Freedman & Schiff, 1970).

METHODS

Euglena gracilis Klebs var. *bacillaris* Pringsheim and w₃BUL, an aplastidic mutant derived from this strain in which plastid DNA is undetectable (Edelman *et al.* 1965) were used in the present study. The methods for cultivation and maintenance of the organisms as well as the techniques for use of streptomycin (Sm) are described in the accompanying paper (Bovarnick *et al.* 1974). Streptomycin was added to 3-day-old resting cells at a final concentration of 0.05 %, immediately before cultures were exposed to light. Chloramphenicol (Cm) was used at a final concentration of 2 mg/ml. For studies of plastid development, sterile resting medium containing 4 mg Cm/ml was added to an equal volume of medium containing 3-day-old resting organisms; control cultures were diluted with an equal volume of resting medium lacking Cm. Cultures were placed in the light immediately after Cm addition. Cycloheximide was used at a final concentration of 15 µg/ml. Chloramphenicol and cycloheximide were sterilized by millipore filtration.

The techniques for determination of chlorophyll, carotenoid and photosynthetic CO₂ fixation, as well as the assays of the NADP-linked triose phosphate dehydrogenase (NADP-TPDase), ribulose diphosphate carboxylase (RUDPCase) and cytochrome 552, are described in the accompanying paper (Bovarnick *et al.* 1974). NAD-linked triose phosphate dehydrogenase was assayed according to the method given for NADP-TPDase, except that NADH was substituted for NADPH. The NAD enzyme retained its full activity for several hours in the ice bath.

For measurements of the protein content of whole organisms the following method was employed. Suspensions were centrifuged for 10 min at 1100 g, and the pellet extracted with acetone at room temperature. After recentrifugation the pellet was resuspended in NaOH, heated in a water bath for 5 min at 60 °C, and then left in the refrigerator overnight or kept in the frozen condition for several days. The suspension was then centrifuged and the supernatant fluid used for protein assays which employed standard procedures (Lowry, Rosebrough, Farr & Randall, 1951) using the alternative of preparing the Na₂CO₃ solution in water rather than in NaOH. If insufficient NaOH was present after adding the protein solution, more was added at this point before proceeding with the usual methodology.

RESULTS AND DISCUSSION

Studies with Sm

Streptomycin has no effect on chlorophyll formation during the first 12 to 14 h of light-induced chloroplast development in dark-grown non-dividing *Euglena gracilis* var. *bacillaris* (Bovarnick *et al.* 1974). After this time, however, further chlorophyll accumulation was inhibited almost completely.

Cytochrome 552

Cytochrome 552, a component of the photosynthetic electron transport chain in *Euglena* chloroplasts (Katoh & San Pietro, 1967) increases during light-induced chloroplast development (Perini *et al.* 1964). Since the assay for this cytochrome is relatively insensitive, especially in the dark-grown organisms where comparable amounts of a closely absorbing

Table 1. *Kinetics of appearance of cytochrome 552 during light-induced chloroplast development in resting Euglena in the presence and absence of streptomycin*

Time in light (h)	$10^{11} \times \text{cytochrome 552}^* (\mu\text{mol/organism})$	
	— Sm	+ Sm
6	0.01	—
12	0.16 ± 0.19	0.14 ± 0.13
24–36	0.61 ± 0.28	0.23 ± 0.19
48	1.90, 1.50	—
68–117	2.80 ± 0.44	0.39 ± 0.23

* Here and in subsequent Tables, each value reported represents the mean of several experiments, $\pm$ the 95 % confidence interval of the mean.

Table 2. *Kinetics of the appearance of ribulose diphosphate carboxylase activity during light-induced chloroplast development in resting Euglena in the presence and absence of streptomycin*

Time in light (h)	Ribulose diphosphate carboxylase ($\mu\text{mol CO}_2$ fixed/mg protein/h)	
	— Sm	+ Sm
0	0.16 ± 0.04	—
6–9	0.19 ± 0.04	—
12	0.23 ± 0.14	—
12–24	—	0.23 ± 0.08
16–24	0.39 ± 0.14	—
24–50	—	0.14 ± 0.04
48	1.63	—
55–115	4.14 ± 0.45	0.14 ± 0.01

c-type mitochondrial cytochrome (cytochrome 556) are present, one cannot say with assurance that it is absent from the dark-grown organisms or that it definitely increases during the first 12 h of development, although this seems to be the trend in the present data and in previous studies (Table 1; Perini *et al.* 1964). When plastid development was carried out in the presence of Sm, comparable levels were found at 12 h in the controls and the Sm-treated organisms but the increase beyond 12 h was blocked (Table 1). Thus the behaviour of cytochrome 552 during development in the presence of Sm resembles that of chlorophyll.

