

# $\beta$ -1,3-GLUCAN: A SOURCE OF CARBON AND ENERGY FOR CHLOROPLAST DEVELOPMENT IN *EUGLENA GRACILIS*

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## Summary

The illumination of dark-adapted cells of *E. gracilis* under non-dividing conditions induced not only the production of chloroplasts but also a rapid breakdown of  $\beta$ -1,3-glucan, the reserve carbohydrate of this organism. The decrease in  $\beta$ -1,3-glucan preceded the synthesis of most of the chlorophyll and was confined to the first 24 hr of illumination, whereas chlorophyll synthesis continued for at least 72 hr.

The metabolic fate of the  $\beta$ -1,3-glucan and its possible relation to chloroplast formation was investigated by preferentially labelling the  $\beta$ -1,3-glucan of dark-adapted cells with  $^{14}\text{C}$  and then determining the redistribution of  $^{14}\text{C}$  in the cell after exposing the cells to light. Of the  $^{14}\text{C}$  released from  $\beta$ -1,3-glucan during the first 24 hr of illumination, 41.5% was recovered in other cell components (24.5% in lipid, 11% in protein) and the rest as  $\text{CO}_2$ . In cells in the dark,  $^{14}\text{C}$  was released much more slowly from  $\beta$ -1,3-glucan than in cells in the light and there was no net increase in the  $^{14}\text{C}$  content of other cell fractions. Determinations made on isolated chloroplasts indicated that the chloroplasts contained most of the  $^{14}\text{C}$  redistributed into lipid.

The results support our earlier hypothesis that  $\beta$ -1,3-glucan is available only as a source of energy in cells kept in the dark, but in cells exposed to light it can be utilized as a source of both energy and carbon for biosynthesis associated with chloroplast development.

## I. INTRODUCTION

$\beta$ -1,3-Glucan is the characteristic reserve carbohydrate of the Euglenophyta (Barras and Stone 1968). In cells cultivated heterotrophically and in the dark,  $\beta$ -1,3-glucan can amount to as much as 50% of the dry weight of *Euglena gracilis*. Light influences the cellular levels of  $\beta$ -1,3-glucan (Dwyer, Smydzuk, and Smillie 1970) and while  $\beta$ -1,3-glucan is slowly hydrolysed in cells maintained in a carbon-depleted medium in the dark (Blum and Buetow 1963), when such cells are exposed to light  $\beta$ -1,3-glucan is rapidly broken down (Smillie, Evans, and Lyman 1963; Dwyer and Smillie 1970). Since light also induces chloroplast formation, the breakdown of  $\beta$ -1,3-glucan may thus be regulated so as to supply carbon for chloroplast development. This suggestion is supported by the observation that the rate of breakdown of  $\beta$ -1,3-glucan is not affected by light in a bleached mutant of *E. gracilis* that does not show a light-induced synthesis of pigments (Dwyer and Smillie 1970).

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The possible role of  $\beta$ -1,3-glucan in the biogenesis of the *Euglena* chloroplast was investigated using dark-adapted cells in which the  $\beta$ -1,3-glucan was preferentially labelled with  $^{14}\text{C}$ . The release of  $^{14}\text{C}$  from  $\beta$ -1,3-glucan and the redistribution of activity into various cell components was followed during chloroplast development.

