Carbohydrate Research, 61 (1978) 239–252

THE REQUIREMENT FOR A PRIMER IN THE *in vitro* SYNTHESIS OF POLYSACCHARIDE BY SWEET-CORN (1 → 4)-α-D-GLUCAN SYNTHASE*

SIGBERT SCHIEFER†, ERNEST Y. C. LEE, AND WILLIAM J. WHELAN
Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33152 (U.S.A.)
(Received August 4th, 1977; accepted for publication in revised form, October 21st, 1977)

ABSTRACT

A mixture of (1 → 4)-α-D-glucan synthases was partially purified from sweet corn. The synthesis of polysaccharide from ADP-D-glucose by the enzyme preparation was dependent on added carbohydrate primer in solutions of low ionic strength, but displayed the phenomenon of being apparently primer-independent at high ionic strength in citrate buffer. This phenomenon was further investigated; treatment of the enzyme preparation with immobilized amylases led to the abolition of the apparently unprimed synthesis. The amylase-treated preparation then showed a normal dependence on (1 → 4)-α-D-glucan primer, branched primers being the most effective. The affinity of the enzyme for a branched primer appeared to be enhanced in the presence of citrate. The polysaccharide product of the unprimed reaction was glycogen-like, having an average chain-length of 14. These studies suggest that the phenomenon of unprimed synthesis in "high salt" is explicable in terms of an enhanced affinity of the enzyme for traces of primer in the enzyme preparation, and not to a "*de novo*" synthesis of polysaccharide that occurs in the absence of a primer.

INTRODUCTION

At present, two types of enzyme are known that form the (1 → 4)-α-D-glucosidic bonds of glycogen and starch *in vitro* from precursors of low molecular weight. Phosphorylase [(1 → 4)-α-D-glucan: orthophosphate α-glucosyltransferase, EC 2.4.1.1] forms (1 → 4)-α-D-glucans from α-D-glucosyl phosphate, whereas starch synthase [ADP-D-glucose: (1 → 4)-α-D-glucan 4-α-glucosyltransferase, EC 2.4.1.21] or glycogen synthase (UDP-D-glucose: glycogen 4-α-glucosyltransferase, EC 2.4.1.11) use ADP-D-glucose or UDP-D-glucose as the glucosyl donor[1–3]. Under normal experimental conditions, it is necessary to add a (1 → 4)-α-D-glucan primer as well as the glucosyl donor in order to observe synthesis of (1 → 4)-α-D-glucan. The dependence of these enzymes on a pre-existing (1 → 4)-α-D-glucan primer raises the

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.
†Present address: Fachbereich Chemie der Philipps Universität, D-3550 Marburg/Lahnberge, German Federal Republic.

question of how the biosynthesis of starch or glycogen is initiated *in vivo*. What is the origin of the primer? This question has been examined on numerous occasions[4]. One view is that phosphorylase and the synthase are capable of synthesizing (1 → 4)-α-D-glucan *de novo* from α-D-glucosyl phosphate or the nucleoside sugar diphosphates, either by condensation between the donor sugars or by transfer of the donor sugar to a non-glucan acceptor. The idea of *de novo* synthesis has arisen from observations that various enzyme-preparations, under certain experimental conditions, are able to catalyze glucan synthesis in the absence of added primer.

One manifestation of "*de novo*" synthesis is that most phosphorylase and starch-synthase preparations display an apparently "unprimed" synthesis after a lag period, simply on incubation with a glucosyl donor. Where the several reports[5-9] of this type of unprimed synthesis by phosphorylase have been subjected to further investigation, it has emerged that the synthesis was either attributable to a trace of primer present in the D-glucosyl phosphate used[10,11], or in the enzyme preparation[12,13]. In addition, the presence of branching enzyme[4,14] or alpha amylase[12,13] markedly increases the rate of such "*de novo*" syntheses, presumably via an autocatalytic mechanism.

A more-striking phenomenon, pointing towards a true "*de novo*" synthesis, was described by Preiss and coworkers as a property of starch-synthase or glycogen-synthase preparations from plant and bacterial sources[14-20]. An apparent "*de novo*" synthesis of starch from ADP-D-glucose was observed under certain special conditions. In the presence of certain salts in high concentration (such as 0.5M sodium citrate), especially if these were augmented with certain proteins [for example 0.05% bovine serum albumin (BSA)], an apparent "*de novo*" synthesis occurred, whereas under conditions of low salt, a strict dependence on primer was exhibited[14-19]. The "*de novo*" synthesis was sometimes more rapid than in low salt in the presence of ample primer, and occurred without any lag period or with only a very short lag. The addition of primer did not stimulate, and even partly inhibited, synthesis in high concentrations of salt[18,20]. Attempts to eliminate "*de novo*" synthesis by using glucoamylase to decompose any endogenous, contaminating primer in the incubation mixture, did not significantly decrease the rate of unprimed synthesis in high concentrations of salt[14,19].

Based on these and other findings, the phenomenon was thought to constitute a true *de novo* synthesis[14-20]. It was suggested that in high concentrations of salt, starch synthase was able to transfer D-glucose to a non-glucan acceptor, possibly a protein, thus obviating the requirement for a glucan primer[14]. Other investigators have recently suggested that protein primers may be involved in α-D-glucan synthesis. An "initiator synthase" is postulated as creating an oligosaccharide primer, covalently attached to a protein, and starch or glycogen synthase then elongates the oligosaccharide[21-25].

