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# β-1,3-Glucan in Developing Cotton Fibers

## STRUCTURE, LOCALIZATION, AND RELATIONSHIP OF SYNTHESIS TO THAT OF SECONDARY WALL CELLULOSE[1]

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### ABSTRACT

**Evidence is presented for the existence of a noncellulosic β-1,3-glucan in cotton fibers. The glucan can be isolated as distinct fractions of varying solubility. When fibers are homogenized rigorously in aqueous buffer, part of the total β-1,3-glucan is found as a soluble polymer in homogenates freed of cell walls. The proportion of total β-1,3-glucan which is found as the soluble polymer varies somewhat as a function of fiber age. The insoluble fraction of the β-1,3-glucan remains associated with the cell wall fraction. Of this cell wall β-1,3-glucan, a variable portion can be solubilized by treatment of walls with hot water, a further portion can be solubilized by alkaline extraction of the walls, and 17 to 29% of the glucan remains associated with cellulose even after alkaline extraction. A portion of this glucan can also be removed from the cell walls of intact cotton fibers by digestion with an endo-β-1,3-glucanase. The glucan fraction which can be isolated as a soluble polymer in homogenates freed of cell walls is not associated with membranous material, and we propose that it represents glucan which is also extracellular but not tightly associated with the cell wall. Enzyme digestion studies indicate that all of the cotton fiber glucan is β-linked, and methylation analyses and enzyme studies both show that the predominant linkage in the glucan is 1 → 3. The possibility of some minor branching at C-6 can also be deduced from the methylation analyses. The timing of deposition of the β-1,3-glucan during fiber development coincides closely with the onset of secondary wall cellulose synthesis. Kinetic studies performed with ovules and fibers cultured *in vitro* show that incorporation of radioactivity from [$^{14}$C]glucose into β-1,3-glucan is linear with respect to time almost from the start of the labeling period; however, a lag is observed before incorporation into cellulose becomes linear with time, suggesting that these two different glucans are not polymerized directly from the same substrate pool. Pulse-chase experiments indicate that neither the β-1,3-glucan nor cellulose exhibits significant turnover after synthesis.**

In our search for a cellulose synthetase in developing cotton fibers, we discovered, instead, a highly active UDP-glucose:β-1,3-glucan synthetase, the properties of which were described recently in detail (4, 6). Previous work from our laboratory (13) as well as a recent report by Huwyler *et al.* (11) indicated that cell walls of cotton fibers harvested at the time of secondary wall cellulose synthesis contain substantial quanties of a 3-linked glucan.[3] The glucan synthetase appears to be responsible for catalyzing the *in vivo* synthesis of this 3-linked glucan. In this communication, we report further on the localization, structure, and synthesis of the β-1,3-glucan of fibers of *Gossypium hirsutum*, Acala SJ-1. We also examine the relationship between the synthesis of this β-1,3-glucan and secondary wall cellulose.

### MATERIALS AND METHODS

**Growth of Plant Material.** *G. hirsutum* L. (Acala SJ-1) was used throughout these experiments. Conditions for plant growth and *in vitro* culture of fibers with their associated unfertilized ovules have been described previously (13).

**Chemicals.** D-[U-$^{14}$C]Glucose (240 mCi/mmol) was purchased from New England Nuclear. Endo-β-(1 → 3)-glucanase purified from *Rhizopus arrhizus* QM 1032 was the generous gift of E. T. Reese, U.S. Army Laboratories, Natick, Mass. Lichenin and *Bacillus subtilis* α-amylase type II-A were purchased from Sigma.

**Preparation of Cell Walls and Soluble β-1,3-Glucan.** Cell walls were prepared from fibers of various ages as described previously (13) but with the following modifications. Fibers were harvested into liquid $N_2$, then placed directly into 0.05 M Tes buffer (pH 7.0) at 4 C and homogenized in a glass tissue homogenizer. This homogenate was centrifuged at 4 C for 10 min at 5,500*g*. The resulting supernatant was brought to 80% (v/v) with absolute ethanol and allowed to stand for at least 2 h at −20 C or overnight at 4 C. The resulting precipitate was washed twice with cold 80% ethanol. This precipitate was the fraction containing the glucan referred to as the soluble β-1,3-glucan. The original cell wall pellet was washed further as described previously (13) and dried to constant weight over $P_2O_5$ under vacuum prior to analyses. In some cases (chemical or enzymic extraction of the cell walls), the walls were not allowed to dry, but following the final acetone wash, were washed again with water and then extracted as described below.