## II. METHODS

### (a) Cells

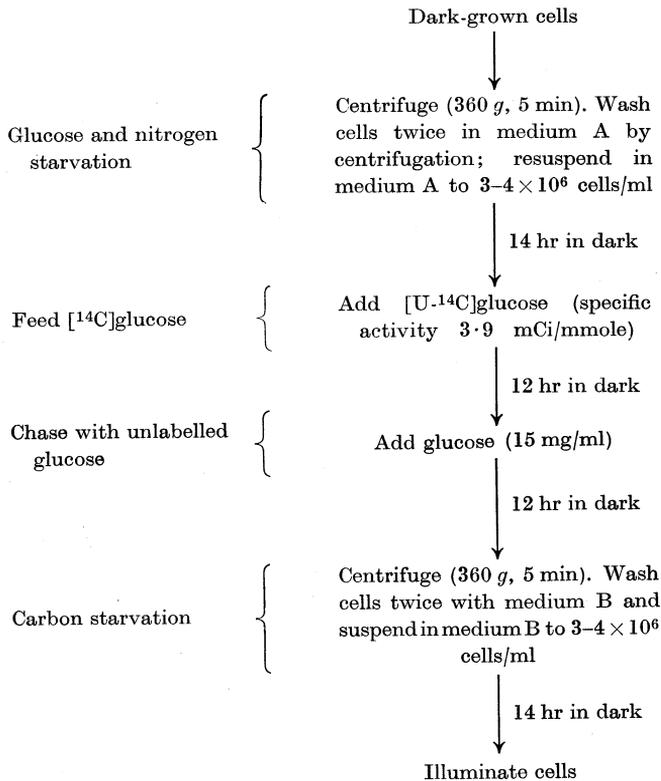
*E. gracilis* strain Z was cultured in the dark as described previously (Dwyer, Smydzuk, and Smillie 1970). Chloroplast development was induced by exposing cells continuously to 1250–1500 lux of white light. Air supplemented with 5%  $\text{CO}_2$  was circulated through the culture flasks.

### (b) Isolation of Chloroplasts

Chloroplasts were isolated using the procedure of Eisenstadt and Brawerman (1964), except that 85% sucrose was substituted for 75% sucrose in the case of cells illuminated for only 22 hr.

### (c) Labelling $\beta$ -1,3-Glucan in vivo

To enable the redistribution of carbon from  $\beta$ -1,3-glucan into other cell components during chloroplast development to be followed, a method for preferentially labelling  $\beta$ -1,3-glucan *in vivo* with  $^{14}\text{C}$  was developed. The final procedure adopted is as follows:



Medium A consists of succinic acid 0.47 g, DL-malic acid 1.0 g, ethylenediaminetetraacetate 0.5 g,  $\text{KH}_2\text{PO}_4$  0.3 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{CaCO}_3$  60 mg, thiamine.HCl 0.6 mg, vitamin  $\text{B}_{12}$  5  $\mu\text{g}$ , and trace metal mix 22  $\mu\text{g}$  (Hutner, Bach, and Ross 1956) in a final volume of 1 litre. Medium B

was prepared by adding  $(\text{NH}_4)_2\text{SO}_4$  (1 g/l) to medium A and omitting the succinic and malic acids. Manipulations involving dark-adapted cells were performed under a dim green safe-light.

In the several experiments in which this procedure was used, 67–90% of the total  $^{14}\text{C}$  taken up by the cells was incorporated into  $\beta$ -1,3-glucan. The total amount of  $^{14}\text{C}$  taken up by the cells varied by as much as 10-fold in the different experiments.

To study the redistribution of  $^{14}\text{C}$  from  $\beta$ -1,3-glucan into other cell components, labelled cells were suspended in a carbon-depleted medium for 14 hr and then exposed to light for 72 hr (see above). During the final period of carbon starvation prior to the illumination any further division of cells following the glucose feeding is completed. Chloroplast development could then be followed in the absence of cell division.

