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UDP-glucose: Glucan Synthetase in Developing Cotton Fibers

I. KINETIC AND PHYSIOLOGICAL PROPERTIES[1]

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ABSTRACT

A uridine diphosphate(UDP)-glucose:glucan synthetase can be demonstrated in detached cotton fibers (*Gossypium hirsutum* L.) and in an isolated particulate fraction from such fibers. When assayed with detached fibers, the kinetics of the glucan synthetase activity with respect to variation in substrate concentration is complex and indicates activation of the enzyme by the substrate. Activity is stimulated by Ca^{2+} or Mg^{2+} and β-linked glucosides; the effect of the β-linked glucosides is to shift the range in which substrate activation occurs to lower concentrations of UDP-glucose. At concentrations of UDP-glucose below 50 μM, addition of uridine triphosphate, in addition to β-linked glucoside, results in significant stimulation of activity. This effect can be explained by the conversion of uridine triphosphate to UDP-glucose by UDP-glucose pyrophosphorylase, thereby raising substrate concentration to the activating range. In detached fibers, glucan synthetase activity is high at all stages of fiber development. The properties of the glucan synthetase of the isolated particulate fraction closely resemble those of the enzyme assayed in detached fibers; however, in contrast to detached fibers, the ability to detect enzyme activity is more dependent on fiber age, showing maximal activity between 16 and 18 days postanthesis, coincident with the time of rapid onset of secondary wall cellulose deposition.

In our studies on cellulose biosynthesis in the developing cotton fiber, we have been investigating the utilization of UDP-glucose by enzyme systems present in the cotton fiber. We have done this because, for a variety of reasons (3), UDP-glucose can be considered as a likely precursor to cellulose. However, UDP-glucose can also serve as a precursor to such compounds as sucrose (4) and steryl glucosides (8, 15) in plants, and it is well known that in a variety of plant tissues, UDP-glucose can also serve as a substrate for the synthesis of β-(1 → 3)-glucan (2, 5, 6, 20-22), or for the synthesis of a glucan with mixed β-(1 → 3) and β-(1 → 4) linkages (14, 19).

We have studied the utilization of UDP-glucose by cotton fibers harvested at the onset of secondary wall cellulose synthesis, determining and comparing the activities both in fibers detached from the ovules ("semi-intact fibers"), and in a particulate fraction isolated from homogenized fibers. Since UDP-glucose can serve as substrate for several different enzyme systems, we have analyzed for a variety of possible reaction products, and by using different UDP-glucose concentrations and addition of cofactors, have attempted to decrease some of the side reactions and to promote the synthesis of alkali-insoluble glucans. In this paper, we shall be discussing the kinetic and physiological properties of an enzyme involved in glucan synthesis from UDP-glucose in cotton fibers without regard to the nature of the linkages of the product(s). Activity for this enzyme is present in high levels in a cell type actively engaged in secondary wall cellulose synthesis. Data in a companion paper (10) show that the predominant product is not cellulose, but rather a noncellulosic glucan with predominately β-(1 → 3) linkages.

MATERIALS AND METHODS

Growth of Plants. Seeds of *Gossypium hirsutum* L. (Acala SJ-1) were obtained from Hubert Cooper, Jr., USDA/ARS, U.S. Cotton Research Station, Shafter, Calif. Plants were grown in growth chambers under the following conditions: 11.5 hr in light (10 hr from fluorescent and incandescent lamps, 1.5 hr from incandescent lamps alone) at 33 C; 12.5 hr in dark at 22 C. Incandescent light alone at the end of the day was found to depress excessive elongation of the plants. On the morning of anthesis, the flowers were fertilized by a gentle brush stroke and labeled. No more than three bolls per plant were allowed to develop. For most experiments, bolls were cut at 16 to 20 days postanthesis, the time of the rapid onset of secondary wall cellulose deposition. Fibers with their associated unfertilized ovules were cultured *in vitro* by the procedure of Beasley and Ting (1).

Chemicals. UDP-[^{14}C]glucose [UL] (224 μCi/μmol) was purchased from New England Nuclear. β-(1→3)-Glucanase purified from *Rhizopus* was the generous gift of E. T. Reese, U.S. Army Laboratories, Natick, Mass. Laminaribiose was prepared by digestion of laminarin (Sigma) with this β(1→3)-glucanase. The disaccharide was purified from the digestion mixture by chromatography on a Bio-Gel P-2 column.