In this paper we report the partial purification of a soluble synthase from sweet corn. This synthase preparation also exhibited a strict primer-dependence in low concentrations of salt, whereas in 0.5M sodium citrate–BSA, the synthesis of

(1 → 4)-α-D-glucan was more rapid, even in the absence of added primer. The results of a detailed investigation of the origin of this phenomenon are presented, together with our conclusion that *de novo* synthesis is not involved. A preliminary account of some of our findings has been published[26].

RESULTS AND DISCUSSION

Purification of sweet-corn synthase. — The overall purification of sweet-corn synthase is shown in Table I. Assays were carried out at each step for both "primed" and "unprimed" activity. Primed activity refers to the assay carried out in low salt with added primer (glycogen), and unprimed activity to assays carried out in high salt (0.5M citrate) without added primer. The purification obtained was 310-fold based on the primed activity-assay and 830-fold based on the unprimed activity-assay. The enzyme preparation was not a homogeneous protein. We have previously observed that crude extracts of maize endosperm contain multiple forms of starch (or glycogen) synthase, as detected by disc-gel electrophoresis and a staining procedure that reveals the enzyme activity in the gel[27]. The purified sweet-corn synthase was

TABLE I

PURIFICATION OF ADP-D-GLUCOSE: (1 → 4)-α-D-GLUCAN 4-α-GLUCOSYLTRANSFERASE (STARCH/GLYCOGEN SYNTHASE) FROM SWEET CORN

Purification step	*Total units*[a]		*Enzyme recovery (%)*		*Specific activity (IU/mg of protein)*		*Purification (fold)*	
	primed	*unprimed*	*primed*	*unprimed*	*primed*	*unprimed*	*primed*	*unprimed*
Crude extract (1 kg kernels)	285	339[a]	100	100	0.0050	0.0059[b]	—	—
20,000*g* supernatant	275	275[b]	97	81	0.0083	0.0083[b]	1.66	1.4
DEAE-Cellulose (batch)	163	181	57	53	0.025	0.028	5.0	4.7
$(NH_4)_2SO_4$ (0–45%)	140	203	49	60	0.051	0.074	10.2	12.5
DEAE-cellulose (column)	102	191	36	56	0.092	0.17	18.4	28.8
Hydroxylapatite (column)	39	124	13.7	36.5	1.55	4.92	310	832

[a]Assays for primed activity were performed in 50mM Bicine buffer with 100 mg/ml of glycogen as a primer (low-salt conditions); assays for unprimed activity were performed in the presence of 0.5M sodium citrate (high-salt conditions) without the addition of a primer, as described in the Methods Section. [b]Although no primer was added, there was a large proportion of endogenous phytoglycogen present in these early stages of purification.

*We have no information on the relative activities of the various isozymes. "Major" refers to the relative intensity of the band of iodine-stained polysaccharide in the gel[27]. However, the colors of the stains vary from band to band, indicating that polysaccharides of different degrees of branching (or no branching) are being synthesized. As the molar extinction coefficients of the iodine complexes of amylose, amylopectin, and glycogen are very different, the relative intensity of the iodine stain of each isozyme band is unlikely to be an accurate reflection of relative enzyme-activity.

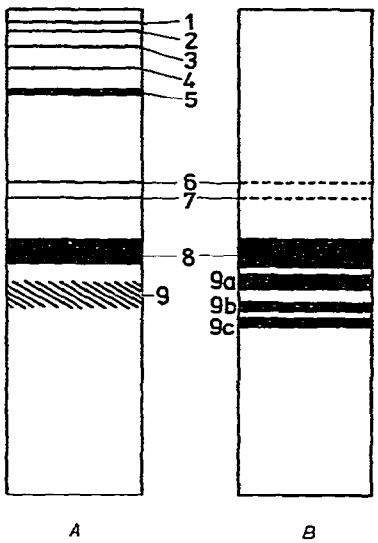

Fig. 1. Disc-gel electrophoretic patterns of (1 → 4)-α-D-glucan synthases from sweet corn. The enzymes were detected by incubation of the gels with ADP-D-glucose and primer, followed by staining the synthetic polymer with iodine, as described previously[27]. (A) Crude extract from kernels; (B) purified enzyme-preparation. The bands are numbered as in ref. 27.

