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Starch biosynthesis: the primer nonreducing-end mechanism versus the nonprimer reducing-end two-site insertion mechanism

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Abstract—Two mechanisms are recognized for polysaccharide chain elongation: (a) the nonreducing-end, primer-dependent mechanism and (b) the reducing-end, two-site insertion mechanism. We recently demonstrated the latter mechanism for starch biosynthesis by pulsing starch granules with ADP-[^{14}C]Glc and chasing with ADPGlc for eight varieties of starch granules. Others have reported the addition of glucose from ADPGlc to the nonreducing ends of maltose, maltotriose, and maltopentaose and a branched maltopentasaccharide. It was concluded that starch chains are biosynthesized by the addition of glucose to the nonreducing ends of maltodextrin primers. In this study, we reinvestigated the maltodextrin reactions by reacting three kinds of starch granules from maize, wheat, and rice with ADP-[^{14}C]Glc in the absence and presence of maltose (G2), maltotriose (G3), and maltodextrin (d.p.12) and found that they inhibited starch biosynthesis rather than stimulating it, as would be expected for primers. The major product in the presence of G2 was G3 with decreasing amounts of G4–G9 and the major products in the presence of G3 was G4 and G5, with decreasing amounts of G6–G9. It was concluded that maltodextrins are acceptors rather than primers. This was confirmed by pulsing the starch granules with ADP-[^{14}C]Glc and chasing with G2, G3, and G6, which gave release of ^{14}C-label from the pulsed granules in the absence of ADPGlc, further demonstrating that maltodextrins are acceptors that inhibit starch biosynthesis by releasing glucose from starch synthase, rather than acting as primers and stimulating biosynthesis.

Keywords: Starch granules; Starch biosynthesis; Starch chain elongation; Reducing-end synthesis; Nonreducing-end synthesis; Maltodextrin primers; Maltodextrin chain terminators; Acceptor reactions; Two-site insertion mechanism; Starch synthase; Starch branching enzyme; Amylose; Amylopectin

1. Introduction

Two general mechanisms are recognized for the biosynthesis of polysaccharides: (a) the primer mechanism in which monomer units are added to the nonreducing-end of the primers and (b) the two-site insertion mechanism in which monomer units are added to the reducing-end of a growing polysaccharide chain[1] (see Fig. 1). The primer mechanism originated from the studies of Cori and Cori[2] and Swanson and Cori[3] on the action of muscle glycogen phosphorylase and from the studies of Hanes[4] on the action of potato starch phosphorylase (EC 2.4.1.1). These investigators found in the 1940s that phosphorylase from the respective sources could transfer D-glucose from α-D-glucopyranosyl phosphate (α-Glc-1-P) to the nonreducing ends of the glucans. For starch biosynthesis by phosphorylase, it was recognized that the synthesis required a primer molecule that was amylose, amylopectin, or a maltodextrin with at least three glucose residues.[3–6]

Phosphorylase is one of a handful of enzymes that catalyze a reaction that is readily reversible. The reaction can be either degradative or synthetic, depending on the starting substrates. It is degradative if the substrate is inorganic phosphate (P_i) or synthetic if it is α-Glc-1-P as shown in the following reaction:

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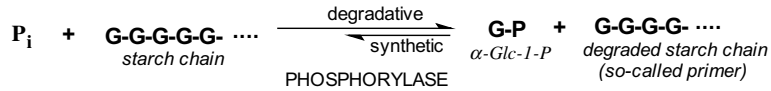

The synthetic elongation of the so-called primer chain will only occur when the ratio of P_i to α-Glc-1-P is less than the equilibrium value, which is 10.8 at pH 5, 6.7 at pH 6, and 3.1 at pH 7.0.[7] This is not attained in vivo, as the concentration of P_i in plant and animal tissues is 20- to 40-fold higher than the concentration of α-Glc-1-P,[7–9] so that the in vivo conditions for phosphorylase greatly favors degradation rather than synthesis.

Because a degraded starch chain is the product of the degradation reaction, it is, thus, a required substrate in the reverse synthetic reaction, as shown in the reaction above. This then was the origin of the primer requirement for starch biosynthesis from the nonreducing-end, and it has pretty much been retained for 65 years.[10–12]

In 1960, de Fekete et al.[13] reported that starch granules incorporated ^{14}C-label after incubation with UDP[^{14}C]Glc. The radioactivity in the starch was converted into ^{14}C-labeled maltose by reaction with the exo-acting beta-amylase. Recondo and Leloir[14] found that ADPGlc was the preferred high-energy glucosyl donor for starch biosynthesis. Experiments similar to de Fekete et al.[13] were performed by Frydman and Cardini,[15] using ADPGlc and giving similar results. Leloir and co-workers[13,14,16] showed that starch granules contained the enzymes necessary for starch biosynthesis: starch synthase [EC 2.4.1.21] for the synthesis of amylose by reaction with ADPGlc and starch branching enzyme [EC 2.4.1.18] for the synthesis of amylopectin by reacting with the amylose chains. Leloir et al.[16] also found that when starch granules reacted with UDP[^{14}C]Glc in the presence of maltodextrins (maltose, maltotriose, and maltotetraose), a single labeled glucose unit was added to the nonreducing ends. From the beta-amylase and the maltodextrin experiments, it was concluded that starch was being synthesized by the addition of glucose to the nonreducing ends of starch chains and like phosphorylase, starch synthase required a primer for synthesizing the starch chains.

The beta-amylase conversion of labeled starch to labeled maltose has been widely considered as proof that glucose is added to the nonreducing ends of starch chains. This conclusion, however, is in error. This is based on the following: if starch chains are synthesized from the reducing end in a de novo synthesis that does not require a primer, as was postulated in our first paper,[17] the synthesized starch chains would have every glucose unit in the chain labeled. The reaction of beta-amylase with this labeled starch chain would, thus, also give ^{14}C-labeled maltose, indicating that ^{14}C-labeled maltose from the action of beta-amylase would be produced when the addition is to either the nonreducing end or to the reducing end. The experiments of Leloir, et al.[13,14] and Frydman & Cardini[15] did not, therefore, show the exclusive addition of glucose to the nonreducing end. There are, however, the experiments in which the added maltodextrins were labeled at the nonreducing end.[16] In more recent experiments, Denyer et al.[18] investigated the reaction of starch granules with ADP[^{14}C]Glc, with the addition of maltose, maltotriose, and maltopentaose. They found that ^{14}C-labeled maltodextrins were formed processively, and like Leloir et al.,[16] the major product from each of the maltodextrins was the addition of a single glucose unit, giving the next higher homologue. In the experiments of Leloir et al.[16] and Denyer et al.,[18] however, starch chains were never synthesized from the added maltodextrins.

