Plant Physiol. (1971) 48, 765–769

Adenosine Diphosphoglucose-Starch Glucosyltransferases from Developing Kernels of Waxy Maize

Received for publication July 22, 1971

J. L. OZBUN,[1] J. S. HAWKER,[2] AND JACK PREISS
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

Two adenosine diphosphoglucose: α-1,4-glucan α-4-glucosyltransferases were extracted from kernels of waxy maize harvested 22 days after pollination and separated by gradient elution from a diethylaminoethyl-cellulose column. Both fractions could utilize amylopectin, amylose, glycogen, maltotriose and maltose as primers. The rate of glucose transfer from adenosine diphosphoglucose to rabbit liver glycogen of fraction II was 78% of the rate of glucose transfer to amylopectin, but with fraction I the rate of transfer of glucose to rabbit liver glycogen was 380% of that observed to amylopectin. Glucan synthesis in the absence of added primer was found in fraction I in the presence of 0.5 M sodium citrate and bovine serum albumin. The unprimed product was a methanol-precipitable glucan with principally α-1,4 linkages and some α-1,6 linkages, and its iodine spectrum was similar to that of amylopectin.

Either sugar nucleotide transferases or phosphorylase could be involved in the initiation and synthesis of starch in higher plants (3, 4, 10, 13–15). Multiple forms of ADP-glucose:α-1,4-glucan α-4-glucosyltransferase have been separated from spinach leaves, one of which in the absence of added primer catalyzes the synthesis of a glucan with principally α-1,4 linkages and some α-1,6 linkages (12). The unprimed activity was stimulated over 1000-fold by BSA[3] and high concentrations of some salts (12). In maize kernels, Tsai and Nelson (15) have found evidence for the presence of multiple forms of phosphorylase, two forms of which are capable of synthesizing a polyglucan in the absence of added α-1,4 glucan primer.

The present communication reports the existence of two forms of ADP-glucose:α-1,4-glucan α-4-glucosyltransferase (ADP-glucose-starch glucosyltransferase) in waxy maize, one of which can catalyze the formation of a polyglucose with properties similar to amylopectin in the absence of added primer. Rates of the unprimed reaction were up to eight times faster than the primed reaction at physiological concentrations of ADP-glucose.

[1] Present address: Vegetable Crops Department, Cornell University, Ithaca, N.Y. 14850.

[2] Present address: Division of Horticultural Research, Commonwealth Scientific and Industrial Research Organization, Adelaide, Australia.

[3] Abbreviations: BSA: bovine serum albumin; DEAE: diethylaminoethyl.

MATERIALS AND METHODS

Materials. ADP-^{14}C-glucose was prepared in the laboratory from α-glucose-1-P (4). Amylopectin was purchased from Calbiochem, maltose was purchased from Mann Research Laboratory, glycogen and β-amylase were purchased from Sigma, amylose was purchased from Corn Products Laboratories, α-amylase was purchased from Worthington, and *Cytophaga* isoamylase (5) was kindly provided by Dr. W. J. Whelan, University of Miami.

Purification of ADP-glucose-Starch Glucosyltransferase. Ears of waxy maize were harvested 22 days after pollination and stored at −15 C until used. Kernels (20 g) were removed and ground to a powder in a mortar with liquid N_2. The powder was stirred with 40 ml of a solution containing 50 mM tris-acetate buffer, pH 7.5, 10 mM EDTA, and 2 mM dithiothreitol for 30 min. All operations were then carried out at 0 to 4 C. Concentration and separation of the ADP-glucose-starch glucosyltransferases on DEAE-cellulose was the same as that described previously (12), except that 65 mg of protein were addd to a 20-ml (resin bed volume) column of DEAE-cellulose and 800 ml of tris-actetate buffer with increasing concentration of KCl (linear gradient 0 to 0.4 M KCl) was passed through the column and collected in 4.4-ml fractions.

