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CONTROL OF CELLULOSE SYNTHESIS IN *Acetobacter xylinum*. A UNIQUE GUANYL OLIGONUCLEOTIDE IS THE IMMEDIATE ACTIVATOR OF THE CELLULOSE SYNTHASE*

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ABSTRACT

The mechanism of GTP-specific activation of the membrane-bound cellulose synthase system of *Acetobacter xylinum* has been further elucidated. The activation by GTP was previously attributed to the presence of a soluble protein factor derived from washed membranes. The protein factor has now been shown to be an enzyme that forms from GTP a low-molecular-weight, heat-stable compound which is highly effective in activating the cellulose synthase. The activator-forming enzyme has been isolated by affinity chromatography on an immobilized GTP column. The heat-stable activator has been purified by ion-exchange chromatography and characterized by labeling experiments, t.l.c., and spectral, chemical, and enzymic analyses. The compound could be labeled with [1-^{32}P]GTP and [8-^{3}H]GTP but not with [3-^{32}P]GTP. The compound contains guanine, ribose, and phosphate in a 1:1:1 ratio, is labile to mild alkali and snake venom phosphodiesterase, but is resistant to alkaline phosphatase, mild acid hydrolysis, and the periodate–β-elimination reaction. The results indicate that the activator is an unusual, cyclic guanyl oligonucleotide composed of GMP residues. The cellulose synthase-containing membranes of *A. xylinum* exhibit a phosphodiesterase-like activity which rapidly degrades the nucleotide activator into 5′-GMP. This activity, however, is strongly inhibited by calcium. It is suggested that intracellular levels of the nucleotide activator, in conjunction with calcium ions, may regulate the rate of cellulose synthesis *in vivo*.

INTRODUCTION

One of the major goals in studies of the mechanism and regulation of cellulose biosynthesis has been to isolate the synthesizing enzyme-assembly from

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cells as a fully active catalytic system. In a cell-free system, the nature of the enzymes and other essential components involved in the process could be studied under defined and controlled conditions, and the regulatory role of likely metabolic effectors could be assessed without the restrictions imposed by selective-permeability factors existing in whole cells.

In spite of many efforts in various laboratories, it has been exceedingly difficult to demonstrate convincingly *in vitro* synthesis by use of extracts derived from organisms capable of ample cellulose synthesis *in vivo*[1–3]. One such organism was *Acetobacter xylinum* which for many years served as a model system for studying cellulose biogenesis. The advantages of using a unicellular organism as a research tool are obvious. Furthermore, unlike the algae and higher plants, the cellulose of *A. xylinum* is not produced as a cell-wall component but rather as an extracellular pellicle of essentially pure cellulose. The cellulose-synthesizing capacity of these cells is most impressive: each bacterium polymerizes 200 000 D-glucose units per second. But even more importantly, cellulose is the major, if not the sole polysaccharide abundantly produced by this organism[4].

But even with this classical model organism, progress in the study of cellulose biosynthesis has been for many years slow and frustrating. It was with membranes derived from *A. xylinum* that convincing *in vitro* synthesis of a cellulosic product from UDP-D-glucose has been demonstrated for the first time 28 years ago by Glaser[5]. However, in this case too, the membrane preparations used by Glaser, and later by other workers, were only capable of (1→4)-β-D-glucan synthesis at rates that were less than 1% of those achieved *in vivo*[6]. This low synthesizing efficiency indicated that all of these *in vitro* systems are far from acting at optimal rate.

Several years ago, we reported on conditions for an extremely efficient transfer of D-glucose from UDP-D-glucose to a cellulosic (1→4)-β-D-glucan by use of membrane preparations derived from *A. xylinum*[7,8]. The key to this success lay in the discovery of a complex GTP–Ca^{2+}–protein factor-mediated regulatory system for the *A. xylinum* UDP-glucose:(1→4)-β-D-glucan 4-β-D-glucosyltransferase (cellulose synthase). We found that the enzyme shows a marked and specific activation by GTP. The ability to demonstrate GTP activation depends upon the presence of a protein factor present in the supernatant derived from washed membranes. The factor was shown to be a high-molecular-weight protein, heat labile and destroyed by protease treatment. In the presence of limiting amounts of this factor, GTP activation was further enhanced by Ca^{2+}. The synthase system has been solubilized with digitonin, while maintaining its regulatory properties[9]. We now report that the protein factor is an enzyme reacting with GTP to form a low-molecular-weight product (GX), tentatively characterized as a cyclic guanyl di- or oligo-nucleotide which is the immediate activator of the cellulose synthase reaction. The activation may be reversed by a membrane-bound enzyme that degrades GX. The latter enzyme is inhibited by Ca^{2+}. It is suggested that the interaction between these enzymes and nucleotide components, mediated by Ca^{2+}, may form the basis for the regulation of cellulose synthesis *in vivo*.

EXPERIMENTAL

Chemicals. — All radiochemicals were from The Radiochemical Centre, Amersham, U.K. Nucleotides, sugar nucleotides, *E. coli* alkaline phosphatase Type III, and venom phosphodiesterase Type VII were from Sigma Chemical Co. Guanosine 5′-*O*-(3-thiotriphosphate) and guanosine 5′-*O*-(2,3-iminotriphosphate) were from Boehringer, Mannheim. Agarose–hexane–GTP and DEAE–cellulose were obtained from Pharmacia. Thin-layer plates of poly(ethylenimine)–cellulose were from Machery Nagel (polygram Cel 300 PEI).

