Chlorophyll Formation in *Euglena gracilis* var. *bacillaris*: Interference by Analogues of Purines, Pyrimidines and Amino Acids

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SUMMARY

Chlorophyll formation in non-growing etiolated cells of *Euglena gracilis* var. *bacillaris* is inhibited by certain antimetabolite analogues of purines, pyrimidines and amino acids. The inhibitory effects of bromo- and nitro-uracils were annulled by uracil and thymine; those of fluoro- and thio-uracils were not. Ethionine inhibition was completely annulled by methionine. All the base analogues were more inhibitory for the dark growth of the alga than in the light and an adaptation to these antimetabolites was apparent during growth. The growth inhibitory effects of some of these base analogues was annulled by several Krebs's cycle intermediates; the latter in themselves stimulated growth of photosynthesizing euglenas although they were not utilized in the dark with any degree of efficiency.

Long-term exposure of non-proliferating euglenas, green or etiolated, to 5-fluoro-uracil had a profound effect on the chloroplast integrity of the organisms. Such euglenas on further subculture in an adequate growth medium were permanently bleached. The effect of 5-bromo-uracil, though similar, was less marked.

INTRODUCTION

Euglena gracilis, a green photosynthetic flagellate, loses its chlorophylls when grown heterotrophically in the dark. Exposure of such colourless euglenas to light brings about the re-synthesis of chlorophyll accompanied by a considerable increase in chloroplast proteins and the RNA of several subcellular fractions (Brawerman & Chargaff, 1959*a*; Brawerman, Pogo & Chargaff, 1962). An additional RNA species associated with the chloroplasts and which is specifically formed during their induced formation in light was demonstrated by Pogo, Brawerman & Chargaff (1962). These results suggest that light induces the associated synthesis of specific types of proteins concomitant with chlorophyll synthesis. The effect of certain antimetabolite analogues of purines, pyrimidines and amino acids was therefore tested to verify such an association.

Euglena gracilis is the only phytoflagellate which can be rendered permanently apochlorotic by an assortment of chemical agents, e.g. streptomycin (Provasoli, Hutner & Schatz, 1948; Jirovec, 1949), antihistamines (Gross, Jahn & Bernstein, 1955), *O*-methylthreonine (Aaronsen & Bensky, 1962), furadantin (McCalla, 1962), erythromycin (Ebringer, 1962) and magnesium starvation (Dubash & Rege, 1967); and physical agents such as heat (Pringsheim & Pringsheim, 1952; Brawerman &

Chargaff, 1959*b*) and ultraviolet radiation (Pringsheim, 1958). This phenomenon of permanent apochlorosis is one of the few instances of a cytoplasmic mutation, with a consequent alteration of phenotype, being induced by environmental changes. In *E. gracilis* var. *bacillaris*, diverse agents operating in unknown ways affect finally the synthesis of chlorophyll or plastid or both. The mechanism of action of any of such agents is not yet fully understood. 5-fluoro-uracil, which inhibits growth and chlorophyll synthesis in *E. gracilis*, has been found to render organisms of this alga permanently apochlorotic. Organisms grown in the dark with sulphanilamide and 5-bromo-uracil and subsequently plated on a basal medium were shown by Scher & Collinge (1965) to be markedly mutated to the apochlorotic form, but although mere exposure to 5-fluoro-uracil can bring about a 100 % mutation, we were not able to show a similar effect with 5-bromo-uracil even on longer exposures.

METHODS

Organisms of the photosynthetic strain of *Euglena gracilis* var. *bacillaris* were grown in the defined medium of Hutner, Provasoli, Schatz & Haskins (1950) as described by Coelho & Rege (1963); the medium for dark-grown cultures was supplemented with 1 % glucose. Growth studies were made essentially by the method described earlier (Coelho & Rege, 1963). For studies on chlorophyll synthesis by non-growing cultures under illumination, etiolated euglenas were obtained by growth in the dark for 7 days; these forms were harvested, washed twice with sterile distilled water under aseptic conditions and then suspended in a measured volume of sterile distilled water. Samples of this suspension were then dispensed in 6 in. $\times$ $\frac{1}{2}$ in. rimless test tubes containing double- (or greater) strength of the 'resting' medium suggested by Brawerman & Chargaff (1959*a*) composed of 1 % glucose, 0.01 M-KH₂PO₄, and 0.01 M-MgCl₂·6H₂O with additions where required. No rigorous sterility precautions needed to be observed as this medium did not favour bacterial growth. The euglenas were then illuminated with frequent shaking for 72 hr at 28° in a transparent glass tank by a pair of daylight fluorescent tubes placed 10 in. from the bottom of the tank.