From this, it was our view that the primer nonreducing-end mechanism has never been satisfactorily demonstrated for starch biosynthesis. We, therefore, recently studied the mechanism of starch chain elongation by pulsing eight varieties of starch granules with ADP-[^{14}C]Glc and chasing them with nonlabeled ADPGlc.[17] In these experiments, the starch granules were solubilized from the pulse and from the chase reactions. The solubilized starch was reduced with $NaBH_4$, and then hydrolyzed by glucoamylase. It was found that the ADP-[^{14}C]Glc pulsed starch gave ^{14}C-labeled D-glucitol and that the starch chased by nonlabeled ADPGlc gave a significant decrease in the amount of ^{14}C-label in D-glucitol. These results indicated that the starch chains were being elongated by the addition of D-glucose to the reducing-end of a growing starch chain rather than being added to the nonreducing-end of a primer, as had been assumed for so many years. We also showed[17] that starch synthase forms a high-energy covalent complex with D-glucose and the growing chain, and it was proposed that the D-glucose is added to the reducing-end of the growing chain by a two-site insertion mechanism, involving high-energy covalent enzyme intermediates. The proposed mechanism gives the de novo synthesis of starch chains without the need of a reducing-end primer, see Figure 1 for a comparison of the primer nonreducing-end mechanism to the non-primer, reducing-end two-site insertion mechanism.

Because the maltodextrin experiments of Leloir et al.[16] and Denyer et al.[18] were diametrically opposed to the results that we obtained in our pulse and chase studies of starch granules, and the interpretations that we made, we have further investigated the reactions of starch granules with ADPGlc and maltodextrins in this study. We report on two kinds of experiments: (1) the kinetics of the reaction of starch granules with ADP-[^{14}C]Glc in the absence and presence of different concen-

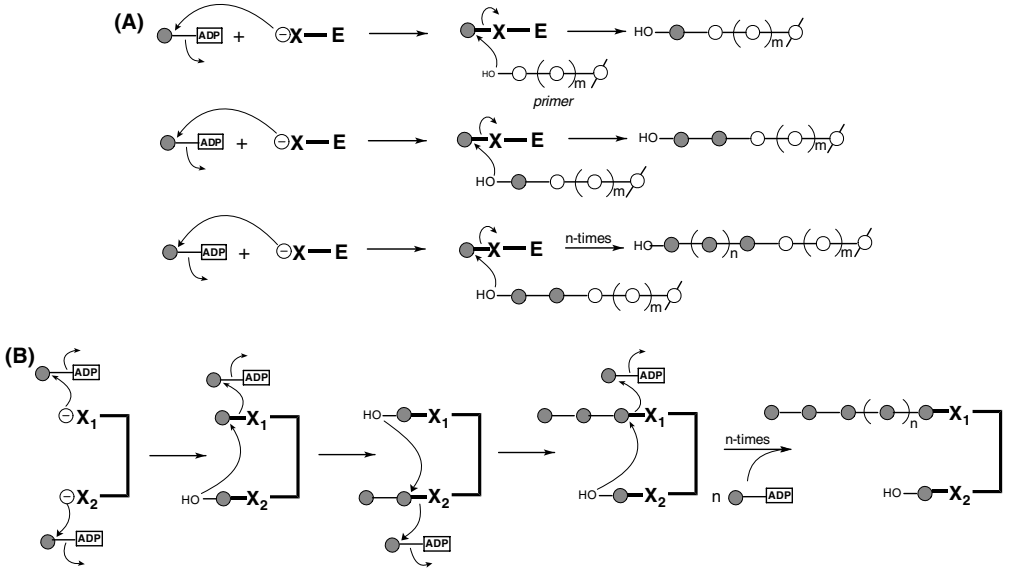

Figure 1. Comparison of the primer nonreducing-end mechanism to the nonprimer reducing-end two-site insertion mechanism for starch chain elongation. (A) The primer mechanism in which D-glucose is transferred from ADPGlc to starch synthase (X–E) and a glucosyl covalent complex is formed. Then a maltodextrin or starch chain primer C-4-OH group attacks C-1 of the glucosyl–enzyme complex, giving transfer of the glucose to the nonreducing-end of the primer. The process continues, giving elongation of the primer and the formation of amylose. (B) The reducing-end two-site insertion mechanism occurs de novo, as two D-glucose units are transferred from two ADPGlc to two catalytic groups (X_1 and X_2) at the active site of starch synthase. Then the C-4-OH group of one of the glucose units makes a nucleophilic attack onto C-1 of the other glucosyl unit, forming an α-(1→4) linkage. Another glucose unit is then added to X_1 and its C-4-OH group makes a nucleophilic attack onto C-1 of the maltosyl unit, giving the transfer of the maltose unit to the glucose and the formation of maltotriose. The process continues back and forth between the two X-groups, giving the synthesis of an α-(1→4)-linked amylose chain. Eventually water hydrolyzes the chain from the active site. ● = ^{14}C-labeled glucose unit; ○ = unlabeled glucose unit; ⟋○ = unlabeled reducing-end glucose unit.

trations of maltose, maltotriose, and d.p.12 maltodextrin and determine the nature of the water-soluble products that result, and (2) the pulsing of starch granules with ADP-[^{14}C]Glc, followed by chasing with various nonlabeled maltodextrins (maltose, maltotriose, and maltohexaose) and other saccharides (isomaltose and cellobiose).

2. Experimental

2.1. Materials

ADP-[^{14}C]Glc (242 mCi/mmol) was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ, USA). Nonlabeled ADPGlc was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Maize starch granules (22% amylose and 78% amylopectin) and rice starch granules (25% amylose and 75% amylopectin) were freshly prepared from maize kernels and rice grain, by steeping them in water at 50 °C for 24 h, without the addition of sodium bisulfite. Wheat starch granules were prepared as previously described.[17]

Maltose was obtained from Sigma Chemical Co. and purified to remove D-glucose and maltotriose contaminants, to give chromatographically pure maltose. Maltotriose and maltohexaose were chromatographically pure maltodextrins obtained from Nihon Shokuhin Kako Co., Ltd (Tokyo, Japan). Isomaltose and cellobiose were obtained from Sigma Chemical Co. and were chromatographically pure. Maltodextrin (d.p.12) was the linear Nägeli Amylodextrin Fraction III, prepared from waxy-maize starch.[19]

Liquid scintillation counting cocktail, containing 5.0 g PPO and 0.1 g PoPoP in 1.0 L of toluene, was used for all heterogeneous liquid scintillation spectrometry. All other chemicals were of the highest grade commercially available and were used without further treatment.

2.2. Reaction of starch granules with ADP-[^{14}C]Glc with varying concentrations of maltose, maltotriose, and maltodextrin [d.p.12]

Starch granules (600 mg containing 10% water) from maize, wheat, and rice were suspended in 2.0 mL of 0.1 mM EDTA/4 mM glycine buffer (pH 8.4), containing 0, 10, 40, and 80 mg of maltose/mL and 1 mM (0.2 μCi) ADP-[^{14}C]Glc.[†] The reaction was allowed to go at 20 °C for 10 h. Aliquots (~350 μL, containing ~100 mg of starch granules) were removed at 60, 120, 180, 300, and

[†] 1 mM ADPGlc was used, as an estimate of the concentration in plastids.

