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β-Glucan Synthesis in the Cotton Fiber[1]

II. Regulation and Kinetic Properties of β-Glucan Synthases

Likun Li and R. Malcolm Brown, Jr.*

Department of Botany, The University of Texas at Austin, Austin, Texas 78713–7640

The regulation and kinetic properties of cellulose synthase as well as β-1,3-glucan synthase have been studied. The cellulose was detected using acetic/nitric acid insolubility as an indicator of cellulose (this product contained only β-1,4-linked glucans; K. Okuda, L. Li, K. Kudlicka, S. Kuga, R.M. Brown, Jr. [1993] Plant Physiol 101: 1131–1142). These studies reveal that (a) β-1,3-glucan synthesis is enhanced up to 31-fold by cellobiose with a K_a of 1.16 mM; (b) cellulose synthesis is increased 12-fold by a combination of cellobiose (K_a = 3.26 mM) and cyclic-3′:5′-GMP (K_a = 100 μM); (c) the common components in the reaction mixture required by both enzymes are cellobiose, calcium, and digitonin; (d) cellulose synthase has an essential requirement for magnesium (K_a = 0.89 mM); (e) cellulose synthase also requires a low concentration of calcium (K_a = 90 μM); (f) the optimal pH for cellulose synthase (7.6–8.0) is slightly higher than that for β-1,3-glucan synthase (7.2–7.6); (g) the K_m for UGP-Glc for cotton (*Gossypium hirsutum*) cellulose synthase is 0.40 mM; (h) the K_m for UDP-Glc for for β-1,3-glucan synthase is 0.43 mM.

Previous enzyme assay methods for cellulose synthase have been based on alkali-insoluble products; however, these products contained both β-1,4-glucan and β-1,3-glucan or only β-1,3-glucan in the presence of magnesium (Hayashi et al., 1987). The AN reagent has been used for isolation of cellulose (Updegraff, 1969), but unfortunately, β-1,4-glucan synthesized in vitro was found to be soluble in this reagent (Bacic and Delmer, 1981; Read and Delmer, 1991). Because of such reports in the literature and the difficulties in securing an ANIP, most investigators have not used the AN reagent in the assay for in vitro cellulose synthesis.

In view of the lack of progress in accomplishing in vitro cellulose biosynthesis from higher plant extracts, we decided to use the valuable experience gained from the *Acetobacter* system and apply it to the cotton (*Gossypium hirsutum*) fiber. Based on numerous trials, we have found that about 4% of the total glucan isolated as the ANIP (Okuda et al., 1993) could be synthesized in vitro with an optimal combination of CB, cyclic nucleotide, magnesium, calcium, and digitonin. We also have found that the ANIP contains exclusively β-1,4-linked glucan, which has been identified as crystalline cellulose. In addition, approximately 25% of the total glucans is found as β-1,4-glucan and 69% as β-1,3-glucan in the EIP fraction synthesized under conditions favoring β-1,4-glucan synthesis (Okuda et al., 1993). In addition, we have discovered that the EIP produced only with CB and calcium as effectors yielded essentially β-1,3-glucan (Okuda et al., 1993).

The research described in this report provides new information for in vitro β-1,4-glucan synthesis from membrane fractions of cotton fibers, including data concerning putative activators, the combination of cofactors, and kinetic properties of β-1,3- and β-1,4-glucan synthases. From these results, we have gained new insight into the perennial and continuing problems underlying in vitro cellulose synthesis in higher plants.

MATERIALS AND METHODS

Chemicals

UDP-[U-^{14}C]Glc (200 mCi/mmol) was purchased from ICN Biochemicals Inc. (Costa Mesa, CA). Cyclic diguanylic acid was a gift from Dr. J.H. van Boom of the Gorlaeus Laboratories (Department of Organic Chemistry, Leiden, The Netherlands). Digitonin was purchased from Serva (Heidelberg, Germany). Nucleotides, CB, octyl-β-glucoside, protease inhibitors, and other chemicals were obtained from Sigma (St. Louis, MO). Gx, an extract from *Acetobacter xylinum* containing cyclic diguanylic acid, was prepared as described by Lin and Brown, 1989.

