Intermediary Steps in *Acetobacter xylinum* Cellulose Synthesis: Studies with Whole Cells and Cell-Free Preparations of the Wild Type and a Celluloseless Mutant

MORDECHAI SWISSA, YEHO SHUA ALONI, HAIM WEINHOUSE, AND MOSHE BENZIMAN*

Department of Biological Chemistry, The Hebrew University of Jerusalem, Institute of Life Sciences, Jerusalem, Israel

Intermediary steps in cellulose synthesis in *Acetobacter xylinum* were studied with resting cells and particulate-membranous preparations of the wild-type strain and of a celluloseless mutant. Exogenously supplied [1-14C]glucose was rapidly converted by resting cells of both types into glucose 6-phosphate, glucose 1-phosphate, and uridine glucose 5′-diphosphate (UDP)-glucose and incorporated into lipid-, water-, and alkali-soluble cellular fractions. The decrease in the level of labeled hexose-phosphates and UDP-glucose upon depletion of the exogenous substrate was accounted for by a continuous incorporation of [14C]glucose into cellulose in the wild type and into the above-mentioned cellular components in the mutant. [14C]glucose retained in the alkali- and water-soluble fractions of pulse-labeled wild-type cells was quantitatively chased into cellulose. Sonic extracts of both strains catalyzed the transfer of glucose from UDP-glucose into lipid-, water-, and alkali-soluble materials, as well as into an alkali-insoluble cellulosic β-1,4-glucan. The results strongly support the sequence glucose → glucose 6-phosphate → glucose 1-phosphate → UDP-glucose → cellulose and indicate that lipid- and protein-linked cellobextrins may function as intermediates between UDP-glucose and cellulose in *A. xylinum*.

Among the true bacteria, substantial cellulose synthesis occurs only in the genus *Acetobacter*, of which the most-studied species has been *A. xylinum* (13, 17, 26).

Some general features of cellulose formation in this organism, pertaining to the present study, are as follows. (i) Synthesis by resting cells occurs from a defined exogenous substrate in a system practically free from net synthesis of any other polysaccharide (6, 14, 27, 38). (ii) The polymer product is accumulated exclusively in the extracellular phase (10, 27). (iii) Synthesis is immediate at onset, varies linearly with cell concentration, and is essentially irreversible (12, 27, 35). (iv) Dissimilation of carbohydrates occurs mainly via the pentose cycle; hexose-phosphate is a common intermediate of cellulose synthesis and glucose oxidation (6, 25, 39, 44, 45). (v) Swirling of growth cultures facilitates overgrowth of the wild-type organism by a mutant which is specifically deficient in the ability to form cellulose (40).

The path of carbon into cellulose has been investigated in higher plants, algae, and bacteria (2, 14, 37, 39, 42). Through analogy with other polysaccharide-synthesizing systems, it has been assumed that phosphorylated sugars and nucleotide sugars are the primary precursors of cellulose. This assumption has not as yet been firmly established, mainly because it has not been possible, to date, to demonstrate convincingly in vitro synthesis of fibrillar cellulose from such compounds. Synthesis of fibrillar cellulose has been achieved with cell-free lysates of *A. xylinum* with glucose as the substrate, but such lysates showed only little activity in converting hexose-phosphates and UDP-glucose into cellulose (M. Benziman and M. Swiss, Abstr. Am. Chem. Soc./Chem. Soc. Japan, Congress 1979, CELL-46). The involvement of lipid- and protein-linked intermediates in cellulose synthesis in *A. xylinum* has been postulated by Colvin and co-workers (29, 30), and the synthesis of glucosyl-phosphoryl-polymer from UDP-glucose, using EDTA-treated *A. xylinum* cells, has been reported (21). It is still impossible to state with certainty what the intermediate compounds are on the pathway from glucose to cellulose and where and how they fit into the overall mechanism, especially with regard to the final stages of the polymerization process.