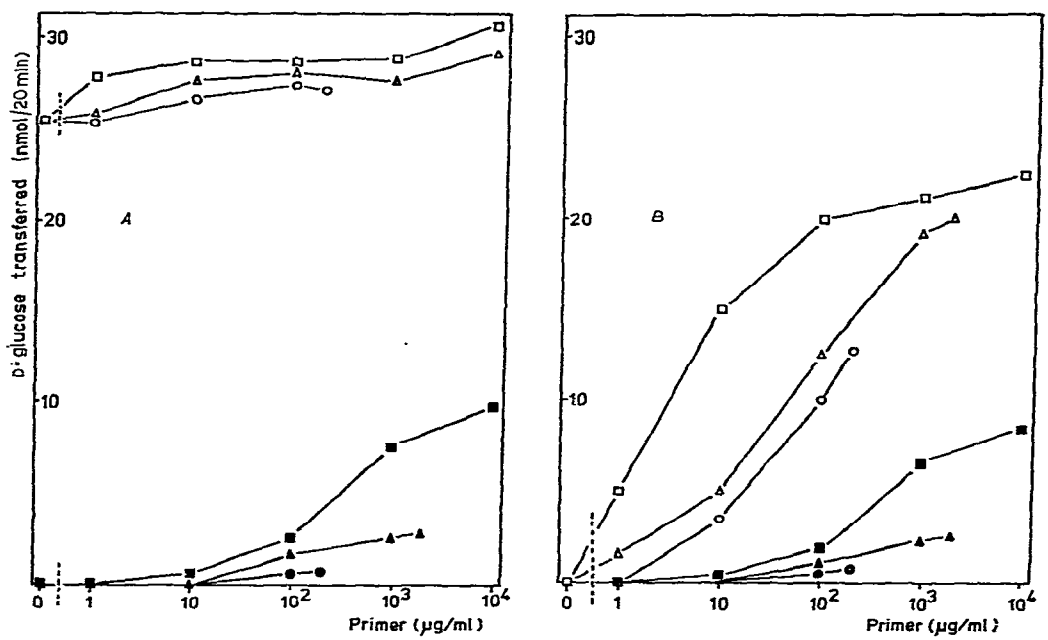

Fig. 2. The primer dependence of sweet-corn synthase in low and high concentrations of salt. Purified synthase activity was assayed in low salt and in 0.5M citrate as described in Methods, with various amounts of added glycogen, amylopectin, or amylose. A, the results obtained with the untreated synthase-preparation; and B, the results obtained after treatment of the enzyme with immobilized amylases. Key: Low-salt assays with glycogen (■), amylopectin (▲), or amylose (●); high-salt assays with glycogen (□), amylopectin (△), or amylose (○).

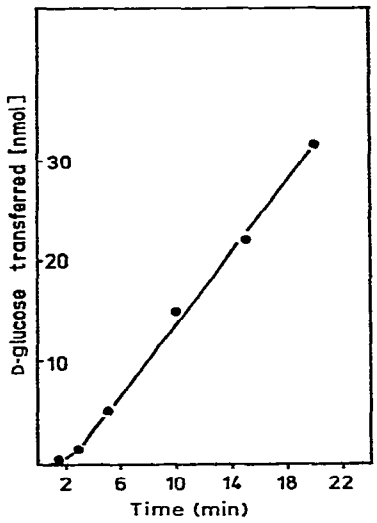

Fig. 3. Time course of the unprimed reaction of sweet-corn synthase. Purified sweet-corn synthase was assayed in 0.5M citrate as described in Methods, for various periods of time.

examined by gel electrophoresis and the pattern of the activity bands obtained is shown diagrammatically in Fig. 1, together with that of the crude extract. The synthase preparation had four major bands, one of which corresponds to band 8, the "major" form* in the crude extract, and three faster-migrating bands that appear to represent an enrichment and resolution of band 9 of the crude extract into three components (9a, 9b, and 9c; Fig. 1). The preparation was free of amylase and phosphorylase activities, but did contain branching-enzyme activity. All attempts to remove the branching enzyme were unsuccessful.

The apparent "de novo" *synthesis.* — The synthase preparation displayed a normal primer-dependence in buffers of low salt (50mM Bicine, incubation 1, Experimental), as shown in Fig. 2A. No synthesis was detected over a period of 20 min in the absence of added primer. (After sufficiently prolonged lag-periods, synthesis did occur, this being a general phenomenon commonly observed with both phosphorylase and synthase preparations.) The reaction rate also appeared to be correlated with the degree of branching of the primer. Thus, glycogen was the best primer, followed by amylopectin and amylose, in that order.

Entirely different results were obtained when the incubations were conducted in high salt (0.5M citrate, 0.5 mg/ml BSA; incubation 2, Experimental). A rapid reaction occurred in the absence of added primer, and the addition of primer had a minimal effect. The unprimed reaction-rate was much higher than the primed rate in low salt under all conditions tested (Fig. 2A), and was essentially linear with time, with only a very short lag-period, if any (Fig. 3). Thus the sweet-corn starch synthase preparation displayed the phenomenon of being primer-dependent in low salt and of being more active and apparently primer-independent in 0.5M citrate. This is the same as the unprimed reaction discovered by Preiss and co-workers[14–20].

The effects on the unprimed reaction of amylase treatment of starch synthase. — The preparation was treated with a mixture of glucoamylase and alpha amylase, as this combination has been shown to cause the complete conversion of branched α-D-glucans into D-glucose[28]. In addition, the enzymes were covalently bound to Sepharose, to eliminate the necessity for additional separation procedures and to allow for rapid treatment of the synthase preparation. The isozyme pattern (Fig. 1B) was not altered by treatment with amylase.

Treatment of the purified, sweet-corn synthase preparation with immobilized amylases completely abolished the unprimed or high-citrate reaction over the 20-min assay period (Table II). Treatment of the other reagents used in the assay had no effect. The addition of primer in the form of glycogen essentially restored the high citrate activity (Table II). The low-salt or primed reaction was essentially unaffected (Table II). Therefore, after treatment of the synthase preparation with immobilized amylases, the ability of the synthase to function in high citrate became dependent on the addition of exogenous primer. It should be noted that the assays were carried out by using both the ethanol precipitation and the ion-exchange resin procedures for the separation of and determination of ^{14}C-labelled products. The resin assay depends on the removal of ADP-D-glucose from the mixture and measurement of the remaining radioactivity. This procedure was used as ambiguous results might have been obtained had there been any contamination of the synthase with the amylases used to remove endogenous primer. The amylases could degrade any synthetic polymer to oligosaccharides that would not be precipitated by ethanol. However, there was no apparent difference in the values obtained by the ethanol and