Plant Materials

The cotton line, *Gossypium hirsutum* Texas marker 1 (TM-1) was used for the experiments. For details, see Okuda et al. (1993).

Preparation of Plasma Membrane-Enriched Fractions

The plasma membrane-enriched fraction of cotton fibers was prepared by a modification of the procedure of Delmer et al. (1984). For details, see Okuda et al. (1993). Protein assays were performed using a modification of the Lowry procedure (Markwell et al., 1978).

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* Corresponding author; fax 1–512–471–3573.

Abbreviations: AN reagent, acetic/nitric acid reagent; ANIP, acetic/nitric acid reagent-insoluble product; CB, cellobiose; EIP, ethanol-insoluble product; Gx, unknown compound made from GTP.

Enzyme Assay

Assay for β-1,4-glucan synthase was performed for 15 min in a reaction mixture containing bis-trispropane-Hepes buffer, CB, magnesium, calcium, c-3′:5′-GMP, digitonin, plasma membrane-enriched fraction, and UDP-[U-^{14}C]Glc (specific activity = 12,5000 cpm/nmol) in a final volume of 100 μL. The concentration of the components in the reaction mixture for particular experiments will be described. The reactions were terminated by addition of 1 mL of AN reagent (Updegraff, 1969) and incubated in a boiling water bath for 30 min. The ANIP was collected by filtration on a GC/F glass filter, washed once with 0.5 N NaOH, three times with distilled water, and once with methanol. The radioactivity retained on the filter was dissolved in a Ready Organic cocktail (Beckman Instruments, Inc., Fullerton, CA) and counted with an LS 6800 Liquid Scintillation System (Beckman Instruments, Inc., Irvine, CA).

The reaction mixture for the β-1,3-glucan synthase reaction was composed of bis-trispropane-Hepes buffer, CB, calcium, digitonin, enzyme protein, and UDP-[^{14}C]Glc in a final volume of 100 μL. The concentrations of the components in the reaction mixture for specific studies will be indicated. The reactions were conducted for 15 min at 25°C, terminated by addition of 2 mL of 66% ethanol with 0.85 mM EDTA (Henry and Stone, 1982), and kept at −20°C for at least 30 min (the EDTA is used to chelate divalent cations essential to the enzyme activity and thus promote the termination of the reaction). The EIP was collected by filtration as described above and washed with three changes (5 mL each) of 66% ethanol and once with methanol:chloroform (1:1, v/v). The radioactivity retained on the filter was counted as described above. Kinetic parameters were obtained from Lineweaver-Burk plots.

RESULTS

Test for Putative Activators

The test for putative activators is summarized in Table I. β-1,3-Glucan is produced under specified conditions without magnesium in the reaction mixture and is collected from the EIP fraction. The ANIP is synthesized under conditions with magnesium in the reaction mixture (for product properties, see Okuda et al., 1993).

The formation of an ANIP from cotton is not enhanced by cyclic diguanylic acid alone. This is different from the in vitro cellulose synthesis by *A. xylinum*, which was enhanced up to 200-fold by cyclic diguanylic acid (Ross et al., 1987).

It is interesting that in vitro cellulose synthesis in cotton is stimulated about 5-fold by CB. In addition, formation of the ANIP is increased 12-fold by a combination of CB and c-3′:5′-GMP, and 11-fold by a combination of CB and cyclic diguanylic acid.

When CB is replaced by octyl-β-glucoside in combination with c-3′:5′-GMP, a similar stimulation is obtained. During a search for activators of cellulose synthase in cotton, we unexpectedly found that thymine monophosphate and ATP stimulated the formation of the ANIP. The mechanism of this stimulation is still unclear.

We can conclude from these experiments in combination with extensive product analysis (Okuda et al., 1993) that the ANIP is a homopolymer with exclusive β-1,4-linkages. Therefore, the conditions that promote the synthesis of the ANIP also favor cellulose assembly. This represents a significant breakthrough in the search to produce cellulose in vitro from eukaryotic cells.