#### (d) Fractionation of Cells and Chloroplasts

Washed cells or isolated chloroplasts were extracted (15 min at 2°C) three times with 10% trichloroacetic acid (TCA-soluble fraction). Residues from this and the following extractions were recovered by centrifuging at 1200 g for 15–30 min between each extraction. The residue from the TCA extraction was extracted successively with boiling methanol–chloroform (1:2 v/v), boiling methanol–ether (1:2 v/v), boiling ethanol–ether (1:2 v/v), and ether, and the supernatant fractions combined (lipid fraction). Nucleic acids were extracted from the residue with 10% NaCl at 100°C for 1 hr (Davidson, Frazer, and Hutchison 1951). The nucleic acids were precipitated and washed with 70% ethanol (nucleic acid fraction). The residue remaining from the extraction with NaCl was washed with water, then absolute ethanol, and finally with ether and allowed to dry. The residue was ground and extracted for several hours with phenol–glacial acetic acid–water (2:1:1 w/v/v) at room temperature (Jennings *et al.* 1968). The extraction was repeated twice (protein fraction). The final residue contains  $\beta$ -1,3-glucan. Some of the protein (5–20%) was not always extracted and, if necessary, corrections were made for this after hydrolysis of the  $\beta$ -1,3-glucan fraction and separation into amino acid and carbohydrate fractions.

#### (e) Analyses

The  $^{14}\text{C}$  content of the various fractions was determined by liquid scintillation counting. Corrections were made for quenching using an internal standard of [ $^{14}\text{C}$ ]n-hexadecane. Insoluble samples were first digested with nitric acid using the procedure of O'Brien (1964). Chlorophyll was determined according to Arnon (1949) and  $\beta$ -1,3-glucan by the method described by Dwyer, Smydzuk, and Smillie (1970). The number of cells was estimated using a Coulter counter.

### III. RESULTS

#### (a) Breakdown of $\beta$ -1,3-Glucan during the Development of Chloroplasts in Cells Labelled with $^{14}\text{C}$

Figure 1 shows the changes in the amount and activity of  $\beta$ -1,3-glucan during chloroplast development in cells prelabelled with  $^{14}\text{C}$  as described in Section II(c). During the prelabelling procedure there was little change in the amount of  $\beta$ -1,3-glucan per cell between the beginning and the end of the period of glucose feeding. During the subsequent period of carbon starvation in the dark the cells divided and the amount of  $\beta$ -1,3-glucan per cell decreased to about 2 mg per  $10^7$  cells. This level of  $\beta$ -1,3-glucan was usually attained in cells after 14 hr in a carbon-depleted medium, but further decreases occurred only slowly provided the cells were kept in darkness (Dwyer, Smydzuk, and Smillie 1970). Illuminating the cells resulted in the rapid breakdown of  $\beta$ -1,3-glucan and the synthesis of chlorophyll (Fig. 1).  $\beta$ -1,3-Glucan breakdown is associated with the early phase of chloroplast development as most of the decrease in  $\beta$ -1,3-glucan occurred prior to the main period of chlorophyll synthesis.

A change in the specific activity of the  $\beta$ -1,3-glucan during chloroplast development indicated that the  $\beta$ -1,3-glucan was unevenly labelled. The typical  $\beta$ -1,3-glucan granule probably consisted of an outer region of low specific activity, which presumably was synthesized last and degraded first, then a region of relatively higher specific activity and finally a region of decreasing specific activity. Observations that granules are synthesized from a central core have been described for other species of *Euglena* (Nath, Dutta, and Dhillon 1960, 1961; Leedale 1967). Contrasting with these conclusions is the observation that the specific activity of  $\beta$ -1,3-glucan in the cells kept in the dark did not increase. This could mean that the mechanism of breakdown of the  $\beta$ -1,3-glucan in the dark and the light is different.

