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Note

Starch biosynthesis: further evidence against the primer nonreducing-end mechanism and evidence for the reducing-end two-site insertion mechanism

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Abstract—Two reactions were studied with three varieties of starch granules from maize, wheat, and rice. In *Reaction-I*, the granules were reacted with 1 mM ADP-[^{14}C]Glc and in *Reaction-II*, a portion of the granules from *Reaction-I* was reacted with 1 mM ADP-Glc. The starch granules were solubilized and reacted with the exo-acting glucoamylase and beta-amylase to an extent of 50% or less of the ^{14}C-label. The amounts of ^{14}C-labeled products from glucoamylase and beta-amylase were nearly equal for *Reaction-I* and *Reaction-II*. If the addition had been to the nonreducing ends of primers, *Reaction-II* would not have given any labeled products from the hydrolysis of glucoamylase and beta-amylase. These results indicate that the elongation of the starch chain is the addition of D-glucose to the reducing end by a de novo two-site insertion mechanism and not by the addition of D-glucose to the nonreducing end of a primer. This is in conformity with previous results in which starch granules were pulsed with ADP-[^{14}C]Glc and chased with nonlabeled ADP-Glc, giving ^{14}C-labeled D-glucitol from the pulsed starch and a significant decrease in ^{14}C-labeled D-glucitol from the chased starch on reducing with $NaBH_4$ and hydrolyzing with glucoamylase [*Carbohydr. Res.* **2002**, *337*, 1015–1022]. It also is in conformity with the inhibition of starch synthesis that occurs when putative primers are added to starch granule–ADP-Glc digests, indicating that the elongation is not by the nonreducing-end primer mechanism [*Carbohydr. Res.* **2005**, *340*, 245–255].
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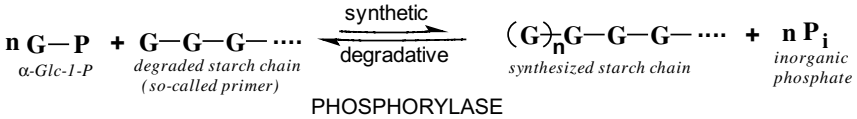
1. Introduction

The first polysaccharide thought to have been biosynthesized was glycogen.[1] It was found that the enzyme, glycogen phosphorylase, catalyzed the transfer of D-glucose from α-D-glucose 1-phosphate (α-Glc-1-P) to the nonreducing ends of the glycogen chains. Shortly after that a similar reaction was reported in which potato phosphorylase transferred D-glucose from α-Glc-1-P to the nonreducing ends of starch chains.[2] The concept of a required primer for these reactions was fully developed by Swanson and Cori[3] a few years later.

Problems were developed for this mechanism. The reaction was reversible, and the synthetic elongation of



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the primer occurred when the ratio of P_i was less than the equilibrium value, which is 3.1 at pH 7.0.[4] However, after the addition of a few glucose units to the primer, the reaction slowed down as the concentration of P_i increased. Further, the concentration of P_i in plant tissue was found to be 20- to 40-fold higher than the concentration of α-Glc-1-P, and the in vivo reaction is degradative rather than synthetic.[4–6]

In 1960, 20 years after the phosphorylase experiments, it was found that UDP-Glc and ADP-Glc were the high-energy glucosyl donors for starch biosynthesis and that active starch-synthesizing enzymes were located inside the starch granules.[7,8] When UDP-[^{14}C]Glc and ADP-[^{14}C]Glc were incubated with starch granules, ^{14}C-labeled D-glucose was incorporated into the starch granules. The radioactivity in the starch granules was converted into ^{14}C-labeled maltose by reaction with the exo-acting beta-amylase,[7–10] and it was assumed that the D-glucose from UDP-Glc and ADP-Glc was being added to the nonreducing ends of starch primers. These experiments have been widely considered as proof that starch is biosynthesized by the primer-dependent nonreducing-end mechanism. This conclusion, however, is not necessarily correct in that if starch chains were synthesized from the reducing end in a de novo synthesis, independent of a primer, the synthesized starch chain would have every glucose unit labeled when the starch granules were reacted with ADP-[^{14}C]Glc. Treatment of this labeled starch with beta-amylase would also give ^{14}C-labeled maltose.

