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Accumulation of Maltose during Photosynthesis in Protoplasts Isolated from Spinach Leaves Treated with Mannose[1]

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ABSTRACT

When mannose was included in the enzyme incubation medium during the preparation of protoplasts from leaves of spinach, maltose was an early product of protoplast photosynthesis and, after 12 minutes, accounted for up to 15% of the ^{14}C incorporated from $^{14}CO_2$. Maltose was not detected in protoplasts prepared in the normal enzyme medium. Rapid separation of cytoplasm and chloroplasts following exposure to $^{14}CO_2$ showed that maltose was present in both fractions. Direct measurements of [^{14}C]maltose uptake indicated transport across the chloroplast envelope at rates similar to the transport of glucose. The source of maltose and site of its initial formation are discussed.

Maltose occurs in only small amounts in leaves (18) but has been detected as a ^{14}C-labeled product of $^{14}CO_2$ assimilation in several species (9, 19, 20, 22, 23, 30). Herold, McGee, and Lewis (13) reported greatly enhanced incorporation of ^{14}C from $^{14}CO_2$ into maltose in sugar beet (*Beta vulgaris* L.) when leaf discs were preincubated in mannose solution. This effect of mannose was not resolved, but advantage has been taken of the increased content of [^{14}C]maltose to examine the routes of maltose synthesis in leaves and its possible role in starch synthesis. Studies of photosynthetic CO_2 assimilation and carbon metabolism were undertaken with isolated protoplasts from spinach, another species in which mannose causes increased maltose content. The distribution of maltose between chloroplast and cytoplasm was determined during the first few min of $^{14}CO_2$ uptake and kinetics of maltose transport across the envelope of isolated chloroplasts established.

MATERIALS AND METHODS

Protoplast and Chloroplast Isolation. Protoplasts were isolated from spinach [*Spinacia oleracea*, United States Hybrid 424, Ferry Morse Seed Co, Mountain View, CA, grown in H_2O culture as described by Lilley and Walker (17)] following the method of Edwards *et al.* (6), except that leaves were sliced finely under 500 mM sorbitol, the enzyme medium contained 500 mM sorbitol, 5 mM Mes (pH 5.5), 1 mM $CaCl_2$, 3% cellulase, and 0.5% pectinase, and incubation was at 28 C. For mannose treatments, the medium contained 250 mM sorbitol and 250 mM mannose. Protoplasts were purified on a sorbitol-sucrose step gradient (6). Chl was measured according to Arnon (1).

Photosynthetic Carbon Assimilation. Protoplasts were added to a medium containing 500 mM sorbitol, 1 mM $CaCl_2$, 25 mM Tricine (pH 7.6), and 5 mM $NaH^{14}CO_3$ (20 Ci/mol) to give 50 μg Chl/ml. The mixture was illuminated in an O_2 electrode (5) at 20 C and photosynthesis was measured by O_2 evolution and by incorporation of ^{14}C (below). The reaction was terminated by the addition of boiling absolute ethanol to give a final concentration of 80% (v/v). This total extract was centrifuged, and the pellet reextracted twice in 80% ethanol and twice in warm H_2O to give an ethanol/H_2O-soluble fraction (13). The remaining pellet was incubated with amyloglucosidase solution (3), and the starch content was estimated by release of [^{14}C]glucose. Samples of the total extract, the ethanol/H_2O-soluble fraction, and the supernatant following amyloglucosidase digestion of the pellet were counted on planchets using a Nuclear Chicago gas flow counter, together with appropriate standards. Free sugars and a base line component (which includes charged compounds and oligosaccharides) in the ethanol/H_2O-soluble extract, and glucose in the amyloglucosidase extract were separated by paper chromatography in ethyl acetate-pyridine-H_2O (8:2:1, v/v) and radioactivity was determined with a Berthold LB 280 scanner. In addition to the earlier methods used for maltose identification (13), extracts were heated with borohydride which reduces maltose to maltitol (18), followed by maltase treatment. Maltitol, sorbitol, and glucose released from it by maltase were identified by chromatography against ^{14}C-labeled standards and by GLC. The identity of sucrose was confirmed as before (26).