TABLE II

PRIMER DEPENDENCE OF SWEET-CORN SYNTHASE AFTER TREATMENT WITH IMMOBILIZED AMYLASES

Assay conditions	*Synthase activity (nmol D-glucose transferred/20 min.)*[a]			
	No treatment		*Pre-treated with immobilized amylases*	
	Ethanol assay	*Resin assay*	*Ethanol assay*	*Resin assay*
50mM Bicine (low salt) No added primer	0	0	0	0
Primer (100 mg/ml glycogen)	10.2	10.1	9.0	9.0
0.5M Citrate (high salt) No added primer	28.1	28.2	0	0
Primer added (100 mg/ml glycogen)	32.0	32.0	28.4	28.2

[a]The synthase was treated with immobilized amylases as described in the Experimental section, and assayed under conditions of low salt (primed assay) and high salt (unprimed assay). The products of the reactions were analyzed both by the ethanol-precipitation procedure and the ion-exchange resin procedure (Experimental).

resin assays (Table II), indicating that there was no overt contamination of the preparation with amylases leached from the Sepharose support.

The primer dependence of the amylase-treated, sweet-corn synthase under conditions of low and high salt concentration was examined in regard to concentration of glycogen, amylopectin, and amylose (Fig. 2B). In the low salt assay-system, the results were essentially identical to those previously obtained with the untreated enzyme-preparation (compare Fig. 2A). In high citrate concentration, the amylase-treated synthase now displayed a concentration dependence on added primer (Fig. 2B). As little as 1 μg/ml of glycogen was now able to elicit a marked response, whereas 10 μg/ml effected almost no response in low salt. The effectiveness of the different primers also followed the same order as for the low-salt activity. Similar results (not shown) to all those described here were obtained when human salivary alpha-amylase was used instead of hog-pancreatic alpha-amylase.

These experiments show that the activity in high citrate, the so-called "unprimed" activity, cannot be ascribed simply to a primer-independent, *de novo* reaction. The amylase-treated enzyme preparation now behaved like other primer-dependent systems. It also now displayed the phenomenon of a lag period in the absence of added primer, in that while no reaction was observed in the absence of added primer over a 20-min incubation period, synthesis did occur after prolonged incubations of several h.

The phenomenon of enzyme preparations capable of "*de novo*" synthesis in high salt, while displaying a normal primer-dependence in low salt, is best explained by our data as a greater affinity of the enzyme for primer in high salt. According to the data of Fig. 2B, the amounts of added primer needed to achieve equal reaction-rates in low and high salt are in the ratio of 100–1000 : 1. It would appear that, before treatment of the synthase preparation with the amylases, there was sufficient endogenous primer to allow a reaction in high salt to be observed, but not enough for a reaction to be seen in low salt.

Structural analysis of the product formed in the unprimed reaction by the (1 → 4)-α-D-glucan synthase preparation. — The enzymes used were beta amylase and two debranching enzymes, pullulanase and isoamylase (for a review on their usage see ref. 29). After treatment with beta amylase, 51 % of the radioactive product remained ethanol-precipitable, indicating a branched structure. Simultaneous treatment with pullulanase and beta amylase completely degraded the polysaccharide, indicating that it consisted entirely of (1 → 4)-α-D-glucan chains joined by (1 → 6)-D-glucosidic branch linkages. The product was distinguished as being related to glycogen rather than to amylopectin from the following experiments, based on the knowledge that pullulanase has only a limited action on glycogen[29], whereas isoamylase is capable of causing complete debranching[30]. Thus, sequential treatment of the product with pullulanase and beta amylase left a resistant dextrin comprising 38 % of the original polysaccharide, whereas sequential treatment with isoamylase and beta amylase left almost no resistant dextrin (0.7 %).

Further confirmation that the radioactive polysaccharide was a glycogen-like polysaccharide came from determination of its average chain-length. This was done

by paper-chromatographic examination of the products of the reaction mixtures in which the material had been treated sequentially with isoamylase and beta amylase, and simultaneously with beta amylase and pullulanase. As expected, the radioactive products were found to be almost exclusively glucose and maltose. Calculation of the average chain-length from the ratios of the radioactive glucose and maltose formed, as described by Lee and Whelan[31], gave values of 14.0 and 14.6 for the products formed by pullulanase/beta amylase and isoamylase/beta amylase, respectively. Chain lengths of glycogens are in the range 10–14, and for amylopectin they are in the range[31] of 20–24. The product formed in the unprimed reaction of the sweet-corn synthase preparation is therefore a glycogen. The enzyme preparation thus contained both branching-enzyme and synthase activities.

The effect of isoamylase treatment on the unprimed synthesis of polysaccharide. — A test was made for branching in the putative primer-contaminant by examining the effect of isoamylase on the unprimed reaction of the synthase in high salt (Table III). Isoamylase added at the beginning of the reaction inhibited synthesis by about 20%, both in the presence and absence of added primer. When the synthase was pre-incubated with isoamylase before addition of ADP-D-glucose, the reaction rate was decreased by about 80% in the absence of added primer. The rate was restored by the addition of glycogen. These data suggest that the endogenous primer was a branched α-D-glucan.