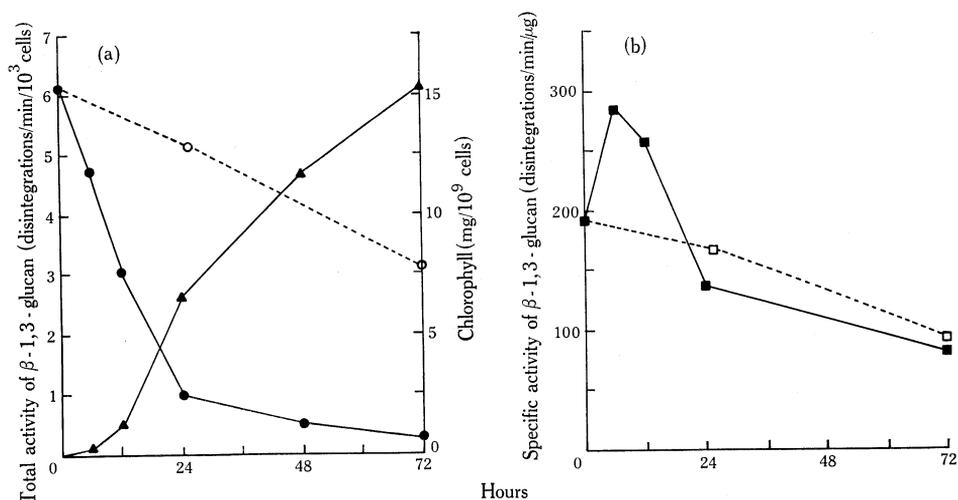


Fig. 1.—Changes in the activity of prelabelled  $\beta$ -1,3-glucan during chloroplast development. Dark-grown cells were labelled with [<sup>14</sup>C]glucose, starved, and exposed to light as described in Section II(c). (a) Changes in chlorophyll (▲) and the total activity of  $\beta$ -1,3-glucan (○, ●). (b) Changes in the specific activity (□, ■) of  $\beta$ -1,3-glucan during the period of illumination. — Cells illuminated. - - - Cells in darkness.

(b) *Redistribution of <sup>14</sup>C from [<sup>14</sup>C] $\beta$ -1,3-Glucan into Subcellular Fractions during Chloroplast Development*

Figure 2 shows light-induced changes in the distribution of <sup>14</sup>C in cells which had been prelabelled with <sup>14</sup>C. Almost all of the decrease in <sup>14</sup>C in  $\beta$ -1,3-glucan occurred during the first 24 hr of illumination. Of this about 1% was excreted into the medium, 41.5% was retained in other cell components, and the remainder was released as CO<sub>2</sub>. In contrast, during this same period of time there was no net movement of <sup>14</sup>C from  $\beta$ -1,3-glucan into other cellular fractions in unilluminated cells.

Figure 3 shows how the <sup>14</sup>C which originated from  $\beta$ -1,3-glucan and which remained in the cells was redistributed during illumination. After 24 hr of illumination, lipid accounted for 59% of this <sup>14</sup>C, protein 27%, and compounds extracted with TCA 14%. A small amount of activity was also found in nucleic acids. The activity in lipid and protein showed small changes between 24 and 72 hr, whereas that of the TCA-soluble fraction reached a maximum during the first 24 hr of illumination and then decreased.