Protoplast Fractionation. Protoplasts were incubated in a medium containing 400 mM sorbitol, 1 mM $CaCl_2$, 25 mM Tricine (pH 7.6), and 5 mM $NaH^{14}CO_3$ (60 Ci/mol). Protoplasts were illuminated and photosynthesis was measured by O_2 evolution and ^{14}C incorporation (27). After 12 min illumination, or at 2-min intervals for time-course experiments, 100-μl protoplast samples of extract (5 μg Chl) were taken and the chloroplastic and cytoplasmic fractions were rapidly separated by centrifugation filtration through nylon mesh and silicone oil (AR 200-AR 20, 19:1), using the technique developed by Robinson and Walker (25, 26). ^{14}C-labeled components of the total acid-soluble extract, the supernatant fraction (cytoplasm), and the pellet (chloroplast) were separated by paper chromatography as described above.

Transport Studies. Chloroplasts were prepared from protoplasts as described by Edwards *et al.* (6). Protoplasts were broken and resuspended in a medium containing 330 mM sorbitol, 10 mM $NaHCO_3$, 10 mM EDTA, 20 mM Hepes (pH 7.6). Measurement of the uptake of glucose and maltose was by the silicone oil centrifugation technique described by Heldt *et al.* (8). Each microtube contained a bottom layer of 25 μl 10% (v/v) $HClO_4$, 70 μl silicone oil, and 100 μl chloroplast suspension (5–10 μg Chl). Incubations were done in the dark at 20 C and uptake initiated by the addition of ^{14}C-labeled sugar. Evaluation of spaces was done using

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[^{14}C]sorbitol and $^{3}H_2O$ (8).

Reagents. Cellulase (Onozuka 35) and pectinase (Macerozyme) were purchased from Yakult Biochemicals Co. Ltd., Nishinomiya, Japan. Amyloglucosidase (grade II from *Rhizopus*) and maltase (α-gluosidase, type I from yeast) were from Sigma. [U-^{14}C]Glucose (291 mCi/mmol), [U-^{14}C]maltose (660 mCi/mmol), [U-^{14}C]sorbitol (333 mCi/mmol), $^{3}H_2O$ (5 mCi/ml), and NAH$^{14}CO_3$ were obtained from the Radiochemical Centre, Amersham. Silicone oil was obtained from Wacker Chemicals (London) Ltd.

RESULTS

On all occasions, photosynthetic rate was lowered by 60 to 70% in protoplasts which had been pretreated with 250 mM mannose. The fraction of ^{14}C from $^{14}CO_2$ incorporated into starch was increased (Table I), although the total starch content was lower than in control protoplasts because of the marked decline in photosynthetic rate. In the ethanol/H_2O-soluble extract, both the proportion of ^{14}C incorporated into sucrose and the absolute amount were decreased, and maltose was present as a ^{14}C-labeled product. Maltose, which was not detected in control protoplasts, contained up to 15% of the total ^{14}C. Figure 1 shows the pattern of accumulation of ^{14}C in maltose and starch in mannose-pretreated protoplasts. After 12 min, maltose contained 6.4% and starch contained 30% of the total ^{14}C incorporated. Table II shows the distribution of ^{14}C in chloroplasts and the cytoplasm in two experiments after 12 min photosynthesis. Sucrose is a product of the cytoplasm and only a trace (<0.5%) appeared in the chloroplast pellet after rapid separation of the two fractions (26). Maltose accounted for 13 to 14% of the total ^{14}C and its presence as a major component of both chloroplasts and the cytoplasm makes contamination of one fraction by the other a very unlikely explanation. After 2 min exposure to $^{14}CO_2$, the shortest time investigated, the chloroplast fraction had a higher [^{14}C]maltose content than did the cytoplasmic fraction but, after 6 min, the chloroplast pool remained relatively constant, whereas the [^{14}C]maltose content of the cytoplasm continued to increase (Fig. 2). Studies of uptake of maltose indicated that it is transported across the chloroplast envelope. Figure 3 shows that the rate of uptake of maltose was about half that of glucose. As with glucose transport, the concentration of maltose in the sorbitol-impermeable space did not exceed the external concentration (2 mM), suggesting that no active uptake was involved. Chromatography of the chloroplast fraction after uptake showed that the label was wholly present as [^{14}C]maltose and that uptake was not, therefore, a consequence of the uptake of glucose derived from external hydrolysis of maltose.

