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A LIGHT-INDUCED β-1,3-GLUCAN BREAKDOWN ASSOCIATED WITH THE DIFFERENTIATION OF CHLOROPLASTS IN *EUGLENA GRACILIS*

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SUMMARY

1. Light induced a rapid breakdown of β-1,3-glucan in carbon-starved cells of *Euglena gracilis*, Strain Z. In contrast, β-1,3-glucan was utilized slowly in cells deprived of carbon growth substrates and maintained in the dark.

2. The breakdown of β-1,3-glucan required continuous light. At a low light intensity (7 ft candles), induction of the breakdown was delayed 12–24 h.

3. Cells showing a rapid breakdown of β-1,3-glucan contained high activities of the enzymes β-1,3-glucan phosphorylase and β-1,3-glucan hydrolase (EC 3.2.1.6) and a low activity of β-1,3-glucan synthetase (EC 2.4.1.12).

4. The light-induced breakdown of β-1,3-glucan appeared to be associated with the development of chloroplasts. A bleached mutant, ZUV-3, which cannot synthesize pigments when exposed to light, did not show a light-induced breakdown of β-1,3-glucan.

5. Chloramphenicol and 5-fluorouracil, inhibitors of the synthesis of a number of chloroplast proteins, did not inhibit the light-induced breakdown of β-1,3-glucan. Cycloheximide, an inhibitor of cytoplasmic protein synthesis, slightly delayed the breakdown.

6. An inhibitor of photosynthesis, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), only partially inhibited chloroplast development during the period of rapid breakdown of β-1,3-glucan. After the store of β-1,3-glucan had been depleted, DCMU completely inhibited chloroplast development. The requirement for either photosynthesis or an endogenous supply of β-1,3-glucan for chloroplast development could be satisfied by supplying glucose exogenously.

INTRODUCTION

Chloroplasts contain unique species of DNA and RNA as well as mechanisms for the synthesis of DNA, RNA and protein[1,2]. There is evidence that chloroplast DNA directs the synthesis of chloroplast rRNA[3,4] and that chloroplast ribosomes

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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are the site of synthesis of many chloroplast proteins[5]. However, the chloroplast cannot be regarded as being independent of the rest of the cell. Many facets of chloroplast development involve nuclear genes[1], and from studies of changes in RNA[6], protein[7] and lipids[8,9] in the cytoplasm of cells containing differentiating chloroplasts, evidence is accumulating that many of the metabolic changes occurring in the cytoplasm of these cells are closely linked with developmental changes in the chloroplast.

Cytoplasmic metabolism may have a direct role in chloroplast development in the provision of carbon and energy during the initial stages of chloroplast differentiation before the rate of photosynthesis becomes appreciable. When cells of *E. gracilis* are grown in the dark, large amounts of a storage carbohydrate, β-1,3-glucan (paramylon), are synthesized[10]. Since chloroplasts rapidly develop when these cells are illuminated, even in the absence of exogenous carbon sources, the β-1,3-glucan may serve as a major source of energy and carbon for synthesis of chloroplast components[7]. The action of light in regulating the utilization of β-1,3-glucan and its effect on the activities of cytoplasmic enzymes catalyzing the breakdown of β-1,3-glucan are reported in this paper. The extent to which light-induced catabolism of β-1,3-glucan is linked to biosynthetic processes in plastids is also considered.

MATERIALS AND METHODS

Cell cultures

The cells used were *E. gracilis*, Strain Z wild type, or the bleached mutants ZUV-1, ZUV-3 (obtained from Dr. H. Lyman) and Tetracycline A-2 (obtained from Dr. S. H. Hutner). These mutants were isolated from cultures of normal cells that were either irradiated with ultraviolet light or treated with tetracycline. The cells were grown heterotrophically in darkness (dark-adapted cells) at 25° in the basal medium of HUTNER *et al.*[11] supplemented with vitamin B_{12} (5 μg/l). The cultures were contained in flasks fitted with cotton wool plugs and were agitated by a horizontal reciprocal shaker.