In the present study, we performed two reactions: (1) the reaction of starch granules from three different sources with 1 mM ADP-[^{14}C]Glc for 30 min and (2) the reaction of the starch granules with 1 mM ADP-[^{14}C]Glc for 30 min, followed by reaction with non-labeled ADP-Glc for 30 min. The starch granules from the two reactions were solubilized and then reacted with beta-amylase and with glucoamylase in separate reactions and the amounts of labeled maltose and labeled glucose determined, respectively, and compared for the two reactions.

2. Experimental

2.1. Materials

ADP-[^{14}C]Glc (333 mCi/mmol) was obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ, USA). Nonlabeled ADP-Glc was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Maize starch granules (28% amylose and 72% amylopectin), wheat starch granules (26% amylose and 74% amylopectin), and rice starch granules (20% amylose and 80% amylopectin) were freshly prepared from maize kernels, wheat berries, and rice grain, by steeping them in water at 50 °C for 24 h as previously described.[11] Barley beta-amylase and *Aspergillus niger* glucoamylase were obtained from Megazyme International Ltd, Bray, Ireland.

Liquid scintillation counting cocktail, containing 5.0 g PPO and 0.1 g PoPoP in 1.0 L of toluene, was used for all liquid-scintillation counting.

2.2. Reaction of maize and wheat starch granules with 1 mM ADP-[^{14}C]Glc and ADP-Glc

Maize and wheat starch granules (5.660 g) were suspended in 15 mL of 0.1 mM EDTA/4 mM glycine pH 8.4 buffer and preincubated at 40 °C. The reactions were started by making the suspension 1 mM (0.3 μCi) in ADP-[^{14}C]Glc and allowed to go 30 min at 40 °C. The suspension digests were centrifuged and the starches were washed five times with 16 mL of pH 8.4 buffer. The solid starch (*Reaction-I*) was divided into two equal parts (~2.83 g).

Part I starches (*Reaction-I*) were washed five times with 8 mL of water, followed by treating five times with 8 mL of acetone and once with 8 mL of ethanol, placed under continuous vacuum at 40 °C for 18 h, and 100.0 mg of the starches were counted in toluene liquid-scintillation cocktail.

Part II starches were suspended in 7.5 mL pH 8.4 buffer and preincubated at 40 °C for 20 min. Reactions were started by making the suspension 1 mM in ADP-Glc and allowed to go 30 min at 40 °C giving *Reaction-II*. The starch suspensions were centrifuged and the starches were washed five times with 8 mL of water, treated five times with 8 mL of acetone, and once with 8 mL of ethanol, placed under continuous vacuum at 40 °C for 18 h, and then 100.00 mg of the starches were counted in toluene liquid-scintillation cocktail.

Reaction-I starch granules (2.500 g) and *Reaction-II* starch granules (2.400 g) were dissolved in 34.5 mL 90/10 Me_2SO–water by constant stirring and gentle warming. The solutions were diluted to a final volume of 250 mL giving 10 mg/mL of starch; two 50 mL aliquots were taken from *Reaction-I* and *Reaction-II* for each starch; one aliquot was made to pH 5.20 for a glucoamylase reaction and the other to pH 6.00 for a beta-amylase reaction; 0.05 units (0.01 U/100 mg of starch) of the glucoamylase and beta-amylase were added to the two aliquots, respectively, for each starch. The reactions were allowed to go 20 min at 40 °C; 2 vol of ethanol were added to stop the reactions and precipitate the starches. The starches were centrifuged and washed five times with 8 mL of 67% v/v ethanol, followed by treatment five times with 8 mL of acetone, and once by 8 mL of ethanol, placed under continuous vacuum at 40 °C for 18 h, and 100.0 mg were counted in 10 mL toluene liquid-scintillation cocktail. The experiments were performed in triplicate.