Cells. — The cellulose synthesizing strain of *Acetobacter xylinum* was the same as that used in earlier investigations[10,11]. Harvesting and washing of cells were performed as previously described[7].

Preparation of washed membranes and crude factor. — Membrane fractions prepared in poly(ethyleneglycol)-4000 (PEG-MF) were obtained by sonication of cells in 50mM Tris · HCl [2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride] (pH 7.5) containing 10mM $MgCl_2$ and mM EDTA (1,2-diaminoethane-*N,N,N′,N′*-tetraacetic acid) (TME buffer) which contained 20% (wt./vol.) PEG-4000 as described in detail earlier[7]. PEG-MF in TME buffer was recentrifuged at 18 000*g* for 15 min at 4°. The supernatant obtained comprises the "crude factor" typically containing 5–6 mg of protein/mL. The pellet was again washed by resuspension in TME buffer and centrifugation; this supernatant was discarded. The washed pellet, resuspended in the same buffer, comprises the "washed membranes" typically containing 15–20 mg of protein/mL.

To obtain Mg^{2+}-free washed membranes, a regular preparation in TME buffer was centrifuged and resuspended in 50mM Tris · HCl (pH 7.5). Mg^{2+}-free crude factor was prepared by adding PEG-4000 to the regular preparation to a concentration of 20% (wt./vol.). The resultant pellet was collected by centrifugation and resuspended in 50mM Tris · HCl (pH 7.5).

Enzyme assays. — *Cellulose synthase.* The activity was assayed by measuring the rate of incorporation of radioactivity from UDP-D-[^{14}C]glucose into alkali-insoluble (1→4)-β-D-[^{14}C]glucan. The standard assay mixture contained, in a final volume of 0.2 mL, 70mM Tris · HCl (pH 8.6), 10mM $MgCl_2$, 1.0mM $CaCl_2$, 0.9mM EDTA, 20μM UDP-D-[^{14}C]glucose (25 c.p.m./pmol), washed membranes (0.3 mg of protein), and additional components as indicated. The incubation was carried out for 5 min at 30°. The reactions were terminated, and the ^{14}C-product formed was determined as described in detail by Aloni *et al.*[7].

RE activity. The activity was assayed by measuring the rate of formation of the heat stable, low-molecular-weight activator of the cellulose synthase (GX) from GTP. The standard assay mixture contained, in a final volume of 0.2 mL, 50mM Tris · HCl (pH 7.5), 10mM $MgCl_2$, mM EDTA, mM GTP, and enzyme (0.1–0.3 mg of protein). The incubation was carried out for 20 min at 30°. The reaction was terminated by heating in boiling water for 3 min, followed by centrifugation. Aliquots (5–40 μL) of the deproteinized supernatant were assayed for stimulatory

activity in a standard, cellulose-synthase assay system. A unit of stimulatory activity is defined as the amount of GX required to increase the cellulose-synthase activity of washed membranes above basal activity, under standard conditions, by 1%.

Analytical procedures. — U.v. spectral measurements were performed in 1-cm path length cuvettes with a Bausch and Lomb Spectronic 2000 spectrophotometer. Ribose was determined by the Dische modification of the orcinol reaction[12]. The total phosphate content after ashing was determined by the method of Ames[13] using GMP, GDP, and GTP as standards. Guanine was estimated by u.v. spectroscopy at 252 nm using a molar extinction coefficient of 13 700 at pH 7.0. The protein content was determined according to Bradford[14]. For periodate oxidation and β-elimination reaction[15–17], the nucleotide preparation was incubated in the dark with a 100-fold molar excess of $NaIO_4$ in M lysine (pH 8.0) for 90 min at 45°. The reaction was terminated by adding excess glycerol (to 2.5%). Under these conditions, 5′-GMP is completely degraded and its phosphate group released as inorganic phosphate, whereas 2′-GMP or 3′-GMP are not reactive.

Enzyme degradations. — For hydrolysis by alkaline phosphatase, ^{32}P-labeled nucleotide samples (0.1–0.4 μmol/mL) were incubated for 30 min at 37° with *E. coli* alkaline phosphatase (2.0 unit/mL) in 50mM Tris · HCl (pH 8.6) containing 10mM $MgCl_2$ and mM $ZnCl_2$. The reaction was terminated by heating for 5 min at 100°. In the case of tests for resistance to enzyme action, the amount of enzyme was increased to 40 units/mL and the incubation time extended to 2 h. Under the latter conditions, 5.0 μmol/mL of either GMP, GDP, or GTP were completely hydrolyzed, as judged by the total release of their phosphate groups as inorganic phosphate.

For hydrolysis by snake venom phosphodiesterase (SVPD), ^{32}P-labeled nucleotide samples (0.1–0.4 μmol/mL) were incubated for 60 min at 37° with SVPD (3 munits/mL) (a unit of activity being defined as the liberation of 1 μmol of *p*-nitrophenol/min from bis-(*p*-nitrophenyl)phosphate at 25°) in 50mM Tris · HCl (pH 8.5) containing 10mM $MgCl_2$. The reaction was terminated by heating to 100° for 5 min.

Ion-exchange t.l.c. was performed on poly(ethyleneimine)(PEI)–cellulose plates (20 × 20 cm) prewashed with 0.5M LiCl, rinsed with redistilled water, and dried before use. Samples (5–50 μL) were applied to an origin-line drawn 2.5 cm above the starting edge, and the plates were developed to a distance of 15–17 cm beyond the origin. The chromatography was improved by dipping the sample-loaded plates into absolute methanol and drying them in a ventilation hood prior to development. Unlabeled markers were included and made visible under short-wave u.v. light. Labeled compounds were detected by autoradiography; the exposure times varied from 4 h to 1 day. When quantitation was necessary, the radioactive spots were cut out from the plate and counted in toluene-based scintillation fluid.