Experiments to study chloroplast development were performed as follows. Cells were grown to about $3 \cdot 10^6$ cells/ml (early phase of growth; maximum growth is $2 \cdot 10^7$–$3 \cdot 10^7$ cells/ml), harvested and centrifuged at 200 × *g* for 5 min. The cells were washed twice by suspension and centrifugation in the above medium from which all compounds containing carbon except EDTA, thiamine and vitamin B_{12} had been omitted. The washed cells were suspended in the same medium to a density of about $3 \cdot 10^6$ cells/ml and were shaken for a further 2 days in the dark. These manipulations were carried out under aseptic conditions and in the presence of green light of low intensity. Continuous exposure to this light for 48 h failed to initiate either β-1,3-glucan breakdown or chlorophyll synthesis. The starved cells were then exposed to white light (Philips warm white fluorescent lights) at 1300 lux, the optimum intensity for chloroplast development[12]. Control flasks were kept in the dark and 5 % CO_2 in air was supplied continuously to all flasks. Where inhibitors or glucose (22.5 mg/ml) were added to the medium, these were added immediately prior to exposing the cells to light. In the experiment where a light intensity of 7 ft candles was used, this was obtained by covering the flasks with a grey filter made of fine mesh. Light intensity was measured with an Eel Lightmaster light meter.

Cell density was determined using a Coulter counter. Chlorophyll was measured

according to the method of ARNON[13]. To determine carotenoids, the cells were extracted with absolute methanol and the absorbance at 450 nm measured.

Determination of β-1,3-glucan

The cells were killed by adding to them a mixture of methanol–chloroform–7 M formic acid (7:5:3, by vol.)[14]. The residues were extracted with methanol (twice) and then with 40 % (v/v) ethanol in water. β-1,3-Glucan was assayed using the anthrone method[15], adapted for use with a Technicon autoanalyzer. The extracted cell residue (from approx. $2 \cdot 10^7$ cells) was drained, cooled to 0°, and 2 ml of 70 % H_2SO_4 (2–5°) were added slowly with stirring and cooling. After the mixture was left standing for 20 min or until the residue had dissolved, the solution was diluted to known volume (50–250 ml) with 30 % H_2SO_4 (2–5°). The sample was fed into the autoanalyzer at a rate of 1.06 ml/min and mixed with a stream of 0.05 % anthrone in 70 % H_2SO_4 (4.06 ml/min). The mixture was pumped through an oil bath maintained at 95° (5 min), cooled, and the absorbance of the developed colour measured using a 620-nm filter.

Enzyme assays

Samples of cells were harvested, washed twice by centrifugation in 0.05 M Tris buffer (pH 7.8), resuspended in 5 ml of the buffer and immediately frozen. The cells were broken by freezing and thawing twice, and the extract was centrifuged at 20000 × *g* for 10 min. The residue was retained for assays of β-1,3-glucan synthetase (EC 2.4.1.12)[16], while the remaining assays were carried out using the supernatant.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed according to KORNBERG AND HORECKER[17], phosphoglucomutase (EC 2.7.5.1) and UDPG pyrophosphorylase (EC 2.7.7.9) according to LARNER AND VILLAR-PALASI[18], and β-1,3-glucan phosphorylase, (β-1,3-oligoglucan:orthophosphate glucosyltransferase) by the method of MARÉCHAL AND GOLDEMBERG[19], except that Tris buffer was used instead of citrate. β-1,3-Glucan hydrolase (EC 3.2.1.6) was assayed using a procedure based on those of FELLIG[20] and CLARKE AND STONE[21]. The extract was incubated at 40° in a reaction mixture containing 0.1 mM $MnCl_2$, 100 mM acetate buffer (pH 5.0) and 0.18% laminarin. Laminarin isolated from *Laminaria cloustoni* was dialyzed against 3 mM acetate buffer for 2 days before use. The reaction was stopped by the addition of Somogyi's reagent and assayed for glucose by the Nelson colorimetric method with the Somogyi alkaline copper reagent[22].

RESULTS

Light-induced breakdown of β-1,3-glucan

Fig. 1 illustrates the light-induced breakdown of β-1,3-glucan in dark-adapted cells. In this and subsequent experiments, the dark-adapted cells were starved for 2 days before illumination. The 2-day starvation period was introduced so that the development of chloroplasts could be followed in the absence of cell division. During the first 12 h of starvation in the dark, cell division occurred and this was accompanied by a decrease in the content of β-1,3-glucan per cell. Thereafter, β-1,3-glucan was utilized only slowly in cells kept in darkness; the levels in these cells between

2 and 5 days of starvation are shown in Fig. 1. In contrast, β-1,3-glucan was rapidly depleted if the cells were illuminated. Most of the β-1,3-glucan was broken down prior to the main period of chlorophyll synthesis. Between 24 and 72 h the rate of decrease in β-1,3-glucan approximated that in the dark and there appeared to be a residual amount of β-1,3-glucan which was not affected by light. β-1,3-Glucan probably does not account for all the insoluble carbohydrate measured in the illuminated cells, since the anthrone assay does not discriminate between β-1,3-glucan and other minor insoluble carbohydrates such as those known to be constituents of the pellicle[23]. However, microscopic examination confirmed the existence of small granules of β-1,3-glucan in cells after 72 h of illumination. The slight apparent increase in cell number shown in Fig. 1 can be attributed mainly to evaporation of water from the culture.

