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

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**ABSTRACT**

The distribution of 14C 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 14CO2. 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 14CO2. Following photosynthesis of *Chlorella* and *Scenedesmus* in 14CO2 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 14CO2 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 redekeri* (11). Jensen also noted maltodextrins in extracts of isolated spinach chloroplasts after photosynthesis in 14CO2 (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-P2 was shown to involve two glucosyl-enzyme intermediates (βG-E and βG-E') by a double displacement mechanism:

\[
\begin{align*}
2 \alpha G-1-P + E + E' & \rightleftharpoons \beta G-E + \beta G-E' + 2 P, \\
\beta G-E + \beta G-E' & \rightleftharpoons \alpha G-\beta G-E' + E \\
\alpha G-\beta G-E' & \rightleftharpoons \alpha G-\alpha G + E'
\end{align*}
\]

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 14CO2 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 Blender for 3 to 5 s in 200 ml of semifrozen buffer containing 0.333 M sorbitol, 0.005 M MgCl2, 0.010 M Na3P2O7, and 0.004 M L-ascorbic acid. The latter was added after adjustment of the icy buffer to pH 6.6.

The mace rate was filtered through cheesecloth into 50 ml centrifuge tubes. The chloroplasts were pelleted at 0°C from rest to 1000g 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 MgCl2, 0.001 M MnCl2, 0.002 M ethylenedinitrilotetraacetic 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*, α-glucose containing C-label; E and E', independent active sites on maltose synthase enzymes (s); βG-E, beta glucosyl intermediate of maltose synthase; αG-βG-E, beta maltosyl enzyme intermediate; αG-αG, alpha maltose; αG-βG-βG-E, beta maltotriose enzyme intermediate; αG-αG-αG*, maltotriose labeled in reducing end; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltononose; 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 thermostatted 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 [14C] NaHCO3 (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 10,000g for 10 min, the green supernatants and white pellets were separated and stored at -15°C. In vivo photosynthesis of spinach leaves in 14CO2 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 impurities, 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-amylolase digestes were rechromatographed as described above for separation of glucose and maltose hydrolysis products. The distribution of radioactivity in the maltose products 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 POPP 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 (G3) and maltotetraose (G4) labeling were nearly identical to each other but quite distinct from the labeling kinetics of maltose (G1) and higher maltodextrins (≥G3). 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 [14C]NaHCO3 into the spinach chloroplast suspension. The radioactivity appearing in maltotriose, 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 (≥G4) and those for the maltodextrins G5, G6, G7, and G8. The kinetics of G1 and G4 were distinct from those of the maltodextrins ≥G3. Label in G3 and G4 continued to accumulate, whereas label in maltodextrins ≥G3 remained at the same level or declined.

The distribution of radioactivity in maltotriose from both experiments was determined. After hydrolysis of G3, with β-amylase, maltose and glucose were products; the reaction is shown in reaction 4.

\[
\alpha G - \alpha G - \alpha G^* \rightarrow \alpha G - \beta G + \alpha G^* 
\]

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 CO2, and the fixation of CO2 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. 1. Kinetics of 14C-labeling photosynthetic products in spinach chloroplasts with continuous illumination. Quantities of radioactivity accumulated with time in maltose (A), maltotriose plus maltotetraose (B), maltopentaose (O), 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 14CO2.](image-url)
we assume that the second reducing-end pentasaccharide (G5), which was labeled in the middle of the reducing-end labeling procedure (cf. Fig. 1), would result in the formation of the reducing-end labeled maltotriose. This was confirmed by the fact that the reducing-end labeled maltotriose formed in the earlier experiment was isolated and used as the substrate for the subsequent experiments described above. The results of these experiments are presented in Table I and show that the reducing-end labeled maltotriose was indeed the predominant product formed in the initial labeling experiment. The distribution of activity in the reducing-end labeled maltotriose was also determined and is presented in Fig. 2. The activity was found to be concentrated in the reducing-end glucose residue, with a smaller amount of activity found in the nonreducing-end glucose residue.

**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 glucose-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

\[
αG-βG-E' + βG*-E \rightleftharpoons αG-αG-βG-E' + E
\]

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

\[
αG-βG-E' + βG*-E \rightleftharpoons αG-αG-βG*-E' + E
\]

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 14CO2 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 maltotriose 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 fact was the result observed from several of the short term 14CO2 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 14CO2 photosynthesis. In the early morning hours between 3 and 9 AM.

**FIG. 2.** Distribution of radioactivity in maltotriose from two photosynthesis experiments. Ratios of 14C appearing in nonreducing end as maltose product of β-amylase hydrolysis (G1) to that appearing in reducing end as glucose product of β-amylase hydrolysis (G1) of maltotriose from equal volume quantities of extracts of spinach chloroplasts removed during course of steady state photosynthesis in 14CO2 (C) and during an experiment with a light-to-dark transition (O) after 6.75 min (i).

**FIG. 3.** Distribution of radioactivity in maltotriose from steady state photosynthesis experiment in 14CO2. Ratios of activity appearing in each glucose moiety of maltotriose with time.
Table I. Probabilistic Analysis of Potential Double Displacement Mechanisms for Maltotriose Biosynthesis

<table>
<thead>
<tr>
<th>Relative Time</th>
<th>Maltosyl $G_{11}G_{22}$</th>
<th>Glucosyl $G_{11}G_{22}$</th>
<th>Mixed $G_{11}G_{22}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t = 1h$</td>
<td>$0$</td>
<td>$0$</td>
<td>$0$</td>
</tr>
<tr>
<td>$t = 10$ a</td>
<td>$1$</td>
<td>$0$</td>
<td>$0$</td>
</tr>
<tr>
<td>$t = 10$ b</td>
<td>$0$</td>
<td>$1$</td>
<td>$1$</td>
</tr>
<tr>
<td>$t = 100$</td>
<td>$1$</td>
<td>$1$</td>
<td>$1$</td>
</tr>
</tbody>
</table>

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.

$$\alpha G^*\beta G + \beta G = \alpha G^* + \beta G^* + \beta G$$  \hspace{1cm} (8)

These results have demonstrated in vivo compatibility 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 enzyme 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.

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 oligosaccharidic moieties may serve as the donor molecule for further transglucosylation reactions. For instance, the transglucosylation involving a maltotetraose-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 amy-
lose. 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 \textit{in vivo} maltotriose
biosynthesis by isolated spinach chloroplasts by results presented
in this paper localizes the activity to the chloroplast.

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distribution studies were conducted in 1968 and also with Professor Otto Kandler
of the Botanisches lnstitut der Universitaet Muenchen where the hypotheses for
the enzymic mechanisms were developed.

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