Liquid chromatography analysis was performed under elevated pressure on a Merck Licrosorb RP-18 column (25 × 0.4 cm) in 0.1M sodium acetate (pH 5.6) at

a constant flow rate of 1 mL/min. Column temperature was maintained at 37° and detection at 252 nm. The longest chromatographic analysis exceeded 40 min (twice the retention time of the GX compound).

RESULTS

Interaction of protein factor with GTP. — On the basis of our previous work, we proposed a relatively simple scheme for the interaction of the protein factor and GTP in stimulating the membrane-bound cellulose synthase of *A. xylinum*[7]. In this scheme, GTP was assumed to bind to the synthase already associated with the factor (which is the supernatant derived from washed-membrane fractions). Another, and now substantiated, possibility considered was that enzyme activation occurs after GTP binds or reacts with the factor. An indication for this interaction was obtained when the crude protein factor was applied to an agarose–hexane–GTP column, and its activity (assayed by its ability to facilitate GTP activation of washed, factor-free synthase) was retained (Fig. 1). Extensive washing of the column with buffer eluted practically all of the protein but very little factor activity. This emerged as a single, sharp peak upon elution with 5mM GTP ("GTP eluate"). Surprisingly the activity found in the "GTP-eluate", unlike that of the crude factor, was both dialyzable and stable to heating at 100° for 20 min (Table I).

These findings led us to a modified working hypothesis which assumes that

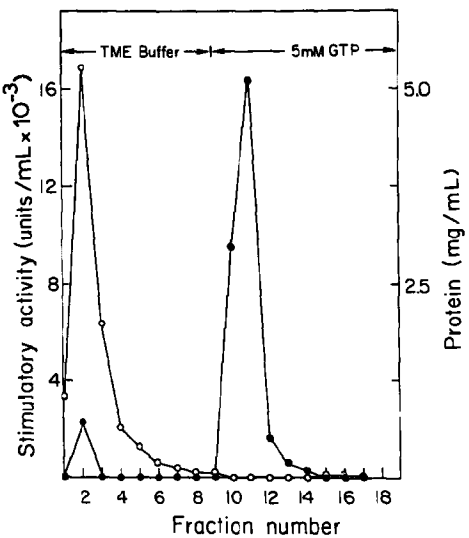

Fig. 1. Chromatography of crude factor on GTP-agarose. The crude factor (5 mL) was applied to a column (2.0 mL) of agarose–hexane–GTP, pre-equilibrated at 4° with TME buffer. After extensive washing with the buffer, the column was eluted with 5mM GTP in TME buffer. Fractions (1 mL) were collected and assayed for protein (—○—○—) and stimulatory activity (—●—●—). For the latter, aliquots (20–50 μL) of each fraction were added directly to the standard cellulose synthase assay system (as described in the Experimental section), supplemented with GTP, to a final concentration of 1.25mM.

TABLE I

THE EFFECT OF PRETREATED CRUDE FACTOR AND "GTP-ELUATE" ON SYNTHASE ACTIVITY[a]

Addition to synthase assay mixture	*Preincubation time (min)*	*Glucose incorporation (c.p.m.)*
None		400
GTP		600
Crude factor + GTP		1500
Boiled crude factor + GTP		600
Crude factor preincubated with GTP and then deproteinized	5	4000
	10	8000
	20	17 000
"GTP-eluate"		2400
Boiled "GTP-eluate"		2100
Dialyzed "GTP-eluate"		400
"GTP-eluate" preincubated and then deproteinized	5	5400
	10	8800
	20	16 000
	30	22 000

[a]Factor preparations are those described in the legend to Fig. 1. The fractions eluted from the GTP-agarose column with 5mM GTP were pooled, and are referred to as the "GTP-eluate". Synthase activity was assayed under standard conditions as described in the Experimental section, supplemented where indicated with crude factor 0.1 mg protein, mM GTP, and "GTP-eluate" (40 μL). Preincubations were at 30° in the presence of mM GTP, for the times specified. Preincubations were terminated by heating at 100° for 5 min. Dialysis, when indicated, was against TME buffer for 20 h.

the synthase is activated by a heat-stable, low-molecular-weight compound, referred to as GX, which is formed from GTP by an enzyme, referred to as RE, present in the crude protein factor. RE binds to the GTP–agarose column and, when the column is eluted with GTP, it is released and reacts with GTP to form GX which is highly potent in activating the synthase. This hypothesis was verified by demonstrating RE activity in the crude factor as well as in the "GTP-eluate" (Table I). Crude factor was preincubated with mM GTP, then heated for 10 min at 100° and centrifuged. The resulting, deproteinized supernatant fraction, when added to the membrane-bound cellulose synthase, greatly increased its activity. The extent of activation was linearly dependent upon the amount of crude factor preincubated with GTP and on the time of preincubation. Similarly, when the "GTP-eluate" was incubated at 30° and then heated to 100°, its capacity to activate synthase activity increased linearly with incubation time at 30°. Such an increase did not occur with a "GTP-eluate" preheated for 2 min at 100°. This procedure of testing a deproteinized preparation, previously preincubated with GTP, for its ability to stimulate the synthase reaction was the basis for further standard assays of RE activity.