600 min of reaction. Maize starch granules were reacted with 3.5, 7, 15, and 22 mg/mL maltotriose, and with 30 and 60 mg/mL maltodextrin [d.p.12] under the same conditions as the reactions with maltose. The starch granules were centrifuged and washed five times with 1 mL of water and then treated five times with 1 mL of acetone to obtain dry starch. Residual acetone was removed under vacuum and the resulting dried starch was weighed and the ^{14}C-incorporated into the granules was measured by adding the granules to liquid scintillation cocktail, followed by heterogeneous counting in a liquid scintillation spectrometer. The amount of ^{14}C in each sample of starch was normalized for 100 mg of dry starch.

2.3. The determination of the products from maize starch granules reacting with ADP-[^{14}C]Glc in the absence and in the presence of maltose and maltotriose

Maize starch granules 427 mg (~10^3 units[‡] of starch synthase) were suspended in 2.0 mL of 0.1 mM EDTA/4 mM glycine buffer (pH 8.4), containing 1 mM (0.2 μCi) ADP-[^{14}C]Glc and maltodextrins [0 mg/mL, 10 mg/mL (30 mM) maltose, and 15 mg/mL (30 mM) maltotriose], and the reaction was allowed to go 10 h at 20 °C. The starch was removed by centrifugation and washed with 1 mL of water. The supernatant and wash were combined, giving 2.4–2.8 mL, and 120 mg (1.2 mL) of DEAE-cellulose (Whatman DE 23) in 40 mM pyridine–acetate buffer (pH 5.0) was added and allowed to stand 30 min to remove unreacted ADP-[^{14}C]Glc; 120 mg (1.2 mL) of DE 23 DEAE-cellulose was added 7-more times and the supernatants were combined and concentrated to 500 μL by rotoevaporation at 40 °C; the concentrate was added along a 15 cm strip in the center and 7.5 cm from the top of a piece of Whatman 3 MM paper (23 × 52 cm) for descending paper chromatography.[20] The chromatogram was irrigated, using 65:35 parts by volume of propanol-1/water for 36 h at 20 °C. The chromatogram was dried and test-strips (3 cm) from each side of the chromatogram, containing D-glucose and maltodextrins (G2–G14), were removed and developed using the silver nitrate dipping method.[20] The ^{14}C-maltodextrins from the reaction digests were located on the chromatograms, using the test-strips, and were cut from the chromatogram and counted by heterogeneous liquid scintillation spectrometry.

2.4. Pulsing of starch granules with ADP-[^{14}C]Glc and chasing with maltodextrins and other carbohydrates

Starch granules (3.0 g containing 10% water) from each of the three starches were suspended in 12 mL of 0.1 mM EDTA/4 mM glycine buffer (pH 8.4). Each of the starches were pulsed with 1 mM (1.0 μCi) ADP-[^{14}C]Glc at 20 °C for 10 min. The suspensions were centrifuged and the starches were washed five times with 10 mL water. Each of the starches were suspended in 14 mL of water and divided into seven equal parts. The starches were centrifuged and one part (representing the pulsed sample) was treated with acetone and centrifuged five times and the residual acetone was removed under vacuum, and the starch was weighed and the amount of ^{14}C determined by heterogeneous liquid scintillation spectrometry. The remaining six samples of the starches were suspended in 1.0 mL of 0.1 mM EDTA/4 mM glycine buffer (pH 8.4), containing buffer for a water chase, and a chase with 30 mM of maltose, maltotriose, maltohexaose, isomaltose, and cellobiose; each chase was allowed to incubate at 20 °C for 30 min. The starches were then centrifuged and washed five times with 1 mL of water; the starches were then treated five times with 1 mL of acetone. Residual acetone was removed under vacuum and ~100 mg of the dried starches were counted by liquid scintillation spectrometry. The amount of ^{14}C-released from the starch granules by the chase compounds was the difference of the chase samples from the pulse sample.

3. Results and discussion

In this study, we have investigated the role that maltodextrins might have as necessary primers in the biosynthetic elongation of starch chains. Starch granules from three diverse sources (maize, wheat, and rice) were reacted with ADP-[^{14}C]Glc in the absence and in the presence of increasing concentrations of maltose (0, 10, 40, and 80 mg/mL). All three of the starches incorporated ^{14}C-labeled D-glucose into their starch granules over a 10 h reaction period without the addition of any added maltose or maltodextrin primers (Figs. 2–4A). The addition of maltose to the starch granule digests produced a decrease in the amount of ^{14}C-labeled D-glucose that was incorporated into the starches. This inhibition of starch synthesis increased as the concentration of maltose was increased (Figs. 2–4A). The final amounts of starch synthesized after 10 h of reaction, as a function of the increasing concentration of maltose, is shown in Figures 2–4B. Similar results were obtained when various concentrations of maltotriose (Fig. 5A and B) and maltodextrin [d.p.12] (Fig. 6A and B) were added to maize starch granules and ADP-[^{14}C]Glc digests, with the exception that maltotriose was a much more potent inhibitor than maltose, inhibiting at significantly lower concentrations (Fig. 5A and B). These experiments show that maltose, maltotriose, and maltodextrin [d.p.12] inhibit the synthesis of starch in the granules. The percents of inhibition for the different concentrations of maltose,

[‡] Where 1 unit = 1 μmol of D-glucose incorporated into 100 mg of starch granules/h from ADPGlc at pH 8.4 and 20 °C.