**Extraction of Cell Walls.** Cell walls (100–200 mg) derived from fibers harvested 19 to 22 DPA,[4] were extracted for 10 min in 10 ml 0.5% (w/v) ammonium oxalate (pH 5.0) at 90 C. Solubilized material was separated from residual material by centrifugation at 5,500*g* for 10 min. The remaining pellet was reextracted in identical fashion, and the combined supernatants represented the hot ammonium oxalate-soluble fractions. The pellet was extracted further by shaking overnight at room temperature in 20 ml 24% (w/v) KOH containing 0.5 M $NaBH_4$. The flask was flushed with $N_2$ and stoppered during the incubation. The solubilized material was separated by centrifugation and designated the alkali-soluble fraction. The residue, washed repeatedly with $H_2O$ to remove

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[3] The terminology for methylated derivatives used throughout is to indicate the deduced linkage by which the glycosyl residue in the original sample was connected to other sugars, rather than by indicating the positions in the derivatives of the methoxy and acetyl groups. For example, 3-linked glucose is equivalent to (1,3,5-tri-*O*-acetyl-2,4,6-tri-)-methylglucitol. Arabinosyl residues have been assigned the furanose ring form, and all other sugars the pyranose ring form.

[4] Abbreviations: DPA: days postanthesis; Rha: rhamnose.

KOH, was designated the cellulosic fraction. Both solubilized fractions were dialyzed extensively against $H_2O$, precipitated at 4 C in 80% ethanol (v/v), washed twice with ice-cold 80% (v/v) ethanol, and dried prior to analyses.

**Enzymic Digestion of Cell Walls.** For digestion of the cell walls of living intact fibers, ovules with their associated fibers cultured *in vitro* for 15 DPA were used. Ovules plus fibers were floated for up to 6 h at 34 C in standard culture medium (13) which was modified by the omission of glucose and the addition of 0.2 mg/ml of *Rhizopus* endo-β-1,3-glucanase. The release of reducing sugars was monitored, and at various times, the submerged fibers were harvested and analyzed for 3-linked glucose by methylation analysis as described below. In other experiments, isolated cell walls prepared from plant-grown fibers harvested at 18 DPA were digested. Five mg of cell walls were suspended in 1 ml 50 mM Na-acetate (pH 5.0) containing 3 mM sodium azide and 0.5 mg of *Rhizopus* endo-β-1,3-glucanase. For incubation with *B. subtilis* α-amylase (5 mg/ml), the incubation buffer was 100 mM K-phosphate (pH 7.0). All incubations were performed in a shaking water bath at 45 C for 18 h. Following incubation, ethanol was added to the reaction mixture to give a final concentration of 80% (v/v) and the mixture was allowed to stand at −20 C for 2 h. The precipitate was collected by centrifugation at 3,000*g* for 10 min and the resulting pellets were washed in 80% ethanol, then with acetone, and then dried for methylation analysis.

**Carbohydrate Analysis.** Reducing sugars were determined by the method of Nelson and Somogyi (8). Total carbohydrate was measured by the anthrone reaction (8). Alditol acetates were prepared by the procedure of Albersheim *et al.* (1) and quantitated by GLC using a (0.2 × 180 cm) glass column packed with Supelco 3% SP-2340 on 100/120 Supelcoport. Inositol was used as an internal standard. Separations were achieved with a temperature program of 4 C/min from 115 to 210 C using a Varian 2100 gas chromatograph.