ASSAY OF ADP-GLUCOSE-STARCH GLUCOSYLTRANSFERASE

Transfer of Glucose to Primer. The reaction mixture contained 140 nmoles of ADP-^{14}C-glucose (500 cpm/nmole), 20 μmoles of Bicine buffer (pH 8.5), 5 μmoles of potassium acetate, 2 μmoles of GSH, 1 μmole of EDTA, 1 mg of amylopectin (amylose free), and enzyme in a final volume of 0.2 ml. The reaction was stopped by adding 2 ml of 75% methanol containing 1% KCl, and the methanol-insoluble polysaccharide was determined by the method of Ghosh and Preiss (4). In experiments where maltose or maltotriose was used as a primer, the reaction was stopped by heating for 1 min at 100 C; 2 ml of a slurry containing 400 mg of anion exchange resin (AG1-X8, 200–400 mesh) were added to absorb the remaining ADP-^{14}C-glucose. After filtration through Whatman No. 1 filter paper, the radioactivity in 0.5 ml of the filtrate was determined in a liquid scintillation counter.

Production of Glucan without Added Primer. Reaction mixtures were as above, except that potassium acetate and amylopectin were replaced by 100 μmoles of sodium citrate and 100 μg of BSA. The reaction was stopped by heating for 1 min at 100 C. Carrier amylopectin was added (1 mg), and radioactivity was determined by methanol precipitation as described for the reaction mixture containing primer.

Product Identification. Radioactive product formed by the unprimed reaction was coprecipitated with carrier amylopec-

Table I. *Purification of ADP-glucose:α-1,4-glucan α-4-Glucosyltransferase from Waxy Maize*

Enzyme was assayed at 30 C for 30 min, as described in "Materials and Methods."

Fraction	Volume	Total Protein	Total Units[1]		Specific Activity	
			Primed	Unprimed	Primed	Unprimed
	ml	*mg*			*units/mg of protein*	
Crude	50	345	1.67	1.05	0.0048	0.0030
45,000*g* supernatant	46	181	1.68	0.67	0.0093	0.0037
$(NH_4)_2SO_4$ (0–40%)	4.5	65	1.45	0.77	0.0223	0.0118
DEAE-cellulose						
fraction I	1.5	0.90	0.034	0.28	0.0378	0.311
fraction II	1.8	2.41	0.205	0.00	0.0851	0.0

[1] One unit = 1 μmole ^{14}C-glucose transferred per min at 30 C.

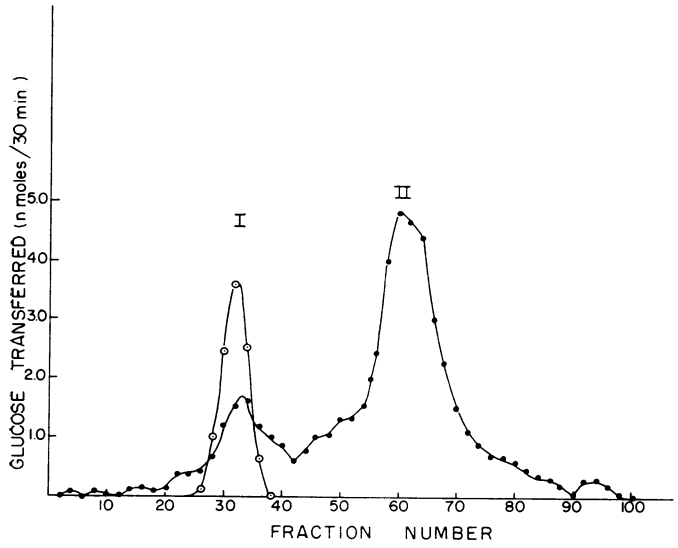

FIG. 1. Elution pattern of waxy maize ADPglucose:α-1,4-glucan α-4-glucosyltransferase from DEAE-cellulose. Fractions were assayed with amylopectin as a primer (●——●) and without amylopectin in the presence of bovine serum albumin and 0.5 M sodium citrate (○——○). Assays were carried out at 30 C for 30 min as described in the Methods section. No unprimed activity was found in the fractions before or after those shown.

tin with methanol-KCl, washed and dissolved in 1 ml of water as above. Aliquots (100 μl containing 6000 cpm) were incubated for 4 hr at 37 C and pH 5.5 with either β-amylase, α-amylase, isoamylase, or isoamylase plus β-amylase. The reaction mixtures were heated for 1 min at 100 C and spotted on Whatman No. 1 paper. Chromatograms were developed in a 1-butanol-pyridine-water solvent (6:4:3) for 40 hr. Radioactive spots on the chromatograms were located with a strip scanner and then cut out and counted by liquid scintillation.