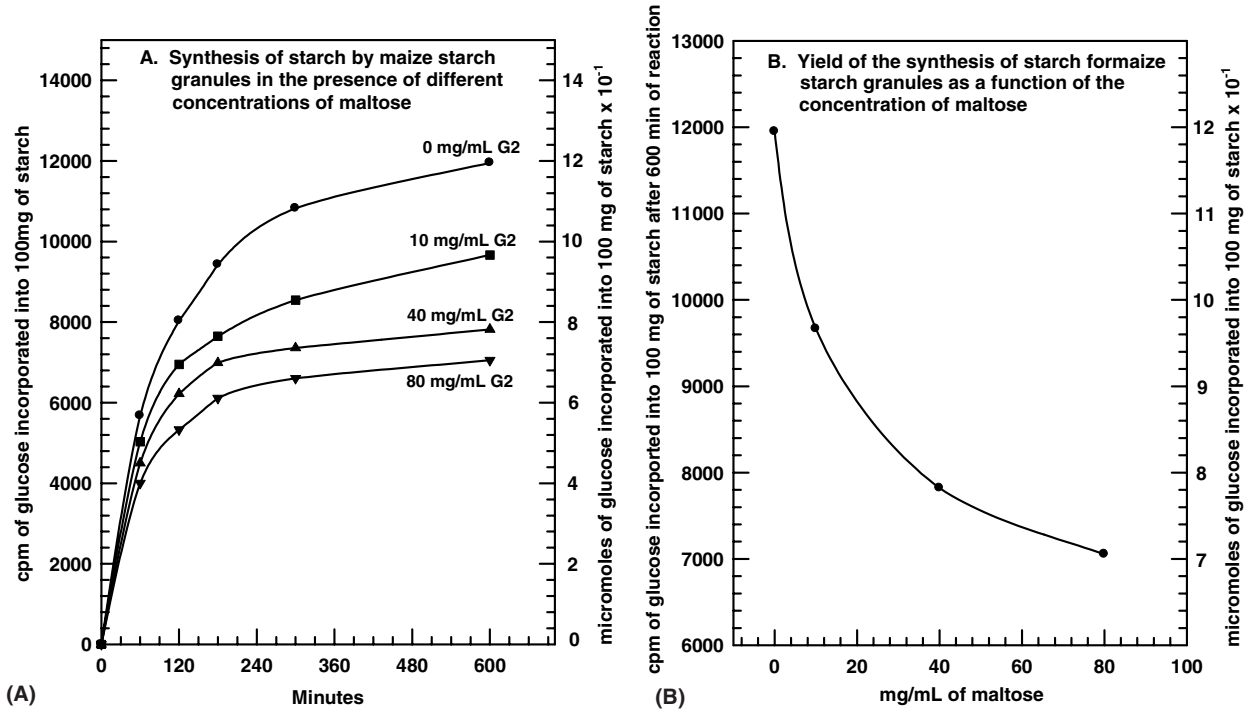

Figure 2. Reaction of maize starch granules with ADP-[^{14}C]Glc in the presence of various concentrations of maltose. (A) Reaction as a function of time. (B) Amount of starch synthesized after 10 h of reaction as a function of the maltose concentration.

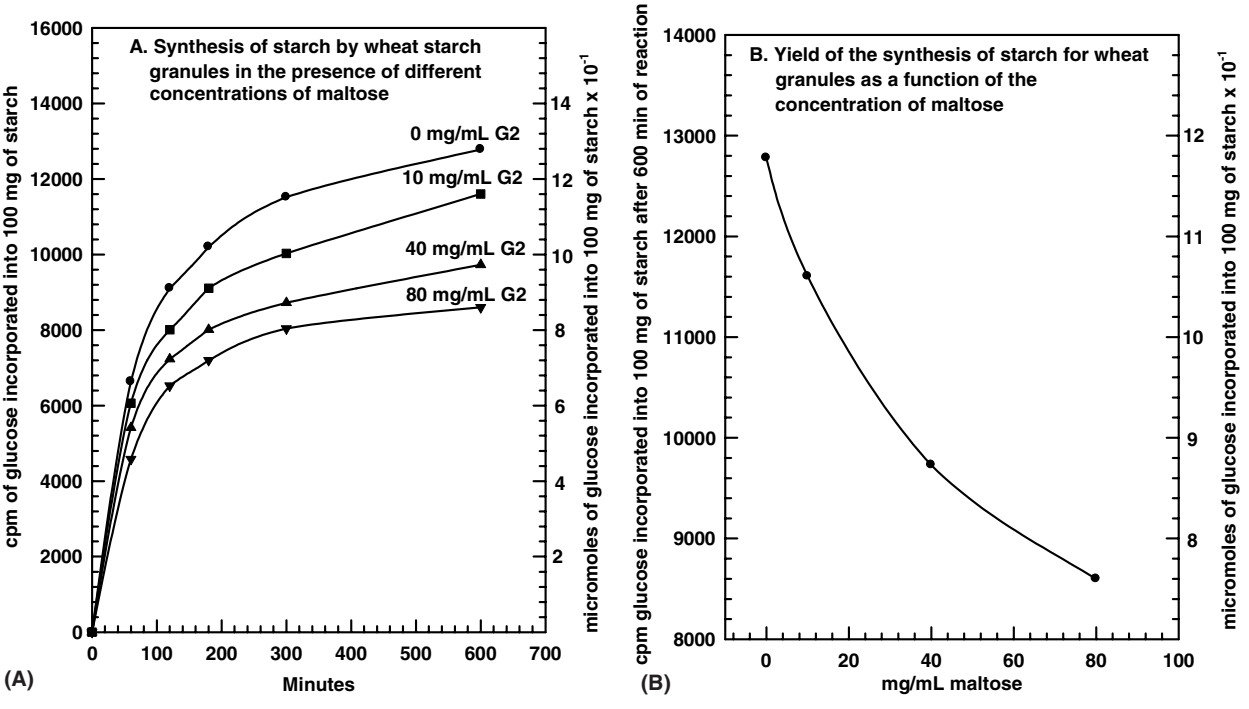

Figure 3. Reaction of wheat starch granules with ADP-[^{14}C]Glc in the presence of various concentrations of maltose. (A) Reaction as a function of time. (B) Amount of starch synthesized after 10 h of reaction as a function of the maltose concentration.

maltotriose, and maltodextrin [d.p.12] are given in Table 1.

If maltose, maltotriose, or maltodextrin [d.p.12] had been required primer substrates in the biosynthesis of starch, it would have been expected, according to usual Michaelis–Menten enzyme kinetics, that the rates and amounts of product (starch in this case) would be proportional to the concentration of the substrate