Methylation of polysaccharides was performed by the method of Hakamori (5) with some modifications. The dried sample (usually 3–5 mg) was suspended in 1.0 ml dry dimethylsulfoxide in a tube which was sealed with a serum-vial cap, and sonicated for 4 h at 60 C; the tube was degassed, and $N_2$ was then continuously circulated through the tube during the reaction of the sample with 0.5 ml of 2 N methylsulfinyl carbanion at room temperature with stirring for 4 to 8 h. The reaction mixtures were then frozen in an ice bath and overlaid with 0.25 ml methyl iodide. The mixtures were allowed to thaw slowly at room temperature with stirring to allow a slow mixing of the methyl iodide into the chilled reaction. The mixture was stirred at room temperature until clear (usually 1 h). The mixture was then dialyzed overnight against running water, and then extracted twice with $CHCl_3$, followed by a third extraction with $CHCl_3$-$CH_3OH$ (2:1). The organic phases were pooled, evaporated to dryness under $N_2$, and hydrolyzed for 90 min at 121 C with 1 ml 2 N trifluoroacetic acid containing inositol as an internal standard. The samples were again evaporated to dryness and then reduced with 1.0 ml $NaBH_4$ (4 mg/ml in 1 N $NH_4OH$) for 90 min at 37 C or overnight at room temperature. When derivatives were to be subjected to GLC/MS, sodium borodeuteride was substituted for sodium borohydride to aid in the identification of stereochemically symmetrical derivatives. (It might prove helpful to the reader to note that our original protocol for borohydride or borodeuteride reduction [same concentration but at room temperature for 60 min] resulted in incomplete reduction which, following acetylation, yielded a significant amount of acetyl glycosides especially of 4-linked glucose. These acetyl glycosides eluted as peaks on the GLC in the region of terminal hexoses and singly linked pentoses.) Residual borohydride was destroyed by the addition of glacial acetic acid. The samples were then evaporated repeatedly with dry methanol until the residue no longer appeared oily. They were then dried under vacuum for at least 2 h prior to acetylation with 0.2 ml acetic anhydride for 90 min at 121 C. The resulting derivatives were then separated on GLC or subjected to GLC/MS as described previously (13).

**Quantitation of Changes in Content of 3-Linked Glucose and Cellulose as a Function of Fiber Age.** Three-linked glucose was quantitated by methylation analysis and cellulose was quantitated by the procedure of Updegraff (19). Calculations of content per mm fiber length also necessitated the measurement of gram fresh weight of fibers harvested, gram cell wall per gram fresh weight of fibers, and fiber length for each age harvested. Fiber lengths were taken from our previous data (13), and calculations were performed essentially as described in that publication.

**Pulse-labeling Studies.** All labeling studies were performed with fibers associated with unfertilized ovules which had been cultured *in vitro* for 15 DPA. Extreme caution was taken to handle the fibers gently. Following careful removal from flasks with forceps, the ovules (plus fibers) were transferred to wash solution in Petri dishes which contained flat plastic strainers with diameters just smaller than those of the Petri dishes. For subsequent washes or labeling, the fibers were lifted with these strainers, blotted through the strainer onto paper towels, and transferred to the solution of choice. Prior to labeling, the ovules were washed three times in a modified culture medium which had the glucose concentration lowered to 10 or 20 mM and which contained, in addition, 80 or 90 mM pentaerythritol as an osmotic substitute for glucose. Labeling with [$^{14}$C]glucose was performed in this same medium as indicated in the figure legends for each experiment. At the time points indicated in the text, ovules (plus fibers) were harvested into liquid $N_2$, and stored at −20 C until processed. Fibers were removed from the ovules with forceps while still frozen and homogenized in 0.05 M Tes buffer (pH 7.0). Cell walls and the soluble β-1,3-glucan fraction were prepared for each sample as described above. β-1,3-Glucan and cellulose were measured by either of two procedures which yielded essentially the same results. The first procedure was to quantitate β-1,3-glucan by methylation analysis, and cellulose was determined on a separate sample by the radioactivity that remained insoluble after treatment with acetic/nitric reagent at 100 C for 1 h (19). In the second method, β-1,3-glucan was measured as radioactivity and reducing sugar released by treatment of cell walls with *Rhizopus* endo-β-1,3-glucanase as described above. In this case cellulose determinations were made as described above with the residue which remained after enzyme digestion. Methylation analyses confirmed that the polysaccharide residue after acetic/nitric digestion contained only 4-linked glucose residues.

