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Starch and Oligosaccharide Synthesis from Uridine Diphosphate Glucose*

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Several reactions in which uridine diphosphate glucose acts as a glucose donor have been described. The products of these reactions are: trehalose phosphate (1), sucrose (2), sucrose phosphate (3), bacterial cellulose (4), callose (5), glucosides (6), and glycogen (7-9). In a previous paper (10), it was reported that the starch granule fraction of plant tissue catalyzes the incorporation of the glucose moiety of uridine diphosphate glucose into starch. The results of enzymic degradation of the reaction product were consistent with the following formulation:

$$\text{UDP-glucose} + \text{acceptor} \rightarrow \text{UDP} + \alpha(1 \rightarrow 4)\ \text{glucosyl-acceptor}$$

Furthermore, it was reported that sucrose, glucose 1-phosphate, and glucose 6-phosphate were either ineffective or inferior to uridine diphosphate glucose as glucose donors. Further work on the subject is reported in this paper.

EXPERIMENTAL PROCEDURE

Substrates—UDP-glucose was obtained according to Pontis *et al.* (11). Radioactive UDP-glucose was prepared by incubation of glucose-6-P-C^{14} with UDP-glucose and *Saccharomyces fragilis* extract and isolated by paper chromatography (12). Malto-oligosaccharides were prepared by hydrolysis of amylose (13) and separation by a charcoal-Celite column (14). Radioactive samples of these oligosaccharides labeled at the reducing glucose unit were obtained from glucose-C^{14} with D-enzyme and soluble starch (15) and separated by paper chromatography.

Preparation of Enzyme—Active extracts have been obtained from young potatoes, sweet corn, and beans. A preparation of the latter was used in all the experiments described in this paper. The procedure was as follows. Freshly harvested immature dwarf beans (*Phaseolus vulgaris* Bountiful) were peeled, and the cotyledons and embryos were ground in a mortar with 2 volumes of water. The coarse material was removed by straining through cheese cloth, and the suspension of starch granules was centrifuged for 5 minutes at 3000 r.p.m. The white sediment was suspended in several volumes of water and recentrifuged. After the procedure had been repeated three times, the starch granules were suspended in 4 volumes of acetone at −15° and centrifuged at 0°. The latter procedure was repeated three times, after which the starch granules were dried in a vacuum.

Measurement of Enzyme Activity—The standard reaction mixture, unless otherwise indicated, contained (in μmoles): UDP-glucose, 0.3; EDTA,[1] 0.1; glycine buffer at pH 8.4, 4; and 5 mg of enzyme preparation. The total volume of fluid was 14 μl. After incubation at 37°, UDP formation, radioactivity, or both, were measured as follows.

UDP Formation—The pyruvate kinase procedure described previously was used (9). When the bean enzyme preparation was heated in order to stop the reaction, starch paste was formed which made difficult the subsequent mixing with the reagents. Therefore, in most experiments, the phosphopyruvate and pyruvate-kinase were added without inactivating the starch-forming enzyme.

Radioactivity Measurements—(*a*) *Starch:* After enzyme action, 0.5 ml of 50% ethanol was added and the suspension was centrifuged. The supernatant fluid containing oligosaccharides was set aside. The white precipitate was washed four times with 0.5 ml of 50% ethanol and then suspended in 0.4 to 0.5 ml of water, heated for 10 minutes at 100° in order to disperse the starch. Suitable aliquots were then plated on aluminum disks and counted with a gas flow counter (Tracerlab, Inc.). No correction was applied for self-absorption.

In experiments in which oligosaccharides were formed, it was observed that they were not removed completely from the starch by the above mentioned procedure. Therefore, in some experiments, the starch granules were first ruptured by heating in 0.2 ml of 0.1% Na_2SO_4 during 10 minutes at 100°, 2 volumes of 95% ethanol were added, and the suspension was centrifuged. The supernatant fluid was used for oligosaccharide estimation. The precipitate was redissolved in dilute Na_2SO_4 as before and reprecipitated. After the procedure was repeated four times, an aliquot was plated for measuring radioactivity.