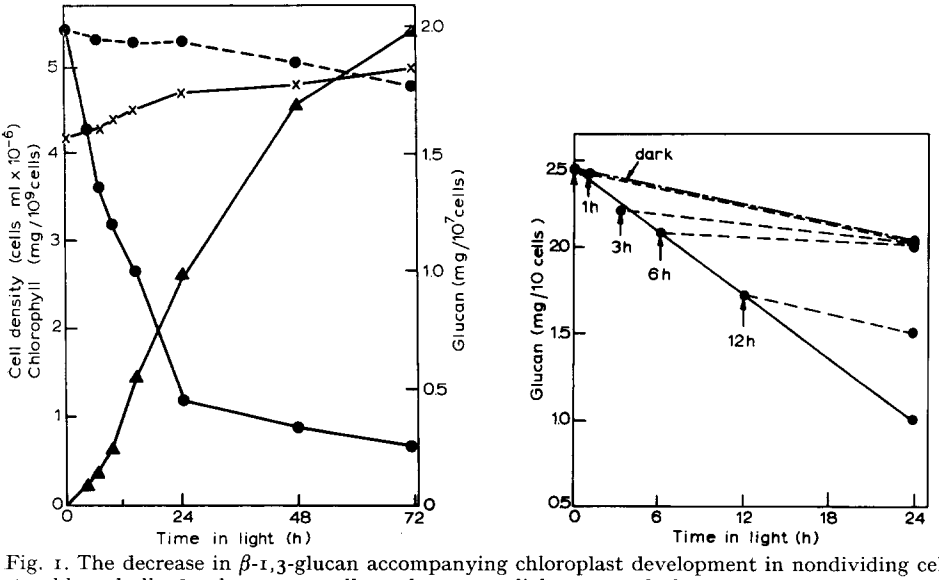

Fig. 1. The decrease in β-1,3-glucan accompanying chloroplast development in nondividing cells. ▲, chlorophyll; ●, glucan; ×, cell number; ——, light; – – –, dark.

Fig. 2. The dependence of β-1,3-glucan breakdown on continuous light. Cells were returned to the dark after 0, 1, 3, 6 and 12 h exposure to light, as indicated by arrows. ——, light; – – –, dark.

Continuous light is required for chlorophyll synthesis and, as can be seen in Fig. 2, this was true also of β-1,3-glucan breakdown. When dark-adapted cells were exposed to continuous light for periods from 1 to 12 h and then returned to the dark, chlorophyll synthesis ceased and the rate of β-1,3-glucan disappearance returned to about that found in cells kept in continuous darkness.

The effect of light of low intensity on β-1,3-glucan breakdown

The maximum rate of development of mature chloroplasts in *E. gracilis* occurs at a light intensity of about 120 ft candles[12]. STERN *et al.*[24] have shown that at 7 ft candles, development of chloroplasts is retarded and plastid differentiation stops at a stage prior to the fusion of membrane discs to form lamellae. At this low intensity, β-1,3-glucan breakdown was also retarded (Fig. 3), but by 72 h, the β-1,3-glucan had fallen almost to the level found in cells illuminated at 120 ft candles.

Breakdown of β-1,3-glucan in bleached mutants

Since the experiments quoted above indicated a close relationship between chloroplast development and light-induced breakdown of β-1,3-glucan, the effect of light on β-1,3-glucan content was studied in bleached mutants which do not form chloroplasts in light. These mutant cells of *E. gracilis* were treated in the same way as normal cells, *i.e.* they were grown in continuous darkness and then transferred to inorganic medium for 2 days before illumination. Two of the mutants showed an appreciable light-induced breakdown of β-1,3-glucan during the first few h of illumination (one of these mutants is shown in Fig. 4), but light had only a slight effect on the β-1,3-glucan content of the third mutant (Fig. 4). The light-induced breakdown appeared to be correlated with the ability of the cells to form carotenoid in the light. The mutants ZUV-1 and Tetracycline A-2 showed both a light-stimulated decrease in β-1,3-glucan and increase in carotenoid (Fig. 4). In contrast, light had little effect on the β-1,3-glucan content of the third mutant (ZUV-3) which synthesized only trace amounts of carotenoid.