Solubility in trichloroacetic acid of the product formed in the unprimed reaction of sweet-corn synthase. — It has been observed that such precursors as ADP-D-glucose, UDP-D-glucose, or α-D-glucosyl phosphate, used with a variety of enzyme systems, have yielded α-D-glucan products that are precipitated by dilute trichloroacetic acid, suggesting that the glucan is bound to protein[14,21–25,32]. The solubility in trichloroacetic acid of the α-D-glucan product formed in the unprimed reaction of sweet-corn synthase was examined in the absence and presence of added carrier glycogen. In both cases, about 15% of the radioactive product was precipitated by

TABLE III

EFFECT OF ISOAMYLASE ON THE ACTIVITY OF SWEET-CORN SYNTHASE IN HIGH CONCENTRATIONS OF SALT

Addition	*Synthase activity*[a] *(nmol D-glucose transferred/20 min)*	
	No primer added	*Primer added*
None	19.8	23.8
Isoamylase	16.2	18.6
Pre-incubated 10 min with isoamylase	4.2	18.0

[a]Synthase activity was determined in high salt (0.5M citrate), as described in the Experimental section, over a 20-min incubation period. Isoamylase was added at a concentration of 0.36 IU/ml. Glycogen (10 mg/ml) was added as the primer at the start of the assay period. Reactions were started by the addition of ADP-D-[^{14}C]glucose, and assays were carried out by the resin procedure (Experimental).

TABLE IV

TRICHLOROACETIC ACID PRECIPITATION OF THE RADIOACTIVE PRODUCT[a] OF THE UNPRIMED REACTION OF SWEET-CORN SYNTHASE

Number of precipitations	*Radioactivity recovered in trichloroacetic acid pellet (% of total)*	
	No carrier	*With carrier*
First precipitate	14.7	14.8
Second precipitate	3.3	1.7
Third precipitate	0.9	0.1

[a]Incubations were carried out under high-salt conditions in the absence of added primer (Experimental). The mixtures (1 ml) contained sufficient purified synthase such that the incorporation of label from ADP-D-[^{14}C]glucose into polymer was 78%, as determined by the ethanol-precipitation procedure (Experimental), after an incubation period of 20 min. The reactions were stopped by the addition of an equal volume of 12.5% trichloroacetic acid. The pellet was recovered by centrifugation, washed with 95% ethanol, and then ether, and dried. For re-precipitation, the pellet was resuspended in 2 ml of 8M urea, and the precipitation by trichloroacetic acid repeated. The radioactivity present in the trichloroacetic acid pellets was determined and the results are expressed as a percentage of the total radioactivity incorporated, as determined by the ethanol-precipitation procedure (Experimental). In the precipitations where a carrier was included, 50 mg of glycogen was added prior to each precipitation with trichloroacetic acid. In all cases, radioactivity in the trichloroacetic acid supernatants could be quantitatively precipitated by 67% ethanol.

trichloroacetic acid (Table IV). In each of two subsequent reprecipitations, the amount of precipitable material was decreased by factors of 3 and 10 for the experiments without and with carrier glycogen, respectively. Therefore, although the product appeared initially to be partially precipitable by trichloroacetic acid, the results suggest that this may have been due to some co-precipitation phenomenon rather than to an inherent property (trichloroacetic acid-insolubility) of the product.

CONCLUSIONS

An extensively purified preparation of (1 → 4)-α-D-glucan synthase from sweet-corn was found to display the phenomenon of apparently being able to synthesize D-glucan from ADP-D-glucose without the need for a primer. Examination of the structure of the polysaccharide formed in the unprimed reaction showed it to be branched, having an average chain-length of 14. Thus the enzyme preparation contained branching enzyme as well as synthase, and the former must have been active in the unprimed reaction.

Our investigation of the phenomenon showed that the "unprimed" reaction was, in fact, dependent on the presence of an endogenous primer in the enzyme preparation. This conclusion is based on the observation that prior treatment of the synthase preparation with a mixture of immobilized alpha amylase and glucoamylase abolished the unprimed reaction, which could then be restored by the addition of

primer. The amount of primer required to stimulate a given degree of synthesis in high citrate by the amylase-treated synthase was much smaller than that required in low salt. A possible explanation of the high-citrate phenomenon has its basis in a markedly higher affinity of the enzyme for primer in the presence of high concentrations of citrate. Seen in this light, the behavior of the synthase preparation in high citrate is not due to a different type of reaction, involving a *de novo*, primer-independent synthesis of polysaccharide, but one involving a change in the affinity of the enzyme for a primer. The alleged "*de novo*" synthesis would result from there being sufficient endogenous primer in the enzyme preparation to stimulate a measurable degree of polymer synthesis in high salt but not in low salt.

Studies of a starch-synthase preparation from spinach leaves by Preiss *et al.* have led to conclusions similar to those reported by us here and earlier[26], namely that the phenomenon they had originally considered to be a *de novo* synthesis could, at least in part, be explained by contamination of the enzyme preparation by endogenous primer[33]. Their results were based on the removal of branching enzyme from the synthase preparation. This resulted in a marked diminution of the unprimed reaction, which could then be restored by the addition of primer. Nevertheless Preiss *et al.*[33] were unable to confirm this conclusion in a direct fashion by destroying the putative primer. Our experiments provide that direct evidence, namely that (*1*) a mixture of alpha amylase and glucoamylase affects the synthase in such a way that synthesis from ADP-D-glucose now becomes dependent on added primer, and (*2*) addition of isoamylase to the synthase changes the characteristics of the unprimed reaction in a manner consistent with the putative contaminant's being a branched (1 → 4)-α-D-glucan.

It remains open to question whether our studies completely eliminate the possibility of a *de novo* synthesis. They do show, however, that what was once believed to constitute *de novo* synthesis is explained in large degree as being due to contamination of the enzyme by a (1 → 4)-α-D-glucan primer.

