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De Novo Maltotriose Biosynthesis from the Reducing End by *Spinacia oleracea* L. Chloroplasts

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ABSTRACT

The distribution of ^{14}C in the various glucose residues of maltotriose was studied as a function of time of photosynthesis of isolated chloroplasts of spinach (*Spinacia oleracea* L.) using $^{14}CO_2$. The distribution of label showed that the reducing-end glucose residue was labeled first and the label subsequently distributed to the second and third glucose residues at approximately equal rates.

A mechanism for the distribution of label and the synthesis of maltotriose from the reducing end is presented. The mechanism has postulated to be the same as that for the maltose synthase recently described by Schilling. Maltose biosynthesis from α-D-glucose-1-phosphate was characterized as involving two glucosyl intermediates by a double displacement mechanism with inversion of configuration. The mode of enzymic action by which maltosyl intermediates were transferred to glucosyl intermediates was consistent with the fractional distribution of radioactivity found in each glucose unit of maltotriose during short term photosynthesis experiments.

Maltodextrins have not been generally recognized as constituents of photosynthetic tissue. Indeed, the maltodextrins were not observed as such until green algae were labeled during photosynthesis in $^{14}CO_2$. Following photosynthesis of *Chlorella* and *Scenedesmus* in $^{14}CO_2$ and extraction of these tissues, Bassham and Calvin (2) prepared radioautograms of two-dimensional chromatograms of the extracts. The radioautograms showed a homologous series of compounds extending from the origin to sucrose. The homologous series was identified by French (6) as maltodextrins (*i.e.* α-1,4-linked D-glucose oligosaccharides). Kandler (12) also observed the maltodextrin series on two-dimensional chromatograms of various plant extracts following $^{14}CO_2$ photosynthesis. Heber (9) reported tri-, tetra-, and pentasaccharides present in chloroplasts of *Nitella*, *Valonia*, and *Chara*. Maltotriose and maltotetraose represented a major percentage of the excretion products of *Oscillatoria redeker* (11). Jensen also noted maltodextrins in extracts of isolated spinach chloroplasts after photosynthesis in $^{14}CO_2$ (10).

Maltose biosynthesis *in vivo* has been shown to involve α-glucose-1-P (18). A maltose synthase isolated from spinach was recently described by Schilling (19) in which maltose formation from αG-1-P[2] was shown to involve two glucosyl-enzyme intermediates (βG-E and βG-E′) by a double displacement mechanism:

$$2\ \alpha\text{G-1-P} + \text{E} + \text{E}' \rightleftharpoons \beta\text{G-E} + \beta\text{G-E}' + 2\ \text{P}_i \quad (1)$$

$$\beta\text{G-E} + \beta\text{G-E}' \rightleftharpoons \alpha\text{G-}\beta\text{G-E}' + \text{E} \quad (2)$$

$$\alpha\text{G-}\beta\text{G-E}' \rightleftharpoons \alpha\text{G-}\alpha\text{G} + \text{E}' \quad (3)$$

Robyt (17) has described a similar mechanism for polysaccharide biosynthesis involving transfer of activated monomer units to the reducing end of a growing polysaccharide chain covalently linked to the active site of the enzyme.

The work reported in this paper describes the relationship of the above enzymic activity to a possible route of maltotriose biosynthesis. The labeling of maltotriose during photosynthesis in $^{14}CO_2$ has been found to originate in the reducing end glucose moiety, and can be explained by extension of the glucosyl intermediate displacement mechanism described above.

MATERIALS AND METHODS

Chloroplast Preparation. Chloroplasts were isolated in pyrophosphate buffer according to methods of Cockburn *et al.* (5) from fresh spinach (*Spinacia oleracea* L.) leaves purchased at the grocery. About 50 g of washed and chilled leaf laminae were homogenized in a Waring Blendor for 3 to 5 s in 200 ml of semifrozen buffer containing 0.333 M sorbitol, 0.005 M $MgCl_2$, 0.010 M $Na_4P_2O_7$, and 0.004 M L-ascorbic acid. The latter was added after adjustment of the icy buffer to pH 6.6.