(*b*) *Oligosaccharides:* The supernatant fluid obtained after the starch was centrifuged off as described above was diluted with 1 volume of water and passed through a column 0.5 cm diameter × 5 cm long of mixed-bed resin (Amberlite MB 3, acetate). The percolate was concentrated and either plated on aluminum disks for measuring the radioactivity or spotted on paper for chromatography. The solvent used was butanol-pyridine-water (6:4:3) (16), and the paper used was Whatman No. 1 or 4. The times of development were, respectively, about 48

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[1] The abbreviation used is: EDTA, ethylenediaminetetraacetate.

and 16 hours. Known samples of oligosaccharides were run at the same time and located with silver-NaOH (17). The radioactivity was measured (*a*) by cutting 1-cm strips, eluting with water, and plating on aluminum disks, (*b*) by introducing the paper strips directly in a gas flow counter, (*c*) by scanning automatically with a Nuclear-Chicago model D-47 gas flow counter fitted to C-100A actigraph II, ($\frac{1}{2}$ in collimator). The relative number of counts obtained in procedures *a*, *b*, and *c* were, respectively, about 100, 40, and 30.

Separation of Starch Components—The starch (5 mg) was defatted by washing three times with 1 ml of hot methanol and then was suspended in 1 ml of water. The suspension was heated at 100° and homogenized intermittently in a tightly fitting small glass homogenizer for 1 hour. A small precipitate was removed by centrifugation, and 0.01 ml of 10% thymol in ethanol (18) was added. After 3 days at room temperature, the precipitate (amylose fraction) was separated from the supernatant (amylopectin fraction).

Bromine Oxidation—The oligosaccharides separated by paper chromatography were eluted with water. An aliquot (0.5 ml) was treated with Br_2 and $BaCO_3$ as described by Smith and Srivastava (19). Excess Br_2 was removed by aeration, the solution was passed through a cation exchange resin (Dowex 50-H^+), and hydrolysis was carried out in 1 N HCl at 100° for 1 hour. The solution was concentrated under reduced pressure and dried in a desiccator over NaOH. After the residue was redissolved and neutralized with KOH, paper chromatography was carried out with butanol-pyridine-water (16) as solvent. The zones corresponding to glucose and gluconic acid were then counted directly on the paper with a gas flow counter.

Borohydride Reduction—The samples obtained as for bromine oxidation were treated with borohydride as described by Walker and Whelan (15), hydrolyzed, and chromatographed on paper with ethyl acetate-boric acid-acetic acid as solvent. The sorbitol and glucose zones were then counted as described before. In this procedure, as with Br_2 oxidation, the losses were considerable when small amounts (about 1 μmole) of oligosaccharides were used. In every case, known samples of labeled oligosaccharides were run at the same time.

RESULTS

Properties of Enzyme—The dried enzyme preparations could be stored for several months at −15° without appreciable loss of activity. Grinding the enzyme in the dry state or in buffer solutions led to complete inactivation. The dry enzyme resisted heating for 5 minutes at 100°. Suspended in water, about 25% of the activity was lost in 5 minutes at 50° and about 80% at 60°. Many attempts to extract the enzyme from the starch granules with different buffers, digitonin, or detergents gave negative results. The addition of EDTA (0.01 M) did not affect the activity and neither did 0.01 M Mg^{++}. The pH optimum was found to be about 8.2 in glycine or glycyl-glycine buffer and about 7.5 in phosphate buffer (Fig. 1). The rate of reaction increased with increasing amounts of enzyme (Fig. 2), but the response was not quite linear. The activity of the enzyme increased with temperatures up to 45° (Fig. 3), at which temperature the rate of reaction fell with time. At 37°, the reaction followed a linear course up to at least 4 hours, when the starch granules were not allowed to sediment either by shaking or by keeping the fluid volume low in relation to the amount of solids.