Ribulose diphosphate carboxylase (RUDPCase)

RUDPCase was already present at substantial levels in the dark-grown organisms and did not increase during the first 12 h of development (Table 2). Thus, Sm had no effect during the first 12 h, but beyond this point the formation of RUDPCase was almost completely inhibited. Again, the pattern of inhibition is like that of chlorophyll and cytochrome 552 formation.

We have recently demonstrated that dihydrostreptomycin binds specifically to 68S *Euglena* chloroplast ribosomes (Schwartzbach *et al.* 1973). Since Sm is an inhibitor of translation on bacterial 70S ribosomes (Modolell & Davis, 1968), it seems likely that Sm specifically inhibits translation on *Euglena* 68S chloroplast ribosomes. The lack of effect on division or viability (Provasoli *et al.* 1948; Bovernick *et al.* 1974) would seem to exclude any inhibitory effect of Sm on the 87S cytoplasmic ribosomes, or on the mitochondrial system (Avadhani & Buetow, 1972*a, b*) since *Euglena* is an obligate aerobe. Since protein synthesis on mitochondrial ribosomes has been shown to be inhibited by Sm and Cm (Avadhani & Buetow, 1972*a, b*), the mitochondria may be impermeable to these antibiotics.

Table 3. *Kinetics of appearance of NADP-linked triose phosphate dehydrogenase during light-induced chloroplast development in resting Euglena in the presence and absence of streptomycin and of cycloheximide*

Time in light (h)	NADP-linked triose phosphate dehydrogenase ($\mu\text{mol NADPH oxidized/mg protein/h}$)		
	– Antibiotics	+ Streptomycin	+ Cycloheximide
0	4.0 ± 0.75	4.0 ± 0.75	2.7
12	9.9 ± 2.80	9.15 ± 1.91	2.6
24	14.7 ± 0.55	12.00, 12.6	1.7
36–48	22.9 ± 4.87	19.9	2.5, 2.0
72–96	27.0 ± 6.83	16.0 ± 6.25	1.3

Table 4. *Specific activity of DPN-linked triose phosphate dehydrogenase during light-induced chloroplast development in resting Euglena in the presence and absence of streptomycin*

Time in light (h)	NAD-linked triose phosphate dehydrogenase ($\mu\text{mol NADH oxidized/mg protein/h}$)	
	– Sm	+ Sm
0	43.5 ± 15.0	43.5 ± 15.0
12–24	33.0 ± 8.0	34.0 ± 14.0
31–55	30.3 ± 4.0	35.0 ± 11.0
60–96	26.8 ± 6.0	32.0 ± 6.0

TPN-linked triose phosphate dehydrogenase (NADP-TPDase)

The NADP-TPDase showed quite a different pattern (Table 3). It developed within an apparent lag and its formation was relatively insensitive to Sm. Although there was some difference between the controls and Sm-treated cells at 72 h, the 95 % confidence ranges of the means overlap and the variability makes it doubtful that even this difference is significant. Table 3 also shows that cycloheximide, an inhibitor of translation on 87S ribosomes, completely inhibited the increase. Thus, this enzyme does not appear to be synthesized on the 68S chloroplast ribosomes and is probably translated on the Sm-resistant cycloheximide-sensitive ribosomes of the cytoplasm. This leaves open the question of whether mitochondrial ribosomes might participate. The electron micrographs of mitochondria in whole organisms show few or no ribosomes (Schiff, 1970), but ribosomes isolated from the mitochondrial fraction are not sensitive to cycloheximide (Avadhani & Buetow, 1972*a, b*). If we assume that mitochondria are permeable to cycloheximide, then the cycloheximide sensitivity of the NADP-TPDase would argue against the mitochondrion being a site of synthesis for this enzyme.

The NAD-linked triose phosphate dehydrogenase (Table 4) from the cytoplasm of *Euglena* (probably the glycolytic enzyme) decreased slightly during plastid development, but Sm appeared to have little or no effect.