The present study was aimed at assessing the intermediary role of the various compounds which may be reasonably considered as potential precursors of cellulose in *A. xylinum*. The experimental systems employed were resting cells.
and particulate-membranous preparations of the wild-type strain and of a celluloseless mutant of \textit{A. xylinum}.

Tracer studies were carried out to evaluate the in vivo flow of carbon from \[^{14}C\]glucose through sugar phosphates and nucleotide sugar pools into cellulose. Time courses of labeling and turnover of these compounds strongly support the following sequence: glucose → glucose 6-phosphate → glucose 1-phosphate → UDP-glucose → cellulose. The pattern of label incorporation into various cellular fractions, which accompanies glucose utilization and cellulose formation, indicates that lipid- and protein-linked celloodextrins may serve as intermediates beyond UDP-glucose on the pathway to cellulose in \textit{A. xylinum}.

**MATERIALS AND METHODS**

Cells. The cellulose-synthesizing strain of \textit{A. xylinum} was the same as in earlier investigations (40, 45). Cells were grown for 24 h at 30°C under static conditions, as previously described (4). The pellicles produced at the liquid-air interface of the medium were transferred to a flask containing 0.05 M phosphate buffer (pH 6.0; 150 ml per pellicle), and the cells were released by shaking vigorously by hand. The suspension was passed through 16 layers of gauze and then centrifuged at 10,000 × g for 15 min. The cells in the pellet were washed once and then suspended in the same buffer. All the stages of harvesting and washing were carried out at 4°C.

The celluloseless mutant of \textit{A. xylinum} was obtained as described by Schramm and Hestin (40) and was grown and harvested after 24 h as described by Schramm et al. (41). The culture medium for both the wild-type and mutant strains, prepared with glass-distilled water, was as follows: 2% succinic acid; 0.5% yeast extract (Difco Laboratories); 0.5% peptone (Difco); and 0.3% KH₂PO₄. The medium was brought to pH 4.0 with NaOH.

**Particulate-membrane enzyme preparation.** Freshly harvested cells were washed and then suspended in Tris-hydrochloride buffer (pH 7.5), containing 10 mM MgCl₂ and 1 mM EDTA, to a density of 30 mg (dry weight) of cells per ml. The cells were treated in the cold in a Raytheon model DF 10 magnetorestrictive oscillator, at 200 W and 10 kc/s, for 20 min. The sonic extract was centrifuged at 15,000 × g for 20 min, and the resulting supernatant was then centrifuged at 150,000 × g for 120 min. The sediment obtained was suspended in the same buffer as described above to a protein concentration of 50 mg/ml.

**Incubation procedure for metabolic changes during glucose utilization.** Cylindrical flasks (30 ml) served as reaction vessels. Reaction mixtures (2 ml) contained 50 mM phosphate buffer (pH 6.0), an appropriate cell suspension, and 3 mM \[^{14}C\]glucose (2 Ci/mol). The mixtures were shaken at 30°C in a gyratory incubation shaker at 120 oscillations per min. After incubation, the reaction was terminated by rapidly injecting 3 ml of precooled (−15°C) chloroform-methanol (2:1, vol/vol), containing 0.05 N HCl, with thorough mixing. The amounts of various metabolites (such as ATP, ADP, AMP, cyclic AMP, fructose 1,6-diphosphate, and citrate cycle intermediates) released from cells treated as described above were similar to those released by treating a similar cell suspension with 2.5 N H₂SO₄ as previously described (1, 8, 45).

**Analysis of products.** All of the steps described below were carried out in 0 to 4°C, unless otherwise specified. Carrier cellulose powder (30 mg) was mixed into the terminated-reaction mixture, and the phases were separated by centrifugation with the lower (organic) and upper (aqueous) phases removed. The interface was suspended in 2 ml of water, and 3 ml of chloroform-methanol (2:1, vol/vol) was added and mixed in. The separated phases were added to the corresponding layers from the previous step. The combined organic layers were washed once with 3 ml of a solution containing 0.5% NaCl, 0.034% MgCl₂, and 0.04% CaCl₂ (18), and then transferred to scintillation vials, dried with a stream of N₂, and counted. This fraction is referred to as "chloroform soluble."