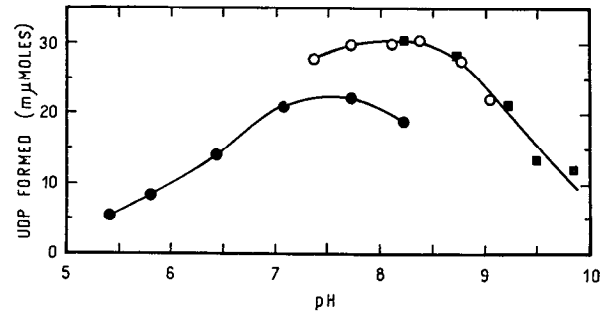

FIG. 1. pH optimum. ○——○, glycyl-glycine; ■——■, glycine; ●——●, phosphate buffer. The enzyme preparation (2 mg) was incubated for 2.5 hours (in μmoles) with: UDP-glucose, 0.32; EDTA, 0.1; and buffer indicated, 2. Total volume was 14 μl. Before UDP was measured, 4 μmoles of glycine buffer of pH 8.4 were added in order to correct for any change of pH that might affect the pyruvate kinase reaction.

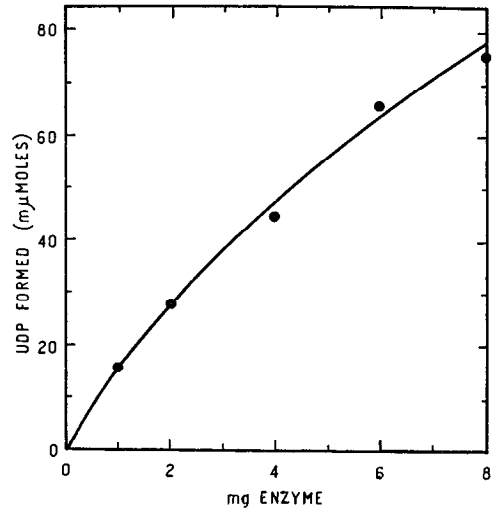

FIG. 2. Proportionality between action and amount of enzyme. The amounts of enzyme preparation indicated were incubated 1.5 hours at 37° with the standard reaction mixture.

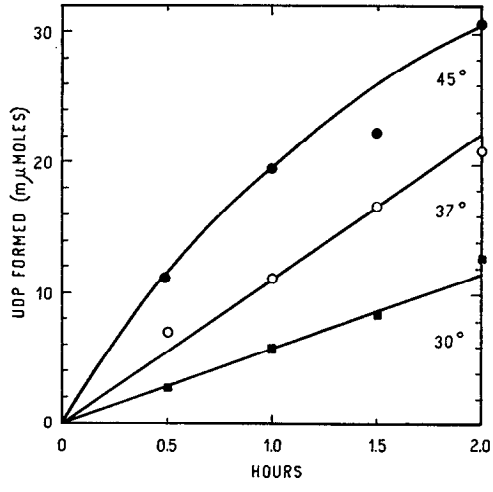

FIG. 3. Effect of temperature. The enzyme preparation (2 mg) was incubated (in μmoles) with: UDP-glucose, 0.32; glycine, pH 8.2, 2; and EDTA, 0.05. Total volume was 16 μl.

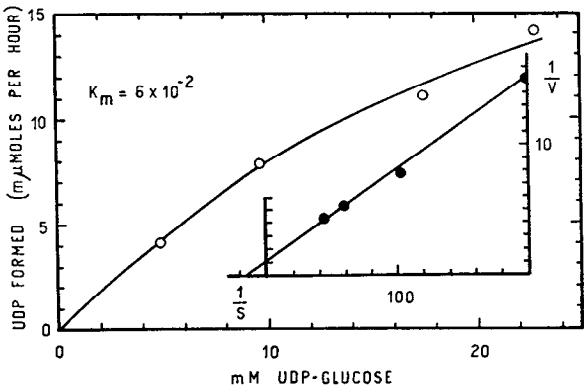

FIG. 4. Effect of UDP-glucose concentration. The standard reaction mixture with 2 mg of enzyme and variable amounts of UDP-glucose was incubated. The rates were calculated from straight lines joining the results of 1-, 2-, and 3-hour incubation periods.