The effect of light on the activities of enzymes involved in the catabolism of β-1,3-glucan

During the period of rapid breakdown of β-1,3-glucan, the activities of several enzymes involved in the catabolism of β-1,3-glucan increased. Fig. 5 shows changes

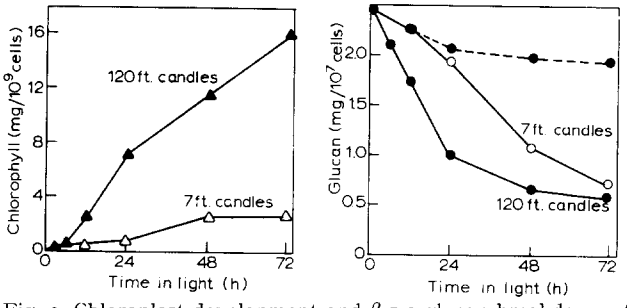

Fig. 3. Chloroplast development and β-1,3-glucan breakdown at low light intensity (7 ft candles). ▲, △, chlorophyll; ●, ○, glucan; ——, light; – – –, dark.

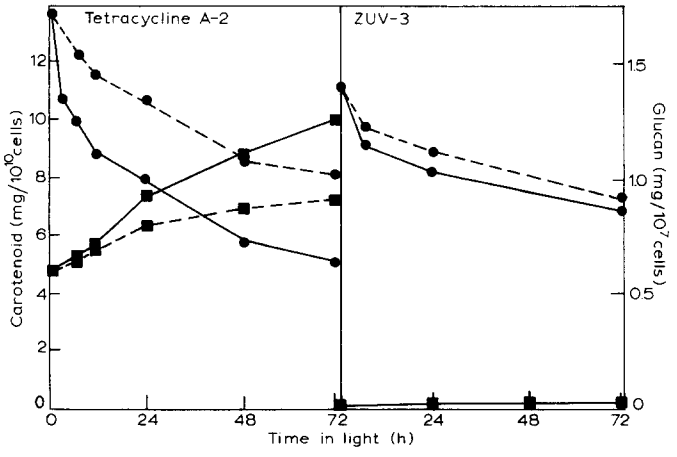

Fig. 4. Light-induced breakdown of β-1,3-glucan in chlorophyll-less mutants Tetracycline A-2 and ZUV-3. ●, glucan; ■, carotenoid; ——, light; – – –, dark.

in the activities of β-1,3-glucan phosphorylase, β-1,3-glucan hydrolase, UDPG pyrophosphorylase, phosphoglucomutase and glucose-6-phosphate dehydrogenase.

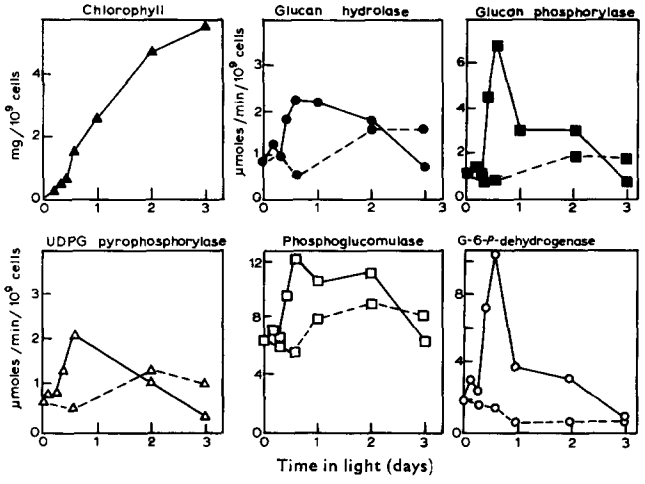

Fig. 5. Changes in levels of chlorophyll and enzymes catabolizing β-1,3-glucan during chloroplast development. ——, light; – – , dark.

The peaks of activity were usually attained during the first 14 h of illumination. The activities then declined and by 72 h had decreased to below those of the dark controls. The magnitude of the initial increase in activity for any one enzyme varied considerably between different populations of cells, but the activities of the various enzymes relative to each other remained fairly constant. β-1,3-Glucan synthetase, which was readily detectable in growing cells, could be found in only trace amounts in illuminated cells showing a rapid net breakdown of β-1,3-glucan.