The iodine absorption spectrum of the product formed in the unprimed reaction was obtained. Enzyme was incubated with unlabeled ADP-glucose in a reaction mixture (with GSH omitted) containing BSA and 0.5 M sodium citrate, as described above. The reaction was stopped by heating at 100 C for 1 min; 50 μl of reaction mixture containing about 25 μg of the product were added to 0.95 ml of the iodine reagent containing saturated $CaCl_2$ (9). The absorption spectrum of the solution and of solutions containing amylopectin and amylose were recorded on a Cary model 14 spectrophotometer.

RESULTS

ADP-glucose-starch glucosyltransferases from developing kernels of waxy maize were purified by ammonium sulfate precipitation and DEAE-cellulose chromatography (Table I). Each column fraction collected was assayed for primed (using amylopectin) and unprimed activity (Fig. 1). Two forms of the enzyme were found and will be referred to as transglucosylase I and II (Fig. 1). Unprimed activity was associated only with transglucosylase I. It showed a 100-fold purification over the crude extract and was 8-fold greater than primed activity with amylopectin as a primer.

Many anions, as well as citrate which was used in the standard assay, in the presence of BSA stimulated the unprimed activity of transglucosylase I (Table II). Chloride ions and anions above it in the chaotropic series (6) were ineffective, but sulfate ions and anions below it in the series stimulated unprimed activity. The latter anions were also found to stimulate the unprimed activity of one of the transglucosylases from spinach leaves (12).

Addition of 200 μg of the primer amylopectin to reaction mixtures containing 0.5 M sodium citrate and BSA reduced the amount of product synthesized to 50% of that formed in the absence of primer. Whether this represents an inhibition of the unprimed reaction, a stimulation of the primed reaction, or a combination of the two is not known at the present time. In any case, it can be seen that the primed reaction is either not stimulated at all or is not stimulated to nearly the same extent as the unprimed reaction.

UDP-glucose was found not to substitute for ADP-glucose as the sugar nucleotide donor in either primed reaction (fractions I and II) or unprimed reaction (fraction I). Glucose-1-P was also inactive. Primed and unprimed reaction rates were linear with protein concentration and time in the range used to obtain the data reported.

Table II. *Effect of Salts on the Activity of ADP-glucose:α-1,4-glucan α-4-Glucosyltransferase from Waxy Maize in the Absence of Added Primer*

Enzyme was assayed at 30 C for 30 min, as described in "Materials and Methods."

Salt	Concentration	BSA (0.5 mg/ml)	Glucose Transferred[1]
	M		*nmoles/30 min*
K acetate	0.025	+	0
K acetate	0.025	−	0
KSCN	1	+	0
$NaClO_4$	1	+	0
KNO_3	1	+	0.2
KI	1	+	0.1
KBr	1	+	0.1
KCl	1	+	0.3
$(NH_4)_2SO_4$	1	+	9.0
K acetate	1	+	17.5
Na acetate	1	+	20.1
NaF	1	+	8.3
Na citrate	0.5	+	23.7
Na citrate	0.5	−	4.1

[1] In the presence of 0.5 M sodium citrate and BSA the rate of glucose transferred was linear with time. Time studies were not carried out with the other salts.