2.3. Reaction of rice starch granules with 1 mM ADP-[^{14}C]Glc and 1 mM ADP-Glc

Rice starch granules (1.320 g) were suspended in 4 mL of 0.1 mM EDTA/4 mM glycine pH 8.4 buffer and pre-incubated at 40 °C for 20 min. The reaction was started by making the suspension 1 mM (0.2 μCi) in ADP-[^{14}C]Glc and allowed to go 30 min at 40 °C. The suspension digest was centrifuged and the starch washed five times with 4 mL of pH 8.4 buffer. The solid starch was divided into two equal parts giving ~660 mg.

Part I (*Reaction-I*) of the starch was centrifuged and washed five times with 2 mL of water, followed by treating five times with 2 mL of acetone and once by 2 mL of ethanol, placed under continuous vacuum at 40 °C for 18 h, and 100.0 mg was counted in 10 mL of toluene liquid-scintillation cocktail.

Part II of the starch (660 mg) was suspended in 2 mL of pH 8.4 buffer and incubated at 40 °C. The suspension was made 1 mM in ADP-Glc and the reaction was allowed to go for 30 min at 40 °C giving *Reaction-II*. The suspension was centrifuged and the starch washed five times with 2 mL of water, treated five times with 2 mL of acetone, and once with 2 mL of ethanol, dried under continuous vacuum at 40 °C for 18 h; 100.0 mg of the starch granules was counted in 10 mL of toluene liquid-scintillation cocktail; 500 mg of the starch granules from *Reaction-I* and *Reaction-II* were dissolved in 8 mL of 90/10 Me_2SO–water and diluted to 100 mL; two 45 mL aliquots were taken from each *Reaction-I* and *Reaction-II* solutions; one aliquot was adjusted to pH 5.20 for a glucoamylase reaction and the other to pH 6.00 for a beta-amylase reaction; 0.05 U (0.01 U/100 mg of starch) of the enzymes were added to the two aliquots, respectively. The reactions were allowed to go 20 min at 40 °C; 2 vol of ethanol were added to stop the reaction and precipitate the starch. The starch was centrifuged and washed five times with 1 mL of 67% ethanol, followed by treating five times with 1 mL of acetone, and once with 1 mL of ethanol, dried under continuous vacuum at 40 °C for 18 h, weighed, and counted in 10 mL of toluene liquid-scintillation cocktail. The experiments were performed in triplicate.

3. Results and discussion

Previously, we pulsed eight different kinds of starch granules with ADP-[^{14}C]Glc and then chased them with nonlabeled ADP-Glc. The starches were gelatinized and reduced with $NaBH_4$ and hydrolyzed completely with glucoamylase. A significant amount of ^{14}C-labeled D-glucitol was obtained from the pulsed starches and there was a significant decrease in the amount of ^{14}C-labeled D-glucitol from the chased starches, indicating that starch was synthesized de novo from the reducing end.[11] More recently, we found that the addition of putative maltodextrin primers (maltose, maltotriose, and DP12-maltodextrin) to starch granules–ADP-Glc digests inhibited starch biosynthesis instead of stimulating it, as primers would be expected to do. The percent inhibition of starch synthesis increased as the concentrations of the maltodextrins were increased. It was also shown that the putative primers released label from pulsed starch granules by acting as limited acceptors that released D-glucose from the active-site of starch synthase, preventing its incorporation into starch chains, showing that synthesis of starch is not by the addition of glucose to the nonreducing ends of maltodextrin or starch chains.[12]

In the present study, the starch synthase activities for the three starches were determined to be the following: maize starch, 296 nmol of glucose/h/100 mg of granules; wheat starch, 317 nmol of glucose/h/100 mg of granules; rice starch, 456 nmol of glucose/h/100 mg of granules. The assay was performed as previously described,[11] using 20 mM (1 μCi) ADP-[^{14}C]Glc. The differences in the reaction conditions used for rice starch granules and those used for maize and wheat starch granules in the present study are due to the differences in the starch synthase activities and only involved differences in amounts of starch, concentration of reagents and reaction volumes.

Reaction-I involved reacting the starch granules with 1 mM ADP-[^{14}C]Glc for 30 min. The starches were solubilized and reacted with the exo-acting glucoamylase and beta-amylase to give ~50% or less hydrolysis of the labeled starch. *Reaction-II* resulted from taking a portion of the labeled starch granules produced by *Reaction-I* and reacting them with 1 mM nonlabeled ADP-Glc for 30 min. The starch granules from *Reaction-I* and *Reaction-II* were solubilized and reacted with the same amounts of exo-acting glucoamylase and beta-amylase for 20 min.