Studies with chloramphenicol (Cm)

Chloramphenicol is another inhibitor which is thought to block translation on bacterial 70S ribosomes and which has no effect on the viability or growth of *Euglena* at the concentrations we employed (Provasoli *et al.* 1948; Smillie *et al.* 1963, 1967). This antibiotic showed essentially the same pattern of inhibition as Sm (Table 5). To ensure that these results were not due to some side effect of Cm, following Ellis (1969) we used the same

Table 5. *Changes in various parameters during light-induced chloroplast development in resting Euglena in the presence and absence of chloramphenicol*

Parameter	No light	Light for 72 h	
		- Cm	+ Cm
Chlorophyll (pg/organism)	0	10.07 ± 1.59	4.95 ± 0.83
Photosynthesis (pmol CO ₂ fixed/organism/10 min)	0	0.265 ± 0.058	0.07 ± 0.02
RUDPCase (μmol CO ₂ fixed/mg protein/h)	0.053, 0.046	2.11, 9.68, 3.32	0.807, 1.43, 0.59
NADP-TPDase (μmol NADPH oxidized/mg protein/h)	6.17, 4.26	31.40, 40.37, 35.75	18.60, 37.34, 28.46

Table 6. *Comparison of various parameters of light-grown resting w₃BUL and of wild-type resting Euglena after 72 h of chloroplast development*

Parameter	w ₃ BUL	Wild type
Structure	Plastid largely absent	Chloroplast
Plastid DNA	Not detectable	Present
Chlorophyll (pg/organism)	0	10.50 ± 0.86
Carotenoids (pg/organism)	0.13	2.62 ± 0.19
10 ² × photosynthesis (pmol CO ₂ fixed/organism/h)	0	28
10 ¹¹ × cytochrome 552 (μmol/organism)	0	2.79 ± 0.39
RUDPCase (μmol CO ₂ fixed/mg protein/h)	0	4.11 ± 0.34
NADP-TPDase (μmol TPNH oxidized/mg protein/h)	4.01	27.00 ± 6.83
NAD-linked triose phosphate (μmol NADH oxidized/mg protein/h)	40.10	27.00 ± 5.42

concentrations of the *L-threo* isomer of Cm, which does not inhibit protein synthesis but shows many of its side effects. This isomer had no effect on chloroplast development in *Euglena* (W. Li and J. A. Schiff, unpublished results).

Investigation of mutant w₃BUL

Mutant w₃BUL is one of several *Euglena* strains in which plastid DNA is undetectable (Edelman *et al.* 1965). Chlorophyll, carotenoids, photosynthetic carbon dioxide fixation, cytochrome 552 and RUDPCase were all undetectable in this mutant even when grown in the light (Table 6). The NAD-linked triose phosphate dehydrogenase was present at normal levels. The NADP-TPDase, however, was present at precisely the same levels in the mutant as in the dark-grown wild-type organisms (compare Table 3). This suggests that NADP-TPDase is not coded in the plastid DNA, which is absent; it is probably coded in the nuclear DNA. The fact that the levels found are the repressed levels of the dark-grown organisms indicates that this mutant lacks the machinery to induce the higher levels of this enzyme formed during chloroplast development. Possibly, the activity in the mutant represents a small non-specificity of the NAD-linked enzyme allowing it to use NADP, although studies in other organisms and in *Euglena* indicate that the NAD enzyme has an extremely high specificity for DPN and no activity with NADP (Pupillo, 1972).

Table 7. *Total protein during chloroplast development in resting Euglena in the presence and absence of streptomycin*

Time in light (h)	Protein content (pg/cell)	
	– Sm	+ Sm
0	130	130
10	121	137
36	131	148
72	117	134

Table 8. *Protein content of dark-grown non-dividing Euglena after 72 h incubation in darkness or in the light*

The S.E.₉₅ was computed as follows. The standard deviation of the measurements (S.D.) was computed in the usual way. The standard error of the mean (S.E.M.) was computed from $S.E.M. = S.D./\sqrt{n}$ and was multiplied by t_{95} for the appropriate number of degrees of freedom to yield S.E.₉₅.

The variances of *A* and *B* were compared by means of the variance ratio (*F* test) where $F_{33}^3 = 1.9$ ($P > 10\%$). Using the combined variance of *A* and *B* the significance of the difference from zero of the mean of the population (*A*–*B*) was determined by means of the usual *t* test where $t = 2.63$ ($P > 5\%$).

Culture number	Protein at 72 h (pg/cell)		
	Light-exposed half of culture (<i>A</i>)	Dark-incubated half of culture (<i>B</i>)	Difference (<i>A</i> – <i>B</i>)
I	177	152	– 25
II	135	197	+ 62
III	150	160	+ 10
IV	141	136	– 5
$\bar{x} \pm S.E._{95}^*$	151 ± 30	161 ± 41	+ 42

* Mean of measurements ($\bar{x}$) plus or minus the standard error of the mean for 95 % confidence level (S.E.₉₅).