Estimates of growth were made, after suitable dilution of cultures, by the method of Hutner, Bach & Ross (1956), by measuring the turbidity with a Klett-Summerson photoelectric colorimeter with a 66 (640–700 m μ) filter. Photosynthetic pigments were routinely determined by extraction of euglenas with 3 $\times$ 5.0 ml. methanol; these extracts were pooled, the volume made to 20 ml. with methanol and the colour intensity read with a Klett-Summerson colorimeter, using the same 66 filter. Absolute values, where given, were determined by the method described earlier (Dubash & Rege, 1967).

Counts of euglenas were made in a Levy blood counting chamber after immobilizing the organisms by adding a drop of chloroform.

RESULTS

Effect of antimetabolites on chlorophyll synthesis

It was observed that all the analogues of uracil tried and 2,6-diamino-purine inhibited the synthesis of chlorophyll in suspensions of the euglena. Benzimidazole was without effect; 6-mercaptopurine showed a slight stimulation; ethionine, at higher concentration, was also inhibitory (Table 1). The effects of 5-bromo- and 5-nitro-

uracil were annulled by uracil and thymine; those of 5-fluoro- and 5-thio-uracil were not (Table 2). The inhibition by ethionine was completely annulled by methionine. The incorporation of ethionine into the euglenas showed a lag period; short-term exposure to this analogue before illumination did not inhibit chlorophyll synthesis (Table 3).

Table 1. *Effect of analogues of purines, pyrimidines and amino acids on chlorophyll synthesis in resting etiolated Euglena gracilis var. bacillaris*

Addition to non-proliferation medium	Concentration of addition	Inhibition of chlorophyll synthesis (%)
None	—	0
5-Bromo-uracil	400 µg./ml.	37.4
5-Thio-uracil		47.5
5-Nitro-uracil		24.6
5-Fluoro-uracil		26.7
2,6-Diaminopurine	10 ⁻³ M	36.1
6-Mercaptopurine		-14.0
Benzimidazole		2.8
Ethionine	2 mg./ml.	69.2

Table 2. *Effect of uracil analogues on chlorophyll synthesis by etiolated Euglena gracilis var. bacillaris: annulment by uracil and thymine*

Addition to non-proliferation medium (400 µg./ml.)	Net synthesis of chlorophyll/mg. dry wt euglenas*/72 hr mµg.	Inhibition (%)	Annulment (%)
None	1812	—	—
5-Bromo-uracil (BU)	1101	37.4	—
5-Thio-uracil (TU)	964	47.5	—
5-Nitro-uracil (NU)	1376	24.6	—
5-Fluoro-uracil (FU)	1330	26.7	—
Uracil (U)	1796	1.0	—
Thymine (Th)	1536	15.5	—
BU + U	1697	6.5	85.0
BU + Th	1468	19.0	49.1
TU + U	1009	44.5	6.4
TU + Th	917	49.5	-4.2
NU + U	1812	0	100
NU + Th	1514	16.6	32.6
FU + U	1399	23.0	13.9
FU + Th	1005	44.5	-66.5

* Dry weights of euglenas were calculated from counts according to the relationship suggested by Kirk (1962).

Effect of antimetabolites on the growth of Euglena gracilis var. bacillaris

In view of the effect of these various antimetabolite analogues on chlorophyll synthesis in non-proliferation conditions, their influence on growth was studied. To verify whether the effect on growth was due only to interference with chlorophyll biosynthesis, the studies were extended to dark-grown cultures as well. Table 4 shows that all purine and pyrimidine analogues tested were more inhibitory in the dark than in the light. The organism thus appeared to use pathways for carbohydrate metabolism which were

different in the heterotrophic and photosynthetic phases. The possibility of light instability of some of the compounds cannot, however, be precluded. Whether such an action indicates that these analogues might hamper in some way the utilization of glucose as a source of energy was examined by experiments designed to test the effects of various breakdown products of glucose and the Krebs cycle intermediates in the presence of these antimetabolites. Table 5 shows the effect of the Krebs cycle intermediates on the growth of *Euglena* in the presence and absence of 8-azaguanine. The