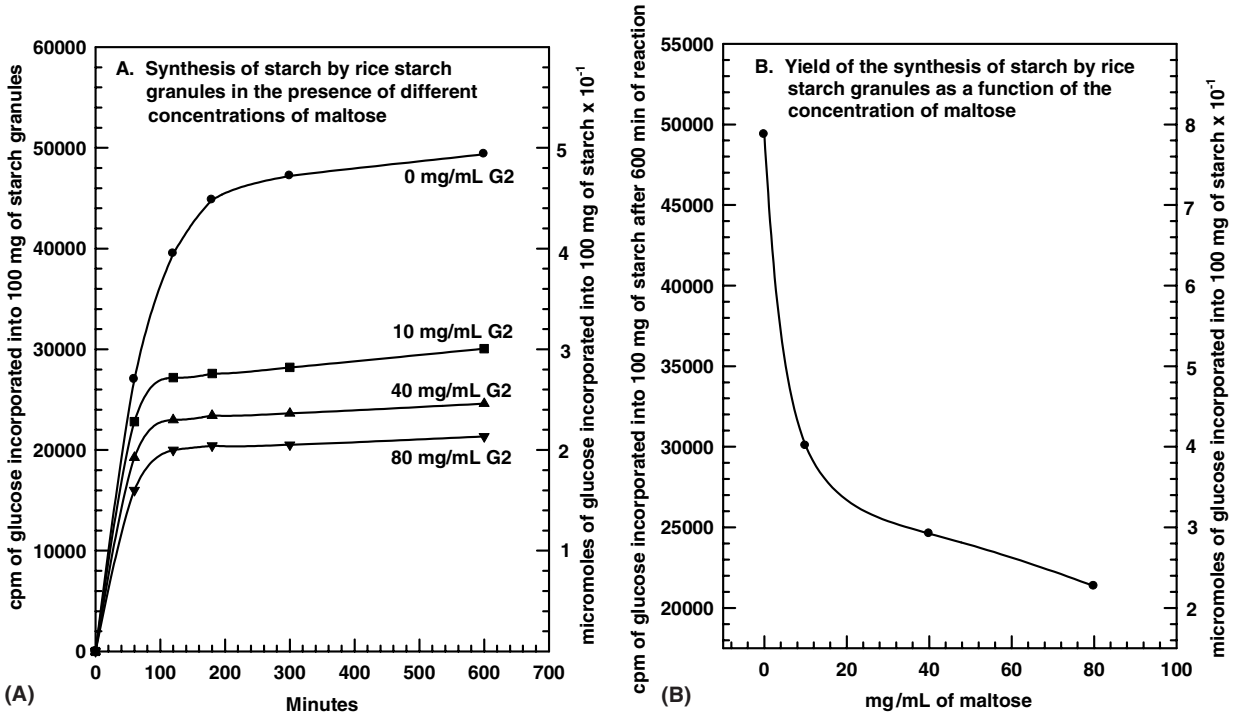

Figure 4. Reaction of rice starch granules with ADP-[^{14}C]Glc in the presence of various concentrations of maltose. (A) Reaction as a function of time. (B) Amount of starch synthesized after 10 h of reaction as a function of the maltose concentration.

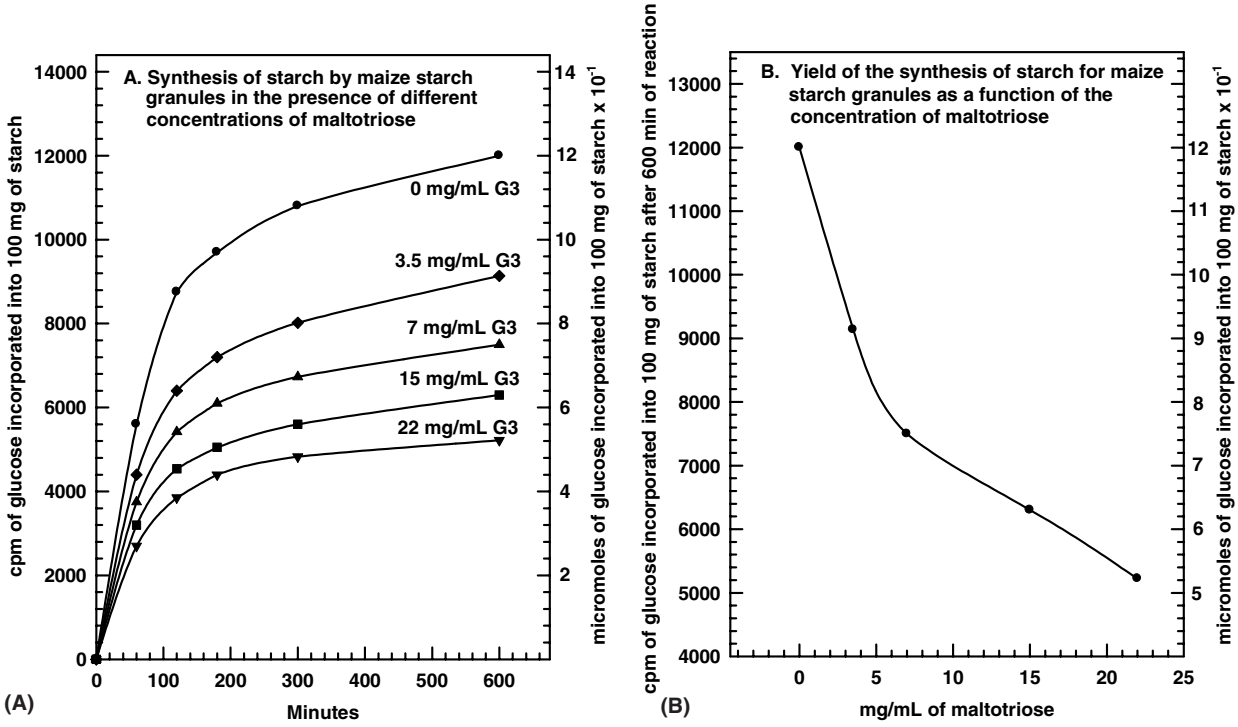

Figure 5. Reaction of maize starch granules with ADP-[^{14}C]Glc in the presence of various concentrations of maltotriose. (A) Reaction as a function of time. (B) Amount of starch synthesized after 10 h of reaction as a function of the maltotriose concentration.

(maltodextrins in this case). Instead, as the concentrations of maltose, maltotriose, and maltodextrin [d.p.12] were increased in the reaction digests, the rates and amounts of starch synthesized were decreased, indicating