As shown in Table I, the formation of an EIP that is largely β-1,3-glucan (Okuda et al., 1993) is greatly enhanced by CB. The stimulation is up to 31-fold compared with the control. Octyl-β-glucoside also activates β-1,3-glucan synthase, but the efficiency is about half that of CB. In some experiments with a low UDP-Glc concentration (20 μM), addition of c-

Table I. *Test for putative activators*

The reaction mixture for EIP contained 10 mM bis-trispropane-Hepes (pH 7.4), 2 mM $CaCl_2$, 0.05% digitonin, 0.5 mM UDP-[^{14}C]-Glc, 10 μg of enzyme proteins, and the tested compound. The reaction mixture for ANIP was composed of 10 mM bis-trispropane-Hepes (pH 7.6), 8 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% digitonin, 0.5 mM UDP-[^{14}C]Glc, 10 μg of enzyme proteins, and the tested compounds. Final volume of the reaction mixture for both assays was 100 μL. The concentrations of the tested compounds: CB, 20 mM; octyl-β-glucoside, 2 mM; crude Gx from *A. xylinum*, 10 μL; heated supernatant, from *A. xylinum*, 10 μL; other compounds, 100 μM.

Tested Compounds	EIP	ANIP
	nmol min^{-1} mg^{-1}[a]	
None	0.9	0.2
CB	28.1	1.0
Octyl-β-glucoside	12.7	—[b]
c-3′:5′-GMP	—	0.9
Cyclic diguanylic acid	—	0.2
Thymine monophosphate	—	0.8
ATP	—	0.8
CB, Octyl-β-glucoside	26.5	—
CB, c-2′:3′-AMP	31.5	1.2
CB, c-2′:3′-CMP	—	1.0
CB, c-2′:3′-GMP	28.4	1.3
CB, c-2′:3′-UMP	—	1.0
CB, c-3′:5′-AMP	29.3	1.2
CB, c-3′:5′-CMP	—	1.0
CB, c-3′:5′-GMP	24.1	2.5
CB, c-3′:5′-thymine monophosphate	—	1.0
CB, c-3′:5′-UMP	—	1.0
CB, cyclic diguanylic acid	26.0	2.2
CB, crude Gx from *A. xylinum*	30.0	—
CB, heated supernatant from *A. xylinum*	30.7	—
CB, AMP	—	1.1
CB, CMP	—	1.0
CB, GMP	—	1.0
CB, thymine monophosphate	—	2.2
CB, UMP	—	1.0
CB, ATP	28.1	1.8
CB, GTP	28.4	1.0
CB, UTP	27.8	1.2
Octyl-β-glucoside, c-3′:5′-GMP	—	2.0

[a] nmol min^{-1} mg^{-1}, nmol of [^{14}C]Glc incorporated min^{-1} mg^{-1} of protein. [b] —, No data, not tested.

2′:3′-AMP, crude Gx from *A. xylinum*, or heated supernatant of *A. xylinum* could increase enzyme activity by about 3-fold based on CB enhancement (data not shown); however, in recent experiments with higher concentrations of substrate (0.5 mM), addition of such a compound gave only a very slight change. These experiments suggest that c-2′:3′-AMP, crude Gx from *A. xylinum*, and the heated supernatant of *A. xylinum* are functioning primarily in lowering the K_m. This conclusion is supported by the observation that the above effectors promote activation only when the substrate concentrations are low.

Combinations of Effectors

A study of effectors working synergistically revealed significant optimal combinations. As summarized in Table II, the common components in the reaction mixture required by both enzymes are CB, calcium, and digitonin. When any one of them is omitted from the reaction mixture, the enzyme activities are significantly depressed. Magnesium is not absolutely necessary for β-1,3-glucan synthase, but it is extremely important for the formation of the ANIP. As shown in Table II, the formation of the ANIP not only is magnesium dependent but also is calcium dependent.

Kinetic Properties of β-1,4-Glucan Synthase

Kinetics data concerning the in vitro functioning of β-1,4-glucan synthase from higher plants has been unavailable because of great difficulties with the enzyme assay. Some work has been done with alkali-insoluble products, but it is now generally recognized that an alkali-insoluble product does not represent an exclusive β-1,4-glucan or cellulose product.

Table II. *Effect of different combinations of effectors on in vitro β-glucan synthesis by cotton fiber glucan synthases*

The concentrations of effectors: Ca^{2+}, 2 mM; Mg^{2+}, 10 mM; CB, 20 mM; c-3′:5′-GMP, 100 μM; digitonin, 0.05%. The reaction mixture was pH 7.4 for the EIP and pH 7.6 for the ANIP.