Table 3. *Effect of ethionine on chlorophyll synthesis in non-proliferating etiolated Euglena gracilis var. bacillaris organisms: annulment by methionine*

Euglenas were grown heterotrophically in the dark for 7 days, harvested, washed and resuspended in water. Equal samples were used for the different treatments. Illumination was for 96 hr; the chlorophylls were extracted with methanol as described in the text. Klett readings were taken with the 66 filter. Pre-exposure was effected by suspending the etiolated *euglenas* in non-proliferation medium containing the additions at 2 mg./ml.; the same concentration was used during illumination.

Treatment	Chlorophyll index (Klett units)	Inhibition (%)	Annulment (%)
1. Blank	162	—	—
2. Non-proliferation medium + ethionine	50	69.2	—
3. <i>Euglenas</i> exposed to ethionine for 15 min., washed and taken up in non-proliferation medium	161	0	—
4. <i>Euglenas</i> exposed to ethionine for 15 min., centrifuged and taken up in non-proliferation medium without washing	93	42.5	—
5. Non-proliferation medium + ethionine + methionine	161	0	100
6. <i>Euglenas</i> exposed to ethionine + methionine for 15 min., washed and taken up in non-proliferation medium	165	0	—
7. <i>Euglenas</i> exposed to ethionine + methionine for 15 min., centrifuged and taken up in non-proliferation medium without washing	165	0	—
8. <i>Euglenas</i> exposed first to ethionine for 15 min., then to methionine for 15 min., washed and taken up in non-proliferation medium	165	0	—
9. <i>Euglenas</i> exposed to ethionine for 15 min., washed and taken up in non-proliferation medium containing methionine	167	0	—
10. Non-proliferation medium + methionine	167	0	—

Table 4. *Effect of purine and pyrimidine antimetabolite analogues on the light and dark growth of Euglena gracilis var. bacillaris*

Analogue added	Concentration ($\times 10^{-3}$ M)	% Inhibition of growth in	
		Light	Dark
None	—	0	0
5-Bromo-uracil	1.048	4.0	43.6
5-Thio-uracil	1.560	2.8	8.5
5-Nitro-uracil	1.272	4.4	5.6
5-Fluoro-uracil	1.526	97.9	100
	0.153	27.2	71.1
8-Azaguanine	1.314	19.4	53.5
8-Azaxanthine	1.304	0	5.0
8-Azaadenine	1.470	19.4	49.3

inhibition due to 8-azaguanine was less evident in the dark in the presence of lactate or α -ketoglutarate and was completely overcome by citrate, although these compounds cannot replace glucose in the medium with growth stimulatory action. In the light, 8-azaguanine was completely ineffective in the presence of α -ketoglutarate and citrate. It was further observed that many of the carbon compounds stimulated growth of the organism in the light although some of these could not be utilized in the dark.

Table 5. *Effect of Krebs cycle intermediates on the 8-azaguanine inhibition of growth of Euglena gracilis var. bacillaris*

Krebs cycle acid added* (1 %)	Growth (Klett units at 660 m μ)					
	Without 8-azaguanine		With 8-azaguanine			
	Dark	Light	Dark	Inhibition (%)	Light	Inhibition (%)
None	79	286	42	46.9	264	7.6
Glucose	298	422	142	52.4	345	18.4
Pyruvate	48	126	23	52.0	114	9.5
Lactate	85	350	70	17.6	305	13.0
Succinate	115	422	53	54.0	252	40.3
Malate	95	422	48	49.5	320	24.1
α -Ketoglutarate	27	194	22	18.6	208	—7.0
β -Glycerophosphate	54	211	36	33.4	100	52.6
Fumarate	168	350	76	54.7	234	33.1
Citrate	65	325	64	0	348	—7.0

* Additions of Krebs intermediates were to the basal medium containing glutamate (1 %) and malate (0.2 %), but without glucose.