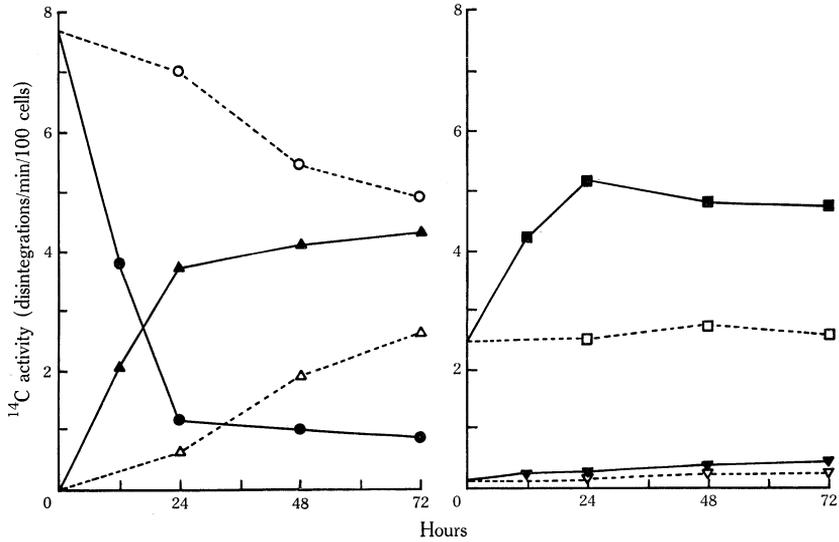


Fig. 2.—Redistribution of  $^{14}\text{C}$  during chloroplast development. Cells were prelabelled with  $^{14}\text{C}$  as described in Section II(c).  $\circ$ ,  $\bullet$   $\beta$ -1,3-Glucan.  $\Delta$ ,  $\blacktriangle$   $\text{CO}_2$ .  $\square$ ,  $\blacksquare$  Cell components other than  $\beta$ -1,3-glucan.  $\nabla$ ,  $\blacktriangledown$  Products excreted into medium. — Cells illuminated. - - - Cells in darkness.

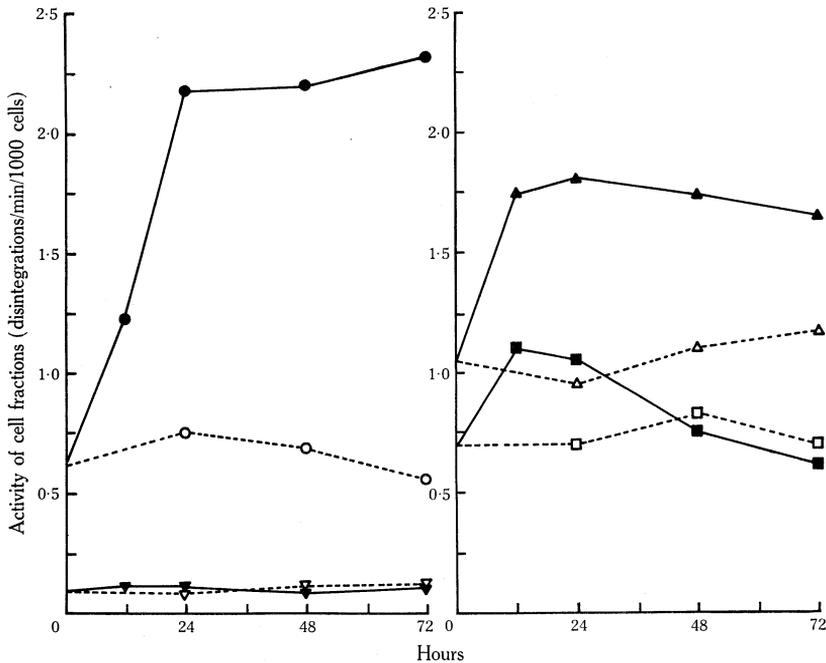


Fig. 3.—Changes in the activity of various cell fractions during chloroplast development. Experimental conditions as in Figure 2.  $\circ$ ,  $\bullet$  Lipid.  $\nabla$ ,  $\blacktriangledown$  Nucleic acids.  $\Delta$ ,  $\blacktriangle$  Protein.  $\square$ ,  $\blacksquare$  TCA-soluble fraction. — Cells illuminated. - - - Cells in darkness.

Further fractionation of the lipids by the method of Dawson (1960) revealed that one-fifth of the  $^{14}\text{C}$  in lipid was contained in a fraction which included glycerol esters and galactosylglycerols, and the remainder in a fraction which contained fatty acids and non-esterified lipids such as chlorophylls and carotenoids.

(c) *Utilization of  $^{14}\text{C}$  from  $\beta$ -1,3-Glucan for Synthesis of Chloroplast Lipid and Protein*

The data shown in Figures 2 and 3 indicate that products of the light-induced breakdown of  $\beta$ -1,3-glucan are utilized for the synthesis of lipid and protein, but they give no indication of the extent to which these products are utilized for the synthesis of chloroplast components. To determine this,  $\beta$ -1,3-glucan of dark-grown cells was labelled with  $^{14}\text{C}$  as described above, and after exposing the cells to light for various times the chloroplasts were isolated and the activity incorporated into their lipids and proteins was determined.