Effect of inhibitors of protein synthesis on breakdown of β-1,3-glucan

The synthesis of chloroplast proteins in nondividing dark-adapted cells after exposure to continuous light is preferentially inhibited by 5-fluorouracil and chloramphenicol[5,7]. The latter inhibitor, in particular, appears to have very little effect at 1 mg/ml on the synthesis of nonchloroplast protein since it fails to inhibit the rate of cell division in cells grown heterotrophically. Fig. 6 shows changes in the β-1,3-

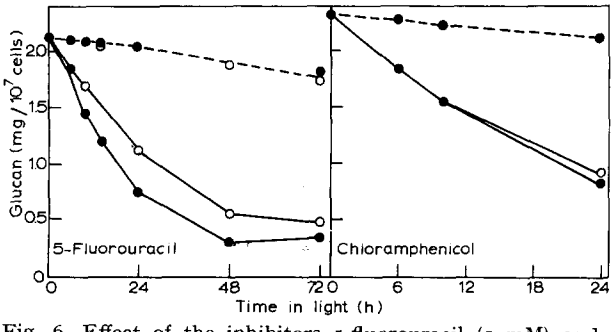

Fig. 6. Effect of the inhibitors 5-fluorouracil (5 mM) and chloramphenicol (1 mg/ml) on the breakdown of β-1,3-glucan during chloroplast development. ○, *plus* inhibitor; ——, light; – – –, dark.

glucan content of cells treated with 5-fluorouracil or chloramphenicol. Although 5-fluorouracil inhibited chlorophyll production, the rate of β-1,3-glucan breakdown, either in the light or dark, was only slightly affected. Similarly, chloramphenicol did not significantly reduce the light-induced breakdown of β-1,3-glucan.

Since chloramphenicol did not inhibit β-1,3-glucan breakdown, the effect of another inhibitor of protein synthesis, cycloheximide, was tested. In contrast to chloramphenicol, cycloheximide inhibits protein synthesis in higher organisms, including *E. gracilis*[25], but not in bacteria. At 5 and 15 μg/ml, cycloheximide showed only a marginal effect on the breakdown of β-1,3-glucan, although there was some retardation of breakdown between 10 and 24 h of illumination.

Effect of added glucose on the cellular levels of β-1,3-glucan during chloroplast development

Fig. 7 shows the effect of adding glucose to the medium prior to exposing cells to light. The effect on chlorophyll synthesis was not large, usually there was a slight stimulation during the first 24 h. In the presence of glucose there was little change in β-1,3-glucan per cell of illuminated cells (*cf.* Fig. 1), while in the cells kept in the dark β-1,3-glucan increased rapidly and by 10 h had reached the level normally found in a growing culture of dark-adapted cells. Hence, even in the presence of an excess of exogenous glucose, a marked difference between the β-1,3-glucan content of cells kept in the light or the dark was preserved.

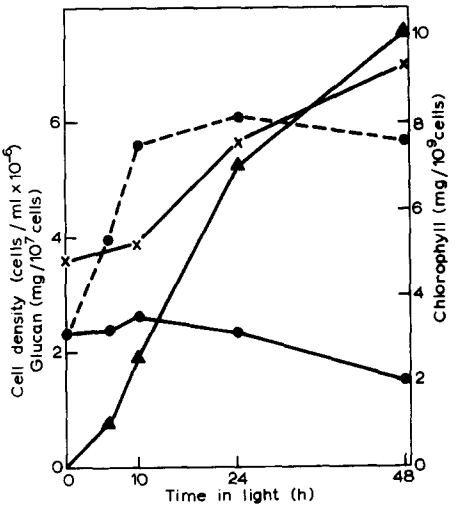

Fig. 7. Changes in β-1,3-glucan during chloroplast development in the presence of added glucose ▲, chlorophyll; ●, glucan; ×, cell number; ——, light; – – –, dark.