Determination of Radioactivity. Samples were dried on paper or filters and counted in toluene scintillation fluid (2.2 g PPO and 176 mg POPOP/l toluene). Aqueous samples, samples in chloroform-methanol, or undried fibers were counted in the same toluene scintillation fluid to which 1/10 volume of Bio-Solve (Beckman) has been added. All samples were counted in a Packard Tri-Carb model 3390 liquid scintillation spectrometer. Quench curves for various counting conditions were constructed, and where appropriate, corrections to dpm were made.

Detached Fiber Assay. Fibers of known age were removed from the boll, divided into 50-mg aliquots, and placed in tubes containing 2 ml 0.01 M TES buffer (pH 7.5) containing 1 mM EDTA. The fibers were agitated on a Vortex mixer with a small spatula in the tube until thoroughly wetted (about 30 sec). The individual aliquots were then removed from the tubes, drained on paper towels, and a single aliquot was placed into each reaction mixture at room temperature to start the enzyme assay.

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For standard assays, under low UDP-glucose concentration (hereafter designated as Low U-CB, UTP), the fibers were incubated in a final volume of 0.25 ml containing 12.5 μmol of TES buffer (pH 7.5), 1.25 μmol $MgCl_2$, 2.5 μmol CB,[4] 0.15 μmol UTP, and 300 pmol UDP-[^{14}C]glucose (356 cpm/pmol). Under high UDP-glucose concentration (hereafter designated as High U-CB), UTP was omitted and the final concentration of UDP-glucose was raised to 1 mM by addition of unlabeled UDP-glucose (final specific radioactivity 472 cpm/nmol). Product production was linear with respect to time for 15 to 20 min at room temperature. Standard assays were run for 10 min and terminated by the addition of 0.75 ml chloroform-methanol (1:2). The tubes were then kept for 1 hr at 37 C, followed by the addition of 0.25 ml chloroform and 0.25 ml 0.9% NaCl in 0.01 N HCl. The resulting mixtures were agitated on a Vortex mixer and centrifuged to separate layers (fibers remained at interface). If chloroform-methanol-soluble products were quantitated, 0.5 ml of the lower layer was counted in 10 ml Bio-Solve scintillation fluid. The fibers were removed from the mixture and thoroughly washed in water and counted in scintillation fluid. Radioactivity quantitated in the fibers by this procedure is referred to as insoluble product. Blank values have been subtracted for the data presented. These values were obtained by terminating reactions in the normal manner at zero time. Similar results were obtained by boiling the reactions at zero time prior to further incubation. For detached fiber assays, blank values ranged from 15 to 75 cpm for all experiments; in the assay of the particulate fraction, blank values were approximately 500 cpm. All data are presented as an average of duplicate or triplicate samples, and all experiments presented were repeated from two to eight times with similar results. Reproducibility of the standard assays was approximately ± 10%. Raw data representative of usual cpm obtained in the assays are shown in Table II.

Preparation and Assay of the Particulate Fraction. Bolls were harvested 16 days postanthesis (unless otherwise stated). The fibers were removed and thoroughly washed in TES-sorbitol buffer (50 mM TES [pH 7.5], 0.1 mM EDTA, 1 mM DTT, and 0.5 M sorbitol), blotted, weighed, and homogenized with a glass tissue homogenizer in 3 ml of TES-sorbitol buffer/g fibers. The homogenate was centrifuged 10 min at 700*g* to remove cell walls and cell debris. The supernatant (crude homogenate) was recentrifuged at 20,000*g* for 20 min. The resulting particulate fraction was resuspended in one-third the original volume of buffer identical to the homogenizing buffer except that the sorbitol was omitted. All procedures were performed at 4 C. For assay, the resuspended particulate fraction was mixed with an equal volume of a mixture of 10 or 40 mM $MgCl_2$ (as indicated), 0.2% digitonin, 20 mM CB, 2 mM UDP-[^{14}C]glucose (472 cpm/nmol). Any variations of this mixture are described in the text. The incubation was carried out at room temperature for 20 min, during which production of insoluble product was linear with time. The reaction was stopped by placing it in a boiling water bath for 10 min. The next day the samples were filtered onto Whatman GF/A glass fiber filters, thoroughly washed with water followed by chloroform-methanol (1:2), and counted in Bio-Solve.

Sucrose Density Gradient Centrifugation. Crude homogenate, prepared as described above, was layered onto a sucrose gradient (15–45% sucrose, w/v) containing TES buffer, DTT, and EDTA in the same concentration as the homogenate. The samples were centrifuged at 4 C for 2 hr at 114,000*g* in a Beckman SW 65 rotor. The gradient was fractionated and assayed as described above except that 10 mg of cellulose was added after boiling.