Effectors	ANIP	EIP
	nmol min⁻¹ mg⁻¹[a]	
Mg^{2+}, Ca^{2+}, CB, c-3′:5′-GMP, digitonin (complete for product produced under conditions favoring the synthesis of β-1,4-glucan)	2.4	24.1
$-Mg^{2+}$	0.1	—[b]
$-Ca^{2+}$	0.2	—
−CB	0.4	—
−c-3′:5′-GMP	0.5	26.7
−Digitonin	0.2	—
$-Mg^{2+}$, −c-3′:5′-GMP	—	28.4
$-Ca^{2+}$, −c-3′:5′-GMP	—	2.0
−CB, −c-3′:5′-GMP	0.2	0.9
$-Mg^{2+}$, $-Ca^{2+}$, −c-3′:5′-GMP	n.d.[c]	Trace
No effectors	n.d.	Trace

[a] nmol min^{-1} mg^{-1}, nmol of [^{14}C]Glc incorporated min^{-1} mg^{-1} of protein. [b] —, No data, not tested. [c] n.d., Not detectable.

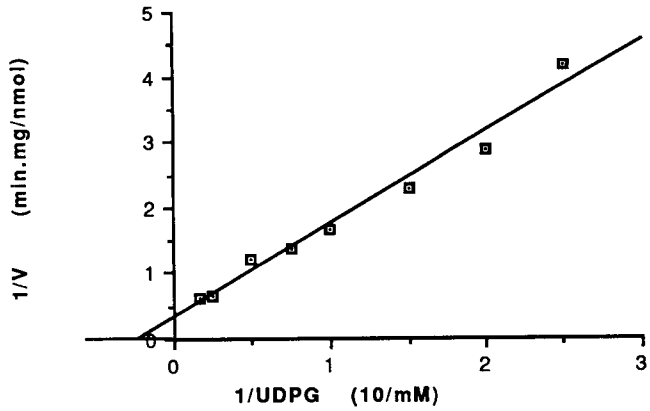

Figure 1. K_m of the ANIP. The reaction mixture contained 10 mM bis-trispropane-Hepes (pH 7.6), 20 mM CB, 8 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM c-3′:5′-GMP, 0.05% digitonin, 10 μg of enzyme protein, and the indicated quantity of UDP-[^{14}C]Glc. K_m = 0.40 mM. V_{max} = 2.8 nmol min^{-1} mg^{-1}.

We now present the kinetic properties based on the ANIP, which is known to contain only β-1,4-linkages according to methylation analysis and enzymic degradation analysis (Okuda et al., 1993). In higher plants, a generally accepted perception has been that β-1,4-glucan synthase requires a lower concentration of substrate than β-1,3-glucan synthase. This notion is not supported by our kinetics data.

Figures 1 and 2 show that the K_m for UDP-Glc for β-1,4-glucan synthase is not greatly different from that for UDP-Glc for the β-1,3-glucan synthase. Both K_m values are about 0.4 mM (0.40 mM for β-1,4-glucan and 0.43 mM for β-1,3-glucan). With an optimal combination of cofactors, the V_{max} for the β-1,4-glucan product can be as high as 2.8 nmol min^{-1} mg^{-1} (Fig. 1), and the V_{max} for the β-1,3-glucan product can reach 45.5 nmol min^{-1} mg^{-1} (Fig. 2). Magnesium, a critical cofactor for the β-1,4-glucan product, activates the enzyme with a K_a of 0.89 mM (Table III). Calcium, a cofactor that is extremely important for β-1,3-glucan synthase and also quite