EXPERIMENTAL

Materials. — ADP-D-glucose and UDP-D-glucose were purchased from Sigma Chemical Co., St. Louis, MO. ADP-D-[^{14}C]glucose was obtained from the Radiochemical Center, Amersham. A malto-oligosaccharide mixture (average degree of polymerization 6) was a gift from Corn Products International (Chicago, IL). Amylose (average degree of polymerization ~300) was purchased from Nutritional Biochemicals Corporation, Cleveland, OH. Amylopectin was prepared from waxy maize as by Schoch[34]. Shellfish glycogen (Sigma) was further purified before use by precipitations with trichloroacetic acid and ethanol, followed by treatment with a mixed-bed, ion-exchange resin [Analytical Grade Mixed-Bed Resin, AG 501-X8(D), Bio-Rad Laboratories, Richmond, CA].

The alpha amylase used was from hog pancreas (di-isopropylphosphofluoridate-treated, Sigma). Glucoamylase was prepared from *Aspergillus niger*[35]; beta amylase was a crystalline preparation from sweet potato[36]; pullulanase was a crystalline,

amylase-free preparation from *Aerobacter aerogenes*[37]; isoamylase, prepared from *Cytophaga*[30], was a gift from Drs. Z. Gunja-Smith and E. E. Smith. These enzymes were of the highest purity available and were free of maltase, alpha amylase, and other interfering activities as required for structural determination[29].

The buffers used were as follows. Buffer 1 contained 50mM Tris–HCl, 1mM dithiothreitol, 2mM EDTA, and 100 mg/ml of sucrose, pH 7.6. Buffer 2 was the same as buffer 1, except that the pH was adjusted to 4.9 and 0.5 mg/ml of BSA was added. Buffer 3 was the same as buffer 2, but the pH was 7.0. Buffer 4 was 50mM Tris-base, with the same additions as in buffer 2. Buffer 5 was 50mM Bicine [N,N'-bis(2-hydroxyethyl)glycine], pH 8.5, with the same additions as in buffer 2.

Assays for starch synthase activity. — Incubations were conducted at 30°C in glass tubes that had previously been washed in chromic acid. Assay procedures were essentially similar to those described by Ghosh and Preiss[38] and by Ozbun *et al.*[18].

Incubation 1: low salt conditions in Bicine buffer, "primed" conditions. The mixture (0.2 ml) contained buffer 5 (50mM), mM ADP-D-[^{14}C]glucose (0.2–2.0 mCi/mmol), 25mM potassium chloride, and the enzyme. Glycogen (100 mg/ml) was added as primer, unless otherwise indicated.

Incubation 2: high salt conditions in citrate–BSA, "unprimed" conditions. The mixture was as for Incubation 1, except that potassium chloride was replaced by 0.5M sodium citrate and primer was omitted, unless otherwise indicated. The formation of a radioactive α-D-glucan was then determined by one of two procedures (Assay A or Assay B, next).

Assay A (ethanol assay). The reaction was stopped by transferring (50-μl) portions of the incubation mixture to filter-paper circles (Whatman 31 ET, 2.4 cm diameter). These were washed five times, each for 15 min, with 67% ethanol, dried, and counted. In experiments where no primer was added to the incubation mixtures, glycogen was added as a carrier to a final concentration of 100 mg/ml immediately before transfer of the incubation mixture to the paper circles.

Assay B (resin assay). The reaction was stopped by adding a sample of the digest (50–100 μl) to a solution of 10 mg/ml of glycogen and 10 mg/ml of malto-oligosaccharides, contained in a centrifuge tube in a boiling-water bath. Heating was continued for a further 2 min. In the case of Incubation 1, 0.2-ml aliquots were applied to an anion-exchange resin (Dowex-1 X8, Cl^- form; 200–400 mesh), packed in Pasteur pipettes (0.5 × 7 cm). The columns were washed with 5 ml of an aqueous solution of 10 mg/ml of glycogen and 10 mg/ml of malto-oligosaccharides. The total radioactivity in the eluate was then determined by liquid-scintillation counting of a 1-ml sample in 10 ml of Aquasol (New England Nuclear). In the case of Incubation 2, the column step was preceded by a batchwise treatment of the 0.2-ml aliquots by a slurry of 0.3 g of the resin in an aqueous solution of 10 mg/ml of glycogen and 10 mg/ml of malto-oligosaccharides.

Preparation of Sepharose-bound amylases. — Sepharose 4B (20 ml) was activated by being stirred at room temperature in a solution of 6 g of cyanogen bromide in 240 ml of water; the pH of the solution was maintained at 11 by dropwise addition

of 3M sodium hydroxide. The activated Sepharose was washed with 300 ml of ice-cold 0.2M sodium hydrogencarbonate, 100 ml of water, and 300 ml of 0.2M sodium phosphate buffer, pH 7.8. Alpha amylase (50,000 IU) or glucoamylase (100 IU) in 40 ml of 0.2M sodium phosphate buffer (pH 7.8) was added and the slurry was stirred for 10 h at 0–4°. The enzyme–Sepharose was then filtered off and washed with 1 liter of buffer 1, containing M potassium chloride, and then transferred to a column and washed with 1 liter of buffer 1 containing 0.5M potassium chloride, followed by 300 ml of buffer 2 containing 0.5M potassium chloride, and finally with 300 ml of buffer 3.