In *Reaction-II*, the reaction with nonlabeled ADP-Glc was conducted to the same extent as *Reaction-I* with ADP-[^{14}C]Glc so as to give a chain of nonlabeled glucose units that was approximately identical to the labeled chain of *Reaction-I*. If the mechanism of glucose addition was to the nonreducing ends of primer chains, *Reaction-I* should have given labeled D-glucose units at the nonreducing ends. *Reaction-II* would then have given the addition of nonlabeled D-glucose to the nonreducing ends of the labeled chains from *Reaction-I*. Glucoamylase and beta-amylase reaction with the products of *Reaction-II*, to the same extent as their reaction with the products of *Reaction-I*, would not have given any labeled D-glucose or maltose if the addition of D-glucose was to the nonreducing ends of the chains.

The results of the reactions of glucoamylase and beta-amylase with the starches from *Reactions-I* and *Reactions-II* are given for the three starches in Table 1. The

Table 1. Reaction of starch granules with 1 mM ADP-[^{14}C]Glc and 1 mM ADP-Glc followed by reactions with glucoamylase and beta-amylase

	Reaction-I[a]			*Reaction-II*[b]		
	cpm[c]	cpm *P*[d]	% *P*[e]	cpm[c]	cpm *P*[d]	% *P*[e]
Maize starch granules						
0	13,045	0	0	13,045	0	0
Beta-A	10,972	2073	15.9	10,657	2388	18.3
GA	10,823	2222	17.0	10,748	2297	17.6
Wheat starch granules						
0	16,000	0	0	16,000	0	0
Beta-A	10,221	5779	36.1	10,664	5336	33.4
GA	11,255	4745	29.7	10,855	5145	32.2
Rice starch granules						
0	18,360	0	0	18,360	0	0
Beta-A	8391	9969	54.0	9514	8846	48.2
GA	8082	10,278	56.0	9388	8972	48.9

[a] *Reaction-I* was the reaction of starch granules with 1 mM (0.3 μCi for wheat and maize starches and 0.2 μCi for rice starch) ADP-[^{14}C]Glc for 30 min. The resulting labeled starches were solubilized and reacted with beta-amylase and glucoamylase.

[b] *Reaction-II* was the reaction of a portion of *Reaction-I* labeled starch granules with 1 mM ADP-Glc for 30 min. The resulting starches were solubilized and reacted with glucoamylase and beta-amylase in amounts identical to what was used in *Reaction-I*.

[c] cpm in the starting solubilized polysaccharide and cpm in the polysaccharide precipitated with 2 vol of EtOH, after reaction with beta-amylase (beta-A) and glucoamylase (GA).

[d] *P* = maltose for the reaction with beta-amylase and D-glucose for the reaction with glucoamylase. The cpm's for these products were determined by subtracting the cpm in the EtOH precipitated polysaccharide after reaction with the enzymes for 20 min from the cpm of the starting polysaccharide.

[e] % *P* = cpm in the product/cpm in the starting polysaccharide × 100.

results show that glucoamylase and beta-amylase released about equal amounts of labeled glucose and labeled maltose from the nonreducing ends of the starch chains from both *Reactions-I* and *Reactions-II* for all three starches for varying degrees of hydrolysis from 17.6% to 48.9%. These results show that the starch chains are not being synthesized by the addition of D-glucose units to the nonreducing ends of putative primer chains in the starch granule but are being added from the reducing end.