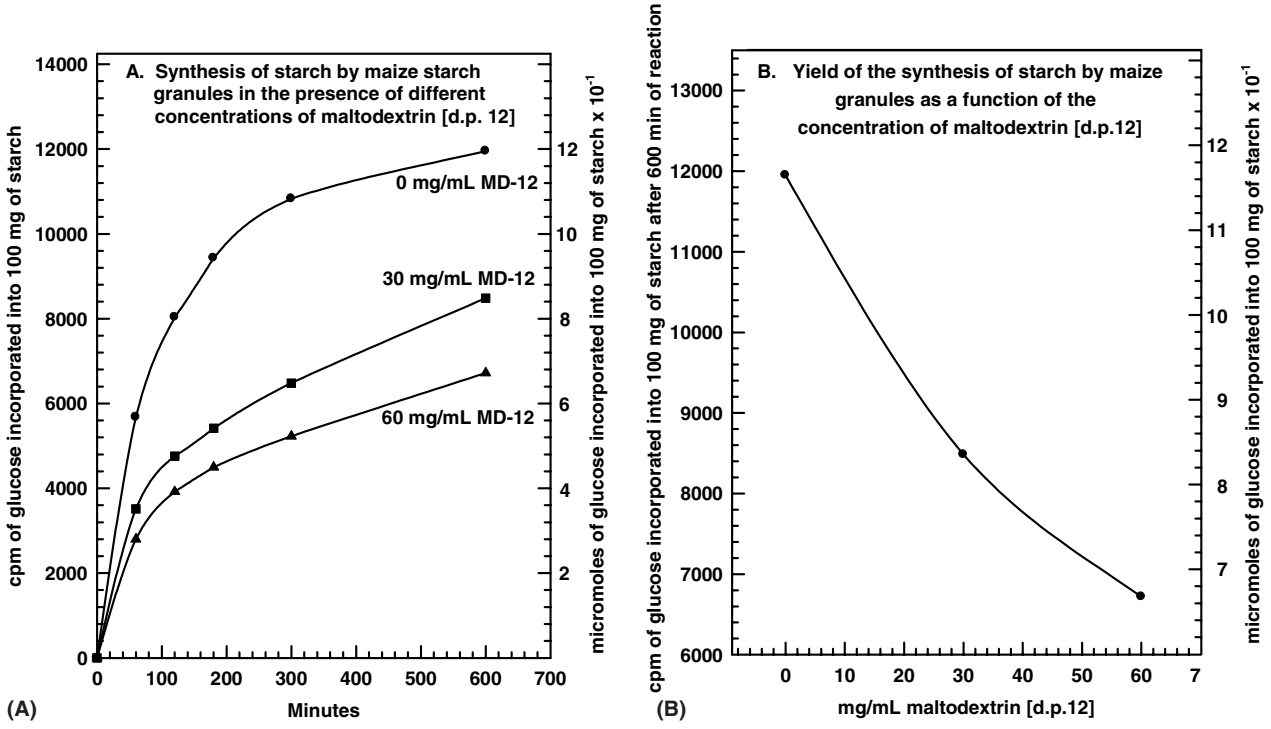

Figure 6. Reaction of maize starch granules with ADP-[^{14}C]Glc in the presence of various concentrations of maltodextrin [d.p.12], abbreviated as **MD**. (A) Reaction as a function of time. (B) Amount of starch synthesized after 10 h of reaction as a function of the maltodextrin (d.p.12) concentration.

Table 1. Percent inhibition of starch synthesis by starch granules in the presence of different concentrations of maltose, maltotriose, and amylodextrin [d.p.12]

Starches	Maltose (mg/mL)			Maltotriose (mg/mL)				Amylodextrin [d.p.12] (mg/mL)	
	10	40	80	3.5	7	15	22	30	60
Maize (%)	20	32	58	25	38	47	57	30	45
Wheat (%)	10	25	33	Nd[a]	Nd[a]	Nd[a]	Nd[a]	Nd[a]	Nd[a]
Rice (%)	39	50	55	Nd[a]	Nd[a]	Nd[a]	Nd[a]	Nd[a]	Nd[a]

[a] Nd = not determined.

that maltose, maltotriose, and maltodextrin [d.p.12] inhibited the biosynthesis of starch. Maltodextrins, therefore, cannot be required primer substrates for the biosynthetic elongation of starch chains by starch synthase. It, thus, is concluded that starch biosynthesis does not require a primer as has been assumed for 65 years.

We also investigated the nature of the soluble products that were formed when starch was biosynthesized by the reaction of starch granules with ADP-[^{14}C]Glc in buffer (water) and in the presence of 10 mg/mL (30 mM) maltose and 15 mg/mL (30 mM) maltotriose. The results are shown in Table 2. Water gave 66.94 mol % D-glucose and 31.96 mol % maltose (G2) and much lower amounts of G3 and G4. The addition of maltose gave 89.25 mol % G3, 6.66 mol % G4, 1.95 mol % G5, 0.90 mol % G6, 0.51 mol % G7, and 0.21 mol % G8, and lesser amounts of higher maltodextrins. The addition of G3 gave greater amounts of the second and third higher homologues than did G2, with 68.6 mol % G4, 18.2 mol % G5, 7.3 mol % G6, and 1.2 mol % G7, with decreasing amounts of G8–G15 (Table 2).

Similar results have been observed for dextran biosynthesis by dextransucrase when maltose, isomaltose, and many other kinds of saccharides were added to the reaction digests.[21,22] When maltose was added to the reaction digests, D-glucose was transferred from sucrose to the nonreducing-end of maltose processively, giving a series of isomaltodextrin homologues attached to the nonreducing-end of maltose, and when isomaltose was added, a series of isomaltodextrin homologues was formed.[23–25] These reactions have been called acceptor reactions in which maltose or isomaltose and other saccharides terminate the synthesis of dextran by releasing D-glucose and the growing dextran chain from covalent, enzyme-intermediate complexes at the active site of

Table 2. Products resulting from the reaction of water, maltose, and maltotriose in the 10 h reaction of maize starch granules with ADP-[^{14}C]Glc

Products	Water			30 mM Maltose			30 mM Maltotriose		
	Measured cpm	Normalized cpm[a]	Mol %[c]	Measured cpm	Normalized cpm[b]	Mol %[c]	Measured cpm	Normalized cpm[b]	Mol %[c]
G1	4400	4400	66.86	0	—	—	0	—	—
G2	4201	2101	31.93	0	—	—	0	—	—
G3	129	43	0.65	11,462	11,462	89.25	0	—	—
G4	115	29	0.44	1,711	856	6.66	6914	6914	68.6
G5	40	8	—	750	250	1.95	3675	1838	18.2
G6	0	0	—	464	116	0.90	2198	733	7.3
G7	0	0	—	332	66	0.51	1243	311	3.1
G8	0	0	—	162	27	0.21	620	124	1.2
G9	0	0	—	102	15	0.12	373	62	0.6
G10	0	0	—	74	9	0.07	231	33	0.3
G11	0	0	—	73	8	0.06	142	18	0.2
G12	0	0	—	67	7	0.05	106	12	0.1
G13	0	0	—	64	6	0.04	103	10	0.09
G14 & G15	0	0	—	73	6	0.04	90	8	0.08
G16 & >	0	0	—	244	14	0.11	502	20	0.2

[a] The normalized cpm for the water reaction was obtained by dividing the measured cpm of the product by the d.p. of the product, so as to obtain one labeled glucose unit per compound.

[b] The normalized cpm for the maltodextrin reactions were obtained by dividing the measured cpm of the product by the d.p. of the product minus the d.p. of the added maltodextrin, so as to obtain one labeled glucose unit per compound.

[c] The mol % was obtained by dividing each of the normalized cpm's by the total cpm's times 100.

dextransucrase.[23,24] The series is formed when the first acceptor product becomes an acceptor and the next higher homologue is formed that in turn becomes an acceptor. With increasing concentrations of maltose, the synthesis of dextran was inhibited and there was an exponential decrease in the amount of dextran formed,[23] very similar to what has been observed in this study when maltose, maltotriose, and maltodextrin were added to the starch synthase reaction with ADPGlc.