[4] Abbreviations: CB: cellobiose; LB laminaribiose; DTT: dithiothreitol.

Identification of Reaction Products Other Than Insoluble Products. Sucrose was identified by filtering the incubation medium through Whatman DE81 paper, thus removing the remaining UDP-[^{14}C]glucose. The solution which passed through the DE81 filters, when separated by paper chromatography, yielded one radioactive spot which migrated coincident with sucrose. Digestion of the filtrate by a highly purified invertase (Sigma, grade X) followed by paper chromatography showed glucose to be the only resulting radioactive product. Sterylglucosides and acetylated sterylglucosides were identified as the sole chloroform-methanol-soluble products by comparing the relative mobilities of these products to reported values (8, 15) after chromatography on silica gel plates (Brinkmann Polygram Sil G) with chloroform-methanol-H_2O (65:25:4) as solvent.

RESULTS

The time course of cotton fiber development has been determined under our growth conditions (13). Briefly stated, the rate of fiber elongation is maximal between 8 and 12 days postanthesis, and elongation ceases at about 24 days postanthesis. When expressed as a per cent by weight of the cell wall, there is an abrupt increase in the content of cellulose in the wall starting at about 16 days postanthesis, as secondary wall synthesis begins. Secondary wall cellulose synthesis is complete by 32 days postanthesis.

Products Formed from UDP-glucose in the Detached Fiber Assay. When detached fibers were incubated in TES buffer with 5 mM $MgCl_2$ and UDP-[^{14}C]glucose at 0.5 μM, they utilized up to 66% of the substrate within 10 min. Under these conditions, only a minor fraction of the substrate was incorporated into insoluble product(s). The substrate was primarily used for the production of sucrose and, to a lesser extent, of chloroform-methanol-soluble products. The ratio of sucrose to chloroform-methanol-soluble to insoluble product(s) under these conditions was 2,600:20:1. TLC of the chloroform-methanol-soluble products gave two peaks with R_F of 0.70 and 0.86, corresponding to sterylglucosides and acetylated sterylglucosides, respectively (8, 15). The possibility that these compounds could serve as intermediates in glucan synthesis seems remote since they do not appear to exhibit turnover of the glucose moiety. If fibers which have synthesized these compounds *in vitro* from UDP-[^{14}C]glucose are subsequently incubated in reactions lacking UDP-[^{14}C]glucose or containing 1 mM unlabeled UDP-glucose, no change in content of the previously synthesized [^{14}C]sterylglucosides is observed. Occasionally, trace amounts of a product with a mobility resembling that of glucosyl-phosphoryl-polyprenols could be detected (expected R_F 0.4, see ref. 7) but in amounts too low and variable to allow further study. Incubation of the fibers with a high UDP-glucose concentration (1 mM) resulted in increased production of all products, but the relative production of insoluble product(s) to other products was greatly enhanced. Thus, the ratio of sucrose to chloroform-methanol-soluble products to insoluble product(s) was now shifted to 18:0.5:1.

Activators of Glucan Synthetase Activity in the Detached Fiber Assay. Synthesis of insoluble product can be markedly increased by the addition of β-linked glucosides and UTP to the incubation mixture. However, the details of the effect were different depending upon the level of UDP-glucose present (Table I). Whereas at high UDP-glucose concentrations addition of β-linked glucosides alone was sufficient to enhance product formation, at low UDP-glucose concentrations it was necessary also to add UTP. Figure 1 shows that the UTP effect is observed only at substrate concentrations below 50 μM. The activating effect of the glucosides was relatively specific for the β linkage. In light of recent observations by Ray (16) and Villemez and Hinman (23) that UDP-xylose stimulates incorporation from

TABLE I

Effect of Various Compounds on Production of Insoluble Product

Fibers (18 days post-anthesis) were assayed using standard detached fiber assay as described in Materials and Methods using variations in components as described below. $MgCl_2$(5 mM) was present in all cases. Nucleoside di- and triphosphate were added at 1 mM; glucosides and glucose at 10 mM. Data are expressed as cpm, representing actual values measured during 10 min incubations, and also have been converted to pmol/hr/50 mg fresh weight of fibers.