Relative roles of β-1,3-glucan and photosynthesis as energy sources for chloroplast development

Since DCMU specifically inhibits the light reaction of photosynthesis, the relative roles of β-1,3-glucan and photosynthesis as sources of carbon and energy for chloroplast development can be ascertained by determining the effect of DCMU on chloroplast formation[26]. As shown in Table I, DCMU only partially inhibited the formation of chlorophyll during the first 24 h when the β-1,3-glucan was available

as a source of carbon and energy for chloroplast development. During the first 6 h, DCMU inhibited glucan breakdown by about 25 % although there was little inhibition subsequently. After 24 h, when the breakdown of β-1,3-glucan in the light had ceased, DCMU severely inhibited further development of the chloroplasts. If an exogenous supply of glucose was provided, DCMU inhibition was largely overcome.

TABLE I

INHIBITION OF CHLOROPLAST DEVELOPMENT BY DCMU (1.25 μM) WITH AND WITHOUT EXOGENOUS GLUCOSE

Time in light (h)	*% Inhibition by DCMU of chlorophyll per cell*	
	No glucose	*+ glucose*
0– 6	33	27
6–10	40	20
10–24	60	34
24–49	82	40
49–72	87	0
72–96	100	0

DISCUSSION

Growing cells of dark-adapted *E. gracilis* accumulate a large store of carbohydrate in the form of β-1,3-glucan which can account for as much as 50% of the dry weight of the cells. When these cells are transferred to a medium lacking carbon growth sources, cell division continues for several hours while the β-1,3-glucan per cell is decreased by about two-thirds. The β-1,3-glucan is then utilized only slowly if the cells are maintained in the dark[27]. Thus these cells contain a control mechanism for preserving a relatively large reserve of carbon even under conditions of carbon starvation. Upon illuminating these cells, most of the reserve of β-1,3-glucan is depleted within 24 h (Fig. 1), a process which appears to be associated with the development of chloroplasts.

The light-induced breakdown of β-1,3-glucan is accompanied by an increase in activity of several enzymes catabolizing β-1,3-glucan, including β-1,3-glucan phosphorylase and β-1,3-glucan hydrolase. According to BARRAS AND STONE[28] at least two separate β-1,3-glucan hydrolases are active in *E. gracilis*. The data do not allow a distinction to be drawn between enzyme activation and synthesis of new protein as the basis for the increases in the activities of these enzymes. The small inhibition of β-1,3-glucan breakdown shown by cycloheximide, however, suggests that if it is new protein synthesis which is involved, then this is not essential for β-1,3-glucan breakdown, although it may result in more rapid mobilization of the β-1,3-glucan reserve.

The action spectrum of the light-induced breakdown of β-1,3-glucan is unknown, but it was established that the process is dependent upon continuous illumination and upon the intensity of the illumination. Light at low intensity (7 ft candles) failed to stimulate breakdown of β-1,3-glucan for several hours, but by 72 h the rate

of breakdown approached values found in cells illuminated at 120 ft candles. Thus the disappearance of β-1,3-glucan appeared to be geared to synthesis of plastid protein and lipid, possibly through a control mechanism based on the concentration of specific metabolites, rather than being a result of a specific and direct effect of light on glucan metabolism. This was further indicated by the studies on bleached mutants. Where light induced carotenoid synthesis, which in these mutants may reflect some light-dependent development of residual defective plastids, the level of β-1,3-glucan fell, although less so than in the normal cells. In Mutant ZUV-3, where only traces of carotenoids were formed in the light, there was little change in the content of β-1,3-glucan.

Although utilization of β-1,3-glucan in illuminated cells appears to be linked to chloroplast development, neither 5-fluorouracil nor chloramphenicol inhibited breakdown of the β-1,3-glucan. However, the inhibitory effects of these two compounds on the synthesis of chlorophyll and chloroplast protein are largely manifested subsequent to the main period of β-1,3-glucan breakdown .This breakdown then is probably related to early light-induced developmental events occurring in the plastid and perhaps also in the cytoplasm.

The experiments with DCMU also attest to the importance of β-1,3-glucan as a source of carbon and energy for the early stages of chloroplast development. Increasing inhibition of chlorophyll synthesis by DCMU after 10–24 h of illumination coincided with depletion of the glucan reserve and the formation of chloroplast lamellae with photosynthetic capability[12]. The almost complete breakdown of β-1,3-glucan at 7 ft candles also suggests that β-1,3-glucan is utilized mainly for biosynthetic reactions occurring prior to the main period of lamellae formation[24]. The requirement for photosynthesis during the later stages of chloroplast development can be met by an exogenous source of carbon such as glucose (Table I), but even under these heterotrophic growth conditions a difference in the levels of β-1,3-glucan in illuminated and non-illuminated cells is still maintained (Fig. 7).

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