DISCUSSION

As in discs taken from leaves of sugar beet (13), mannose induces accumulation of [^{14}C]maltose during photosynthetic $^{14}CO_2$ incorporation by protoplasts from spinach (Table I). Fractionation experiments (Fig. 2) suggest that maltose appears in the chloroplast first and transport studies (Fig. 3) indicate that export of maltose from the chloroplast could account for its subsequent appearance in the cytoplasm. However, the possibility of a second synthetic pathway in the cytoplasm analogous to synthesis of sucrose via sucrose phosphate synthetase (2) cannot be ruled out.

Within the chloroplast (Fig. 4), maltose could be a product of starch degradation by amylase (9, 15, 16, 21, 23), the activity of which is likely to become increasingly important where concentration of Pi, a substrate for the alternative degradative pathway via phosphorylase, is decreased due to sequestration by mannose (3, 11, 12). Amylase activity, combined with inhibition of maltose phosphorylase phosphorolysis where [Pi] is low, would result in maltose accumulation. [An increased [^{14}C]maltose content during mobilization of ^{14}C-starch in the presence of mannose has, in fact, now been established for *Plantago major* (E. E. M. McGee and D. H. Lewis, unpublished data)]. This would imply rapid turnover of starch (4, 14) in the experiments described here since [^{14}C]maltose was identified after only 1 min photosynthesis in $^{14}CO_2$ (Fig. 1). Alternatively, or additionally, maltose phosphorylase may function in a synthetic role (Fig. 4), as has been demonstrated for spinach (18, 29). In the presence of mannose, decreased cytoplasmic [Pi] causes accumulation of phosphorylated compounds inside the chloroplast (9, 12, 31). A high phosphorylated sugar to Pi ratio is likely to stimulate synthesis of both starch [see Preiss and Levi (24) for review] and maltose (Fig. 4), and hexose phosphates will also be a source of free glucose if appropriate phosphatases are present. Cleavage of maltose by maltase (Fig. 4) would further contribute to the increased pool of free glucose previously reported in mannose-treated leaves (13) if activity of this enzyme in chloroplasts is established (see ref. 21 for discussion). Export of photosynthetic triose phosphates to the cytoplasm causes temporary consumption of Pi (31). Synthesis of starch and maltose releases Pi for recycling within the chloroplast (Fig. 4) and thus may be important where [Pi] is low.

Table I. *Assimilation of $^{14}CO_2$ and Distribution of ^{14}C in Control and Mannose-pretreated Spinach Protoplasts*

Mannose pretreatment involved incubation in an enzyme medium containing 250 mM mannose and 250 mM sorbitol rather than the usual 500 mM sorbitol. Photosynthesis (50 μg Chl/ml) was for 12 min, under the conditions described, in a medium containing 500 mM sorbitol, 25 mM Tricine (pH 7.6), 1 mM $CaCl_2$, and 5 mM NaH$^{14}CO_3$ (20 Ci/mol). The reaction was terminated by addition of boiling ethanol and the protoplast pellet was re-extracted in ethanol and warm H_2O. The radioactivity of the ethanol/H_2O-soluble and the ethanol-insoluble fractions was determined from planchets. Components of the ethanol/H_2O-soluble fraction were separated by paper chromatography in ethyl acetate-pyridine-H_2O (8:2:1, v/v). Starch was estimated by release of [^{14}C]glucose during incubation of the ethanol-insoluble fraction in amyloglucosidase. Each value is the average of two preparations and the results are typical of several experiments.