The interface fraction was washed five times with 2 ml of water, and the washings were added to the combined aqueous layers from the previous step. A portion of the aqueous fraction was supplemented with a mixture of 5 mM each glucose, gluconate, glucose 6-phosphate, glucose 1-phosphate, 6-phosphogluconate, UDP-glucose, and fructose 6-phosphate and then dialyzed for 48 h against 6 liters of water, with intermittent changes of the water. The dialysate was concentrated in a lyophilizer and then counted. This fraction is referred to as "water soluble." The remainder of the undialyzed aqueous fraction was kept for the analysis of hexose-phosphates, UDP-glucose, and glucose, as described below.

The washed interface fraction was treated with 3 ml of 0.5 N NaOH for 20 min in a boiling-water bath. The mixture was centrifuged, and the pellet was washed five times with water. The washings and the alkali supernatant were combined and counted. This fraction is referred to as "alkali soluble." The washed alkali-treated precipitate was dried at 60°C and counted and is referred to as the "cellulose fraction."

**Analytical methods.** The labeled glucose 6-phosphate, glucose 1-phosphate, 6-phosphogluconate, and UDP-glucose, within a mixture, were determined by measuring the \[^{14}C\]O₂ released in the sequential conversion of each of these compounds into ribulose 5-phosphate. This was accomplished with the aid of the following enzymes: UDP-glucose pyrophosphorylase, phosphoglucomutase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. (M. Swissa, Ph.D. thesis, The Hebrew University of Jerusalem, Jerusalem, Israel, 1978). Glucose was determined with glucose oxidase and peroxidase (9).

Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Values were corrected for quenching by the use of standard-\[^{14}C\]labeled material was counted in a scintillation liquid containing 1 part of Triton X-100 to 2 parts of standard, toluene-based scintillation fluid.

**Thin-layer chromatography.** Celloodextrins and monosaccharides were separated on silica plates, with
isopropanol-ethyl acetate-water (5:3:2, vol/vol/vol) as the solvent (36). After chromatography the plates were dried, and sections of 1 cm width were scraped off and counted. Sugars were detected with the aniline-glacial acetic acid reagent (19).

Enzymatic treatment of alkali- and water-soluble fractions. The alkali-soluble fraction was dialyzed for 48 h against water, with intermittent changes of the water. Samples of radioactive water-soluble and dialyzed alkali-soluble fractions were lyophilized to dryness and then dissolved in water. These preparations were subjected to the action of cellulase and pronase under the following conditions: (i) cellulase from \textit{Trichoderma viride} (5 mg of Onozuka-R-10) or from \textit{Aspergillus niger} (15 mg of Sigma type I) in 0.03 M acetate buffer (pH 4.5), incubated for 72 h at 37°C; (b) pronase (2 mg of Calbiochem grade B) in 0.05 M phosphate buffer (pH 7.3), incubated for 72 h at 37°C. After the first 24 h, an additional 2 mg of pronase was added. Incubations were carried out in the presence of toluene to prevent microbial growth. Reactions were terminated by heating in a boiling-water bath for 10 min. Samples treated similarly but in the absence of the enzymes served as controls. Reaction mixtures and controls were either dialyzed, as described above, or chromatographed on a Sephadex G-50 column (1.0 by 25 cm). Radioactivity remaining in the dialysis bag or eluting with the void volume was considered as nonhydrolyzed material, and the radioactivity released from the dialysis bag or included in the column (V) was considered as hydrolyzed material.