Additions to Assay	Low UDP-glucose (1.2 μM)		High UDP-glucose (1.0 mM)	
	cpm	pmol (hr·50mg fibers)$^{-1}$	cpm	pmol (hr·50mg fibers)$^{-1}$
None	260	4.4	2,610	33,110
CB	370	6.3	6,190	78,740
LB	380	6.4	7,090	90,090
β-Me-glucoside			4,770	60,560
α-Me-glucoside			2,860	36,350
UTP	540	9.0	1,190	15,140
CB,UTP	8,500	143.2	3,550	45,150
LB, UTP	10,430	175.8	5,740	73,000
β-Me-glucoside, UTP	6,340	106.8		
α-Me-glucoside, UTP	1,220	20.5		
Sucrose, UTP	1,100	18.6		
Maltose, UTP	2,020	34.0		
Glucose, UTP	3,800	64.0		
CB, ATP	860	14.5		
CB, GTP	360	6.1		
CB, TTP	350	5.9		
CB, CTP	1,220	20.5		
CB, UDP	560	9.4		

UDP-glucose into an insoluble product, we have assayed our preparations with a range of UDP-xylose concentrations added, but in no case did we observe any stimulation.

Characteristics of the Activated Glucan Synthetase in Detached Fibers. In the standard assays at either low UDP-glucose concentration (1.2 μM plus CB and UTP) or high UDP-glucose (1 mM, plus CB only), the following properties were determined. Production of insoluble product was linear with time at 23 C for 15 to 20 min and proportional to fiber concentration up to 50 mg fresh weight fibers. The reactions had a broad pH optimum, being fully active in the range of pH 6.8 to 8.4. The reactions required divalent cations (5 mM) for optimal activity (see Table III). Magnesium and calcium ions are equally effective at high UDP-glucose concentrations; however, under Low U-CB, UTP conditions, only magnesium ions were effective. Strong support for the concept that UDP-glucose is being directly used for the synthesis of glucan comes from our observations that no substantial incorporation into insoluble product was observed when [^{14}C]sucrose, [^{14}C]glucose, or [^{14}C]glucose-1-P, either in the micromolar or millimolar range, was substituted for UDP-[^{14}C]glucose as substrate. (Data presented and discussed later will show, however, that [^{14}C]glucose-1-P can serve as a precursor in the presence of UTP, but this is not interpreted as direct utilization.) GDP-glucose, at a variety of concentrations, neither stimulated nor inhibited incorporation from UDP-[^{14}C]glucose.

The effect of varying the CB and UTP concentration on production of insoluble product in the detached fiber assay is shown in Figure 2. Activity increases with increasing cellobiose concentrations and no inhibition is observed even at high concentrations of cellobiose. Optimal UTP concentration was found to be approximately 0.6 mM. For standard assays, we have chosen to use 10 mM cellobiose and 0.6 mM UTP.

Kinetics with respect to UDP-glucose concentration was also examined with the detached fiber assay in the presence or absence of CB. At low substrate concentrations, the kinetic data do not follow the standard Michaelis-Menten form and suggest substrate activation (Fig. 3A). The effect of CB is to shift the range of this activation to lower substrate concentrations. Since enzyme activity remains constant throughout the time course of these experiments even in the absence of CB, stabilization, rather than activation, by CB can be ruled out. At high concentrations of UDP-glucose, kinetic data approach the standard Michaelis-Menten form. Lineweaver-Burk plots of data obtained at high UDP-glucose concentrations (Fig. 3B) yield an identical V_{max} with or without cellobiose; the apparent K*m* for UDP-glucose without CB is 5 mM and with CB is 1.7 mM.

Possible Explanation of the CB-UTP Effect. The unusual requirement for both CB (or other β-linked glucosides) and UTP at very low UDP-glucose concentrations could be explained in several ways. One possibility is that there is a single glucan synthetase with a low affinity for UDP-glucose and stimulated only by CB, and that UTP is required because it inhibits competing reactions such as sucrose synthesis, or because it serves as a substrate for the UDP-glucose pyrophosphorylase reaction and thereby raises the concentration of UDP-glucose to a range where substrate activation and thus, higher product production occurs. A second possibility is that there are two glucan synthetases, one with a high affinity for UDP-glucose and requiring both CB and UTP for activity, and a second with a low affinity for UDP-glucose and stimulated only by CB. Because

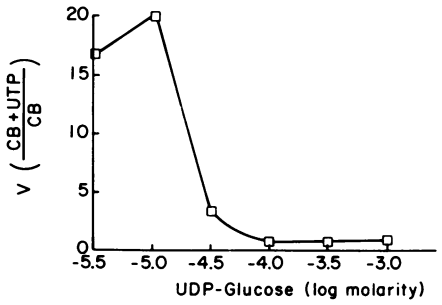

FIG. 1. UTP effect as a function of UDP-glucose concentration. The ratio of reaction velocity in the presence of CB and UTP to that in the presence of CB alone is plotted as a function of UDP-glucose concentration.