The RE reaction. — The GX-forming enzyme, RE, is recovered in the "GTP-eluate" at a purification of more than 1000-fold (a minimum figure is given, as the

TABLE II

EFFECT OF Mg^{2+} AND Ca^{2+} ON THE ACTIVITY OF RE[a]

Cation added	*Stimulatory activity (units)*
None	20
Mg^{2+} (10mM)	1725
Ca^{2+} (2mM)	32
Mg^{2+} (10mM) + Ca^{2+} (2mM)	1735

[a]Mg^{2+}-free crude factor (see Experimental section) was incubated at 30° for 20 min with mM GTP and the cations, as indicated. The reactions were terminated by heating at 100° for 5 min, followed by centrifugation. Aliquots (60 μL) of the supernatant were assayed for stimulatory activity, as described in the Experimental section.

amount of protein in the eluate was too low to be measured). The eluate contained 70–80% of the RE activity originally present in the crude factor. The purified enzyme was rather labile, but partially retained its activity for several days at 4° in the presence of GTP. Formation of GX was optimal at pH 7.5–8.0, had an absolute requirement for Mg^{2+}, and was not affected by Ca^{2+} (Table II). The RE reaction was highly specific for GTP. Other nucleotides tested (for complete list, see Table II in ref. 8) were incubated with crude factor (see Experimental section) at concentrations of 0.01 to 1.0mM, and the deproteinized reaction mixtures were assayed for their ability to stimulate the synthase reaction. Of the various nucleotides tried, only guanosine 5′-*O*-(3-thiotriphosphate) was reactive and able to form a heat-stable product having high-stimulatory activity. Other analogs of GTP, such as guanosine 5′-*O*-(2,3-iminotriphosphate) and 2′-deoxy-GTP, were not reactive.

Nature of the GX compound. — Radioactive labeling and purification of GX. The requirement for GTP as the sole exogenous substrate for the RE reaction suggested that it might form an integral part of the GX product. The following guanosine derivatives, at concentrations up to 1mM, could not substitute for GX in stimulating synthase activity of washed membranes, nor did their presence affect GX stimulatory activity: GTP, GDP, GMP, guanosine-3′:5′-cyclic monophosphate, diguanosine tetra- or penta-phosphate, guanosine 3′-diphosphate-5′-diphosphate, and guanosine 3′-diphosphate-5′-triphosphate. An attempt was made to label GX by incubating the crude protein factor with [1-^{32}P]GTP (Fig. 2). Purification of the activator was achieved by ion-exchange chromatography on a DEAE-cellulose column by use of a step-wise increasing gradient of $(NH_4)_2CO_3$. After extensive washing of the column with 0.2M $(NH_4)_2CO_3$ to remove residual labeled GTP and other labeled by-products lacking synthase-stimulatory activity, GX activity was eluted at higher salt (0.4M) concentration. Fractions were monitored for radioactivity and for absorbance at 252 nm, and, after desalting, for the ability to stimulate synthase activity of washed membranes. In the presence of a GTP regenerating system, as much as 30% of the GTP label was incorporated into 252-nm absorbing material, which was copurified with stimulatory activity. The ratio of

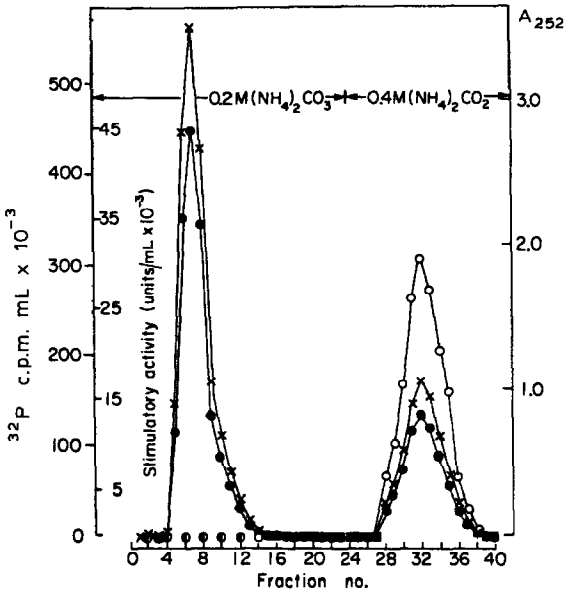

Fig. 2. Purification of GX activity by column ion-exchange chromatography. Crude factor (35 mg of protein) was incubated for 1 h at 30° in the presence of 50mM Tris · HCl (pH 7.5); 10mM $MgCl_2$; mM EDTA; mM [1-^{32}P]GTP (1.7×10^6 c.p.m./μmol); and a GTP regenerating system composed of 2.5mM creatine phosphate and 150 units of creatine phosphate kinase, in a final volume of 7.5 mL. The mixture was then brought to a concentration of 0.6M $HClO_4$ and centrifuged at 5000*g* for 10 min, and the supernatant was made neutral with dilute K_2CO_3 and recentrifuged. Both precipitates were discarded. The supernatant was applied to a DEAE-cellulose column (1.2 × 20 cm) pre-equilibrated with 0.2M $(NH_4)_2CO_3$. The column was eluted first with 0.2M $(NH_4)_2CO_3$ (125 mL), followed by 0.4M $(NH_4)_2CO_3$ (90 mL). Fractions (5-mL) were collected, and sampled for radioactivity (—●—●—) and 252 nm absorbance (—×—×—). The fractions were lyophilized to remove salts, then redissolved in water (1 mL), and aliquots assayed for ability to stimulate cellulose synthase activity of washed membranes (—○—○—) (as described in the Experimental section).

^{32}P-label-to-u.v. absorbance at 252 nm in the purified fraction was identical to that of the labeled substrate.