Determination of Sepharose-bound enzyme activity. — Sepharose–alpha amylase or Sepharose–glucoamylase was stirred in 6 ml of buffer 3, containing 10 mg/ml of glycogen, for 30 min at 25°. Hydrolysis of the glycogen was stopped by removal of the enzyme–Sepharose by filtration. Reducing sugar formed by alpha amylase[39] or the D-glucose formed by glucoamylase[40] was determined in the filtrate. One unit of Sepharose-bound alpha-amylase or glucoamylase was defined as one μmol of reducing sugar (glucose or maltose) formed per min under the conditions described.

Treatment of assay components with insolubilized amylases. — Starch synthase was treated with insolubilized enzymes as follows: The pH of 2 ml of the enzyme solution (in buffer 1, containing 0.5 mg/ml of BSA) was shifted to pH 7.0 by the addition of buffer 2 (~4 ml). Insolubilized enzymes (1 unit of Sepharose–alpha amylase and 0.1 unit of Sepharose–glucoamylase) were added and the suspension was stirred at 25°. After 30 min, the insolubilized enzymes were removed by filtration under suction on a sintered-glass funnel. The pH of the filtrate was brought to pH 7.6 by the addition of buffer 4. This preparation could be stored frozen at −20°. Treatments with insolubilized enzymes, and all experiments performed subsequent to these treatments, were carried out in glassware that had been cleaned with chromic acid. This was found to be essential for the reproducibility of the experiments.

The structure of the product of the "unprimed" reaction. — An incubation of starch synthase with ADP-D-[^{14}C]glucose was carried out in high concentration of citrate without added primer, as already described for Incubation 2, except that a 5-fold larger volume was used and sufficient enzyme was added so that the incorporation of ADP-D-[^{14}C]glucose into the ethanol-precipitable material was 83% after 20 min. The reaction was stopped by the addition of an equal volume of 12.5% trichloroacetic acid containing 4% of glycogen. The radioactive product was recovered from the trichloroacetic acid supernatant by ethanol precipitation (67%, v/v) and reprecipitated by ethanol three times. The final precipitate accounted for 80% of the labelled D-glucose used in the incubation. Portions (5 mg each) of the product were dissolved in 1 ml of 50mM sodium acetate buffer (pH 7.0) and were treated exhaustively (15 h) with beta amylase (100 IU), pullulanase (0.5 IU), and isoamylase (0.5 IU), respectively. After incubation, ethanol was added to a final concentration of 67% (v/v) and the radioactivity in the supernatant and the precipitate were determined.

Purification of starch synthase from sweet corn. — A commercial sweet corn (Golden Bantam Iowa Belle 104) was used as the enzyme source. The corn was

harvested at the milky stage (20–22 days after self-pollination) and stored at −70°. Extraction and purification of the enzyme were performed at 0–4°. Kernels (1 kg) were removed from the cobs and homogenized in 500 ml of buffer 1. The homogenate was passed through cheesecloth (crude extract) and was then centrifuged at 20,000*g* for 30 min.

The supernatant was stirred with 200 g of DEAE-cellulose (Whatman DE 52) previously equilibrated with buffer 1 for 30 min. The DEAE-cellulose was removed by suction and the filtrate was treated a second time with 100 g of DEAE-cellulose. Usually 70–80% of the total activity was adsorbed by this procedure. The DEAE-cellulose was repeatedly washed with 300-ml portions of buffer 1 on a Büchner funnel until the filtrate was no longer opalescent (about 15 times), indicating the removal of phytoglycogen. The DEAE-cellulose was then stirred with two 1-liter portions of buffer 1, containing 0.07M potassium chloride, for 30 min and then filtered. The filtrate, containing about 10% of the total activity, was discarded. The enzyme was released with two 500-ml portions of buffer 1 containing 0.25M potassium chloride. After removal of the DEAE-cellulose by filtration under suction, the filtrate was centrifuged at 20,000*g* for 30 min and the supernatant was brought to 45% saturation with solid ammonium sulfate. After stirring for 1 h, the precipitate was collected by centrifugation at 20,000*g* for 10 min and redissolved in 200 ml of buffer 1.

The solution was dialyzed against buffer 1 and then applied to a column (2.5 × 30 cm) of DEAE-cellulose. After washing the column with 400 ml of buffer 1, the enzyme was eluted with a linear gradient of 0.07–0.4M potassium chloride in 300 ml of the same buffer. Synthase activity was eluted as a single peak at 0.13M potassium chloride. The appropriate fractions were pooled and applied to a hydroxylapatite column (Bio-Gel HTP, Bio-Rad, 2.5 × 20 cm) that had been washed with buffer 1 until the phosphate concentration in the eluate was less than 2mM. The column was washed with 200 ml of buffer 1, and a linear gradient of potassium phosphate (0–0.2M) in 300 ml of the same buffer was applied. The enzyme was eluted between 5mM and 22mM phosphate. The active fractions were pooled, saturated with ammonium sulfate, and kept overnight at 0–1°. The precipitate was collected by centrifugation, redissolved, and dialyzed against buffer 1. (In the experiments described in this paper, 0.5 mg/ml of BSA was added to the enzyme preparation prior to dialysis.) The enzyme was stored frozen at −20°, and was stable over a period of several months. The presence of 10% sucrose was found to be essential for the stability of the enzyme.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation (GB 27491 and PCM 7500492). E.Y.C. Lee is an Established Investigator of the American Heart Association.