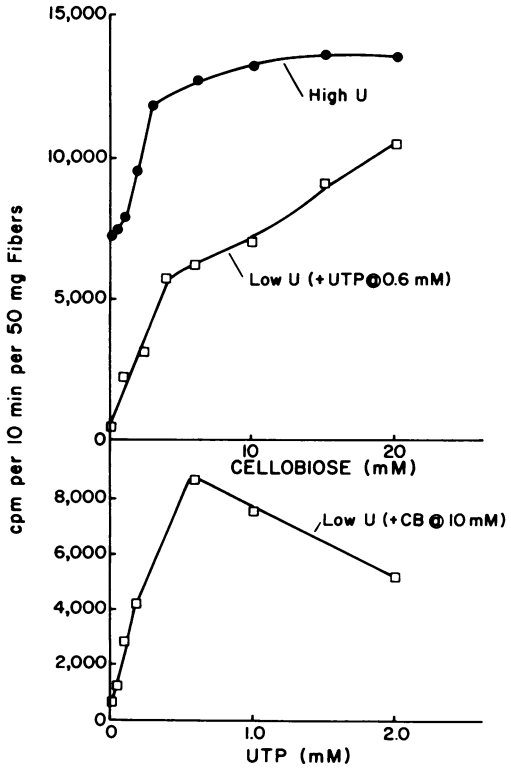

FIG. 2. Effect of varying concentration of effectors on glucan synthetase activity in the detached fiber assay. Standard assays were performed as described under "Materials and Methods" with the exception that the CB or UTP concentration was varied as indicated. Low U = 1.2 μM UDP-glucose; High U = 1 mM UDP-glucose.

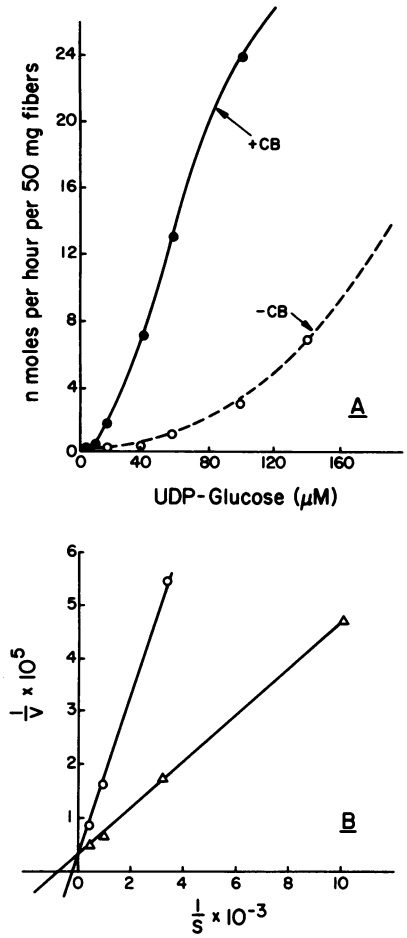

FIG. 3. Kinetics of glucan synthetase with varying UDP-glucose concentrations. Standard detached fiber assays were run as described under "Materials and Methods" with the exception that the UDP-glucose concentration was varied and initial reaction rates were determined for each substrate concentration. V = nmol product formed/hr·50 mg fibers; A: V *versus* S plot at low UDP-glucose concentrations; B: Lineweaver-Burk plot at high UDP-glucose concentrations; (O——O): no effectors; (Δ——Δ): CB (10 mM) present.

the enzyme(s) studied are by no means purified, no definitive conclusion can be given, but the following data strongly indicate that the explanation is most likely the one involving UDP-glucose pyrophosphorylase. First, no significant inhibition by UTP of competing reactions (sucrose or sterylglucoside synthesis) has been observed (data not shown). Second, the linkages in the glucan product obtained either under Low U-CB, UTP or High U-CB conditions appear to be, within the limits of our analyses, identical, indicating although not proving, a single enzyme. Third, if [^{14}C]glucose-1-P is substituted for UDP-[^{14}C]glucose at low substrate concentrations (Table II), incorporation of radioactivity into acetylated and nonacetylated sterylglucosides occurs only when UTP is present. CB neither activates nor inhibits this activity. Incorporation of radioactivity into insoluble product, however, requires both CB and UTP. Such a result is entirely consistent with the conversion of UTP and glucose-1-P into UDP-glucose and PPi. The UDP-glucose thus produced can be used either for the synthesis of sterylglucosides (± CB) or for glucan (+ CB). Under the Low U-CB, UTP assay conditions, endogenous glucose-1-P must be present to support such a reaction sequence.