Sample	Photosynthesis			Distribution of ^{14}C in Ethanol/H_2O-soluble Fraction		
	CO_2	O_2 (maximum rate)	Starch as Total ^{14}C	Charged compounds and Oligosaccharides	Maltose	Sucrose
	μmol/mg Chl·h		%		%	
Control	40	72	15	51.5	0	48.5
Mannose-pretreated	15	24	23	65.3	14.7	20.0

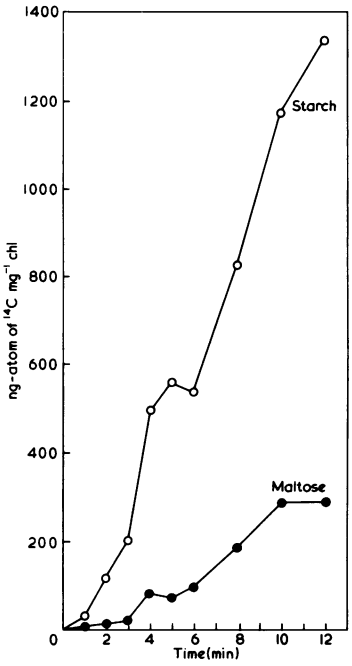

FIG. 1. Incorporation of ^{14}C from $^{14}CO_2$ into starch and maltose in protoplasts isolated from spinach leaves and pretreated with mannose. After 12 min, maltose contained 6.4% and starch contained 30% of the total ^{14}C incorporated. Maltose was not detected in untreated protoplasts and starch accounted for a lower fraction of the total ^{14}C (Table I).

Table II. *Major Distribution of ^{14}C in Acid-soluble Fraction following Photosynthetic Assimilation of $^{14}CO_2$ for 12 min by Mannose-pretreated Spinach Protoplasts*

Protoplasts (50 μg Chl/ml) were illuminated in an O_2 electrode in a medium containing 400 mM sorbitol, 25 mM Tricine (pH 7.6, 1 mM $CaCl_2$ and 5 mM $NaH^{14}CO_3$ (60 Ci/mol). After 12 min, 100-μl samples (5 μg Chl) were centrifuged through 20-μl nylon mesh, which breaks the protoplasts, and silicone oil, which effects separation of chloroplast and cytoplasm (25). Perchloric acid (final concentration, 0.3–0.5 N) was used to terminate the reactions of both fractions. Further samples were centrifuged in tubes from which oil was omitted to give a "total extract." Components of the acid-soluble extract were separated chromatographically (Table I).

	Incorporation of ^{14}C		
	Charged Compounds and Oligosaccharides	Maltose	Sucrose
		%	
Experiment 1			
Total extract	58.0	13.7	28.3
Distribution[a]			
Chloroplastic fraction	26.2	7.2	0
Cytoplasmic fraction	26.6	6.1	25.3
Experiment 2			
Total extract	62.3	9.5	28.2
Distribution[b]			
Chloroplastic fraction	32.7	3.3	0
Cytoplasmic fraction	30.3	5.7	28.4

[a] Recovery of ^{14}C during fractionation procedure, 92.0%.
[b] Recovery of ^{14}C during fractionation procedure, 100.4%.

When discs from leaves of *B. vulgaris* and spinach are floated on mannose solution for several days, the electrolyte content of the medium increases markedly after the initial 8 to 12 h, suggesting changes in cell membrane permeability (10). It seemed likely that mannose might have a similar effect on the chloroplast envelope membranes, so allowing uncontrolled influx of cytoplasmic metabolites as substrates for enhanced maltose synthesis. However, photosynthetic studies using chloroplast obtained from mannose pretreated protoplasts (A. Herold and P. H. McNeil, unpublished data) do not support this proposal. Although the photosynthetic rates of protoplasts were decreased by mannose pretreatment, the rates of isolated chloroplasts were always equal to those of chloroplasts released from control protoplasts. This implies that the integrity of the chloroplast envelope was maintained, making unregulated leakage of Calvin cycle intermediates impossible, or, alternatively, that the properties of the envelope alter during the isolation procedure. There also was no evidence that the 3-h period of mannose pretreatment affected the permeability of the plasma membrane in isolated protoplasts.