From the results of this study, it appears that maltose and other maltodextrins participate in acceptor reactions with starch synthase in which they terminate and thereby inhibit the synthesis of starch chains. To confirm this hypothesis, we designed experiments in which the starch granules were pulsed with 1 mM ADP-[^{14}C]Glc, washed to remove unreacted ADPGlc, and then chased with buffer (water), maltose, maltotriose, maltohexaose, isomaltose, and cellobiose. The experiments were to test whether the chase molecules would release label from the pulsed starch synthase, contained in the starch granules, in the absence of the high-energy glucosyl donor, ADPGlc. The results are presented in Table 3. Water was an acceptor, releasing 2.9–9.3% of the pulsed label from the individual starches (Table 3). When maltose, maltotriose, maltohexaose, isomaltose, and cellobiose were added to the pulsed starch granules, the amount of label released by water was first removed by washing the granules extensively. The carbohydrates were then added to the washed, pulsed starch granules, and a significantly higher percentage of the pulsed label was released from all three of the starches (Table 3). The maltodextrins released the highest percentages of the pulsed label. This release of the label from the starch granules took place in the absence of ADPGlc and thus was not due to synthesis but due to acceptor reactions. When the acceptors encounter the active site of starch synthase, they release glucose and the growing starch chain, which become attached to the acceptors. These results further demonstrate that maltodextrins react with the covalent intermediates of starch synthase as acceptors and not as primers. See below for a discussion of the mechanism of the acceptor reactions.

It should be noted that in addition to maltodextrins, water, isomaltose, and cellobiose, also released ^{14}C-label from the pulsed starch granules and were acceptors (Table 3). These same compounds were acceptors for dextransucrase, but like starch synthase, they were not as good acceptors as was maltose.[23,24] The acceptor reactions with water were slow for dextransucrase and much faster for starch synthase, but not as fast as were the maltodextrin acceptor reactions with starch synthase. The acceptor reactions with water are necessary for the release of starch and dextran from their covalent complexes at the active sites of their respective enzymes, so that new glucan chains can be synthesized.

The pulse of starch granules with ADP-[^{14}C]Glc and the chase with ADPGlc that we previously presented[17] showed that D-glucose and a growing starch chain are covalently attached to the two sites at the active site of a single starch synthase enzyme and that the C-4-OH group of the covalently linked D-glucose makes a nucleophilic attack onto the C-1-carbon of the reducing-end glucose unit of the covalently linked starch chain. This reaction thereby transfers the starch chain to the glucose

Table 3. ADP-[^{14}C]Glc pulsed starches and ^{14}C-label released from pulsed starches by chase with water, maltose, maltotriose, maltohexaose, isomaltose, and cellobiose

Pulse starch and chase by various substrates and percent ^{14}C released	Starches		
	Maize starch granules	Wheat starch granules	Rice starch granules
cpm pulsed starch[a]	11,930	8855	13,769
cpm in H_2O chased starch[a]	10,965	8030	13,370
cpm released by H_2O[c]	965	825	399
% Released by H_2O[d]	8.1	9.3	2.9
(G2)[b] cpm in chased starch[c]	9670	6495	12,221
cpm released by G2[e]	2260	2360	1548
% Released by G2[f]	20.6	29.4	11.6
(G3)[b] cpm in chased starch[c]	9885	6810	11,692
cpm released by G3[e]	2035	2045	2077
% Released by G3[f]	18.6	25.5	15.5
(G6)[b] cpm in chased starch[c]	10,310	7265	12,599
cpm released by G6[e]	1620	1590	1170
% Released by G6[f]	14.8	19.8	8.8
(Im)[b] cpm in chased starch[c]	10,455	7585	13,039
cpm released by Im[e]	1475	1270	730
% Released by Im[f]	13.5	15.8	5.5
(Cel)[b] cpm in chased starch[c]	10,515	7335	13,270
cpm released by Cel[e]	1415	1520	499
% Released by Cel[f]	12.0	18.9	3.7

[a] The radioactivity is reported as cpm/500 mg of dry weight of starch granules.
[b] G2 = maltose, G3 = maltotriose, G6 = maltohexaose, Im = isomaltose, Cel = cellobiose.
[c] cpm's remaining in 500 mg of starch after chasing with the various substrates.
[d] % Released by water = [(cpm of pulsed starch) (cpm in chased starch)] ÷ (cpm of pulsed starch).
[e] cpm's released from 500 mg of starch after chasing with water (buffer), 30 mM maltodextrins, 30 mM isomaltose, and 30 mM cellobiose = [(cpm of pulsed starch) (cpm released by water)] (cpm in chased starch).
[f] $\%\ \text{Released by saccharides} = \dfrac{\text{cpm released from pulsed starch by the saccharide chase}}{(\text{cpm of pulsed starch}) - (\text{cpm released by water chase})} \times 100.$

unit, giving the addition of glucose to the growing starch chain by a two-site insertion mechanism that synthesizes starch chains de novo without the need of a primer.

Acceptor reactions occur when maltodextrins or other oligosaccharides enter the active site of starch synthase and their C-4-OH, nonreducing-end group, makes a nucleophilic attack onto the C-1-reducing-end group of either the covalently linked glucose unit or the covalently linked starch chain to give their release from the active site and their attachment to the nonreducing ends of the maltodextrin acceptors. When maltodextrins were incubated with ADP-[^{14}C]Glc/starch granule digests, low molecular weight maltodextrin products were formed by the addition of one to three or four glucose units to the maltodextrin, giving the next higher homologues in which the addition of one glucose unit gave the predominant homologue product (Table 2). The starch chain covalently linked at the active site can also be released from the enzyme by the acceptor, but it primarily stays within the granule because of its larger size and intermolecular association with other starch chains in the granule. The mechanism for the acceptor reactions of starch synthase is shown for maltotriose in Figure 7.

Leloir and co-workers[13,14,16] and Frydman and Cardini[15] showed that the incubation of starch granules with maltodextrins and UDPGlc or ADPGlc transferred D-glucose to the nonreducing ends of maltose, maltotriose, and maltotetraose primarily to give the next higher homologues: maltotriose, maltotetraose, and maltopentaose, respectively. While they found that UDPGlc and ADPGlc did transfer glucose to starch, they never showed that it was transferred exclusively to the nonreducing ends of the starch in the granule.

Denyer et al.[18] more recently investigated the reaction of starch synthase in starch granules with ADP-[^{14}C]Glc and the addition of maltose, maltotriose, and maltopentaose and found that ^{14}C-labeled maltodextrins were processively formed. Their data showed that the major product from each of the maltodextrins was the addition of one glucose unit to the maltodextrin, forming the next higher homologue, with the other synthesized maltodextrin homologues being formed in much smaller and decreasing amounts, as we have observed in this study (Table 2). It should be noted that Denyer et al.[18] never showed that starch chains were synthesized from the maltodextrins that were added to the reaction digests. Nevertheless, Denyer and co-workers[12,18] concluded that their study showed that maltodextrins were primers for starch biosynthesis by the nonreducing-end, primer mechanism.