## RESULTS

**Detection of 3-Linked Glucan in Fiber Cell Walls by Methylation Analysis.** Figure 1, A and B, shows the GLC profile of the partially methylated, partially acetylated sugar derivatives obtained from derivatized cell walls of cotton fibers harvested during the stage of primary wall synthesis (10 DPA) or secondary wall synthesis (20 DPA). Peak 9 has been identified as 3-linked glucose by virtue of its fragmentation pattern in MS and its retention time which is identical to that of 3-linked glucose derived from laminaribiose or laminarin. This peak is much more prominent in cell walls derived from older fibers engaged in secondary wall cellulose synthesis.

**Evidence for Existence of a Soluble Three-Linked Glucan.** In addition to the 3-linked glucose in the derivatized cell wall fraction of older cotton fibers, we could also detect significant quantities of a buffer-soluble 3-linked glucan. When cotton fiber homogenates, freed of cell walls by centrifugation, were brought to 80% (v/v) ethanol, a precipitate formed which, by methylation analysis, was shown to contain 3-linked glucose (Fig. 1, C and D). The 3-linked glucose in this fraction was substantially higher at 20 DPA than at 10 DPA. Very little 4-linked glucose, indicative of starch

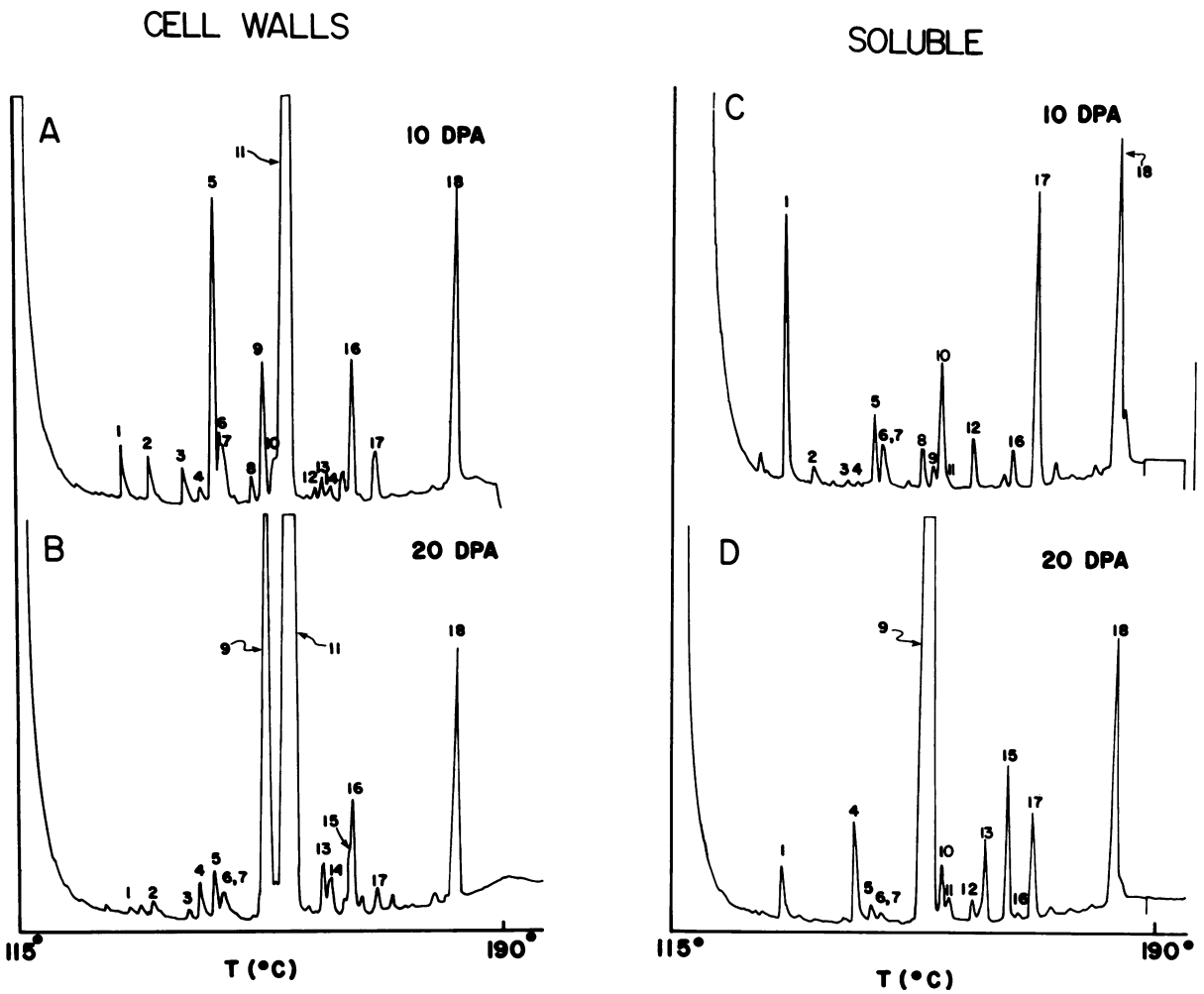


FIG. 1. GLC profiles of partially methylated, partially acetylated derivatives derived from the carbohydrates of cotton fibers. A and B: derivatives obtained from permethylation of cell walls. C and D: derivatives obtained from ethanol precipitable carbohydrates present in the homogenates of fibers freed of cell walls. Numbers by the peaks identify them as the following: 1: terminal Ara; 2: terminal Xyl; 3: 2-linked rha; 4: terminal Glc; 5: 5-linked Ara and terminal Gal; 6: 2-linked Xyl; 7: unidentified; 8: 2,4-linked Rha; 9: 3-linked Glc; 10: 3-linked Gal; 11: 4-linked Glc; 12: 6-linked Gal; 13: 3,4-linked hexose (probably Glc); 14: 2,3-linked hexose (probably Glc); 15: 3,6 linked-Glc; 16: 4,6-linked Glc; 17: 3,6-linked Gal; 18: fully acetylated inositol (internal standard). Separations were