REFERENCES

1 L. F. LELOIR AND C. E. CARDINI, *J. Am. Chem. Soc.*, 79 (1957) 6340–6341.
2 L. F. LELOIR, J. M. OLAVARRIA, S. H. GOLDEMBERG, AND H. CARMINATTI, *Arch. Biochem. Biophys.*, 81 (1959) 508–520.
3 E. RECONDO AND L. F. LELOIR, *Biochem. Biophys. Res. Commun.*, 6 (1961) 85–88.
4 W. J. WHELAN, *Trends Biochem. Sci.*, 1 (1976) 13–15.
5 B. I. BROWN AND D. H. BROWN, *Methods Enzymol.*, 8 (1966) 395–403.
6 B. ILLINGWORTH, D. H. BROWN, AND C. F. CORI, *Proc. Natl. Acad. Sci. U.S.A.*, 47 (1961) 469–478.
7 D. H. BROWN, B. ILLINGWORTH, AND C. F. CORI, *Proc. Natl. Acad. Sci. U.S.A.*, 47 (1961) 479–485.
8 C. Y. TSAI AND O. E. NELSON, *Plant Physiol.*, 44 (1969) 159–167.
9 E. SLABNIK AND R. B. FRYDMAN, *Biochem. Biophys. Res. Commun.*, 38 (1970) 709–714.
10 M. ABDULLAH, E. H. FISCHER, M. Y. QURESHI, K. N. SLESSOR, AND W. J. WHELAN, *Biochem. J.*, 97 (1965) 9P.
11 E. Y. C. LEE AND J. J. BRAUN, *Arch. Biochem. Biophys.*, 156 (1973) 276–286.
12 A. KAMOGAWA, T. FUKUI, AND Z. NIKUNI, *J. Biochem. (Tokyo)*, 63 (1968) 361–369.
13 T. FUKUI AND A. KAMOGAWA, *J. Jpn. Soc. Starch Sci.*, 17 (1969) 117–129.
14 J. FOX, L. D. KENNEDY, J. S. HAWKER, J. L. OZBUN, E. GREENBERG, C. LAMMEL, AND J. PREISS, *Ann. N.Y. Acad. Sci.*, 210 (1973) 90–103.
15 J. PREISS, J. L. FOX, L. D. KENNEDY, E. D. GREENBERG, AND C. LAMMEL, *Federation Proc.*, 32 (1973) 603.
16 J. L. OZBUN, J. S. HAWKER, AND J. PREISS, *Biochem. Biophys. Res. Commun.*, 43 (1971) 631–636.
17 J. L. OZBUN, J. S. HAWKER, AND J. PREISS, *Plant Physiol.*, 48 (1971) 765–769.
18 J. L. OZBUN, J. S. HAWKER, AND J. PREISS, *Biochem. J.*, 126 (1972) 953–963.
19 J. S. HAWKER, J. L. OZBUN, AND J. PREISS, *Phytochemistry*, 11, (1972) 1287–1293.
20 J. S. HAWKER, J. L. OZBUN, AND J. PREISS, in R. PIRAS AND H. G. PONTIS (Eds.), *Biochemistry of the Glycosidic Linkage*, Academic Press, New York, 1972, pp. 529–539.
21 C. R. KRISMAN, *Biochem. Biophys. Res. Commun.*, 46 (1972) 1206–1212.
22 C. R. KRISMAN AND R. BARENGO, *Eur. J. Biochem.*, 52 (1975) 117–123.
23 R. BARENGO, M. FLAWIA, AND C. R. KRISMAN, *FEBS Lett.*, 53 (1975) 274–278.
24 N. LAVINTMAN AND C. E. CARDINI, *FEBS Lett.*, 29 (1973) 43–46.
25 N. LAVINTMAN, J. TANDECARZ, M. CARCELLER, S. MENDIARA, AND C. E. CARDINI, *Eur. J. Biochem.*, 50 (1974) 145–155.
26 S. SCHIEFER, *Fed. Proc.*, 32 (1973) 603.
27 S. SCHIEFER, E. Y. C. LEE, AND W. J. WHELAN, *FEBS Lett.*, 30 (1973) 129–132.
28 J. J. MARSHALL AND W. J. WHELAN, *FEBS Lett.*, 9 (1970) 85–88.
29 E. Y. C. LEE AND W. J. WHELAN, in P. D. BOYER (Ed.), *The Enzymes*, Academic Press, New York, 1971, 3rd edn., Vol. 5, pp. 191–234.
30 Z. GUNJA-SMITH, J. J. MARSHALL, E. E. SMITH, AND W. J. WHELAN, *FEBS Lett.*, 12 (1970) 96–100.
31 E. Y. C. LEE AND W. J. WHELAN, *Arch. Biochem. Biophys.*, 116 (1966) 162–167.
32 J. TANDECARZ, N. LAVINTMAN, AND C. E. CARDINI, *Biochim. Biophys. Acta*, 399 (1975) 345–355.
33 J. S. HAWKER, J. L. OZBUN, H. OZAKI, E. GREENBERG, AND J. PREISS, *Arch. Biochem. Biophys.*, 160 (1974) 530–551.
34 T. J. SCHOCH, *Methods Enzymol.*, 3 (1957) 5–17.
35 M. Y. QURESHI, Ph.D. Thesis, University of London (1967).
36 S. NAKAYAMA AND S. AMAGASE, *J. Biochem. (Tokyo)*, 54 (1963) 375–377.
37 C. MERCIER, B. M. FRANTZ, AND W. J. WHELAN, *FEBS Lett.*, 12 (1970) 96–100.
38 H. P. GHOSH AND J. PREISS, *Biochemistry*, 4 (1966) 1354–1361.
39 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375–380.
40 J. B. LLOYD AND W. J. WHELAN, *Anal. Biochem.*, 30 (1969) 467–469.