Thus, with 15 μl of liquid for 5 mg of dry enzyme preparation, no visible sedimentation occurred during incubation.

The K_m for UDP-glucose was found to be about 6×10^{-2} (Fig. 4).

The main component of the enzyme preparation is starch. The protein content was found to be 3 μg per mg when measured with the method of Lowry *et al.* (20) after heating the starch granules 10 minutes at 100°. If the heating was omitted, the results were about 50% lower.

Separation of Amylose and Amylopectin—In the previous paper (10), it was reported that after incubation of UDP-glucose-C^{14} with the enzyme preparation, the radioactivity was transferred to starch and could be recovered as maltose by treatment with α- or β-amylase. These results showed that the glucose transferred from UDP-glucose becomes joined in α(1 → 4) linkage but did not give information as to which of the two starch components, amylose or amylopectin, was the glucose acceptor. An experiment designed to clear this point is shown in Table I. Some difficulty was experienced in achieving the complete solubilization of the starch granules. By simultaneous heating and mixing in a small glass homogenizer, solubilization was fairly good, and practically no radioactivity remained in the insoluble fraction. Subsequent separation with thymol gave the results appearing in Table I. It may be observed that the approximately equal amounts of radioactivity were found in the amylose and amylopectin fractions. The specific activity was about 3-fold higher in the former. However, it does not seem that any clear-cut conclusion can be drawn from these experiments, as to whether amylose is the precursor of amylopectin, or *vice versa*, or if both act as direct glucose acceptors from UDP-glucose.

Glucose Transfer to Oligosaccharides—It was found that the bean enzyme preparation catalyzed the transfer of glucose from UDP-glucose to malto-oligosaccharides. The process was studied in two ways, (*a*) with labeled UDP-glucose and unlabeled oligosaccharides, (*b*) with labeled oligosaccharides and unlabeled UDP-glucose.

(*a*) *Labeled UDP-glucose*—As shown in Fig. 5, the addition of maltose or maltotriose to the enzyme and UDP-glucose-C^{14} was found to lead to the formation of substances which migrated on paper as the corresponding higher oligosaccharides. Thus, addition of maltose gave mainly maltotriose and some tetraose and pentaose. Likewise, addition of maltotriose led to the formation of mainly tetraose and some pentaose and hexaose.

In other experiments, the radioactivity in the total oligosaccharides was measured by first removing the starch by precipitation with ethanol and the UDP-glucose-C^{14} with an ion exchange resin. One such experiment is shown in Table II. Addition of maltotriose to the enzyme and UDP-glucose-C^{14} increased UDP, led to the appearance of oligosaccharides, and decreased incorporation of radioactivity into starch. Glucose also decreased starch formation, but without giving rise to oligosaccharides. Other saccharides such as fructose, sucrose, cellobiose, gentiobiose, and salicin did not lead to oligosaccharide formation (Table II). In other experiments, glycogen was tested as glucose acceptor with negative results.

The different saccharides of the maltose series from glucose to maltopentaose were tested with the results shown in Table III. In that experiment, the starch was solubilized and reprecipitated several times in order to free it completely from oligosaccharides. All of the added saccharides were found to decrease the incorporation of glucose in starch and with the exception of glucose they all led to the formation of oligosaccharides. It appears therefore that glucose inhibits the enzyme and that the di- and

TABLE I

Separation of starch components

The standard reaction mixture, containing 0.32 μmole of UDP-glucose-C^{14} (17,000 c.p.m.), was incubated for 3 hours at 37°. The starch granules were washed with 50% ethanol and treated as described in "Experimental Procedure" for the separation of starch components. The two fractions were then analyzed for radioactivity and glucose content (21).