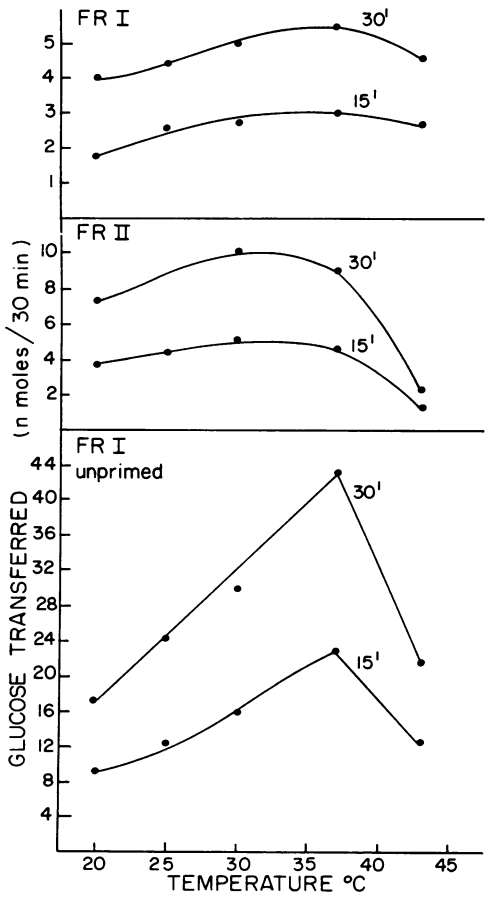

FIG. 2. Effect of temperature on the activity of ADPglucose-starch glucosyltransferases. Assay conditions were as described in the Methods section.

Temperature response curves for 15 and 30 min are shown in Figure 2. Rates of the primed reaction changed only slightly in going from 20 to 37 C, with an optimum of about 37 C for fraction I and 30 C for fraction II. Fraction II was more sensitive to high temperature than fraction I. Unprimed activity of fraction I was greatly stimulated by increasing temperature, with an optimum at about 37 C and a 50% lower rate at 42 C. Each fraction had a broad pH optimum from about pH 7.5 to 8.5 (Fig. 3).

The *Km* for ADP-glucose in primed reactions was 0.10 mM for transglucosylase I and 0.12 mM for transglucosylase II. The unprimed reaction was nonlinear with time at low concentrations of ADP-glucose, but the enzyme appeared to have high affinity for this substrate (Fig. 4). At 82 μM ADP-glucose, the unprimed reaction was 5-fold faster than the transglucosylase activity observed with amylopectin as a primer.

Activity of transglucosylases I and II with different primers is given in Table III. Glycogen, particularly rabbit liver glycogen, was a much better primer than amylopectin for fraction I but was a poorer primer for fraction II. The reaction rate with rabbit liver glycogen for transglucosylase I approached that of the unprimed reaction. The activity with other primers was relatively alike for both fractions.

Treatment of the unprimed product with α-amylase and β-amylase showed that the product was an α-1,4 glucan with some branch points (Table IV). Isoamylase treatment with and without β-amylase showed that the branches were α-1,6 linkages. The degree of hydrolysis with amylases was similar to that obtained with amylopectin (7). The small amount of glucose present after hydrolysis by isoamylase plus β-amylase may result from a maltase contaminant in the β-amylase. The iodine spectrum of the unprimed product was similar to that of amylopectin (Fig. 5).

DISCUSSION

Waxy maize was chosen for this study because it contains soluble ADP-glucose-starch glucosyltransferase due to the low content of amylose in the kernels (2, 8).

The two glucosyltransferases separated from waxy maize have many properties in common with the glucosyltransferases from spinach leaves (12). In both tissues, activity in the absence of added primer was associated with only one form of the enzyme. Some differences have been observed, the main ones being the absorption and elution of the activities from DEAE-cellulose, the ratio of unprimed to primed activity, and the composition of the product. The relative position of the enzyme eluted from DEAE-cellulose which is active in the absence of added primer was reversed in maize compared to that found in spinach leaves. With these fractions, the ratio of unprimed activity (with amylopectin) in maize was about eight, whereas in spinach it was less than three. However, using rabbit liver glycogen as a primer, the ratio of unprimed to primed was nearly the same for both tissues. Thirdly, the product of the unprimed reaction with maize enzyme had properties similar to amylopectin, while the product with spinach enzyme contained fewer α-1,6 linkages than amylopectin (unpublished data).