The macerate was filtered through cheesecloth into 50 ml centrifuge tubes. The chloroplasts were pelleted at 0°C from rest to 1000*g* to rest in approximately 90 s. The supernatant was discarded and the pellet was suspended in 5 ml of ice-cold solution containing 0.333 M sorbitol, 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, 0.002 M ethylendinitrilotetracetic acid (EDTA), and 0.050 M Hepes. The pH of this buffer was 7.6 at 20°C. The pellet was washed and centrifuged as above three times in this buffer at 0°C.

Photosynthesis Procedures. Photosynthesis by the isolated chloroplasts was conducted in the pH 7.6 Hepes buffer following the procedure of Bassham *et al.* (4). Potassium bicarbonate and sorbitol were added to the suspension of chloroplasts so that

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[2] Abbreviations: αG-1-P, α-D-glucose-1-phosphate; αG*, alpha glucose containing ^{14}C-label; E and E′, independent active sites on maltose synthase enzyme(s); βG-E, beta glucosyl intermediate of maltose synthase; α-G-βG-E, beta maltosyl intermediate of enzyme; αG-α-G, alpha maltose; αG-αG-βG-E, beta maltotriosyl enzyme intermediate; αG-αG-αG*, maltotriose labeled in reducing end; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_7, maltoheptaose; G_9, maltononaose; DP, degree of polymerization.

respective concentrations would remain 0.010 M in bicarbonate and 0.333 M in sorbitol after addition of radioactive bicarbonate. The 5 ml of chloroplast suspension were placed in a 3.3-cm diameter, 24/25 standard taper, round bottom flask with side arm (Thomas, Philadelphia) and were kept suspended by the gentle motion of a wrist-action shaker. The bottom of the flask was suspended in water thermostated at 20°C. Illumination through the water was a bank of six 61-cm daylight fluorescent bulbs. Photosynthesis was conducted for 15 to 20 min before injection of 0.2 ml (13 μmol) of high specific activity [^{14}C] $NaHCO_3$ (40 μCi/μmol) into the suspension from a hypodermic syringe by means of 20 cm of 1 mm polyethylene tubing which passed through the side arm of the flask. Samples of 0.4 ml were removed by the same means after intervals of time and injected into 1.6 ml of absolute methanol at 4°C. After centrifugation at 10,000*g* for 10 min, the green supernatants and white pellets were separated and stored at −15°C. *In vivo* photosynthesis of spinach leaves in $^{14}CO_2$ was conducted as described earlier (12).

Paper Chromatography. For the analysis of the products of photosynthesis in the alcoholic extracts in algae and chloroplasts, two-dimensional descending paper chromatography, as described by Bassham and Kirk (3), was employed. To obtain the neutral maltodextrins free from amino acids, organic phosphates and other ionic materials, the alcoholic extracts were deionized with Amberlite MB-3 mixed-bed ion-exchange resin which had been saturated with glucose prior to use to reduce nonspecific binding of maltodextrins to the resin. Following concentration, the samples were chromatographed on water-washed Whatman No. 1 or Whatman 3 MM chromatography paper by multiple ascents in sealed stainless steel tanks at 65°C as described by French *et al.* (7) in solvent containing 70% *n*-propyl alcohol:water (7:3, v/v). Radioautograms were prepared using Kodak No-Screen x-ray film. Chromatograms were developed for reducing compounds by dipping in silver nitrate-acetone and alkali-methanol reagents (20). The distribution of radioactivity between the glucose units of maltotriose was estimated following β-amylolysis either on paper by two-dimensional chromatography interspersed with β-amylase as described by French *et al.* (8) or in test tubes following elution of the radioactive maltotriose from the paper chromatograms. The β-amylase digests were rechromatographed as described above for separation of glucose and maltose hydrolysis products. The distribution of radioactivity in the maltose product was determined following reduction with sodium borohydride and acid hydrolysis. Descending chromatography on Whatman 3 MM paper in solvent containing ethyl acetate:acetic acid: saturated boric acid solution (9:1:1, v/v/v) was used to separate glucose and sorbitol (21). Areas from the chromatograms which corresponded to radiographic spots were cut out and counted by scintillation spectrometry. Scintillation solution containing 4.0 g PPO and 0.2 g POPOP per liter of toluene was used for counting radioactivity on paper in a Packard Tri-Carb scintillation spectrometer.