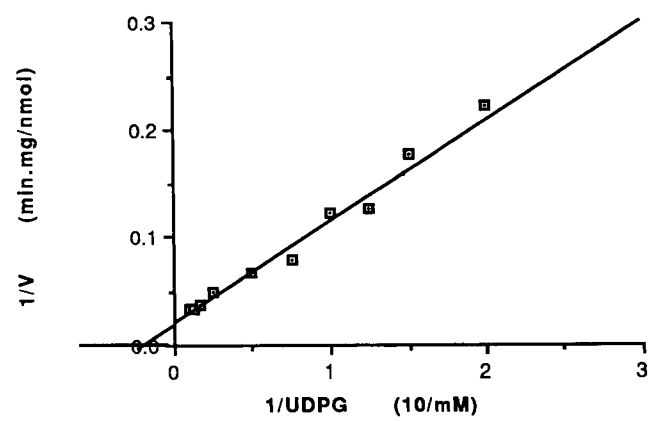

Figure 2. K_m of the EIP. The reaction mixture contained 10 mM bis-trispropane-Hepes (pH 7.4), 20 mM CB, 2 mM $CaCl_2$, 0.05% digitonin, 10 μg of enzyme protein, and the indicated quantity of UDP-[^{14}C]Glc. K_m = 0.43 mM. V_{max} = 45.5 nmol min^{-1} mg^{-1}.

Table III. *K_a of effectors for ANIP and EIP*

Effectors	ANIP	EIP
	mM	
Mg^{2+}[a]	0.89	–[b]
Ca^{2+}[c]	0.09	0.25
CB[d]	3.26	1.16
c-3′:5′-GMP[e]	0.1	–

[a] Components of the reaction mixture. For ANIP, 10 mM bis-trispropane-Hepes (pH 7.6), 20 mM CB, 100 μM c-3′:5′-GMP, 1 mM $CaCl_2$, 0.05% digitonin, 10 μg of enzyme protein, 0.5 mM UDP-[^{14}C]Glc and the indicated quantity of Mg^{2+}. [b] –, No data. [c] Components of the reaction mixture. For ANIP, 10 mM bis-trispropane-Hepes (pH 7.6), 20 mM CB, 8 mM $MgCl_2$, 100 μM c-3′:5′-GMP, 0.05% digitonin, 10 μg of enzyme protein, 0.5 mM UDP-[^{14}C]Glc, and the indicated quantity of calcium; for EIP, 10 mM bis-trispropane-Hepes (pH 7.4), 20 mM CB, 0.05% digitonin, 10 μg of enzyme protein, 0.5 mM UDP-[^{14}C]Glc, and the indicated quantity of Ca^{2+}. [d] Components of the reaction mixture. For ANIP, 10 mM bis-trispropane-Hepes (pH 7.6), 8 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM c-3′:5′-GMP, 0.05% digitonin, 10 μg of enzyme protein, 0.5 mM UDP-[^{14}C]Glc, and the indicated quantity of CB; for EIP, 10 mM bis-trispropane-Hepes (pH 7.4), 2 mM $CaCl_2$, 0.05% digitonin, 10 μg of enzyme protein, 0.5 mM UDP-[^{14}C]Glc, and the indicated quantity of CB. [e] Components of the reaction mixture. For ANIP, 10 mM bis-trispropane-Hepes (pH 7.6), 20 mM CB, 8 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% digitonin, 10 μg of enzyme protein, 0.5 mM UDP-[^{14}C]Glc, and the indicated quantity of c-3′:5′-GMP.

important for β-1,4-glucan synthase, showed activation for both enzymes with a K_a of 0.25 mM for β-1,3-glucan synthase and a K_a of 90 μM for β-1,4-glucan synthase (Table III). CB is required by both enzymes, which yield a K_a of 3.3 mM for the β-1,4-glucan synthase and a K_a of 1.2 mM for the β-1,3-glucan synthase (Table III).

c-3′:5′-GMP, an activator for the β-1,4-glucan synthase, showed a K_a of 100 μM (Table III). For β-1,3-glucan synthase, octyl-β-glucoside served a function similar to CB but with a lower K_m (0.3 mM) and a lower V_{max} (16.9 nmol min^{-1} mg^{-1}), as well as a lower K_a (80 μM).

The effect of pH on the β-glucan synthases from cotton fibers is presented in Figures 3 and 4. The β-1,4-glucan synthase requires a higher pH (optimal pH 7.6–8.0), whereas the β-1,3-glucan synthase requires a slightly lower pH, in the range of 7.2 to 7.6.

A test for the optimal concentration of digitonin is summarized in Figure 5, which shows that a concentration of 0.05% is the best (the data in Fig. 5 came from assays on the ANIP).