Additional attempts to label the GX compound were made by incubating the crude factor with [8-^{3}H]GTP and [3-^{32}P]GTP. Table III summarizes the results obtained from three experiments carried out in parallel in the presence of either (1-^{32}P]-, [3-^{32}P]- or [8-^{3}H]-GTP. In order to avoid a possible dilution of the 3-^{32}P label and for comparative purposes, a GTP regenerating system was not included in these experiments. The pattern of incorporation of the ^{3}H label into the high-salt eluates bearing the stimulatory activity was similar to that of the 1-^{32}P label. Moreover, the ratio of GMP content to stimulatory activity, as calculated from either 1-^{32}P or ^{3}H label, in the high-salt eluates were in good agreement. On the other hand, when [3-^{32}P]GTP was utilized, no radioactivity could be detected in the u.v.-absorbing material coeluted with stimulatory activity.

In order to establish that u.v. absorbance, ^{32}P label, and stimulatory activity

TABLE III

GX PREPARED IN THE PRESENCE OF [1-^{32}P]-, [3-^{32}P]-, AND [8-^{3}H]GTP[a]

$(NH_4)_2CO_3$ eluate	*Substrate*		
	[1-^{32}P]GTP	*[8-^{3}H]GTP*	*[3-^{32}P]GTP*
0.2M			
Absorbance (A_{252})	2.13	2.25	2.09
Radioactivity (c.p.m./mL × 10^{-5})	2.43	3.04	14.40
Stimulatory activity (unit/mL × 10^{-3})	0	0	0
0.5M			
Absorbance (A_{252})	0.330	0.345	0.320
Radioactivity (c.p.m./mL × 10^{-3})	40	55	0.5
Stimulatory activity (unit/mL × 10^{-3})	13.0	13.5	12.0

[a]The crude factor (32 mg protein) was incubated for 1 h at 30° in the presence of 50mM Tris · HCl (pH 7.5), 10mM $MgCl_2$, mM EDTA, and either mM [1-^{32}P]GTP (1.6×10^6 c.p.m./μmol), mM [8-^{3}H]GTP (2.0×10^6 c.p.m./μmol), or mM [3-^{32}P]GTP (9.0×10^6 c.p.m./μmol) in a final volume of 20 mL. The reactions were terminated, and the products deproteinized and applied to a DEAE-cellulose column as described in the legend to Fig. 2. The column was eluted first with 0.2M $(NH_4)_2CO_3$ until the eluate was free of radioactive or u.v.-absorbing material (125 mL), followed by elution with 0.4M $(NH_4)_2CO_3$ (40 mL). The eluates were then monitored for radioactivity, 252-nm absorbance, and stimulatory activity as described for Fig. 2.

are all due to the same material, fractions from high-salt eluates of an [1-^{32}P]GTP experiment were combined and analyzed by t.l.c. The samples were applied to PEI-cellulose plates and developed in various solvent systems. In all cases, a single spot having a distinct mobility was detected by autoradiography (Table IV). Extracts of the cellulose from the pertinent areas of the chromatograms exhibited a ratio of radioactivity- or u.v. absorbance-to-stimulatory activity identical to that of the material applied (Table V).

Liquid-chromatography analysis of combined, GX-active, DEAE-cellulose fractions using reverse-phase C18 columns and 0.1M acetate (pH 5.6) revealed a single, discrete 252-nm absorbing peak having a retention time exceeding that of GTP, GDP, GMP, and 3′,5′-cyclic GMP.

Spectroscopic properties. Table VI shows the u.v.-absorbancy characteristics of DEAE-cellulose-purified GX at acid, alkaline, and neutral pH values. The spectrophotometric ratios and wavelengths of minimal and maximal absorption are almost identical with those of GTP.

Chemical analysis. The results of chemical analyses of GX (Table VII) indicated that the compound contains guanine, ribose, and phosphate in a molar ratio of 1:1:1.

Enzymic and chemical characterization. — To further characterize the activator, ^{32}P-GX (prepared and purified from [1-^{32}P]GTP) was subjected to various chemical and enzymic treatments. The products were analyzed by t.l.c. in four different solvent systems (1.5M KH_2PO_4, pH 3.65; 1.25M LiCl, pH 5.0; 0.2M NH_4HCO_3, pH 7.8; and 1.3M LiCl in saturated $H_3B_4O_7$, pH 7.0) and assayed for

TABLE IV

CHROMATOGRAPHIC CHARACTERISTICS OF GX[a]

Compound	*Solvent*		
	0.2M NH_4HCO_3 (pH 7.8)	*1.25M LiCl (pH 5.0)*	*1.5M KH_2PO_4 (pH 3.65)*
GX	0.19	0.37	0.31
GTP	0.09	0.17	0.40
GDP	0.30	0.34	0.58
GMP	0.50	0.56	0.61

[a]R_F values are given for ion-exchange t.l.c. (see Experimental section) of DEAE-cellulose-purified, ^{32}P-labeled GX.