It might be argued that the conjectures made are not correct in that there are a very large number of starch chains present in starch granules and that most of the ends of the chains should be able to accept D-glucose from ADP-Glc in a primer catalyzed mechanism, rather than being added to starch chains that previously had been labeled with ^{14}C-glucose. This might be true, if the starch and the starch synthase were dissolved in an aqueous solution and the mechanism was from the nonreducing end of the starch chains. But, the starch synthase molecules are entrapped in the starch granules and they do not freely diffuse through the starch granules. In addition, the starch chains in the granules also do not diffuse throughout the granules and encounter the enzymes. The only starch chains in the starch granules that starch synthase has access to are those in the near proximity of the active-site of starch synthase, greatly reducing the accessibility of the enzymes for the nonreducing ends of the starch chains. But, starch synthase does not need proximity to any starch chain, if the synthesis is de novo and does not require a primer starch chain for reaction. This fact in itself might be an argument for the synthesis of starch chains by the two-site insertion mechanism from the reducing end, as starch is relatively freely synthesized by starch granules, having given significant chain lengths, of avg. d.p. 827 for maize, avg. d.p. 476 for wheat, and avg. d.p. 467 for rice in the ADP-[^{14}C]Glc pulse reactions of starches.[12]

It should be noted, however, that small molecules (e.g., buffers, salts, ADP-Glc, oligosaccharides, maltose, maltotriose to DP12-maltodextrin and possibly larger) do diffuse through starch granules.[13,14] Further, enzymes that are added to the solution in which starch granules are suspended also can enter and diffuse through the granules.[15–19] These enzymes, α-amylase,[19] glucoamylase,[15–17] isoamylase,[16] and cyclomaltodextrin glucanyltransferase,[18] however, have varying degrees of accessibility to the starch chains, with the former two giving greater reaction than the latter two.

The present study, thus, confirms that starch is not biosynthesized by the addition of D-glucose from ADP-Glc to the nonreducing ends of primer chains. The data also confirm that starch chains are biosynthesized from the reducing end rather than the nonreducing end. Figure 1 shows the various possibilities of the structures of the products that would result from *Reactions-I* and *Reactions-II* for both the nonreducing-end primer mechanism and the reducing-end two-site insertion mechanism and how these products would react with glucoamylase. Figure 1A shows that the nonreducing-end primer mechanism products would give labeled