RESULTS

The kinetics and distribution of labeling of maltotriose from two photosynthesis experiments were studied. During both experiments, one conducted with continuous illumination and the other with a light-dark transition, the kinetics of maltotriose (G_3) and maltotetraose (G_4) labeling were nearly identical to each other but quite distinct from the labeling kinetics of maltose (G_2) and higher maltodextrins ($\geq G_5$). This is illustrated in Figure 1 for which data from the continuous illumination experiment is presented. The counts of radioactive carbon appearing in maltose, maltotriose plus maltotetraose, maltopentaose, and maltodextrins with DP > 8 are plotted as a function of time following injection of [^{14}C]$NaHCO_3$ into the spinach chloroplast suspension. The radioactivity appearing in maltohexaose, maltoheptaose, and maltooctaose, which followed the same kinetics as that in maltopentaose, were not plotted for clarity. Radioactivity appeared in maltose rapidly and continued to accumulate at the same rate throughout the 45-min sampling period. Similar kinetics were demonstrated for glucose, fructose, and sucrose (data not presented), whereas the rate of radioactivity accumulation in the maltodextrins other than maltose declined dramatically at about 15 min. There was similarity in curves for the chromatographically unresolved maltodextrins ($>G_8$) and those for the maltodextrins G_5, G_6, G_7, and G_8. The kinetics of G_3 and G_4 were distinct from those of the maltodextrins $\geq G_5$. Label in G_3 and G_4 continued to accumulate, whereas label in maltodextrins $\geq G_5$ remained at the same level or declined.

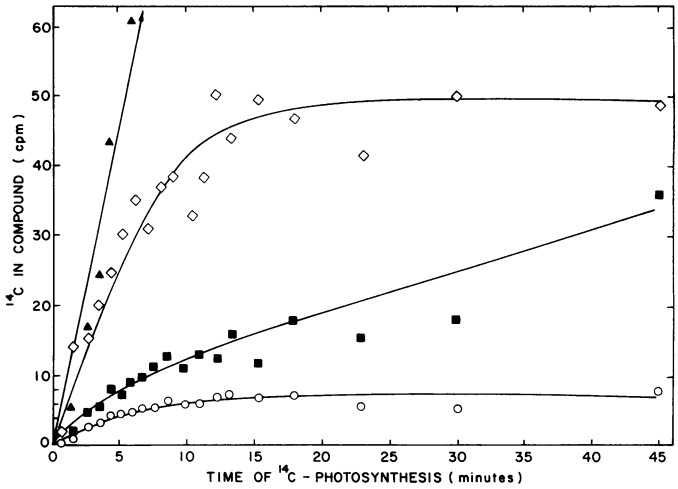

FIG. 1. Kinetics of ^{14}C-labeling photosynthetic products in spinach chloroplasts with continuous illumination. Quantities of radioactivity accumulated with time in maltose (▲), maltotriose plus maltotetraose (■), maltopentaose (○), and maltodextrins with DP > 8 which were not chromatographically resolved (◇), from equal volume quantities of extracts of spinach chloroplasts removed during a photosynthesis experiment in $^{14}CO_2$.