Chemicals. Radioactive compounds were obtained from the Radiochemical Centre, Amersham, England. Nucleotides, sugar phosphates, and most of the enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. Cellulase Onozuke-R-10 was obtained from Yakult Biochemicals Co. Ltd., Japan, and pronase, grade B, was from Calbiochem, La Jolla, Calif.

RESULTS

Metabolic changes within the cell and in the medium during cellulose synthesis by resting wild-type cells. Resting cells were incubated with limited amounts of [14C]glucose. At various time intervals (5 s to 60 min), the cells were extracted and analyzed for 14C-labeled hexose-phosphates and UDP-glucose and for 14C incorporation into various cell fractions. In addition, the 14C-labeled cellulose formed and the glucose concentration in the medium were also determined. Since carbohydrate oxidation in \textit{A. xylinum} occurs by way of the pentose cycle (39, 44), labeling of cellular substances not directly related to carbohydrate metabolism was prevented by using [1-14C]glucose as the substrate in these experiments, since this substrate loses its label upon entry into the pentose cycle. The incorporation of 14C from this substrate into cellulose and its potential precursors, therefore, represents label transfer from noncycled hexose moieties derived directly from exogenous substrate.

14C-labeled glucose 1-phosphate, glucose 6-phosphate, 6-phosphogluconate, and UDP-glucose appeared within 5 s after exposure of the cells to [1-14C]glucose (Fig. 1), the level of glucose 6-phosphate being the highest. Except for 14C-labeled 6-phosphogluconate, the level of which remained practically unchanged after 30 s, the level of labeled hexose-phosphates and UDP-glucose reached a maximum after 5 min and then declined as the exogenous substrate was being exhausted. Incorporation of glucosyl moieties into various cellular fractions, namely, chloroform-, water-, and alkali-soluble fractions, was observed within 60 s, whereas the amount of label incorporated into these fractions reached a plateau within 5 min. Incorporation of label into cellulose was detected at a later stage and increased continuously, even when the level of labeled hexose-phosphates and UDP-glucose decreased. The disappearance of the intermediates is almost completely accounted for by the continuous incorporation of [1-14C]glucose into cellulose.

Metabolic changes during glucose utilization by a celluloseless mutant. A direct relationship between cellular hexose-phosphate and UDP-glucose levels and the glycosylation of various cellular fractions during glucose utilization was indicated in similar time course studies with an \textit{A. xylinum} mutant specifically deficient in the ability to form cellulose (Fig. 2). As with the wild-type strain, a rapid increase in 14C-labeled hexose-phosphates and UDP-glucose occurred during utilization of [1-14C]glucose. However, the pattern of glycosylation of cellular fractions accompanying glucose utilization by the mutant was significantly different. Thus, all three fractions, namely, the chloroform-, water-, and alkali-soluble fractions of the mutant, were glycoylated to a much higher level than the corresponding fractions in the wild type. In this case, again, the labeling of the chloroform-soluble fraction leveled off after 15 min but at a level 10-fold higher than that observed with the wild type. On the other hand continuous incorporation of [1-14C]glucose into the water- and alkali-soluble fractions was observed, reaching a level four to five times higher than that in the wild type. Moreover, in this case no cellulose synthesis occurred, and the disappearance of labeled hexose-phosphate and UDP-glucose was fully accounted for by concurrent [1-14C]glucose incorporation into the water- and alkali-soluble fractions.

Incorporation of glucose from UDP-glucose into various fractions by a particulate
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Enzyme system. Particulate fractions obtained from sonic extracts of A. xylinum catalyze the incorporation of \([^{14}C]\)glucose from UDP-\([^{14}C]\)glucose into a water- and alkali-insoluble product. We demonstrated this activity, originally reported by Glaser (22) for the cellulose-forming strain, with preparations of the celluloseless mutant. The material synthesized by preparations from both sources was identified as a \(\beta\)-1,4-glucan. More than 95\% of the radioactivity of the product was released as water-soluble material after digestion with cellulase. Thin-layer chromatography of the radioactive material released indicated that it was composed mainly of glucose and partly of celllobiose.