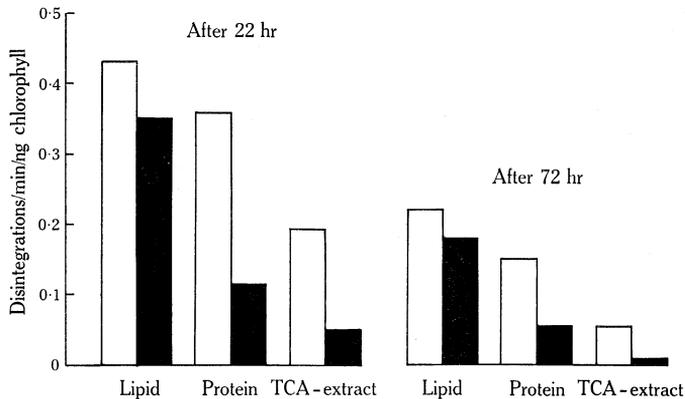


Fig. 4.—Comparison of the distribution of  $^{14}\text{C}$  in the whole cell (open histograms) and in chloroplasts (solid histograms). Cells were treated as described in Section II(c) and exposed to light for 22 or 72 hr. The experimental procedure is described in the text.

Figure 4 shows the proportion of  $^{14}\text{C}$  in fractionated chloroplasts obtained from cells illuminated for 22 and 72 hr. Following illumination, each culture was divided into two and chloroplasts were isolated from the cells contained in one of the portions. The isolated chloroplasts and the whole cells were then each fractionated into lipid, protein, and compounds soluble in 10% TCA (as described in Section II), and the  $^{14}\text{C}$  in each fraction was determined. The values obtained with isolated chloroplasts and whole cells are compared on a chlorophyll basis since this allows a direct estimate of how much of the total activity in a given fraction is present in the chloroplasts. For example, if all of the  $^{14}\text{C}$  in lipid in the cell is due to chloroplast lipid, then the activity of the whole cell and chloroplast lipid fractions should be the same when expressed per milligram of chlorophyll.

After 22 hr of illumination the estimated percentage of the total activity of the lipid and protein fractions was 81 and 32% respectively (Fig. 4). This percentage altered only slightly (to 82 and 36.5% respectively) after a further 50 hr of illumination. Thus after 22 hr most of the activity in the lipid fraction was due to chloroplast lipids, but less than half of  $^{14}\text{C}$  redistributed into protein was recovered in chloroplast protein.

## IV. DISCUSSION

$\beta$ -1,3-Glucan appears to be an important source of both energy and carbon for the early stages of chloroplast development. When dark-adapted cells were exposed to continuous light under our experimental conditions the rate of synthesis of chlorophyll did not reach a maximum until after 12 hr, yet during this first 12 hr more than half of the  $\beta$ -1,3-glucan was broken down and most of that remaining was degraded during the following 12 hr. The experiments with prelabelled cells suggest that during these periods most of the products of  $\beta$ -1,3-glucan hydrolysis not converted to  $\text{CO}_2$  were used for the synthesis of chloroplast lipid, and to a lesser extent, for protein. Thus during the first 24 hr of illumination when the [ $^{14}\text{C}$ ] $\beta$ -1,3-glucan was hydrolysed there was an increase in  $^{14}\text{C}$  in small molecules extracted with TCA and in the lipid and protein fractions. During the next 48 hr there was little change in the activity in the lipid and protein fractions and the activity in the TCA-soluble fraction declined. In the case of the lipid fraction, the analyses on isolated chloroplasts [Fig. 4; Section III(c)] revealed that most of the  $^{14}\text{C}$  which was redistributed into this fraction was contained in the chloroplasts. These organelles accounted for much less of the total  $^{14}\text{C}$  which was redistributed into protein. However, the value obtained for protein was probably low and may have represented largely insoluble chloroplast protein since many soluble chloroplast proteins (e.g. cytochrome 552) were very likely lost from the chloroplasts during their preparation. If most of the soluble protein was lost and assuming that this protein had about the same specific activity as the insoluble protein and accounted for about 55% of the total chloroplast protein (Kirk and Tilney-Bassett 1967), then the chloroplasts would have accounted for the majority of the  $^{14}\text{C}$  which was redistributed into labelled protein. This assumes that products arising from the hydrolysis of  $\beta$ -1,3-glucan were not preferentially utilized for the synthesis of insoluble chloroplast protein compared with the soluble protein.