	Radioactivity	Glucose content	Specific activity
	c.p.m.	*μmoles*	*c.p.m./μmole of glucose*
Amylose.....	1100	6.6	166
Amylopectin....	980	19.0	51

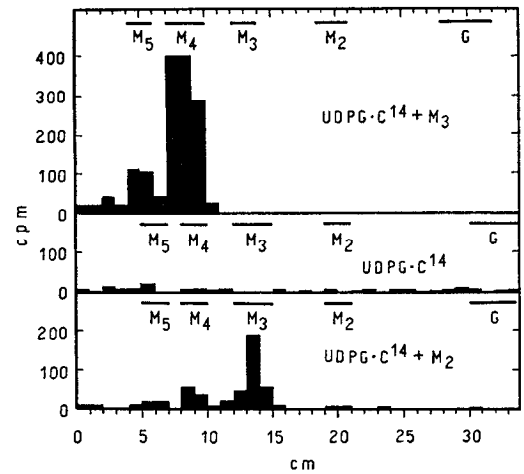

FIG. 5. Labeled UDP-glucose and unlabeled maltose and maltotriose. The standard reaction mixture (in μmoles) with: 0.22 of UDP-glucose-C^{14} (12,000 c.p.m.) plus 5 of maltose or 1 of maltotriose was used. The incubation lasted 1.5 hours except the maltotriose samples which were incubated 3 hours. After paper chromatography, the different zones were eluted and counted. The abbreviations used are: M_2, maltose; M_3, maltotriose; M_4, maltotetraose; and M_5, maltopentaose.

TABLE II

Glucose transfer from UDP-glucose-C^{14} to saccharides

The standard reaction mixture, containing 0.22 μmole of UDP-glucose-C^{14} (12,000 c.p.m.), was incubated for 3 hours with 1 μmole of additions indicated. Results are in mμmoles.

Experiment No.	Addition	Starch formed*	Oligosaccharides formed†	Sum	UDP formed‡
1	None	8.3	2.6	10.9	9.2
	Glucose	4.2	1.0	5.2	5.7
	Maltotriose	4.7	16.2	20.9	21.5
2	None	14.0	3.0	15.0	
	Salicin	10.3	0.9	11.2	
	Sucrose	12.1	2.6	14.7	
	Fructose	12.6	3.3	15.0	
	Gentiobiose	7.7	0.7	8.4	
	Cellobiose	8.2	4.6	12.8	

* Calculated from radioactivity of starch washed with 50% ethanol.

† Calculated from radioactivity of the fraction soluble in 50% ethanol and not removed by mixed-bed resin.

‡ Measured with pyruvate kinase.

TABLE III

Glucose transfer from UDP-glucose-C^{14} to oligosaccharides

The standard reaction mixture, containing 0.53 μmole of UDP-glucose-C^{14} (7300 c.p.m.), was incubated for 3 hours. The starch was heated and precipitated with ethanol as described in "Experimental Procedures." The background counts were subtracted.

Additions	Starch	Oligosaccharides	Sum
	c.p.m.	*c.p.m.*	
None (t = 0)	0	48	48
None	700	64	764
Glucose	304	58	362
Maltose	610	775	1385
Maltotriose	284	930	1214
Maltotetraose	370	894	1264
Maltopentaose	304	634	938

higher saccharides compete as acceptors with the starch present in the granules.

(*b*) *Labeled Oligosaccharides*—Many experiments were carried out with the use of labeled glucose, maltose, maltotriose, and maltopentaose, with and without unlabeled UDP-glucose.

Incubation with labeled glucose, with or without added oligosaccharides, did not give rise to the incorporation of radioactivity in starch or in oligosaccharides.

Incubation of the enzyme with labeled maltose, maltotriose, or maltotetraose and UDP-glucose gave rise to a definite formation of the corresponding higher saccharides. None were formed without UDP-glucose.

A representative experiment with radioactive maltotriose is shown in Fig. 6, and one with maltotetraose appears in Fig. 7.

Incubation with radioactive maltotriose either with or without UDP-glucose did not lead to any incorporation of radioactivity in the starch. Therefore, oligosaccharides do not seem to be intermediates in the transfer of glucose from UDP-glucose to starch.