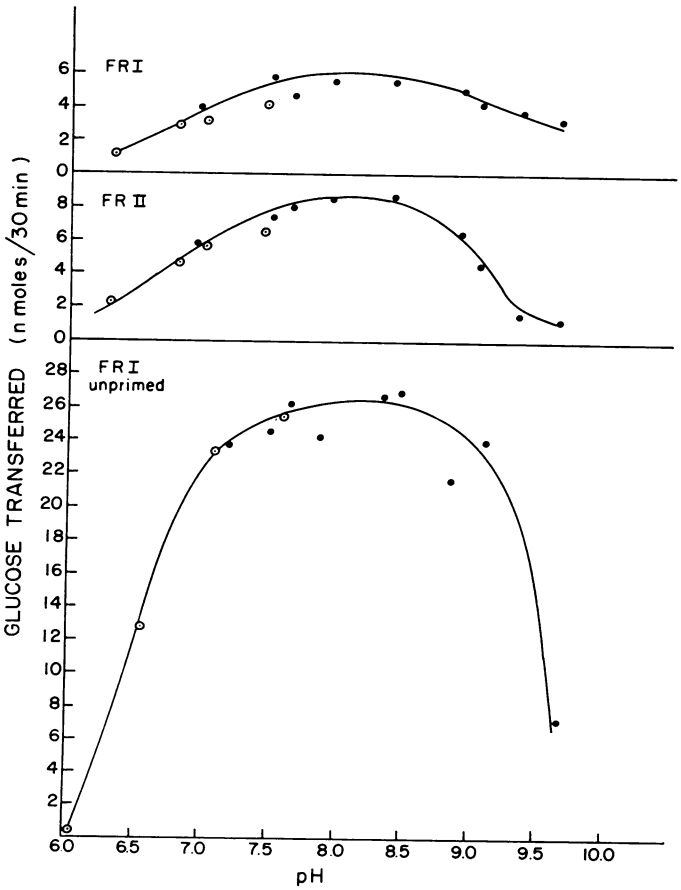

FIG. 3. Effect of pH on the activity of ADPglucose-starch glucosyltransferase. Assays were as described in the Methods section in the presence of Bicine buffer (●) or Hepes buffer (○).

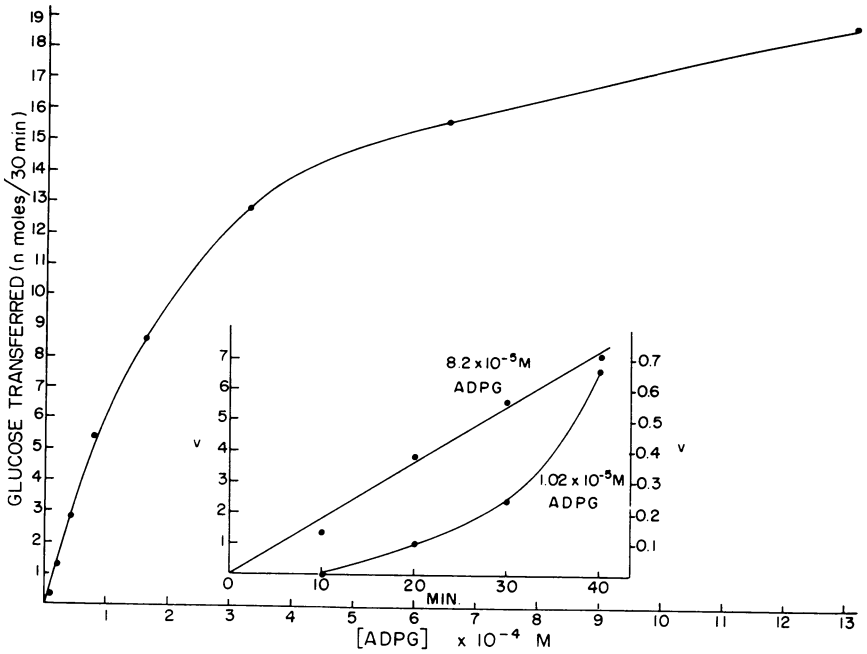

FIG. 4. Saturation curve for ADPglucose-starch glucosyltransferase (Fraction I) in the absence of added primer. Assay was carried out at 30 C for 30 min as described in Methods section. The insert is a time course with 10.2 μM ADPglucose (right hand ordinate) and 82 μM ADPglucose (left hand ordinate).

Table III. *Activity of ADP-glucose:α-1,4-glucan α-4-Glucosyltransferase from Waxy Maize*

Activity was assayed at 30 C for 30 min, as described in "Materials and Methods."

Substrate	Final Concentration	DEAE-cellulose Fractions	
		I	II
Amylopectin	5 mg/ml	100[1]	100
Amylose	5 mg/ml	96	73
Rabbit liver glycogen	5 mg/ml	380	78
Oyster glycogen	5 mg/ml	133	43
Maltose	0.1 M	15	11
	0.5 M	71	79
	1.0 M	160	122
Maltotriose	0.05 M	37	34
	0.10 M	90	51

[1] Percentage of rate observed with amylopectin.