Adaptation to the presence of antimetabolites

When euglenas grown in presence of 8-azaguanine were used to study their chlorophyll-synthesizing capacity, it was observed that 8-azaguanine was not as inhibitory to the synthesis of chlorophyll as it was to euglenas grown in absence of this analogue. Tables 6 and 7 show that not only were the analogues ineffective in most of the cases tried, but 8-azaderivatives of guanine and xanthine, and *S*-methylcysteine actually stimulated the synthesis of chlorophyll when euglenas grown in the dark in the presence of the antimetabolite analogue were illuminated in a non-proliferation medium in the continued presence of the same (Table 6). The effect of 5-bromo-uracil addition to the growth medium in the dark was to enhance synthesis of chlorophyll when etiolated euglenas were illuminated in a non-proliferation medium with or without this analogue; 5-bromo-uracil, however, inhibited chlorophyll synthesis in normal etiolated euglenas (Table 1). With 5-nitro-uracil, with 8-azaderivatives of guanine and xanthine, and with *S*-methylcysteine there was only an increase in chlorophyll synthesis when the illumination in non-proliferation medium was also continued in the presence of the inhibitor. It was thought that this effect may have been due to some sort of adaptation of the euglenas to the inhibitor during the growth phase. After 7 days' growth in the dark in the presence of inhibitor a second transfer in the same medium brought about a decrease in the degree of inhibition in the case of 8-azaxanthine and *S*-methylcysteine, whereas the inhibition was increased in the case of 8-azaguanine. These results are given in Table 7.

Table 6. *Effect of continued presence of purine and pyrimidine antimetabolite analogues on chlorophyll synthesis in non-proliferating etiolated organisms of Euglena gracilis var. bacillaris grown in the presence of the analogue: adaptation to certain analogues*

Analogue	Concentration in the growth and non-proliferation medium ($\times 10^{-3}$ M)	Dark Growth (Klett)	% Chlorophyll synthesized per euglena in 72 hr in non-proliferation medium	
			Alone	+ Analogue
None	—	142	100	100
5-Bromo-uracil	1.048	80	128.1	126.0
5-Thio-uracil	1.560	130	78.3	77.0
5-Nitro-uracil	1.272	134	65.8	72.0
5-Fluoro-uracil	1.526	0	0	0
8-Azaguanine	1.314	66	96.0	129.5
8-Azaxanthine	1.304	135	72.8	85.5
8-Azaadenine	1.470	72	91.3	78.3
S-Methylcysteine	1.480	124	77.3	90.4

Table 7. *Effect of continued presence of antimetabolite growth inhibitors on the growth of Euglena gracilis var. bacillaris*

Dark-grown euglenas in presence of the inhibitor were diluted to the same cell count and used as inocula for growth in presence or absence of the inhibitor as indicated.

Growth studies were done in the dark on a glucose-supplemented growth medium for 7 days.

Addition	Concentration in the growth medium ($\times 10^{-3}$ M)	Dark Growth (Klett)	Inhibition of growth (I) (%)	DG* euglenas inoculated in			
				Basal medium		Basal medium + addition	
				Growth (Klett)	I (%)	Growth (Klett)	I (%)
None	—	156	0	298	0	298	0
8-Azaxanthine	1.304	143	8.5	315	—7	289	3
8-Azaguanine	1.314	116	25.6	286	4	134	55
S-Methylcysteine	1.480	135	13.5	284	5	269	10

* DG = dark-grown euglenas in presence of inhibitor.

Mutagenic effect of prolonged exposure to antimetabolite analogues of certain bases

Scher & Collinge (1965) demonstrated that 5-bromo-uracil gets incorporated into the euglenas during chloroplast replication leading to mutagenic changes and giving rise to bleached colonies. The effect of 5-fluoro- and 5-bromo- analogues of uracil was studied on non-proliferating suspensions of *Euglena* in the light. Long exposures to these base analogues brought about certain mutagenic changes in *Euglena* as evinced when these organisms were subcultured in a glucose-supplemented basal medium in the light. Exposure of green or etiolated euglenas for 144 hr to 5-fluoro-uracil gave pure white cultures when a small population of the euglenas undergoing treatment was inoculated into a glucose-containing growth medium; these cultures were 100 % bleached. The same effect was also observed with 5-bromo-uracil, but the bleaching was not 100 %. The 5-fluoro-uracil treated cultures were permanently bleached and never regained even a part of their chlorophyll when subsequently grown

in the light for many transfers. On the other hand, 5-bromo-uracil-treated cultures, or cultures treated with 5-fluoro-uracil for less than 144 hr were not completely or permanently bleached and returned to the original green state gradually after the analogue was withheld from the growth medium. The results of this experiment are summarized in Table 8.