Successive Action of UDP-Glucose and Maltose—Since it seemed possible that the transfer from UDP-glucose to oligosaccharides might occur in two steps, that is first from UDP-glucose to starch and then from starch to oligosaccharides, the experiment shown in Table IV was carried out. In a first incubation, the starch was made with radioactive with UDP-glucose-C^{14}, and it was then washed and incubated with maltose. It may be observed in Table IV that no radioactivity was transferred to maltose from starch (tube 1) and that the control (tube

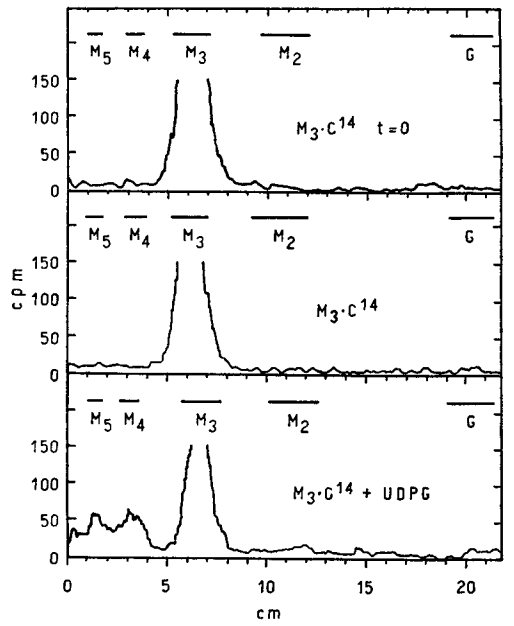

FIG. 6. Labeled maltotriose and unlabeled UDP-glucose. Standard reaction mixture plus 0.045 μmole of maltotriose-C^{14} (5600 c.p.m.). Time of incubation was 3 hours. After paper chromatography, the radioactivity was scanned automatically. Abbreviations are as in Fig. 5.

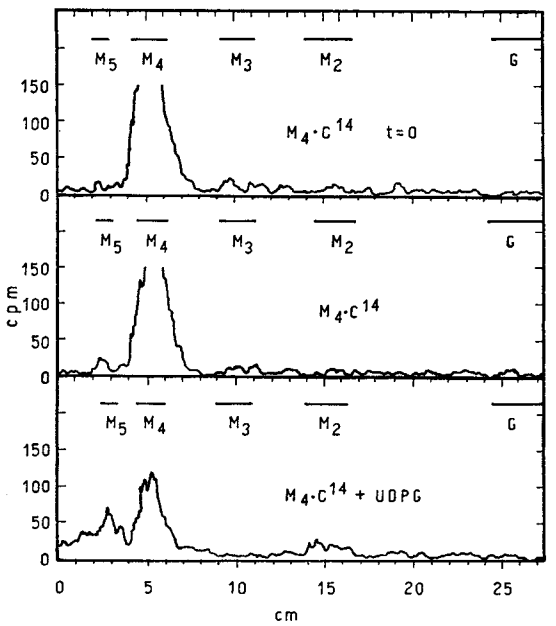

FIG. 7. Labeled maltotetraose and unlabeled UDP-glucose. Conditions as in Fig. 6, with 0.04 μmole of maltotetraose-C^{14} (5000 c.p.m.) instead of maltotriose-C^{14}. Abbreviations are as in Fig. 5.

3) showed that the enzyme was active during the second incubation.

Structure of Oligosaccharides—Inasmuch as the oligosaccharides produced by enzyme action were formed from $\alpha(1 \rightarrow 4)$-linked acceptors and not from others and because their behavior during chromatography on paper was identical with that of the $\alpha(1 \rightarrow 4)$ series, it seems reasonable to assume that they all belong to that series.

Some experiments were carried out in order to find out whether the glucosyl group added from UDP-glucose became attached to the reducing or to the nonreducing end of the acceptor. The reducing sugar unit is transformed into sorbitol by borohydride reduction and hydrolysis and into gluconic acid by bromine oxidation and hydrolysis. The results of these procedures applied to oligosaccharides obtained by enzyme action (Table V) are those expected if the glucosyl group from UDP-glucose became attached to the nonreducing end of the acceptor.