Further support for this explanation is our finding that pyrophosphate is a potent inhibitor of glucan synthesis under Low U-CB, UTP conditions, and that there are very high levels of UDP-glucose pyrophosphorylase in cotton fiber extracts at all stages of fiber development (3). Table III shows further supporting evidence. Under High U-CB conditions, production of glucan is stimulated either by Ca^{2+} or Mg^{2+}. However, under Low U-CB, UTP conditions, only Mg^{2+} is effective, and the same is true when [^{14}C]glucose-1-P is substituted for UDP-[^{14}C]glucose as radioactive substrate. We have also recently directly observed the production of both UDP-[^{14}C]glucose and [^{14}C]sucrose from UTP and [^{14}C]glucose-1-P, and this production is much higher in the presence of $MgCl_2$ than in the presence of $CaCl_2$, suggesting that the specificity for Mg^{2+} is associated with UDP-glucose pyrophosphorylase and not glucan synthetase.

Activity as a Function of Fiber Age. Fibers of various ages from 8 to 32 days postanthesis were assayed with the detached fiber assay under standard conditions (Low U-CB, UTP or High U-CB). Because protein content, fresh weight, dry weight, and fiber length are changing throughout development, it is difficult to know how best to standardize the activities. One rather unique way of expressing the activity is as a function of fiber length; this may be the most valid means of comparing changes in the level of a potential cell surface activity. However, regardless of whether activity is expressed per fresh weight of fiber or as activity per unit length of fiber, values do not vary by more than a factor of 2.5 as a function of fiber age throughout this developmental time.

Comparison of Activity in Detached and Attached Fibers. The fact that a variety of activities utilizing UDP-glucose can be demonstrated with the detached fiber assay raises the question of whether these activities are surface-localized (cell wall or outer plasmalemma) or whether the fibers must be at least partially

TABLE II

[^{14}C]-Glucose-1-P as a Substrate in the Detached Fiber Assay

Assay conditions are as described in Materials and Methods for standard Low U-CB, UTP assays except as indicated below and that [^{14}C]-glucose-1-P (0.92 μM; 381 cpm per pmole) was substituted for UDP-[^{14}C]-glucose as indicated. Fibers used were harvested 18 days post-anthesis.

Radioactive Substrate	Effectors Present	Products of Assay: Sterylglucosides and Acetylated Sterylglucosides	Products of Assay: Insoluble Product
		cpm	
UDP-[^{14}C]-glucose	CB	1,618	588
UDP-[^{14}C]-glucose	CB, UTP	1,532	5,292
[^{14}C]-glucose-1-P	none	72	230
[^{14}C]-glucose-1-P	CB	130	240
[^{14}C]-glucose-1-P	UTP	1,919	380
[^{14}C]-glucose-1-P	CB, UTP	2,014	5,872

TABLE III

Effects of Divalent Cations on Production of Insoluble Product

Standard detached fiber assays were performed as described in Materials and Methods with the exceptions of variations in divalent cations and substitution of [^{14}C]-glucose-1-P (0.92 μM; 381 cpm per pmole) for UDP-[^{14}C]-glucose as indicated. Fibers were harvested 18 days post-anthesis.

Assay Condition	Cation at 5 mM	Insoluble Product Produced (cpm)
High U-CB	none	3,387
High U-CB	$MgCl_2$	9,753
High U-CB	$CaCl_2$	9,005
Low U-CB, UTP	none	448
Low U-CB, UTP	$MgCl_2$	6,028
Low U-CB, UTP	$CaCl_2$	666
As for Low U-CB, UTP except that [^{14}C]-glucose-1-P substituted for UDP-[^{14}C]-glucose	none	162
	$MgCl_2$	7,468
	$CaCl_2$	574

damaged in order to exhibit activity. Certainly damage does occur in removing the fibers from the ovules, and since fibers developing in the boll are tightly interwoven, it is infeasible to assay them in an undamaged state. With fibers cultured *in vitro* (1), the submerged portion of fibers is easily assayed intact by simply removing them from the culture medium, gently washing in buffer to remove this medium, and then placing ovule plus fiber in the assay, having only the lower fibers submerged. At the end of the incubation, the lower fibers are cut off and extracted as for the standard assay, and incorporation is compared to similar incubations where the lower fibers were cut from the ovule prior to assaying. In our first attempts at these experiments, substantial incorporation into insoluble product (40% of cut fiber activity) was observed; however, as we became more careful in our washing and handling of the fibers, we now observe that activity under Low U-CB, UTP or High U-CB conditions in uncut fibers is never more than 10% of the activity obtained with a comparable amount of fibers cut from the ovule before the assay. Thus, it appears that the fibers do require some damage in order for activity to occur.