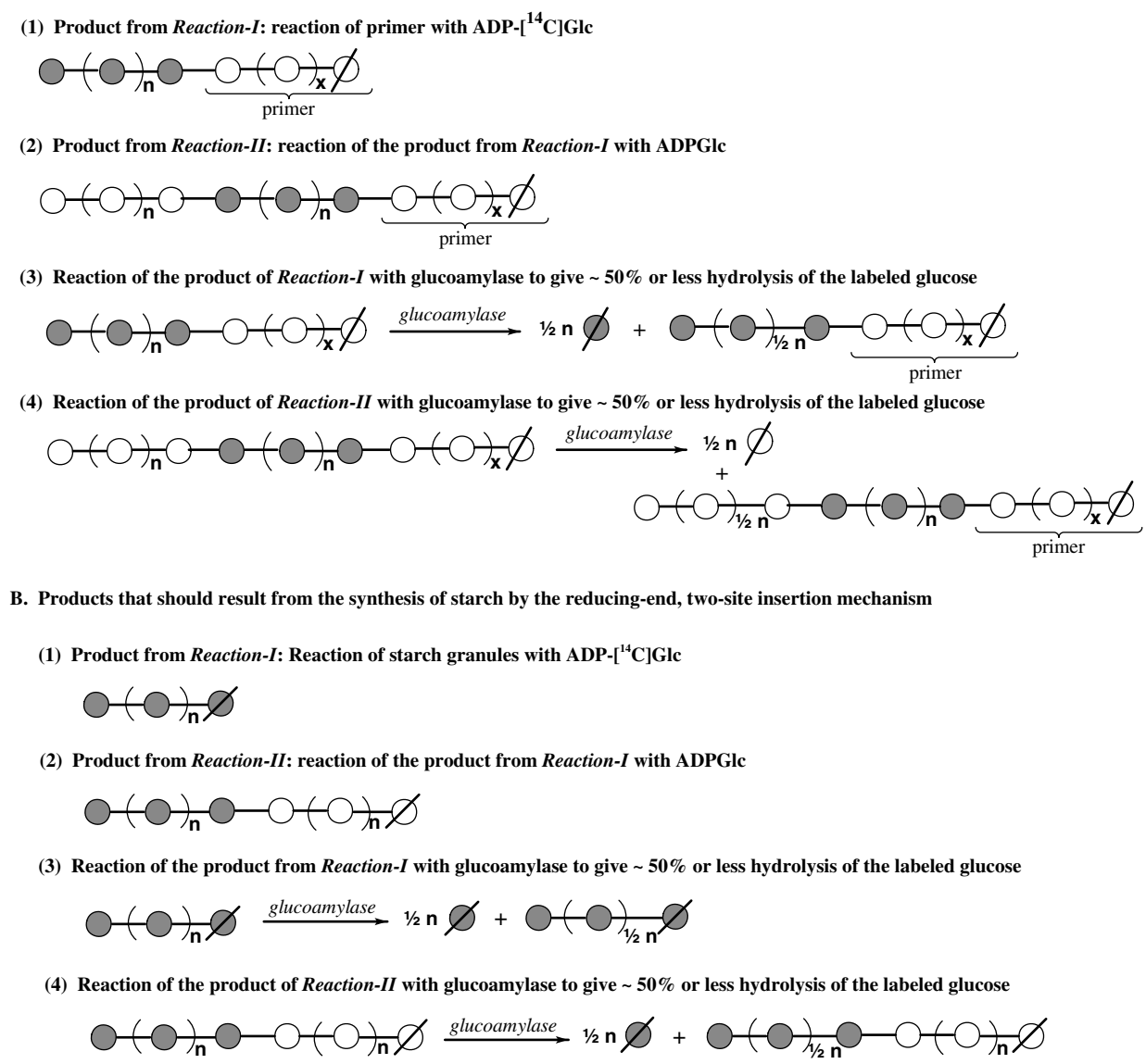

Figure 1. Possible products resulting from *Reactions-I* and *Reactions-II* by (A) the nonreducing-end primer mechanism and their reaction with glucoamylase and by (B) the reducing-end, two-site insertion mechanism, and their reactions with glucoamylase. ● represents ^{14}C-labeled glucose unit; ○ represents nonlabeled glucose unit; the slash through the symbol represents a reducing-end glucose unit. The proposed products from the synthesis of a starch chain from the reducing end by the two-site insertion mechanism, shown in B, both give labeled D-glucose from the reaction of glucoamylase. If the elongation was by the nonreducing-end primer mechanism, the product of *Reactions-II*, shown in **A**, would not give any labeled D-glucose by the action of glucoamylase. The results of the study show that labeled D-glucose was produced when glucoamylase reacted with the products from *Reactions-I* and *Reactions-II*, indicating that the mechanism of starch chain elongation is from the reducing end rather than from the nonreducing end.

products from *Reaction-I* but would not give any labeled products from *Reaction-II*; Figure 1B shows that the reducing-end two-site insertion mechanism products would give labeled glucose from both *Reactions-I* and *Reactions-II*, as was found in this study. A similar figure could be presented for the reaction of beta-amylase in which the only difference would be that the product would be maltose rather than glucose. Both enzymes have specificity to react exclusively at the nonreducing ends of starch chains.

In 2002, Zeeman et al.[20] reported a $^{14}CO_2$ pulse-chase study of Arabidopsis was not accompanied by a significant turnover of starch in that they did not observe a significant release of ^{14}C-label in different intensities of light. It was concluded that Arabidopsis leaf starch is not turned over by the action of exo-enzymes and D-glucose was not transported to other parts of the plant, which is common in a diurnal process. This interpretation was apparently based on the synthesis of starch by the nonreducing-end primer mechanism of starch

synthesis. Alternatively, if the mechanism of starch synthesis is from the reducing end, the ^{14}C-labeled D-glucose units would be located more toward the reducing ends of the starch chains and hence would not be released by the exo-acting enzymes and it would then appear that the starch was not turned over because there was no loss of label, when in fact the nonlabeled starch was being turned over.

In conclusion, the experimental results of the present study show that labeled products were produced when both glucoamylase and beta-amylase reacted with *Reaction-I* products and with *Reaction-II* products, indicating that the mechanism of starch chain synthesis is from the reducing end by a de novo two-site insertion mechanism, rather than by the nonreducing-end primer mechanism.

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