The distribution of radioactivity in maltotriose from both experiments was determined. After hydrolysis of G_3 with β-amylase, maltose and glucose were products; the reaction is shown in reaction 4.

$$\alpha G\text{-}\alpha G\text{-}\alpha G^* \rightarrow \alpha G\text{-}\beta G + \alpha G^* \quad (4)$$

The ratio of radioactivities in maltose to glucose is plotted in Figure 2 as a function of time. Initially, the glucose was more highly labeled than maltose. In fact, the curves obtained from both experiments extrapolate to zero in which case maltose would have no label at zero time, and the reducing end of the molecule would have acquired label almost immediately after injection of radioactivity in the system. This would indicate synthesis of maltotriose from the reducing end. It appeared from these observations that the labeling of maltotriose was very specific.

The differences between the rate of labeling of maltotriose in the two experiments may have been a result of the light-dark transition after 6.75 min of steady state photosynthesis in the latter experiment. However, the initial slopes of the two curves were unequal, which may indicate physiological differences in the chloroplasts of the two preparations. The extent of labeling is apparently dependent upon the ability of the chloroplast to fix CO_2, and the fixation of CO_2 is said to be dependent on the structural integrity of the chloroplasts (1).

The complete distribution of label in maltotriose from the steady state experiment was determined. The maltose, which had been hydrolyzed from maltotriose by β-amylase, was eluted from

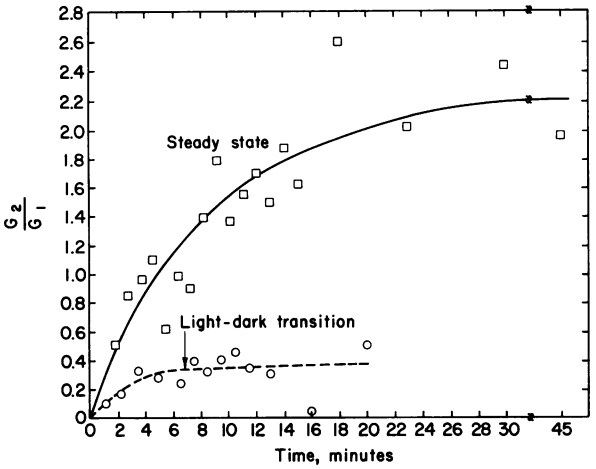

FIG. 2. Distribution of radioactivity in maltotriose from two photosynthesis experiments. Ratios of ^{14}C appearing in nonreducing end as maltose product of β-amylase hydrolysis (G_2) to that appearing in reducing end as glucose product of β-amylase hydrolysis (G_1) of maltotriose from equal volume quantities of extracts of spinach chloroplasts removed during course of steady state photosynthesis in $^{14}CO_2$ (□) and during an experiment with a light-to-dark transition (○) after 6.75 min (↓).

the chromatogram, reduced with sodium borohydride, hydrolyzed by acid, chromatographed, and radioautographed. The radioactivity in the resulting glucose and sorbitol was analyzed. The glucose corresponded to the nonreducing end and the sorbitol to the second glucose unit of the original maltotriose. The fraction of radioactivity in the three glucose units of the molecule is plotted *versus* time in Figure 3. Two minutes after injecting label into the system, 65% of the radioactivity was found in the reducing glucose. At 12 min, equal fractions of radioactivity (37%) were found in the reducing glucose and in the center glucose moieties; and, from that time on, the label in the center glucose moiety was greater than that in the reducing glucose, which was greater than that in the nonreducing glucose. In the course of steady state metabolism, cold maltotriose was first replaced by reducing end labeled maltotriose. As time passed, radioactivity of the G_3 pool gradually was found in the middle glucose residue of G_3 and finally in the nonreducing glucose residue.