performed on a Varian 2100 gas chromatograph using a glass column (0.2 × 180 cm) packed with 0.2% polyethylene glycol adipate, 0.2% polyethylene glycol succinate, and 0.4% silicone XF-1150 on Gas-chrom P (100–120 mesh). Temperature programing was at 2 C/min from 115 to 190 C with a helium flow rate of approximately 30 ml/min.

or cellulose, was found in this fraction of the fiber homogenate at any age. In addition to 3-linked glucose in this soluble fraction, we also consistently observed the presence of terminal arabinose and 3,6-linked galactose, 3-galactose, and minor amounts of 6-galactose, indicative of the presence of a soluble arabinogalactan in the fibers. This polymer was more abundant in younger fibers.

To study the localization of this buffer-soluble glucan further, fibers were broken gently by chopping finely with scissors in a medium designed to preserve organelles (0.05 M Tes [pH 7.0] containing 0.4 M sorbitol, 1 mM $MgCl_2$ and $CaCl_2$, and 0.1 mM DTT). The homogenate was freed of cell walls by squeezing it through several layers of cheesecloth and then separated into a soluble and particulate fraction by centrifugation for 1 h at 100,000*g*. When these fractions were subjected to methylation analysis, it was found that the soluble and the particulate fraction each contained only 0.1% of the total buffer-soluble glucan, although each fraction contained about 7% of the total arabinogalactan. This indicates that although breakage and release of soluble and membrane fractions was not complete by such a gentle extraction, nevertheless significantly larger quantities of soluble arabinogalactan than soluble glucan were released. If one uses the release of arabinogalactan as a measure of breakage and release (~14%), then it appears that if breakage had been complete, less than 2% of the total soluble glucan would have been freely dissociable from the cell walls by gentle extraction. When the cell wall residue after gentle extraction was broken up rigorously by homogenization in a glass grinder, then 99.8% of the total buffer-soluble β-1,3-glucan was released from the cell walls.