**Fig. 1.** Time course of metabolic changes during cellulose synthesis by wild-type cells. Reaction mixture (2 ml) contained 100 \(\mu\)mol of phosphate buffer (pH 6.0), 20 mg (dry weight) of wild-type cells, and 6 \(\mu\)mol of labeled glucose (2 \(\mu\)Ci/\(\mu\)mol). The mixtures were incubated for the indicated time periods. Incubation conditions, termination of the reaction, and analysis of the various components were as described in the text. G6P, Glucose 6-phosphate; G1P, glucose 1-phosphate; 6PG, 6-phosphogluconate.

**Fig. 2.** Time course of metabolic changes during glucose utilization by the celluloseless mutant. Reaction mixtures (2 ml) contained 100 \(\mu\)mol of phosphate buffer (pH 6.0), 20 mg (dry weight) of celluloseless mutant cells, and 6 \(\mu\)mol of labeled glucose (2 \(\mu\)Ci/\(\mu\)mol). The mixtures were incubated for the indicated time periods. Incubation conditions, termination of the reaction, and analysis of the various components were as described in the text. G6P, Glucose 6-phosphate; G1P, glucose 1-phosphate; 6PG, 6-phosphogluconate.
The incorporation of the glycosyl moiety of UDP-glucose into the β-1,4-glucan was accompanied by glycosylation of various fractions. Figure 3B represents the time course of label transfer from UDP-[14C]glucose, using a preparation of the wild type. As was the case with glucose and whole cells, incorporation from UDP-glucose into the chloroform-, water-, and alkali-soluble fractions was rapid during the first 10 to 15 min and then declined, approaching a plateau; however, surprisingly, in this case the incorporation into the alkali-insoluble fraction did not increase continuously but rather reached a plateau at an early stage. However, a markedly different course of label transfer was observed with a preparation of the mutant (Fig. 3A). In this case, incorporation into all of the fractions continued to increase over the period studied, and the amounts incorporated were about three times larger than in experiments with the wild type, except for the incorporation into the cellulose fraction, which in the mutant was 5 to 10 times smaller than in the wild type.

The early decline in label transfer from UDP-[14C]glucose to the different products observed with preparations of the wild type could be due to progressive enzyme inactivation during the incubation period. This was suggested by the loss of activity observed after preincubation of the enzyme at 30°C before substrate addition. Thus, glucose incorporation into the alkali-soluble or insoluble fraction during the first 5 min after addition of UDP-[14C]glucose decreased by 40 to 50%, 60 to 70%, and 70 to 80% after preincubation periods of 5, 10, and 20 min, respectively. On the other hand, label transfer by preparations of the mutant were less affected by such preincubation, and the corresponding decreases in the initial rate of incorporation did not exceed 30 to 40%.

**Incorporation products.** When the labeled chloroform-soluble fraction was treated with 0.1 N HCl at 100°C for 10 min, about 30% of the radioactivity was released as water-soluble material. The radioactivity released migrated coincident with cellobiose and glucose upon thin-layer chromatography. When submitted to mild alkaline treatment (0.08 N NaOH at 37°C for 30 min), only 10% of the radioactivity of the lipid-soluble material was released as water-soluble material. After incubation of suspensions of the lipid fractions with β-glucosidase (5 U, Sigma) or with cellulase (5 mg of Onozuka R-10), 50 and 30% of the radioactivity became water soluble by the action of β-glucosidase and cellulase, respectively.