The distribution of radioactivity in the other odd-numbered chain-length maltodextrins was also determined using β-amylase. At the inception of photosynthesis in $^{14}CO_2$ maltopentaose (G_5), maltoheptaose (G_7), and maltononaose (G_9) formed in isolated spinach chloroplasts were initially labeled most heavily in the nonreducing end (Linden, unpublished results). Similarly, the initial asymmetric labeling of maltose most heavily in the nonreducing end has been described (14). These facts are mentioned to emphasize the uniqueness of the initial reducing-end labeling distribution of maltotriose during photosynthesis in $^{14}CO_2$ of isolated spinach chloroplasts.

DISCUSSION

The specific asymmetric labeling of maltotriose from the reducing end has remained an enigma since the experiments described above were conducted at Iowa State University in 1969 by the first author in the laboratory of the late Dexter French. The hypothesis put forth by Schilling (18) for retention of configuration by a double displacement mechanism finally offers an explanation for the results. In maltose formation, the two glucosyl-enzyme sites may be equivalent, *i.e.* the products of reaction 2 could just as well have been αG-βG-E + E′. However, if we assume that instead of hydrolysis as shown in reaction 3, the E site could react again with labeled G*-1-P. The reactants for maltotriose formation could proceed either as in reaction 5

$$\alpha\text{G-}\beta\text{G-E}' + \beta\text{G*-E} \rightleftharpoons \alpha\text{G*-}\alpha\text{G-}\beta\text{G-E}' + \text{E} \qquad (5)$$

if the enzyme-linked disaccharide acts as acceptor, or as in reaction 6

$$\alpha\text{G-}\beta\text{G-E}' + \beta\text{G*-E} \rightleftharpoons \alpha\text{G-}\alpha\text{G-}\beta\text{G*-E} + \text{E}' \qquad (6)$$

if the activated monosaccharide acts as acceptor. In order for biosynthesis of reducing-end labeled maltotriose to occur, reaction 6, *i.e.* transfer of the glycosyl moiety of greater chain length to the newly formed glucosyl-enzyme, must be favored.

The mode of action described above would be consistent with the initial and specific labeling of the reducing end of maltotriose. Furthermore, the determination of the fractional distribution of radioactivity in each glucose unit of maltotriose showed that there was rapid equalization of acquired label between the three glucose units (Fig. 3). Again, such a labeling pattern would be achieved if the maltosyl moiety were transferred rather than the glucosyl moiety. To demonstrate this point, Figure 4 shows four hypothetical conditions which may exist at three consecutive times following initiation of $^{14}CO_2$ photosynthesis. The model predicts that there would be equal rates of label accumulation in the center glucose and in the nonreducing end glucose following the initial reducing end labeling of maltotriose.

The probabilistic approach to evaluation of the double displacement mechanism for maltotriose biosynthesis shows that maltosyl transfer is solely involved. The analysis presented in Table I shows that glucosyl transfer would result initially in nonreducing and labeled maltotriose. Mixed maltosyl- and glucosyl-transfer would result in initial labeling of both reducing and nonreducing ends of the molecule. Data presented has shown very specifically that initial labeling occurs in the reducing end glucose, and that label distributes at approximately equal rates between the center glucose and nonreducing end glucose.

The accumulation of maltose and therefore the hydrolysis of the maltosyl intermediate following step 1 of the above reactions occurred at a faster rate than maltotriose synthesis (*cf.* Fig. 1). Statistically, the maltosyl intermediate would be equally labeled in the two glucose residues. This in fact was the result observed from several of the short term $^{14}CO_2$ photosynthesis experiments conducted at various times of the day as shown in Table II. From 9 AM through the day until midnight, the symmetry in maltose labeling was observed during the first 5 min of *in vivo* $^{14}CO_2$ photosynthesis. In the early morning hours between 3 and 9 AM,