Presumably, once the  $\beta$ -1,3-glucan supply is exhausted the chloroplasts have developed sufficiently to provide the requisite energy and carbon for their continued growth. This is supported by experiments in which a specific inhibitor of photosynthesis, 3(3,4-dichlorophenyl)-1,1-dimethylurea, was used to show that photosynthesis was not important for chloroplast development during the first hours of illumination, but subsequently it became increasingly important as a source of carbon for further growth of the chloroplasts (Dwyer and Smillie 1970). Amino acids released from the hydrolysis of cytoplasmic proteins are possibly also utilized for chloroplast protein synthesis (Smillie, Evans, and Lyman 1963).

Illuminating dark-adapted cells also resulted in a more rapid respiration of products arising from the hydrolysis of  $\beta$ -1,3-glucan when compared with cells maintained in the dark (Fig. 2). Some of the  $\text{CO}_2$  released in the light would be associated with lipid synthesis, as one  $\text{CO}_2$  is given off for every two carbons incorporated into fatty acids. Schiff (1963) had observed earlier that light increased the rate of respiration of dark-adapted cells. Presumably, much of the increased energy made available in the illuminated cells was utilized for synthesis of chloroplast components. Since, however, during the first hours of chloroplast development there was an increased turnover of cytoplasmic RNA (Zeldin and Schiff 1967) and increases in the activities of cytoplasmic enzymes such as  $\beta$ -1,3-glucan phosphorylase (Dwyer and Smillie 1970) and glucose-6-phosphate dehydrogenase (Smillie, Evans,

and Lyman 1963), part of this energy may have been used for biosynthetic reactions in the cytoplasm.

An estimate of the amount of carbon derived from  $\beta$ -1,3-glucan in the lipid and protein of mature chloroplasts (in cells illuminated for 72 hr) can be obtained if a number of assumptions and approximations are made. These are: (1) the ratio of lipid:water-insoluble protein:chlorophyll in 3-day-old chloroplasts of *E. gracilis* is the same as the corresponding ratio for spinach chloroplasts given by Lichtenthaler and Park (1963) (495,000:465,000:103,000 moles/mole Mn); (2) the carbon in protein is equivalent to 50% of the weight of protein; and (3) the carbon in chloroplast lipids is 70% of the weight of lipid: this is the actual value for monogalactosyl diglyceride, the major lipid in *E. gracilis* chloroplasts (Rosenberg and Gouaux 1967). In three separate experiments the percentages of carbon derived from  $\beta$ -1,3-glucan in the lipids of mature chloroplasts were 24, 34, and 47% and for protein (mostly insoluble protein?) the corresponding values were 9, 10, and 10%.

Our data then indicate that  $\beta$ -1,3-glucan contributes to the initial stages of chloroplast development by provision of energy and carbon for the synthesis of chloroplast materials. This and our other studies which indicate that *E. gracilis* contains a control mechanism which prevents rapid utilization of  $\beta$ -1,3-glucan for biosynthetic reactions unless the cells are illuminated (Dwyer, Smydzuk, and Smillie 1970; Dwyer and Smillie 1970) suggest that  $\beta$ -1,3-glucan is not a general reserve of carbon for cell biosynthesis, but instead has a specific role as a source of carbon and energy in the biogenesis of the chloroplast. The possession of such a control mechanism may be a distinct advantage to the organism's survival in fluctuating environmental conditions which are unfavourable for continued heterotrophic growth.

#### V. ACKNOWLEDGMENT

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