Table IV. *Analysis of Product Formed by Waxy Maize ADP-glucose:α-1,4-glucan α-4-Glucosyltransferase in the Absence of Added Primer*

Product Treatment	Percentage of Total ^{14}C Incorporated		
	Origin	Maltose	Glucose
None	100	0	0
β-Amylase	48	52	0
α-Amylase	28[1]	47	25
Isoamylase	100	0	0
Isoamylase plus β-amylase	1	90	9

[1] Seventy-five per cent of this activity had moved a short distance from the origin, suggesting oligosaccharides 6 to 10 glucose units long.

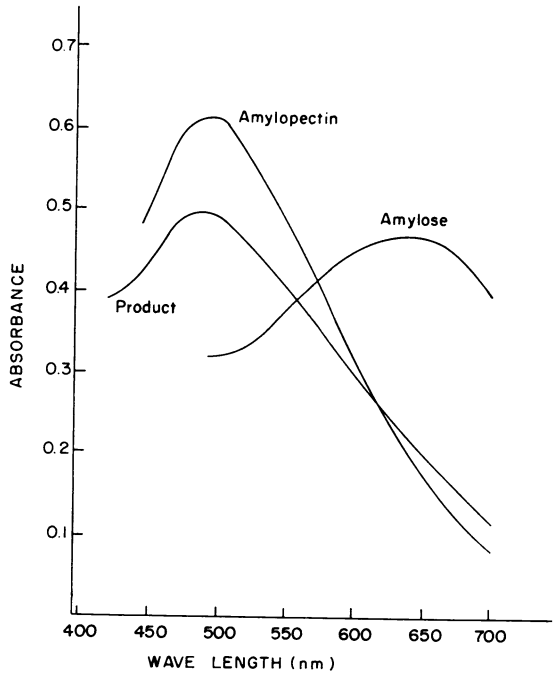

FIG. 5. Iodine absorption spectrum in saturated $CaCl_2$ for amylose, amylopectin and ADPglucose-starch glucosyltransferase product formed in the absence of added primer.

The possibility that a primer is associated with the form of the enzyme which synthesizes glucan in the absence of added primer cannot be ruled out. However, if an endogenous primer is present, it is not effective under normal assay conditions but requires changes in contents of the reaction mixture (*e.g.*, 0.5 M sodium citrate plus BSA).

The presence of a glucose-containing glucan in the spinach leaf transferase fraction that catalyzes the unprimed reaction

has been shown to be at a level of 1 μg anhydroglucose units per mg of protein or less (12). This determination has not been done with the similar maize fraction, since only small amounts of enzyme have been available. However, preliminary results with sucrose density gradient centrifugation (11) indicate that the enzymes from both sources behave as proteins with molecular weights of about 60,000 to 80,000. Thus, no high molecular weight polymer such as amylopectin or amylose appears to be bound to the enzyme. The enzyme fractions also give no detectable stain with I_2 according to the assay procedure of Krisman (9).

At present, the mechanism of action of the salts or BSA in the unprimed reaction is unknown. One possibility is that for initiation of glucan synthesis the enzyme must be in a different conformation than that required for the primed reaction. It is also possible that citrate and BSA have the same effect as α-glucan primers in inducing the active conformation of the transferase. It is thought that those anions of the chaotropic series that are active in the unprimed reaction are anions that tend to aggregate proteins (6). However, no evidence for aggregation by 0.5 M sodium citrate has been obtained in preliminary experiments with sucrose density gradient centrifugation.

The product of the unprimed reaction has been demonstrated to contain α-1,6 linkages; this is most probably due to the presence of branching enzyme activity in the transferase fraction (unpublished data). The presence of this activity in the transferase fraction that catalyzes the unprimed reaction raises the question of the role of branching enzyme in the stimulation of the unprimed reactions. Brown and Brown (1) have shown that mammalian branching enzyme increases the rate of polysaccharide formation from glucose-1-P catalyzed by muscle phosphorylase. Using a purified ADP-glucose:α-glucantransferase from *Streptococcus mitis*, Walker and Builder (16) have shown that glucose transfer from ADP-glucose to a primer endogenous to the enzyme preparation is stimulated about 4-fold by addition of branching enzyme isolated from the same microorganism. Thus, branching enzyme activity may also stimulate the unprimed reaction catalyzed by the transferase by formation of a branched polymer able to accept glycosyl residues at a multiple number of nonreducing ends. Attempts to separate branching enzyme activity from transferase activities are being made in order to demonstrate the above possibility.