Cell protein during development

The total protein/organisms did not change significantly during light-induced chloroplast development in resting *Euglena*, and this was unaffected by Sm (Tables 7 and 8). This is consistent with previous findings that total RNA content does not change significantly during plastid development in resting organisms (Zeldin & Schiff, 1968).

Since many new proteins are presumably made during plastid development, and since the overall protein content of the cells does not change, there must be considerable protein turnover during plastid development. This is not unexpected since these organisms are resting and starving; turnover under conditions of starvation is a widespread phenomenon in micro-organisms. The turnover mechanism must be carefully controlled by the algae since there is no change in cellular protein content even when plastid protein synthesis is blocked by Sm.

General conclusions

The *Euglena* cell contains at least three different DNA genomes localized in the nucleus, mitochondrion and chloroplast respectively (Schiff, 1971; Edelman *et al.* 1965; Nass & Ben-Shaul, 1972), and at least two and perhaps three sets of ribosomes (Rawson & Stutz, 1968; Scott & Smillie, 1969), the 68S of the plastid, the 87S of the cytoplasm and perhaps a mitochondrial species (Avadhani & Buetow, 1972*a, b*). The mitochondrial DNA is small, with a molecular weight of only about 3×10^6 (Ray & Hanawalt, 1964; Shori, Ben-Shaul &

Edelman, 1970); it therefore possesses a very limited amount of potential information, less than that required to build a mitochondrion, and it seems reasonable to think that mitochondrial DNA would not contribute significantly to the formation of proteins for the plastid. The nucleus and chloroplast contain relatively large amounts of DNA and are good candidates for the sources of information for plastid biosynthesis. Although cytochrome 552 and RUDPCase are absent from a mutant (w_3 BUL) in which plastid DNA is undetectable, this cannot be interpreted as unequivocal evidence that they are coded or controlled in the plastid DNA. Plastid ribosomal RNA hybridizes selectively with plastid DNA (Stutz & Vandrey, 1971) indicating that the ribosomal RNA is coded in the plastid DNA. Since plastid DNA is undetectable in w_3 BUL, it is probable that the ribosomes are deleted as well (Cohen & Schiff, 1973) and along with them the ability to translate messages. It is possible that a message coming from elsewhere fails to be translated owing to the absence of plastid ribosomes in this mutant. Regulatory interactions as well are not ruled out. However, since Cm and Sm inhibit the formation of cytochrome 552 and RUDPCase, these must be synthesized on plastid ribosomes.

The plastid NADP-TPDase, however, is formed even in the presence of Sm and Cm and its appearance is completely inhibited by cycloheximide, indicating that it is not synthesized on plastid ribosomes but on the 87S ribosomes of the cytoplasm. Similar observations and conclusions have been reported for two other enzymes induced during chloroplast development: *Euglena* alkaline DNase (Egan & Carell, 1972) and the chloroplast phenylalanyl-tRNA synthetase (Reger, Fairfield, Epler & Barnett, 1970). We surmise, therefore, that enzymes such as NADP-TPDase, *Euglena* alkaline DNase and chloroplast phenylalanyl-tRNA synthetase are coded [in nuclear DNA. Thus these enzymes seem to be synthesized in the cytoplasm but later transported to the plastid, a situation reminiscent of the mitochondrial cytochrome *c* of yeast where the structural gene has been shown to be nuclear (Mounolu, Jacob & Slonimski, 1966; Huang, Briggs, Clark-Walker & Linnane, 1966; Sherman *et al.* 1968). The possibility that the NADP-TPDase arises by a minor modification of the cytoplasmic NAD-linked triose phosphate dehydrogenase has been suggested for other organisms where coenzyme-induced modifications or other conformational changes are suggested for the conversion (Hudock & Fuller, 1965; Muller, 1970; Melandri, Pupillo & Baccarini-Melandri, 1970; Baccarini & Melandri, 1970). The synthesis of NADP-TPDase, *Euglena* alkaline DNase, and chloroplast phenylalanyl-tRNA synthetase are repressed to the same extent in both wild-type dark-grown organisms and in w_3 BUL. If control of these enzymes is at the level of a nuclear gene, normal conditions of derepression by light involve either a signal from the proplastid to the nucleus, or a separate non-chloroplast photoreceptor as previously suggested in connection with RNA synthesis (Schiff & Zeldin, 1968; Schiff, 1971; Cohen & Schiff, 1973). Once the light is turned on, the chloroplasts may exert control over the amount of enzyme available to enter the plastids.

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