TABLE IV

Successive action of UDP-glucose and maltose

The standard reaction mixture, containing 0.15 μmole of UDP-glucose-C^{14} (8000 c.p.m.), was used. After the first incubation (2.5 hours), the enzyme was washed twice with 0.5 ml of water and reincubated for 3 hours with buffer and the different additions. Finally, 0.5 ml of 60% ethanol was added. The soluble fraction was passed through mixed-bed resin and counted (oligosaccharide fraction). The insoluble fraction was washed 4-fold with 60% ethanol (starch fraction).

Tube	Additions during		Starch	Oligosaccharide fraction
	First incubation	Second incubation		
			c.p.m.	
1	UDP-glucose-C^{14}	Maltose	620	4
2	UDP-glucose-C^{14}		560	20
3		UDP-glucose-C^{14} + maltose	400	260
4		UDP-glucose-C^{14}	520	70

TABLE V

Borohydride reduction and bromine oxidation of oligosaccharides

The samples obtained by incubating maltotriose with UDP-glucose-C^{14} (Fig. 5) or maltotriose-C^{14} with UDP-glucose (Fig. 6) with the enzyme were treated as described in "Experimental Procedure." The sample of maltotriose-C^{14} prepared as described under substrates was labeled at the reducing glucose unit.

Substance studied	Radioactivity		
	Glucose	Sorbitol	Gluconic acid
	c.p.m.	c.p.m.	c.p.m.
Borohydride reduction			
M_4* from UDP-glucose-C^{14} + M_3	112	13	
M_5 from UDP-glucose-C^{14} + M_3	70	16	
M_4 from UDP-glucose + M_3-C^{14}	17	262	
M_5 from UDP-glucose + M_3-C^{14}	14	147	
M_3-C^{14} (4000 c.p.m.)	16	417	
Bromine oxidation			
M_4 from UDP-glucose + M_3-C^{14}	12		82
M_5 from UDP-glucose + M_3-C^{14}	12		120
M_3-C^{14} (1000 c.p.m.)	10		290

* The abbreviations used are: M_3, maltotriose; M_4, maltotetraose; and M_5, maltopentaose.

DISCUSSION

There is evidence that in animal tissues, glycogen is synthesized by glucose transfer from UDP-glucose (8) and not from glucose-1-P as was believed. It has been pointed out that at least in some plant tissues (22), the ratio of inorganic phosphate to glucose-1-P is too high for the synthesis of starch via phosphorylase. Therefore, the finding of an enzyme which transfers glucose from UDP-glucose to starch may lead to a reinterpretation of present knowledge, and it may turn out that phosphorylase is mainly involved in starch breakdown.

The bean enzyme preparation which consists mainly of starch and has a very low protein content (3 μg per mg) was found to catalyze the transfer of glucose from UDP-glucose to starch and also to added maltose, maltotriose, or maltotetraose. The preparation appeared to be free from D-enzyme because it did not catalyze an exchange of radioactivity between glucose and oligosaccharides (15).

The K_m for UDP-glucose was found to have a very high value (6×10^{-2}) in relation to other enzymes; for instance, a value of 5×10^{-4} was obtained for UDP-glucose glycogen transglucosylase (9). However, it should be noted that the starch-synthesizing system consists of particles so that diffusion factors may be involved.

An interesting feature of the process is that it takes place in whole grains in which presumably the structural relation between enzyme and polysaccharide is the same as in the intact plant tissue.

SUMMARY

An enzyme has been detected in a bean starch granule preparation which catalyzes the following reaction, in which UDP is uridine diphosphate:

UDP-glucose + acceptor → UDP + $\alpha(1 \rightarrow 4)$ glucosyl-acceptor

The acceptor may be starch or a di- or oligosaccharide of the maltose series. Other disaccharides such as sucrose, cellobiose, gentiobiose, salicin, fructose, or glucose did not act as acceptors. Glucose inhibited enzyme activity.

The conditions for maximal activity have been determined.

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