The water- and alkali-soluble fractions were treated with either cellulase or pronase. About 50% of the radioactivity from the water-soluble fraction and 35% from the alkali-soluble fraction were hydrolyzed by pronase, whereas 80 to 90% of the radioactivity in these fractions was hydrolyzed by cellulase (Table 1). These findings strongly suggest that the water- and alkali-soluble fractions are composed of glycoproteins containing β-1,4-linked glucoses. The relatively small decrease in radioactivity after dialysis or chromatography of the pronase digests may indicate either that the glucoproteins of these fractions are heterogeneous with respect to the molecular weight of their cellodextrin moiety or, alternatively, that these fractions, in addition to glucoprotein, also contain non-protein-linked high-molecular-weight cellodextrins.

Samples of the water-soluble fraction were incubated with 0.1 N NaOH for 4 days at 25°C (β-elimination), but no dialyzable radioactivity

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**Fig. 3.** Time course of incorporation of glucose from UDP-glucose into various fractions by a particulate system. Reaction mixtures contained the following components in a final volume of 0.05 ml: UDP-[U,14C]glucose, 1 nmol (800,000 cpm); MgCl₂ 0.5 μmol; EDTA, 0.1 μmol; Tris-hydrochloride buffer (pH 8.0), 2.5 μmol; and particulate enzyme preparations from the celluloseless mutant (A) and from wild-type cells (B), 1.5 mg of protein. The mixtures were incubated at 30°C for the indicated time periods. Termination of the reaction and analysis of the various components were as described in the text.
TABLE 1. Enzymatic digestion of alkali- and water-soluble fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Enzyme treatment</th>
<th>Non-hydrolyzed material cpm</th>
<th>Hydrolyzed material cpm</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble</td>
<td>Cellulase</td>
<td>1,000</td>
<td>13,000</td>
<td>93</td>
</tr>
<tr>
<td>Water soluble</td>
<td>Pronase</td>
<td>5,760</td>
<td>7,240</td>
<td>52</td>
</tr>
<tr>
<td>Alkali soluble</td>
<td>Cellulase</td>
<td>1,620</td>
<td>7,380</td>
<td>82</td>
</tr>
<tr>
<td>Alkali soluble</td>
<td>Pronase</td>
<td>6,030</td>
<td>2,970</td>
<td>33</td>
</tr>
</tbody>
</table>

* Samples of radioactive water-soluble and dialyzed alkali-soluble fractions were treated with the enzymes indicated, and the digests were dialyzed or chromatographed on Sephadex G-50 columns as described in the text. Radioactivity remaining in the dialysis bag or eluting with the void volume is considered to be nonhydrolyzed material, and the radioactivity released by dialysis or included in the column (Vr) is considered to be hydrolyzed material. The recovery of radioactivity was 95 to 100%.

was released by such treatment. It appears, therefore, that the carbohydrate-peptide linkage is not an α-glycosidic link to serine or threonine (31).

Glucose transfer from glucoprotein to cellulose. A precursor-product relationship between the water- and alkali-soluble products and cellulose was indicated by an in vivo pulse-chase experiment (Table 2). Cells were first incubated for 30 min with [1-14C]glucose at 0°C. The cells were diluted, centrifuged, and reincubated at 30°C either with an excess of unlabeled glucose or in buffer. At the end of the first incubation, some cellulose was formed, and the alkali- and water-soluble fractions of the cells were the ones mainly labeled. The distribution of radioactivity remained unchanged after a second incubation period at 30°C in buffer. However, when the cells were reincubated with unlabeled glucose, radioactivity was quantitatively chased, initially from the alkali-soluble glucoprotein fraction and subsequently from the water-soluble glucoprotein into cellulose.

DISCUSSION

Previous studies (38, 39, 44) have indicated that glucose catabolism in A. xylinum occurs mainly by way of a pentose cycle, that cells acting on glucose rapidly form gluconate, and that, although glucose supports a much more rapid initial cellulose synthesis than does gluconate, the bulk of glucose carbon is assimilated to cellulose via a pentose cycle operating on intermediary-formed gluconate. Predicated upon these findings, [1-14C]glucose appeared to be the substrate of choice for studying the kinetics of labeling and turnover of 14C-containing compounds that are potential precursors of cellulose. A sequence of events similar to those occurring during a pulse-chase experiment would ensue with limited amounts of this substrate. A rapid influx on the pathway to cellulose of labeled noncycled hexose moieties derived directly from exogenous substrate would occur from the outset and would shortly be followed by a flow of unlabeled moieties arising from pentose cycle intermediates.