Table 8. *Effect of 5-fluoro-uracil and 5-bromo-uracil on non-proliferating Euglena gracilis var. bacillaris: loss of the photosynthetic apparatus on prolonged exposure to 5-fluoro-uracil*

The euglena populations were exposed to the base antimetabolite analogues during the non-proliferation period whereafter 2 drops of a 1/50 diluted euglena suspension was used as inoculum.

			Colour of cultures after 7-day growth in light.			
Type of euglenas suspended in non-proliferation medium	Addition to non-proliferation medium	Concentration of addition (mg. %)	Time of exposure to light (hr)*			
			0	24	72	144
			Colour†			
Dark-grown etiolated	None	—	G	G	G	LG
	5-Fluoro-uracil	200	G	G	G	W‡
		50	G	G	G	W
	5-Bromo-uracil	200	G	G	G	LG
		50	G	G	G	LG
	Light-grown green	None	—	G	G	G
5-Fluoro-uracil		200	G	G	G	W
		50	G	G	G	W
5-Bromo-uracil		200	G	G	G	PY
		50	G	G	G	PY

* Time of exposure to analogue in the light in non-proliferation medium before transferring a 1/50 diluted inoculum to a glucose-supplemented growth medium.

† G = green; LG = light green; PY = pale yellow; W = white.

‡ White cultures permanently bleached.

DISCUSSION

Lyman, Epstein & Schiff (1961) suggested the implication of self-reproducing cytoplasmic nucleoproteins during the formation of chloroplasts in *Euglena gracilis*. Ultraviolet radiation prevents the transmission of the chloroplasts of *Euglena* to their progeny; the process, which shows peaks of effectiveness at 260 and 280 m μ , can be photo-reactivated and these cytoplasmic entities are replicated at division and control the formation of chloroplasts (Schiff, Lyman & Epstein, 1961). This cytoplasmic factor was thought to be DNA (Leff, Mandel, Epstein & Schiff, 1963); subsequent work has definitely established the presence of chloroplastic DNA in *Euglena* (Edelman, Cowan, Epstein & Schiff, 1964; Brawerman & Eisenstadt, 1961; Ray & Hanawalt, 1964, 1965; Edelman, Schiff & Epstein, 1965; Ray, 1965). The inhibition of chlorophyll synthesis in resting *Euglena* by actinomycin-D suggests a DNA-dependant synthesis of RNA during chloroplast development (McCalla & Allan, 1964).

The inhibition of chlorophyll synthesis by 5-bromo-uracil and its annulment by uracil or thymine shows that there is possibly a *de novo* synthesis of nucleoproteins

occurring during the development of chloroplasts in resting etiolated euglenas and this in some manner is closely associated with the synthesis of chlorophyll. These nucleoproteins are presumably involved in the synthesis of specific proteins: either the enzymes required for photosynthesis, and/or the protein involved in the chlorophyll-protein complex. The inhibition of greening by ethionine and its annulment by methionine also shows that such proteins are possibly being formed *de novo* during illumination of dark-grown organisms. However, according to Gibson, Neuberger & Tait (1962*b*), the inhibition of chlorophyll synthesis by ethionine, in *Rhodopseudomonas spheroides*, is likely to be due to inhibition by competition with methionine of the formation of the methyl ester group of chlorophyll. The formation of NADP-requiring glyceraldehyde-3-phosphate dehydrogenase during chloroplast formation was shown by Fuller & Gibbs (1959) and Brawerman & Konigsberg (1960). This enzyme is characterized by its absence from dark-grown colourless euglenas and its synthesis is blocked by any agent, chemical or physical, which blocks chloroplast formation. The *de novo* synthesis of the protein involved in the chlorophyll-protein complex is also suggested since any blockage in the synthesis of this protein, as with ethionine, would prevent chlorophyll formation simply because the chlorophyll would have no protein to which to attach. A similar mechanism of inhibition by streptomycin was suggested by Kirk (1962). The breakdown of existing proteins and peptides to amino acids which are then utilized for synthesis of chloroplast protein was also suggested by Kirk (1962) in contrast to a hypothesis suggested by Brawerman & Chargaff (1959*a*) that there was an actual transfer of intact proteins from the soluble fraction to the chloroplast fraction. Gibson *et al.* (1962*a, b*) suggested that the porphyrin excretion by *Rhodopseudomonas spheroides* in the presence of ethionine was due to its interference with the utilization or synthesis of methionine.