TABLE V

ANALYSIS OF ^{32}P-GX FOLLOWING ION-EXCHANGE, THIN-LAYER CHROMATOGRAPHY[a]

Solvent	*Material*	*A_{252}*	*Radioactivity (c.p.m. × 10^{-3})*	*Stimulatory activity (units × 10^{-3})*	*Radioactivity/ A_{252}*	*Stimulatory activity/ A_{252}*
1.5M KH_2PO_4 (pH 3.65)	applied	4.5	50	162.6	11	36
	spot	4.9	49	165.0	10	34
0.2M NH_4HCO_3 (pH 8.0)	applied	4.5	50	180.4	11	40
	spot	5.0	51	180.0	10	37

[a]A solution of DEAE-cellulose-purified, ^{32}P-labeled GX was concentrated by lyophilization, and samples were applied to two PEI-cellulose plates. The plates were chromatographed in the designated solvents, and the single radioactive spot detected by autoradiography on each was scraped off and eluted with 0.5M $(NH_4)_2CO_3$. The eluates were desalted and analyzed for absorbance, radioactivity, and stimulatory activity as described in the Experimental section. For the experiments designated "applied", the plates were developed before application of samples. One A_{252} unit is the amount of material that has an absorbance of 1.0 when dissolved in 1 mL of water at pH 7.0 and measured in a 1-cm cuvette at 252 nm.

stimulatory activity. With all the treatments tested, chromatographic mobility and biological activity were affected in parallel.

GX was resistant to digestion by bacterial alkaline phosphatase. It was, however, digested with SVPD which yielded 5′-GMP as the only detectable product. This digestion product was identified by its chromatographic mobility and by the total release of its label as inorganic phosphate, upon treatment with either bacterial alkaline phosphatase or by the periodate–β-elimination reaction (Fig. 4). GX was resistant to mild acid treatment (0.1M HCl, 100°, 15 min). In addition, such mild acid treatment did not affect the compound resistance to alkaline phosphatase. Mild alkaline treatment (0.2M NaOH, 37°, 24 h) hydrolyzed GX to give two labeled products comigrating chromatographically with 2′-GMP and 3′-GMP (Fig. 4). The GX compound was not affected by the periodate–β-elimination reaction under conditions that completely degrade GMP.

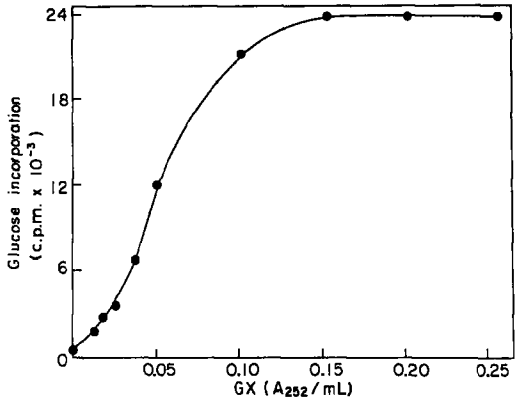

Fig. 3. Effect of GX on cellulose synthase activity of washed membranes. Washed membranes were assayed for cellulose synthase activity in the presence of increasing amounts of DEAE-cellulose-purified GX, as described in the Experimental section. One A_{252} unit is defined as in the legend to Table V.

TABLE VI

U.V. ABSORBENCY CHARACTERISTICS OF GX[a]

Compound	*pH*	λ_{max}	λ_{min}	*Absorbency ratio*		
				250/260	*280/260*	*290/260*
GX	1	255.8	227.0	0.95	0.61	0.43
	7	251.3	225.6	1.14	0.64	0.30
	10	256.6	231.7	0.93	0.63	0.17
GTP	1	256	228	0.96	0.67	0.50
	7	252	223	1.17	0.66	0.28
	10	258	230	0.92	0.59	0.14

[a]For absorbency measurements (see the Experimental section), aqueous solutions were adjusted to the desired pH with dilute NaOH or HCl.

TABLE VII

CONTENT OF RIBOSE, PHOSPHATE, AND GUANINE IN GX

Sample	*Phosphate (nmol)*	*Ribose (nmol)*	*Guanine (nmol)*
1	55	45	50
2	93	94	100
3	187	184	195

[a]DEAE-cellulose fractions bearing GX activity were combined and desalted by lyophilization, and samples analyzed as described in the Experimental section.

Fig. 4. Analysis of the products of SVPD and mild alkaline treatment by thin-layer chromatography t.l.c. ^{32}P-Labeled GX, purified by DEAE-cellulose, was digested with SVPD or by mild alkali (for conditions, see the Experimental section). The figure is an autoradiogram made from a PEI-cellulose plate developed in 1.5M KH_2PO_4 (pH 3.65): Lane (1) ^{32}P-labeled GX; (2) SVPD degradation product of ^{32}P-GX; (3) the SVPD degradation product subjected to the periodate oxidation–β-elimination reaction; (4) mild alkaline hydrolysis products of ^{32}P-GX; (5) ^{32}P-labeled inorganic phosphate; (6) 5′-GMP; (7) 2′-GMP; and (8) 3′-GMP. Lanes 5–8 are markers.

GX concentration and synthase activity. — The aforementioned data strongly suggest that GMP residues are an integral part of the biologically active GX compound. The effect of varying concentrations of GX on cellulose synthase activity is shown in Fig. 3. The apparent activation constant, K_a (the concentration for half-maximal activation), for GX is 0.05 A_{252} units/mL which, depending on the molar proportion of guanine in GX, is estimated as being $\leqslant 3.5\mu$M.

Degradation of GX by a membranous enzyme. — Upon incubation of GX with washed-membrane preparations of *A. xylinum*, the ability of the compound to activate the cellulose synthase was rapidly lost (Table VIII). The inactivation was probably due to a membrane-bound enzyme, referred to here as GXase. The activity of GXase is heat sensitive, absolutely dependent on Mg^{2+}, and is inhibited by Ca^{2+}. GX degradation was linear with time and with the amount of membrane preparation. The products of the GXase reaction utilizing ^{32}P-labeled GX have been analyzed by t.l.c. (Fig. 5). The final product was 5′-GMP, identified by its mobility and by the total release of its label as inorganic phosphate, by treatment with either alkaline phosphatase or by the periodate–β-elimination reaction. An as yet unidentified compound, devoid of stimulatory activity, was transiently formed during the course of the reaction.