DISCUSSION

CB has been reported to be an activator for β-1,3-glucan synthase from higher plants (Delmer et al., 1977, 1984; Callaghan et al., 1988), and it may be a substitute for β-furfuryl-β-glucoside, a native activator identified recently (Ohana et al., 1992). In our investigations, a significant stimulation of the synthesis of β-1,3-glucan by CB has been observed. One interesting point is that CB enhances not only

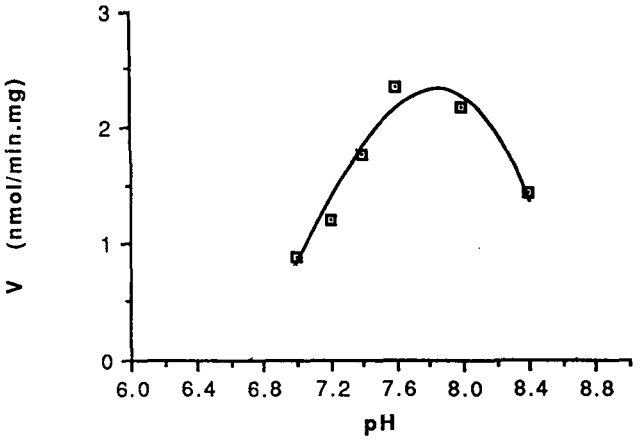

Figure 3. Effect of pH on the ANIP. The reaction mixture contained 10 mM bis-trispropane-Hepes (pH as indicated), 20 mM CB, 8 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM c-3′:5′-GMP, 0.05% digitonin, 10 μg of enzyme protein, and 0.5 mM UDP-[^{14}C]Glc.

the synthesis of β-1,3-glucan but also the synthesis of β-1,4-glucan. This suggests that there are differences between CB and β-furfuryl-β-glucoside; the latter has been reported to be a specific activator only for β-1,3-glucan synthase (Ohana et al., 1992).

The greatest stimulation of the synthesis of the ANIP was observed by a combination of CB with c-3′:5′-GMP, even though c-3′:5′-GMP has been reported to be ineffective in the activation of cellulose synthase from *A. xylinum* (Ross et al., 1987). Activation of the bacterial enzyme is highly specific for the dimeric structure of cyclic diguanylic acid (Ross et al., 1987).

A cyclic diguanylic acid-binding polypeptide was discovered recently in cotton (Amor et al., 1991), and the authors also discussed some stimulation of cyclic diguanylic acid found in their investigations. When it is used alone as an activator in the reaction mixture, cyclic diguanylic acid is

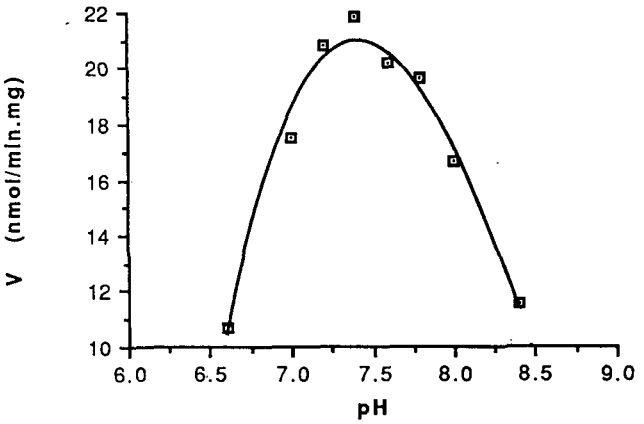

Figure 4. Effect of pH on the EIP. The reaction mixture contained 10 mM bis-trispropane-Hepes (pH as indicated), 20 mM CB, 2 mM $CaCl_2$, 0.05% digitonin, 10 μg of enzyme protein, and 0.5 mM UDP-[^{14}C]Glc.