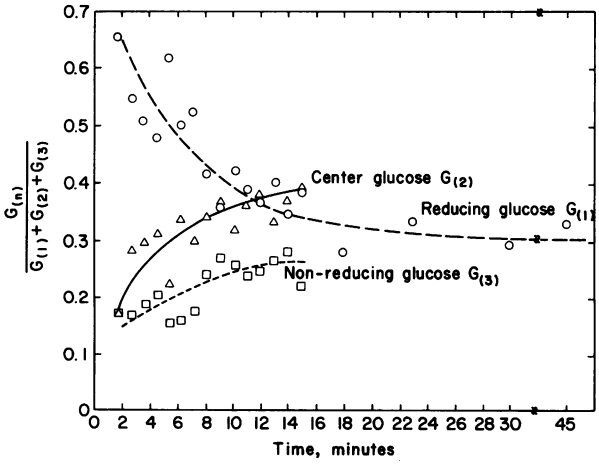

FIG. 3. Distribution of radioactivity in maltotriose from steady state photosynthesis experiment in $^{14}CO_2$. Ratios of radioactivity appearing in each glucose moiety of maltotriose with time.

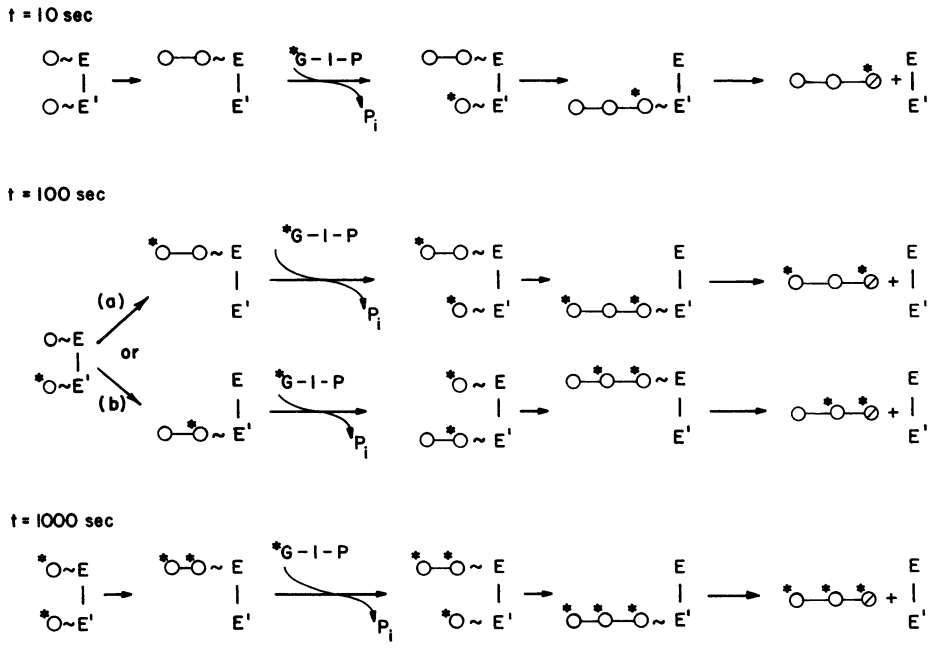

FIG. 4. Representation of enzyme active site(s) of the maltose synthase enzyme and reactions leading to biosynthesis of maltotriose in manner consistent with label distribution in maltotriose observed during photosynthesis experiments in $^{14}CO_2$. (O), Glucosyl moiety; (O), reducing end glucosyl moiety; (—), α-1,4-glucosyl bond; (~), β-1,4-glucosyl linkage with the enzyme at two active sites E and E'; (*O), ^{14}C label of glucosyl moiety.

Table I. *Probabilistic Analysis of Potential Double Displacement Mechanisms for Maltotriose Biosynthesis*

Glucosyl moieties of maltotriose are represented as reducing end glucose, $G_{(1)}$, center glucose, $G_{(2)}$, and nonreducing end glucose, $G_{(3)}$. Symbols 0 and 1, respectively, represent lack of and potential for labeling of glucosyl moiety at given relative time with each possibility of maltosyl-, glucosyl-, or mixed maltosyl- and glucosyl-transfer in maltose synthase according to model presented in Figure 4.