All the antimetabolite analogues of bases tested here were more inhibitory to the growth of *Euglena gracilis* var. *bacillaris* in the dark than in the light. This suggests that the observed effect on growth was due more likely to the inability of the euglenas to utilize glucose rather than to their ability to photosynthesize it. It is apparent that the metabolic pattern of the organisms is markedly influenced by the mode of life, photosynthetic or otherwise. How far the stability of the antimetabolite compounds, or the permeability of the euglenas to these is affected in light is not known. In the light, ATP is generated by the process of photosynthetic phosphorylation and the organisms do not require to metabolize glucose to derive energy. In the dark, where ATP formation depends entirely on glucose utilization, this inhibition is more pronounced. The dark-inhibition by 8-azaguanine was overcome to some extent by certain Krebs's cycle intermediates like lactate and α -ketoglutarate and completely by citrate. During the formation of chloroplasts by non-proliferating etiolated *Euglena gracilis* organisms, the chlorophyll-less euglenas in the initial stages have to rely on exogenous glucose as sole source of ATP until such time as they synthesize enough chlorophyll to derive energy from other pathways such as photosynthetic phosphorylation.

The work of Scher & Collinge (1965) which suggested the incorporation of 5-bromouracil into the plastid material during chloroplast replication provides another explanation to the mode of inhibition of greening by these base analogues. That such an incorporation takes place is manifested by the formation of bleached colonies, indicating mutagenesis. 5-Fluoro-uracil-treated euglenas also give rise to bleached cultures, although 5-fluoro-uracil does not totally inhibit the greening of resting etio-

lated euglenas at a concentration at which this analogue exerts a permanent bleaching effect on cultures derived even from green euglenas. This suggests that the site of action of 5-fluoro-uracil is at a stage before the accumulation of the 'dark-precursor' of chlorophyll. It is, however, not clear whether 5-fluoro-uracil interferes with the formation or functioning of the chloroplastic satellite DNA of *Euglena* or produces drastic metabolic changes by affecting the nuclear DNA. 5-Fluoro-uracil is known to inhibit DNA synthesis by being converted to the deoxyribotide which is a potent inhibitor of thymidylate synthetase activity (Heidelberger, 1963). Thymidine has been shown to annul the inhibition of growth of *Escherichia coli* by 5-fluoro-uracil (Mankodi, 1964). 5-Fluoro-uracil is also known to be incorporated into RNA, rendering it inefficient in directing normal protein synthesis (Nakada & Magasanik, 1964). 5-Bromo-uracil had a similar effect, though less pronounced with *Euglena gracilis* var. *bacillaris*, and further exposures up to 312 hr failed to bring about complete bleaching. The 5-fluoro-uracil bleaching of *Euglena* is analogous to the bleaching effects previously observed by exposures to streptomycin (Provasoli *et al.* 1948; Jirovec, 1949; Provasoli, Hutner & Pintner, 1951), antihistamines (Zahalsky, Hutner, Keane & Burger, 1962), furadantin (McCalla 1962), *O*-methylthreonine (Aaronson & Bensky, 1962), erythromycin (Ebringer, 1962), heat (Brawerman & Chargaff, 1959b), short-term ultraviolet radiation (Lyman *et al.* 1961; Schiff *et al.* 1961), and Mg^{2+} starvation (Dubash & Rege, 1967). Although the site of action of each of these mutagens may be different, in all these cases the permanent loss of photosynthetic ability is preceded by a reversible lesion.

Evidence has been presented for the operation of the Krebs cycle in *Euglena* by Danforth (1953); the reversal of 8-azaguanine inhibition by several Krebs intermediates, both in light as well as in dark, indicates the involvement of this cycle. However, the organism appears to employ pathways for carbohydrate metabolism which are fundamentally different in the dark and photosynthetic phases. A possibility of greater turnover of nucleic acids during the dark adaptive phase is also indicated. The increased formation of chlorophyll on the continued presence of the analogues during chloroplast formation after the cells are first grown in the dark in the presence of the analogue indicates a degree of adaptation of the cells during the growth phase towards the inhibitor.

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