Glucan Synthetase Activity in an Isolated Particulate Fraction. In an attempt to determine the localization of the glucan synthetase activity in the cotton fibers, high activity for incorporation of radioactivity from UDP-[^{14}C]glucose into insoluble product was found in an isolated particulate fraction (prepared from crude homogenate) sedimenting between 1,000 and 20,000*g*. Cell walls, the particulate fraction recovered between 20,000*g* and 100,000*g*, and the 100,000*g* supernatant were virtually free of activity. Sucrose synthesis was not demonstrable under any condition in the 1,000 to 20,000*g* fraction, but high activity for sterylglucoside synthesis was observed in the absence, but not in the presence, of digitonin. Comparison of average glucan synthetase activity of the particulate fraction with average rates observed at 1 mM UDP-glucose (plus CB) with detached fibers indicates that we routinely recover between 7 and 15% of the activity measurable in the detached fiber assay. Table IV shows the activating effects of various glucosides. These results are similar to those observed with detached fibers where the β-linked glucosides are better activators. As for detached fibers under High U-CB conditions, UTP is not required and is actually inhibitory. Only slight stimulation was demonstrated under Low U-CB, UTP conditions, a fact which could be caused either by low levels of UDP-glucose pyrophosphorylase activity in this fraction (a large part of this activity is soluble under our extraction conditions) or by lack of the second substrate, glucose-1-P. The latter certainly appears to be a contributing factor since addition of CB, UTP and glucose-1-P does stimulate activity, and as for detached fibers, [^{14}C]glucose-1-P, in high concentrations, can serve optimally as substrate only in the presence of both CB and UTP. Table IV also shows that Mg^{2+} and digitonin are essential for maximal activity.

TABLE IV

Effect of Various Compounds on Production of Insoluble Product by the Particulate Fraction

Particulate fraction was prepared from fibers harvested 18 days postanthesis and assayed essentially as described in Materials and Methods. $MgCl_2$ was present at 5.0 mM and digitonin at 0.1% except where indicated. Other components were added to give following concentrations: UTP, 1.0 mM; glucose-1-P (unlabeled), 1.0 mM, glucosides or glucose, 10.0 mM. Activity is expressed as pmol product produced per hr per aliquot of particulate fraction equivalent to that obtained from 50 mg fresh weight of original fibers.

Additions to Assay	Radioactive Substrate			
	UDP-[^{14}C]-glucose		[^{14}C]-glucose-1-P	
	1.2 μM	1.0 mM	0.92 μM	1.0 mM
None	0.57	2,960		
CB	0.84	10,830	1.6	510
LB	0.70	13,370		
β-Me-glucoside		9,840		
α-Me-glucoside		3,530		
Maltose		6,970		
Glucose		8,680		
UTP	0.65	2,070		840
CB, UTP	1.58	7,790	3.7	2,170
CB, glucose-1-P	0.66			
CB, glucose-1-P, UTP	3.00	4,920		
LB, glucose-1-P, UTP	4.82			
CB, glucose-1-P, UTP, minus $MgCl_2$	0.54			
CB minus $MgCl_2$		340		
CB, glucose-1-P, UTP minus digitonin	0.66			
CB minus digitonin		990		