TABLE VIII

GXASE ACTIVITY IN WASHED MEMBRANES[a]

Incubation mixture	*GXase pre-treatment*	*Residual GX activity (stimulatory units)*	*GX degraded (%)*
Complete	None	0	100
Complete	Boiled	2200	0
Minus Mg^{2+}	None	1800	10
Plus Ca^{2+} (1.0mM)	None	2000	0

[a]The complete system contained, in a final volume of 0.25 mL, 50mM Tris · HCl (pH 7.5); Mg^{2+}-free washed membranes (0.45 mg of protein); 0.15 A_{252} units of ^{32}P-GX; and 10mM $MgCl_2$. The incubation was for 10 min at 30°. The reactions were terminated by heating to 100° for 5 min, followed by centrifugation. The supernatant (25 μL) was assayed for its ability to stimulate synthase activity, as described in the Experimental section. For determination of GX degradation, 5 μL of the supernatant were aplied to PEI-cellulose plates which were developed in 1.5M KH_2PO_4 (pH 3.65). The region corresponding to GX was extracted and the radioactivity counted.

DISCUSSION

The results presented herein clarify the role of the soluble protein factor, previously shown to be essential to demonstrate GTP activation of the membrane-bound cellulose synthase of *A. xylinum*[7,8]. The protein factor contains an enzyme (RE) which reacts with GTP to form a low-molecular-weight, heat-stable compound (GX) which is highly potent in activating the synthase. The formation of GX by RE has been characterized as a separate and independent reaction from that of

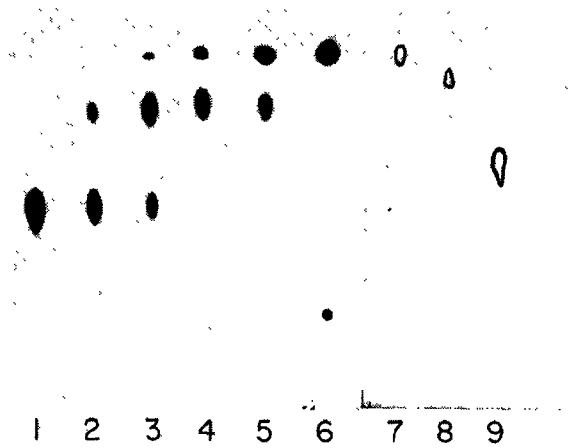

Fig. 5. Kinetics of GXase activity in washed membranes. ^{32}P-Labeled GX (0.3 A_{252}) was incubated with washed membranes (0.14 mg protein) at 30° in a final volume of 0.25 mL containing 50mM Tris · HCl (pH 7.5); 10mM $MgCl_2$; and 0.9mM EDTA. At the times indicated, aliquots of the reaction mixture were boiled for 2 min, centrifuged, and analyzed by t.l.c. Presented is an autoradiogram of a PEI-cellulose plate developed in 1.5M KH_2PO_4 (pH 3.65). Lanes 1–6: 0, 2, 5, 10, 20, and 40 min of reaction times. Lanes 7–9 are markers: 5'-GMP, GDP, and GTP.

the synthase by use of either the crude factor or a highly purified protein fraction isolated from the factor by affinity chromatography. The RE reaction was highly specific for GTP, as is the activation of the cellulose synthase in unwashed, crude factor-containing membrane preparations[8].

Although the mechanism of the RE reaction is still unclear, a single product with stimulatory activity appears to be formed in this reaction. The product was separated from residual GTP and by-products lacking stimulatory activity by DEAE-cellulose chromatography (Fig. 2), and its purity ascertained by ion-exchange thin-layer (Tables IV and V) and liquid chromatography. Evidence that GMP forms an integral part of the biologically active GX molecule is as follows: (a) GTP is the only added substrate needed for GX formation; (b) GX can be labeled by [^{3}H]GTP or [1-^{32}P]GTP but not by [3-^{32}P]GTP (Table III); (c) label experiments indicated that [^{3}H]- and [1-^{32}P]-GMP are incorporated from GTP into GX in the ratio of 1:1 (Fig. 2, Table III); and (d) chemical analyses indicated that GX contains guanine, ribose, and phosphate in the ratio of 1:1:1 (Table VII). A modification of the guanine component of GX appears unlikely, since the u.v. spectral properties of the compound highly resemble those of other guanosine phosphates. It also appears unlikely that synthesis of the GX compound requires stoichiometric amounts of any low-molecular-weight component other than GTP (up to 30% of which is converted into the product), since the activator was synthesized by a highly purified enzyme preparation and its rate of formation remained linear for extended periods of time (Table I).

GX is evidently not any of the various guanosine phosphate derivatives known to have regulatory functions in other biological systems, such as 3′:5′-cyclic GMP, diguanosine tetra- and penta-phosphate, guanosine 3′-diphosphate-5′-diphosphate, and guanosine 3′-diphosphate-5′-triphosphate, since none of these compounds has any effect on the synthase reaction.