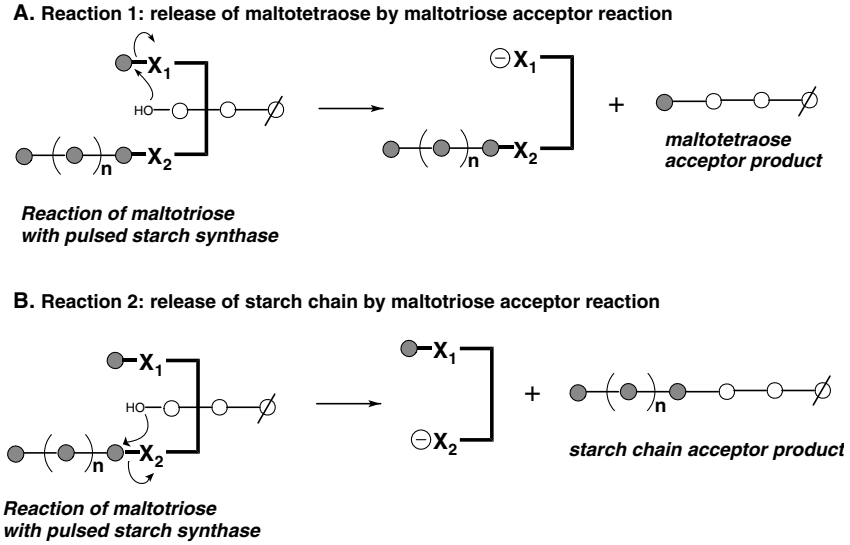

Figure 7. Mechanism for the acceptor reaction of maltotriose with starch synthase. (A) Reaction of maltotriose to give the release of glucose, which is attached to the nonreducing-end of maltotriose to give the formation of maltotetraose, the next higher homologue. (B) Reaction of maltotriose to give release of the starch chain, which becomes attached to the nonreducing-end of maltotriose. ● = ^{14}C-labeled glucose unit; ○ = unlabeled glucose unit; Ø = unlabeled reducing-end glucose unit.

Damager et al.[26] recently reported the chemical synthesis of a branched pentasaccharide, 6^3-α-maltosyl-α-methyl maltotrioside, and investigated it as a primer for starch synthase, using starch granules. They found that both of the nonreducing ends were each and separately extended by one glucose unit, to give two hexasaccharides, as the primary products in nearly equal amounts. Here again, only a single glucose unit was added to the so-called primer and starch chains were not formed. From the evidence presented in the present study, it is clear that the formation of the maltodextrin products observed by Denyer et al.[18] and Damager et al.[26] were due to acceptor reactions and not due to the added maltodextrins acting as primers for starch chain biosynthesis.

Denyer et al.[27] also claimed that ^{14}C-glucose from ADP-[^{14}C]Glc was incorporated primarily into amylopectin, but when soluble extracts from pea embryos and potato tubers were added to starch granules, there was an increase in the incorporation of ^{14}C-glucose into amylose. They further claimed that it was maltodextrins in the extracts that stimulated the incorporation of glucose into amylose by acting as primers. We would now interpret these experiments as the maltodextrins acting as acceptors, releasing a water-soluble, relatively low molecular weight amylose from starch synthase and the granules.

Tomlinson and Denyer[12] recently commented about the reducing-end mechanism for starch biosynthesis. They stated that the elongation of the single reducing-end of an amylopectin molecule rather than the many nonreducing ends of the constituent amylopectin chains seems unlikely on theoretical grounds and it is not supported by the experiments of Denyer et al.[18,27] and Damager et al.[26,28] with maltodextrins. What seems to have been forgotten by Tomlinson and Denyer is that starch synthase catalyzes the synthesis of linear amylose chains and that it is the *starch branching enzymes* that transform the linear amylose chains into branched chains to give amylopectin, with the linear amylose chains being synthesized from the reducing-end and not the amylopectin chains per se.

Tomlinson and Denyer's statements further are not supported by their argument that the reducing-end mechanism is unlikely on theoretical grounds because there are more nonreducing ends than the single reducing-end in the amylopectin molecule. In this connection, it should be noted that *Leuconostoc mesenteroides* B-512F dextran is a glucan with the same degree (5–6%) of branching as amylopectin, and therefore with the same number of branched chains. Dextran has been shown to be synthesized from the reducing-end by the two-site insertion mechanism,[24,29] and based on this, the identical degrees of branching of dextran and amylopectin, and on the results of the pulse and chase study of starch biosynthesis,[17] the reducing-end synthesis of the chains in the amylopectin molecule would not be unlikely on theoretical grounds, as stated by Tomlinson and Denyer,[12] and is supported on experimental grounds.

There are at least three major isoforms of starch synthase.[30] The C-terminal domains, where the active site is located, have been conserved. Because the active sites are conserved, these isoforms most probably have identical mechanisms for starch chain elongation. This is supported by the fact that it has been shown that eight diverse kinds of starch granules synthesize starch by the reducing-end, two-site insertion mechanism,[17] and by the fact that maltodextrins act as acceptors and not primers for three diverse kinds of starches, as shown

in this study. The C-terminal domains of the isoforms include two ADPGlc binding sites and a glucosyl transferase site,[12,30] both of which are consistent and would be required for the reducing-end, two-site insertion mechanism.[17]

In conclusion, we have shown in this study: (1) three diverse kinds of starch granules are capable of incorporating D-glucose from ADPGlc into their starches in the absence of any added maltodextrin primers, suggesting that the synthesis does not require a primer; (2) when maltose was added to the three kinds of starch granules, with ADPGlc, the synthesis of starch was inhibited and the degree of inhibition increased with increasing concentrations of maltose, also indicating that maltose is not a primer; (3) when maltotriose and maltodextrin [d.p.12] were added to maize starch granules/ADPGlc digests, they also inhibited starch synthesis, which was increased as the concentrations were increased, also indicating that they are not primers; (4) the primary water-soluble products from the reaction of maltose and maltotriose with maize starch granules and ADPGlc were the next higher homologues, maltotriose and maltotetraose, respectively; (5) when the three starch granules were pulsed with ADP-[^{14}C]Glc and then chased with maltose, maltotriose, and maltohexaose, a significant percent (8.8–25.5%) of the pulsed label was released from the pulsed granules, indicating that they are acceptors that terminate starch synthesis; (6) when the three kinds of pulsed starch granules were chased with water, isomaltose, and cellobiose, a lesser, but still significant percent (3.7–9.3%) of the pulsed label also was released; (7) it is concluded that the maltodextrins inhibit starch biosynthesis by acting as acceptors that terminate synthesis rather than acting as primers, and the acceptor reactions are very different from the primer reactions, which should stimulate starch synthesis rather than inhibit it; (8) all of the so-called primer reactions that have been used to assay starch synthase are not measuring starch synthesis but instead are measuring a limited transfer reaction of D-glucose to the nonreducing ends of an acceptor, which has been called, a primer; and (9) from the data, we reaffirm that the biosynthesis of starch chains occurs de novo with the addition of two glucose units from ADPGlc to the active site of starch synthase in a nonprimer, reducing-end, two-site insertion mechanism.

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