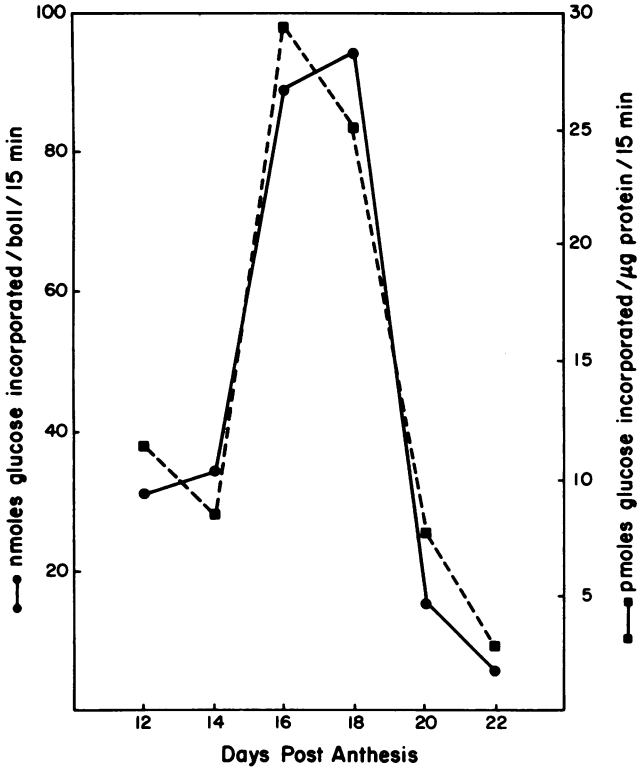

FIG. 4. Glucan synthetase activity in the particulate fraction as a function of fiber age. Particulate fractions were prepared from fibers harvested from bolls of different ages and assayed as described under "Materials and Methods." $MgCl_2$ was present in assay at 20 mM.

The broad distribution of the activity obtained on a sucrose density gradient indicates that under our conditions of extraction, the activity is associated with a mixture of different organelle fractions.

The particulate fraction is most active between 16 and 18 days postanthesis, just at the time of onset of secondary wall synthesis and just before the cell walls show a distinct birefringence (Fig. 4). A similar curve is obtained regardless of whether the activity is expressed as activity per boll, per μg protein, per mm fiber length, or per mg fresh weight (the latter two not shown). In contrast to the situation in detached fibers where activity remains high at much later stages of development, the activity in the particulate fraction has dropped sharply by day 22. Whether the activity may rise again after this time has not been tested because the older fibers are difficult to break.

DISCUSSION

Any discussion of the physiological significance of the noncellulosic glucan synthetase(s) of cotton fibers is best deferred until data which describe the details of the linkages of the product are presented (10). Discussion here will thus be limited to an appraisal of the kinetic and physiological properties of the enzyme(s).

It is clear that a variety of products can be synthesized from UDP-glucose in this system. With detached fibers, sucrose is the

only detectable water-soluble product. We have no evidence for the production of glucosylinositol which has been reported in *Phaseolus aureus* (11, 12). The predominant products soluble in chloroform-methanol are acetylated and nonacetylated sterylglucosides. These are relatively acid-stable and show no evidence of turnover in our experiments, although activity for synthesis of these compounds remains high throughout fiber development, increasing somewhat with fiber age up to about 30 days postanthesis. Occasionally, we observed the production of trace (< 1%) amounts of a product with an R_F corresponding to a glucosyl-phosphoryl-polyprenol of the type reported by Forsee and Elbein (7). None of the data presented here seem to implicate a role for this compound as an intermediate in the synthesis of noncellulosic glucan, although it is impossible to exclude such an intermediate if it turns over rapidly and has a very low steady-state level.

Some of the data presented could indicate that there is more than one enzyme system involved in the production of noncellulosic glucan: (*a*) the complexity of the kinetics with respect to UDP-glucose concentration; (*b*) the requirement for UTP at low, but not at high UDP-glucose concentrations; and (*c*) the effectiveness of Ca^{2+} in stimulating activity at high, but not at low UDP-glucose concentrations. However, analyses of the products obtained at both high and low concentrations of UDP-glucose show them to be quite similar (10). The data described in Tables III and IV strongly indicate that the requirement for UTP at low UDP-glucose concentrations is based on the action of UDP-glucose pyrophosphorylase which raises the level of UDP-glucose in the assay to more optimal concentrations. Nevertheless, an analysis of the data of Figure 3A shows that if a single glucan synthetase is present, it shows substrate activation by UDP-glucose in the lower concentration ranges (0.01–0.1 mM). The glucan synthetase described here seems to resemble closely the β-(1→3)-glucan synthetase in *Avena* coleoptiles (22) which showed stimulation by CB and substrate activation by UDP-glucose. It differs in that the *Avena* enzyme is not stimulated by divalent cations as is the cotton fiber enzyme.

The level of activity of the noncellulosic glucan synthetase(s) in detached cotton fibers is quite high. A moderately high percentage (up to 15%) of the High U-CB activity was also recovered in an isolated particulate fraction. This is in contrast to the cell surface glucan synthetase activity described by Shore and MacLachlan in pea stems (17, 18); in that case a lower percentage (3–5%) of a similar activity was detected in cell-free homogenates. The initial rates of activity in cotton fibers at early stages of development exceed our calculated rates of *in vivo* cellulose deposition; however, these rates are obtained using 1 mM UDP-glucose as substrate, a concentration which could exceed the endogenous levels (9, 19).

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