The complete insensitivity of GX to hydrolysis by alkaline phosphatase and its complete digestion by SVPD to 5′-GMP indicated that the phosphate groups in GX are not monoesterified but rather diesterified. The susceptibility of GX to mild alkaline hydrolysis to yield 3′-GMP and 2′-GMP indicated that 3′,5′ or 2′,5′-phosphoric diester bonds were hydrolyzed and excluded di- or poly-phosphate or inverted (5′,5′)-linkages in GX (ref. 16 and 18). This conclusion is compatible with the resistance of the compound to mild acid hydrolysis. After such acid treatment, the molecule still retained its resistance to alkaline phosphatase, which appears to exclude the presence of cyclic 2′:3′-terminal phosphoric diester bonds[16]. GX was resistant to the periodate oxidation–β-elimination reaction, which indicated that a terminal ribose residue possessing a *cis*-glycol conformation is not a feature of its molecular structure[16]. This, considered together with the absence of phosphatase-susceptible phosphate groups, suggested a nonterminal or cyclic structure.

Thus far, we can attribute all of the described properties of the GX compound to a cyclic nucleotide structure containing GMP residue(s) having SVPD- and alkali-labile phosphoric diester linkages. However, charge considerations

based on the low mobility of the compound on ion-exchange thin-layer and column chromatography suggest that more than one GMP residue is present in GX. Its precise structure remains to be determined, but the results suggest that it is an unusual nucleotide which is highly effective at low concentrations (Fig. 3) in the activation of (1→4)-β-D-glucan synthesis *in vitro*. Its unique structure and apparent stability may well fit its being an intracellular messenger regulating cellulose synthesis *in vivo*.

With GX being the actual activator of the cellulose synthase, the question arises whether this activation is reversible. We have good indications that this might be the case. The cellulose synthase-containing membrane preparations similarly contain an enzyme (GXase) that rapidly degraded GX into 5′-GMP. Hence, this enzyme presumably acts as a phosphodiesterase. Its pattern of degradation, however, contrasts with that observed with the classical venom phosphodiesterase in that its conversion of GX to 5′-GMP occurred *via* an as yet unidentified intermediate. Given that GX is a cyclic nucleotide, the action of GXase may then be similar, in its presumed physiological role, to that of the specific phosphodiesterases acting on 3′,5′-cyclic AMP or GMP. In this connection, the sensitivity of the GXase to Ca^{2+} appears to be of special significance. Since RE formation of GX is not affected by Ca^{2+}, the inhibition of GXase by this cation may well account for our earlier findings that GTP-induced stimulation of the membrane-bound cellulose synthase of *A. xylinum* is markedly enhanced by Ca^{2+}, and lends further support to the suggestion that this cation may play a role in the regulation of cellulose synthesis *in vivo*[9].

A scheme summarizing the proposed interrelationship between the several components of the complex system regulating the cellulose synthase of *A. xylinum* is shown in Figure 6. The control is of the type in which a metabolically stable activator is enzymically synthesized and then enzymically degraded. The mediator, in our case, is an unusual guanyl nucleotide. Its unusual structure is of special

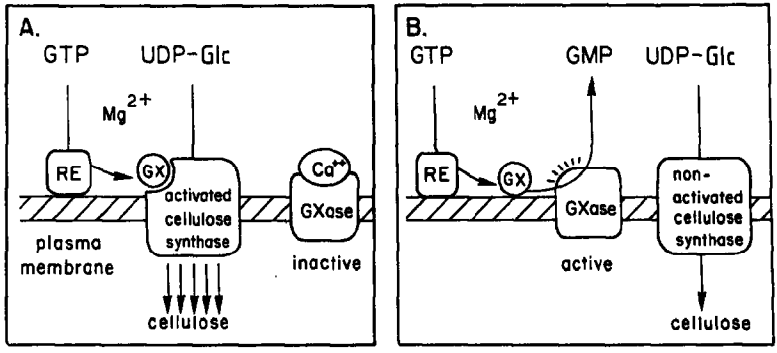

Fig. 6. Proposed model for the regulation of cellulose synthesis in *A. xylinum*. (A) In the presence of GTP and Ca^{2+}, GX is generated and activates cellulose synthase. GXase is inactivated by Ca^{2+}. (B) In the presence of GTP but in the absence of Ca^{2+}, GX is generated but is rapidly degraded by GXase before its effect on the synthase is exerted.

interest in the light of recent reports on the involvement of modified nucleotides in various cellular activities[19–24]. Conclusive characterization of GX, its formation, degradation, and mode of action are currently pursued and should certainly advance the understanding of the mechanism and regulation of cellulose biogenesis.

NOTE ADDED IN PROOF

Recently obtained evidence (manuscript in preparation) strongly suggests that the GX compound is a cyclic dinucleotide composed of two GMP residues linked by 3′,5′-phosphoric diester bonds. Mass spectroscopic measurements (performed by Dr. H. Schwarz, Technical University, Berlin) indicated a molecular weight of 690 for the activator compound, which corresponds to the molecular weight of such a cyclic diguanylic acid structure. Chemically synthesized bis(3′,5′)-diguanylic acid (prepared by Dr. H. J. van Boom, State University, Leyden, The Netherlands) is identical to the GX compound with regard to stimulation of the cellulose synthase in its membrane-bound and solubilized forms, and to degradation by the membrane-bound phosphodiesterase (GXase). Although the mechanism of cellulose synthase activation by the cyclic dinucleotide is still unclear, it is meaningful that the (1→4)-β-D-glucan product of the GX-activated synthase of *A. xylinium*, recently analyzed by electron microscopy and electron diffraction[25], has been shown to consist of fibrillar cellulosic material (probably cellulose I), thus directly supporting our proposition that the *in vitro* system, as described herein, has a close bearing on the biochemical and morphological setting under which native cellulose synthesis occurs.

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