Although the conditions for obtaining optimal rates of unprimed reaction appear to be nonphysiological, it is quite possible that these conditions simulate the environment or milieu *in situ*. The concentration of enzyme required for the *in vitro* unprimed reaction is about 1/30 the concentration of transferase present in the cell, assuming even distribution throughout the cell. Since it has been shown that the rate of endosperm starch formation in maize is 10 nmole glucose equivalent/g·min (15), the maximum rate of unprimed reaction found is 5-fold greater than the *in vivo* rate. The rate of unprimed reaction catalyzed by maize phosphorylase with glucose-1-P is much less than the *in vivo* rate observed for starch synthesis in maize (15).

The present results and those with spinach (12) suggest that ADP-glucose starch glucosyltransferases might initiate and maintain starch synthesis in plants.

Acknowledgments—The authors thank Dr. R. Creech for the waxy maize. This work was supported in part by United States Public Health Service National Institute of Health Grant AI 05520 and South East Asia Treaty Organization Fellowship VII (J. S. H.).

LITERATURE CITED

1. Brown, B. I. and D. H. Brown. 1966. *In:* E. F. Neufeld and V. Ginsburg, eds., Methods in Enzymology, Vol. 8. Academic Press, New York. pp. 395–403.
2. Creech, R. G. 1965. Genetic control of carbohydrate synthesis in maize endosperm. Genetics 52: 1175–1186.
3. Frydman, R. B. and C. E. Cardini. 1965. Soluble enzymes related to starch synthesis. Biochem. Biophys. Res. Commun. 17: 407–411.
4. Ghosh, H. P. and J. Preiss. 1966. Biosynthesis of starch in spinach chloroplasts. J. Biochem. 4: 1354–1361.
5. Gunja-Smith, Z., J. J. Marshall, E. E. Smith, and W. J. Whelan. 1970. A glycogen-debranching enzyme from *Cytophaga*. Fed. Eur. Biochem. Soc. Letters 12: 96–100.
6. Hatefi, Y. and W. G. Hanstein. 1969. Solubilization of particulate proteins and nonelectrolytes by chaotropic agents. Proc. Nat. Acad. Sci. U. S. A. 62: 1129–1136.
7. Kjolberg, O. and D. J. Manners. 1962. α-1,4-Glucosans. XV. Structural analysis of glycogens on a milligram scale. J. Chem. Soc. 4596–4600.
8. Kramer, H. H., P. L. Pfahler, and R. L. Whistler. 1965. Gene interactions in maize affecting endosperm properties. Agron. J. 50: 207–210.
9. Krisman, C. R. 1962. A method for the colorimetric estimation of glycogen with iodine. Anal. Biochem. 4: 17–23.
10. Leloir, L. F., M. A. R. Defekete, and C. E. Cardini. 1961. Starch and oligosaccharide synthesis from uridine diphosphate glucose. J. Biol. Chem. 236: 636–641.
11. Martin, R. G. and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236: 1372–1379.
12. Ozbun, J. L., J. S. Hawker, and J. Preiss. 1970. Multiple forms of α-1,4-glucan synthetase from spinach leaves. Biochem. Biophys. Res. Commun. 43: 631–636.
13. Recondo, E. and L. F. Leloir. 1961. Adenosine diphosphate glucose and starch synthesis. Biochem. Biophys. Res. Commun. 6: 85–88.
14. Slabnik, E. and R. B. Frydman. 1970. A phosphorylase involved in starch biosynthesis. Biochem. Biophys. Res. Commun. 38: 709–714.
15. Tsai, C. Y. and O. E. Nelson. 1969. Two additional phosphorylases in developing maize seeds. Plant Physiol. 44: 159–167.
16. Walker, G. J. and J. E. Builder. 1971. Metabolism of the reserve polysaccharide of *Streptococcus mitis*. Properties of branching enzyme and its effect on the activity of glycogen synthetase. Eur. J. Biochem. 20: 14–21.