Relative Time	Maltosyl			Glucosyl			Mixed		
	$G_{(3)}$	$G_{(2)}$	$(G_{(1)}$	$G_{(3)}$	$G_{(2)}$	$(G_{(1)}$	$G_{(3)}$	$G_{(2)}$	$(G_{(1)}$
$t = 1.$	0	0	1	1	0	0	1	0	1
$t = 10.$ a	1	0	1	1	0	0	1	0	1
$t = 10.$ b	0	1	1	1	0	1	1	1	1
$t = 100.$	1	1	1	1	1	1	1	1	1

the maltose formed during the first 5 min of such experiments was asymmetrically labeled in the nonreducing end. The mobilization of photosynthate by the hydrolyzing enzymes in the chloroplast, which were described by Ponratz and Beck (16), leads to the accumulation of glucose and dextrins during the nighttime hours. The resulting pool then allows for the catalyzed reaction of the maltose glucosyltransferase (15) between labeled maltose and glucose and yields asymmetrically labeled maltose as shown in reaction 8.

$$\alpha G^*\text{-}\beta G^* + \beta G = \alpha G^*\text{-}\beta G + \beta G^* \quad (8)$$

These results have demonstrated *in vivo* compatability of maltose synthase and maltose glucosyltransferase activities. Maltotriose biosynthesis which occurs by further transglucosylation reactions of maltose synthase is also compatible with the demonstrated activity of the purified maltose glucosyltransferase. The latter enzymic activity exhibited glucosyl transfer with maltose and maltodextrins of DP ≥ 5 but not with either maltotriose or maltotetraose as donors (15). Schilling was unable to separate maltose synthase and maltose glucosyltransferase activities by protein purification procedures (19). The possibility that the two activities reside with the same protein raises interesting questions with regard to substrate specificity and interrelationships of reactions.

Table II. *Distribution of Radioactivity in Maltose Formed in Spinach Leaves during the First 5 Minutes of Photosynthesis in $^{14}CO_2$ at Different Times of Day*

The quotients of ^{14}C in nonreducing glucose units to ^{14}C in reducing end glucose units in maltose are given.

Time of Day	$^{14}CO_2$ Photosynthesis	Quotient
	min	
3 AM	2	2.19
	5	1.32
	10	1.22
9 AM	2	1.41
	5	1.34
	10	1.09
12 noon	2	0.95
	5	1.01
	10	0.98
4 PM	2	1.01
	5	1.09
	10	0.95
7 PM	2	0.92
	5	0.95
	10	1.09
12 midnight	2	0.95
	5	1.02
	10	1.04

The most significant conclusion of the work is the *in vivo* evidence of oligosaccharide formation from the reducing end. The insertion mechanism given for maltotriose biosynthesis is distinguished from general oligosaccharide polymerization reactions at the nonreducing end, or in the case of sucrosyl oligosaccharides at the end of the chain distal from sucrose in the case of sucrosyl oligosaccharides (13). The results have also demonstrated that enzyme-bound oligosyl moieties may serve as the donor molecule for further transglucosylation reactions. For instance, the transglucosylation involving a maltosyl-enzyme

intermediate yielded maltotriose; the transfer of a maltotriosyl-enzyme complex, which would yield maltotetraose, has been demonstrated (19). Maltotetraose is the smallest oligosaccharide which may serve as primer for polymerizations leading to amylose. On the other hand, the insertion mechanism described here may well be the synthetic route of amylose and not just primer for other polymerization reactions.

Finally, neither maltose synthase (19) nor maltose glucosyl transferase activities (15) were specifically isolated from extracts of spinach chloroplasts—whole leaf extracts were used in each case. The implication of maltose synthase in *in vivo* maltotriose biosynthesis by isolated spinach chloroplasts by results presented in this paper localizes the activity to the chloroplast.

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