Hexose-phosphates and sugar nucleotides may be considered to be likely primary precursors of cellulose. Enzyme systems which could affect formation of such precursors have been shown to be present in extracts of A. xylinum. These include an ATP-linked constitutive glucokinase catalyzing the phosphorylation of glucose to glucose 6-phosphate (7), a phosphoglucomutase promoting the interconversion of the latter compound to glucose 1-phosphate (23), and a UDP-glucose pyrophosphorylase reacting UDP with glucose 1-phosphate to form UDP-glucose. In our experience, the latter is the only nucleoside diphosphate glucose pyrophosphorylase present in these extracts (Swissa, Ph.D. thesis). The actual in vivo occurrence of this sequence of reactions in cells actively synthesizing cellulose is the subject of an ongoing investigation.

TABLE 2. Transfer of [1-14C]glucose from the alkali- and water-soluble fractions into cellulose

<table>
<thead>
<tr>
<th>Incubation*</th>
<th>[1-14C]glucose incorporation (cpm)</th>
<th>Cellulose</th>
<th>Alkali soluble</th>
<th>Water soluble</th>
<th>Chloroform soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>First incubation, 0°C</td>
<td>[1-14C]glucose (3 mM)</td>
<td>33</td>
<td>182</td>
<td>219</td>
<td>5</td>
</tr>
<tr>
<td>Second incubation, 30°C</td>
<td>(a) Glucose (40 mM)</td>
<td>30 min</td>
<td>131</td>
<td>83</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>60 min</td>
<td>291</td>
<td>39</td>
<td>126</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>90 min</td>
<td>377</td>
<td>15</td>
<td>44</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(b) Buffer</td>
<td>90 min</td>
<td>30</td>
<td>179</td>
<td>225</td>
</tr>
</tbody>
</table>

* Reaction mixtures for the first incubation contained, in a final volume of 0.5 ml, the following components: 1.5 μmol of [1-14C]glucose (2 × 10^7 cpn), 2.5 μmol of potassium acetate, 25 μmol of sodium phosphate buffer (pH 6.0), and 5 mg (dry weight) of wild-type cells. The mixtures were incubated at 0°C in a shaking bath for 30 min. After the incubation, the mixtures were each diluted with 6 ml of 0.05 M phosphate buffer (pH 6.0) and then centrifuged in the cold. The supernatants were removed, and the pellets were suspended, each in 2 ml of phosphate buffer. One of the suspended pellets was extracted with 3 ml of chloroform-methanol (2:1, vol/vol), and the remainder were incubated at 30°C for the times indicated: (a) with 80 μmol of unlabeled glucose and (b) with no addition. At the end of the second incubation, the reaction was terminated by the addition of 3 ml of chloroform-methanol (2:1, vol/vol). Terminated-reaction mixtures from the two incubations were fractionated and analyzed as described in the text.
ing cellulose was indicated here by the rapid incorporation of C-1 of glucose into glucose 6-phosphate, glucose 1-phosphate, and UDP-glucose (Fig. 1). The pattern of labeling and turnover of these 1-14C-labeled compounds, when related to the kinetics of glucose C-1 incorporation into cellulose in resting whole cells described here, indicate that glucose 6-phosphate, glucose 1-phosphate, and UDP-glucose are indeed the primary precursors of cellulose in *A. xylinum*.