**Structural Characterization of Three-Linked Glucan.** Evidence that the 3-linked glucan is β-linked was obtained by glucanase digestion studies. Following methylation analysis and GLC separation, the 3-linked glucose peak was nearly absent in cell walls which had been treated with a *Rhizopus* endo-β-1,3-glucanase (data not shown). Cell walls treated similarly with buffer lacking the enzyme showed no loss of 3-linked glucose compared with untreated walls. The soluble 3-linked glucan was also digested completely by treatment with this glucanase (data not shown). Evidence that the glucan was not a mixed-linked glucan (typical of cell walls of monocotyledonous plants, 14) containing both 1,3 and 1,4 linkages was obtained by an experiment in which cell walls were treated overnight with a preparation of *B. subtilis* α-amylase (Sigma, type IIA) which is reported to be highly contam-

TABLE I

Chemical Extraction of Cotton Fiber Cell Walls

Cell walls, derived from bolls harvested 19, 20, or 21 DPA (Expts 1, 2, and 3, respectively) were used. Procedures for wall extraction and preparation and analysis by GLC of sugar derivatives are described in MATERIALS and METHODS. Separations were performed as described in the legend to Fig. 1. Values given are not corrected for specific losses during extraction or derivitization because suitable standards are not available, but are calculated relative to the recovery of the internal standard inositol. However, values of 3-linked glucose quantitated methylation analysis are similar to the values obtained by β-1,3-glucanase digestion of walls. By contrast, the quantitation of cellulose (4-linked glucose) by methylation analysis yields a severe underestimate due to the difficulty of completely methylating this polymer. NS = no significant amount detected; ND = not determined. Values in parenthesis indicate the percent of total cell wall 3-linked glucose found in the fraction.

| | Amount of Derivative (μmoles/mg original cell wall) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Hot Ammonium Oxalate Soluble | | | 24% KOH Soluble | | | 24% KOH Insoluble | | |
| Sugar Derivative | Exp.1 | Exp.2 | Exp. 3 | Exp.1 | Exp.2 | Exp.3 | Exp. 1 | Exp.2 | Exp.3 |
| T-xyl | NS | NS | ND | 1.4 | 1.7 | ND | NS | NS | ND |
| T-glc | 0.8 | NS | ND | 2.0 | 3.1 | ND | NS | NS | ND |
| 5-ara | NS | NS | ND | 0.7 | 1.2 | ND | NS | NS | ND |
| 2-xyl | NS | NS | ND | 1.3 | 2.3 | ND | NS | NS | ND |
| 3-glc | 17.0(17) | 2.1(1.6) | 84.0(32) | 58.3(58) | 88.3(69) | 134.0(51) | 25.5(25) | 36.7(29) | 45.0(17) |
| 4-glc | 1.0 | NS | ND | 6.1 | 7.7 | ND | 878.5 | 1352.0 | 1128.0 |
| 3,4-hexose | NS | NS | ND | NS | NS | ND | 28.9 | 36.5 | ND |
| 2,3-hexose | 1.2 | NS | ND | 3.4 | 3.6 | ND | 16.9 | 21.8 | ND |
| 3,6-glc x 4,6-glc | 1.4* | NS | ND | 6.3 | 9.9 | ND | 65.5 | 55.1 | ND |

*Primarily 3,6-glc in this fraction as judged by GLC/MS

inated with a glucanase specific for cleaving such mixed-linked β-glucans (10). No significant loss of 3- or 4-linked glucose was obtained by this treatment. This enzyme preparation was quite active in causing release of reducing sugars from a commercial preparation of the mixed-linked-glucan lichenin (data not shown). Further indications that the insoluble glucan associated with the cell wall fraction is truly extracellular comes from our observation that about 50% of this glucan can be removed from *intact* cotton fibers by treatment for 2 h with the *Rhizopus* endo-β-1,3-glucanase (data not shown). This removal was correlated with the release of reducing sugar into the medium. Evidence that fibers remained intact during this treatment comes from our observation that the fibers maintained the ability to accumulate neutral red.