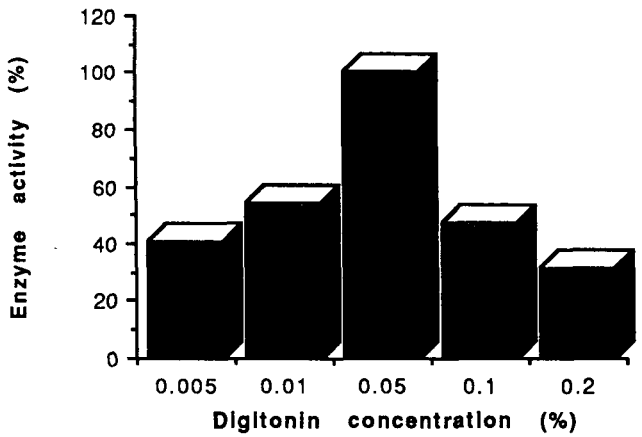

Figure 5. Effect of digitonin concentration on the ANIP. The reaction mixture contained 10 mM bis-trispropane-Hepes (pH 7.6), 20 mM CB, 8 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM c-3′:5′-GMP, 10 μg of enzyme protein, 0.5 mM UDP-[^{14}C]Glc, and the indicated quantity of digitonin.

ineffective; however, the inefficiency of cyclic diguanylic acid changes dramatically when it is combined with CB. This indicates that as an allosteric enzyme, cellulose synthase from higher plants might have two or more active sites for different activators: at least one for CB (or analogs) and one for the cyclic nucleotide (c-3′:5′-GMP, cyclic diguanylic acid, or their analogs). From all information available, we can consider that cyclic diguanylic acid or an analog (such as c-3′:5′-GMP) might be a potential activator for cellulose synthase in higher plants, but for effective action, the nucleotide appears to require an association with some other component.

To achieve maximal activity of cellulose synthase, a combination of CB, c-3′:5′-GMP, magnesium, calcium, and digitonin was found to be absolutely necessary. If any one of these components was omitted from the reaction mixture, the enzyme activity would be significantly depressed (Table II). We emphasize that magnesium functions as a switch that promotes the synthesis of the ANIP (Table II; Okuda et al., 1993) as well as a critical component necessary for the photolabeling of the 37-kD polypeptide, which has been identified as the best candidate for the catalytic subunit of cotton cellulose synthase (Li et al., 1993). In addition, it has been reported that magnesium can promote the aggregation of and thereby change the solubility of the in vitro product (Hayashi et al., 1987). Calcium, which is well known for its function in callose synthesis, also is very important to the synthesis of the ANIP.

The data presented in this study suggest that cellulose synthesis in cell-free cotton preparations not only is dependent on magnesium but also is dependent on calcium. The function of digitonin also is significant. An explanation for its activation is that it functions as a detergent that can remove enzymes from the membrane so that the substrate can make better contact with the enzyme active sites (Read and Delmer, 1987).

Many investigators have reported that relatively more β-1,4-glucan is produced from a low (1–10 μM) UDP-Glc concentration, and relatively more β-1,3-glucan is produced from a high (1 mM) UDP-Glc concentration (Ray, 1979; Henry and Stone, 1982; Henry et al., 1983; Amino et al., 1985). Our kinetics studies reveal a K_m of 0.4 mM for β-1,4-glucan synthase from cotton fibers, which is far from the low UDP-Glc concentrations used above; yet, our K_m is within the same order as the K_m for the cellulose synthase from *A. xylinum* (Bureau and Brown, 1987; Ross et al., 1987).

The reported values of the K_m for β-1,3-glucan synthase have shown a series of changes since enzyme assay conditions have been greatly improved. A high K_m (millimolar level) was reported in earlier work (Delmer et al., 1977; Ray, 1979; Henry and Stone, 1982; Henry et al., 1983; Amino et al., 1985). In recent years a much lower K_m (0.2–0.3 mM) was reported by Delmer et al. (1984) and Hayashi et al. (1987). Our K_m for β-1,3-glucan synthase (0.43 mM) is somewhat higher but essentially the same as theirs. Our V_{max} for the β-1,3-glucan is 45.4 nmol min^{-1} mg^{-1}. This is comparable to the V_{max} for cellulose synthase from *A. xylinum* (52.4 nmol min^{-1} mg^{-1} [Bureau and Brown, 1987]).

We have described the activities of β-1,4-glucan and β-1,3-glucan synthases from cotton fiber membrane fractions under specified conditions. In Li et al. (1993), we will describe which polypeptides are associated with specific glucan products.

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