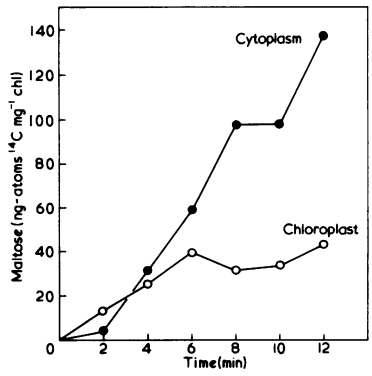

FIG. 2. Distribution of [^{14}C]maltose during photosynthetic $^{14}CO_2$ incorporation by mannose-pretreated protoplasts. Samples of extract equivalent to 5 μg Chl were withdrawn from the incubation vessel at 2-min intervals and the chloroplastic and cytoplasmic fractions were rapidly separated.

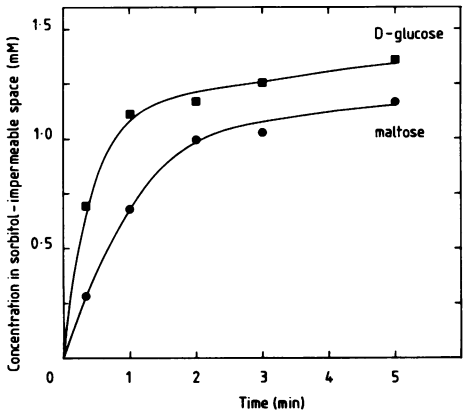

FIG. 3. Uptake of maltose and glucose into the sorbitol-impermeable H_2O space of spinach chloroplasts. The space was equal to 25.1 μl mg^{-1} Chl. The concentration of sugars supplied was 2 mM. Rates estimated from the first 20 s of uptake were 3.1 and 1.3 μmol h^{-1} mg^{-1} Chl for glucose and maltose, respectively.

To our knowledge, this is the first occasion on which studies of maltose uptake by chloroplasts have been reported (see ref. 21 for discussion). Uptake of maltose, comparable to that of glucose and some other sugars (28), contrasts with that of sucrose, where the flux across the chloroplast envelope is negligible (7). Thus, maltose transport across the chloroplast envelope might account for the appearance of maltose in the extra-chloroplastic compartment (Fig. 2). However, in the normal situation, since the content of maltose in leaves is usually very low, its movement into the cytoplasm is likely to be of minor significance in relation to total carbon traffic.

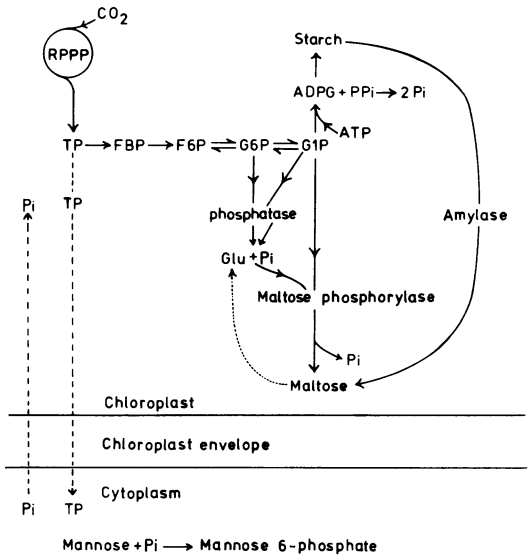

FIG. 4. Proposed routes for synthesis of maltose in spinach chloroplasts in the presence of mannose. Cleavage of maltose by maltase (····) has still to be confirmed (21). FBP, fructose bisphosphate; F6P, fructose 6-phosphate; Glu, glucose; GlP, glucose 1-phosphate; G6P, glucose 6-phosphate; RPPP, reductive pentose phosphate pathway; TP, triose phosphate; ADPG, adenine diphosphate glucose.

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