When cell walls of older fibers were subjected to chemical extraction procedures, the β-1,3-glucan showed a varying degree of solubility (Table I). A variable fraction of the total cell wall 3-linked glucose could be extracted from the cell wall by treatment with hot (100 C) ammonium oxalate. (Subsequent studies have shown that hot water is equally effective.) This fraction appeared to represent a nearly pure β-1,3-glucan. A small amount of terminal and 3,6-linked glucose was present in this relatively pure fraction, and may indicate that a small degree of branching exists in this polymer. (The data of Fig. 1, C and D, also show that a rise in terminal and 3,6-linked glucose occurs concomitantly with the rise in 3-linked glucose in older fibers and may also indicate that the soluble glucan has limited branching. However, since this preparation is relatively less pure, this conclusion can only be considered tentative.) Further extraction of the cell walls with 24% KOH resulted in further solubilization of 3-linked glucose as well as other noncellulosic polymers, but even after this rigorous extraction, a portion of the 3-linked glucose remains associated with the insoluble cellulosic residue.

Both the soluble glucan and glucan solubilized from cell walls by hot water extraction were subjected to chromatography on Bio-Gel P-100 using 0.05 M $NH_4HCO_3$ as solvent. Both glucan fractions eluted in the void volume, indicating a relatively high mol wt (data not shown).

**Variation in Content of β-1,3-Glucan as a Function of Fiber Age.** Cell walls derived from fibers of various ages were prepared, and the content of insoluble β-1,3-glucan was measured by quantitation of the 3-linked glucose obtained in methylation analyses. Complete methylation of cellulose was difficult, and so cellulose was quantitated independently by the method of Updegraff (19). Figure 2 shows the time course of deposition of cellulose in the cell wall. As discussed in a previous publication (13), the shape of the curves is rather different depending upon whether the data are expressed as per cent by weight cellulose or as grams cellulose per mm fiber length. The abrupt increase in per cent by weight cellulose between 16 and 20 DPA best defines the *onset* of secondary wall cellulose deposition. As indicated by the alternate method of plotting the data, the maximum rate of cellulose deposition per unit length of fiber occurs somewhat later in development. Figure 2 shows how the amount of soluble and insoluble β-1,3-glucan per unit length of fiber changes during development. The area under these curves is summed in Figure 2 which shows the calculated *total* β-1,3-glucan per unit length of fiber. This figure also shows a plot of the per cent of the cell wall which is composed of 3-linked glucose as a function of fiber age. The content of both the soluble and insoluble β-1,3-glucan in the cotton fiber was low during the period of elongation and primary wall synthesis, and rose abruptly at approximately the time of onset of secondary wall cellulose synthesis. The content declined somewhat later in development. Although the decline in content at later ages was reproducible, we could not rule out the possibility that the polymer merely becomes more inaccessible to methylation as the fibers mature.

**Occurrence of β-1,3-Glucan in Other Cultivars of Cotton.** Methylation analyses of mature fibers of other cultivars of cotton besides Acala SJ-1 showed that every cultivar examined contained β-1,3-glucan in small amounts (0.1–0.3% of dry weight at maturity)