The possible involvement of UDP-glucose or glucose 1-phosphate or both in cellulose synthesis by *A. xylinum* has previously been suggested by Cooper and Manley (14) from studies on the concentration behavior with time of these compounds during cellulose synthesis. Surprisingly, these authors report that glucose 6-phosphate is not present in detectable amounts during cellulose synthesis. The disparity between our observations and those of these authors could be due to differences in methods of extraction and of assay, as well as to the circumstance that our cells had a 100-fold-higher synthesizing activity.

Incorporation of glucosyl moieties into lipid-, water-, and alkali-soluble cellular materials has been shown to occur during cellulose synthesis by *A. xylinum*. The water- and alkali-soluble products have been identified as glycoprotein(s) containing β-1,4-linked glucoses. The glucolipid product appears to contain in its carbohydrate portion glucosyl moieties linked in a β-configuration. The time course of formation of the glucosylated products compared with that of cellulose suggest that lipid-linked oligosaccharides and protein-linked oligosaccharides may function as intermediates beyond UDP-glucose on the pathway to cellulose in this organism. Such intermediates have been postulated by Khan and Colvin (29) and might be similar to the lipid- and protein-linked intermediates of cellulose synthesis proposed by Hopp et al. for the alga *Prototheca zopfii* (28). These two intermediates, correspondingly, could functionally be analogous to the lipid-bound sugar intermediates involved in the synthesis of bacterial cell wall components (34) and to the glucoprotein precursors of glycogen and starch (32, 33).

A precursor-product relationship between the presumed glucoprotein intermediate and cellulose is suggested by the transfer into cellulose of [14C]glucose retained in the water- and alkali-soluble fractions of pulse-labeled cells (Table 2). In turn, an interaction between the synthesis of hexose-phosphates, UDP-glucose, glucolipid, and glucoprotein is indicated by comparing the kinetics of glucose transfer reactions in the cellulose-less mutant with that in the wild type. In the wild type (Fig. 1), the intermediates beyond UDP-glucose appear to be in a steady state, and the flow of hexose-phosphate and UDP-glucose carbon into cellulose is continuous and undisturbed. On the other hand, in the mutant (Fig. 2), only the glucolipid reached a plateau, which can be interpreted as a steady state, and the carbons of hexose-phosphate and UDP-glucose were incorporated into the glucoprotein fractions which, unable to donate their glucose moieties to form cellulose, continued to accumulate during the incubation.

In accordance with this reasoning, it follows that the mutant is blocked in the stages of cellulose production beyond glucolipid and glucoprotein formation. This conclusion is compatible with the effect of coumarin (cf. 17, 24), which specifically inhibits cellulose synthesis in the wild type and, in a similar manner, affects the glucosylation of endogenous acceptors in both the wild-type and mutant strains (M. Swissa and M. Benziman, unpublished data). In this respect, our mutant is different from the celluloseless mutants reported by other laboratories, in which lipid glycosylation does not occur during glucose utilization (16, 21, 43).

The low specific activity of the cell-free system, which is about 0.2% of cellulose synthesis from glucose by whole cells, and the progressive loss of activity of these particulate preparations could be related to their lack of a specific acceptor or some structural factor of the cell wall or membrane and reflect the dependence of effective cellulose synthesis on the physical integrity of essential enzymatic and structural components of the synthetic system (3, 11, 16). Such a deficiency of the particulate preparation could also explain why more glucose of UDP-glucose was incorporated into the glucoprotein than in the presumed final product, the β-1,4-glucan (Fig. 3).

Another possibility to be considered (10, 15) is that the synthetic system consists of a multienzyme complex, the components of which are tightly coupled and react efficiently in a concerted manner. Studies on multienzyme complexes indicate that intermediary substrates in a multiple reaction sequence catalyzed by such complexes are not always used with great efficiency (20). In our case, an intermediate such as UDP-glucose, normally derived from glucose, the first substrate of the sequence leading to cellulose, remains enzyme bound and does not exchange freely with the UDP-glucose when supplied in solution.

**LITERATURE CITED**

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