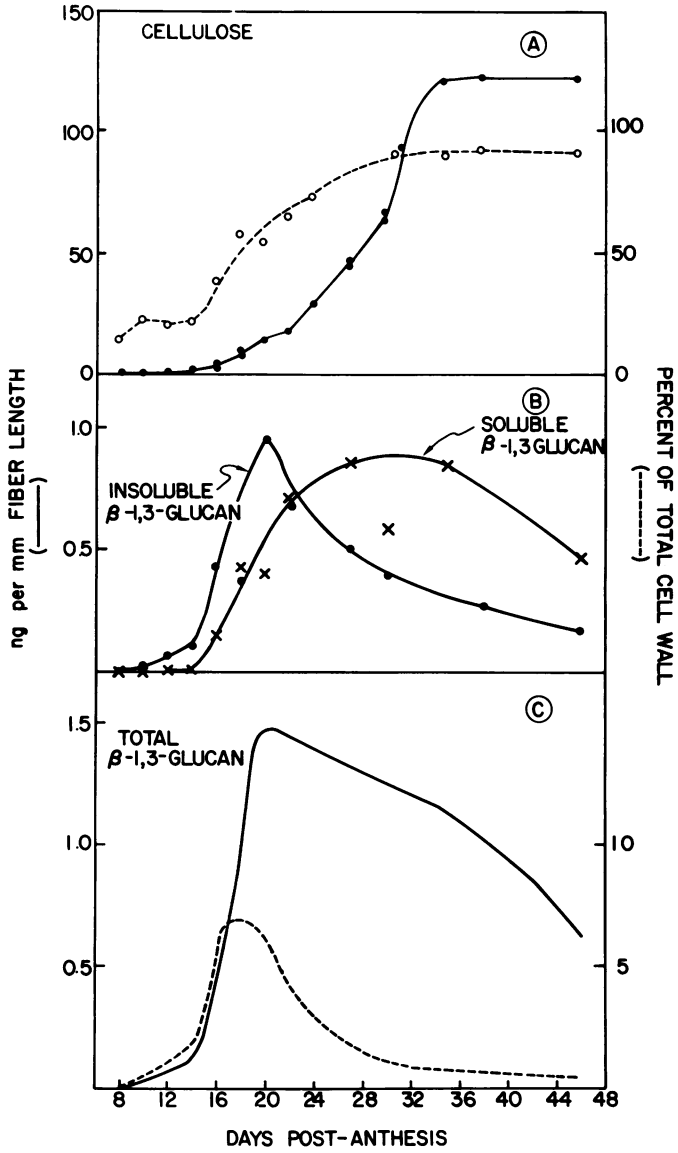


FIG. 2. Variation in content of cellulose and β-1,3-glucan in cotton fibers as a function of fiber age. The total β-1,3-glucan (C) was calculated as a sum of the areas under the curves in B. Each point is the average of duplicate or triplicate determinations.

comparable to those found in Acala SJ-1. The other cultivars of *G. hirsutum* L. examined included: Delta pine 16, Ambassador, Stoneville 213, Delcot 277, and T-59-538. Also examined was a pima cotton (*G. barbadensis*) and a hybrid (H-330) of *G. hirsutum* × *G. barbadensis*.

**Kinetics of Synthesis and Turnover of β-1,3-Glucan and Cellulose *in Vivo*.** The coordinate deposition of β-1,3-glucan and secondary wall cellulose prompted us to examine further the relationship between the synthesis of these two polymers. Since the fibers contain little starch and since there are reports in the literature which suggest that noncellulosic glucans associated with cell walls may show turnover (see 4 and references cited therein), we wondered whether the β-1,3-glucan might serve as a source of extracellular glucose for use in cell surface cellulose synthesis, and in this sense, represent a precursor to cellulose. To study the synthesis and turnover of these polymers, we have monitored the kinetics of [$^{14}$C]glucose incorporation into the glucans of cotton fibers cultured with their associated ovules. Other studies in our laboratory (13) have shown that the biochemical changes occurring in the cell wall of cultured fibers during development parallels quite closely those which occur on fibers growing on the plant. The onset of secondary wall synthesis does occur earlier, however, and the cultured fibers used in these studies (15 DPA) have already begun secondary wall cellulose synthesis. Methylation analyses show that the cultured fibers do contain both soluble and insoluble β-1,3-glucan in quantities similar to those found in plant-grown fibers.

Figure 3 shows the short term kinetics of initial labeling of soluble and insoluble β-1,3-glucan and cellulose. Incorporation of label into the soluble β-1,3-glucan was linear almost from the onset of incubation. Between 50 and 100 min, the rate declined, and incorporation continued at a slower rate for the duration of the experiment (450 min; data are shown only up to 180 min). Incorporation into insoluble β-1,3-glucan in this particular experiment showed a short lag phase before it became linear, although in other experiments the lag was less prominent or not observed at all. In contrast, a lag phase was always observed before incorporation into cellulose became linear with respect to time. (Linear incorporation, once established, remained so throughout the 450 min of the experiment.) The length of the lag phase was temperature-dependent (compare the 30- to 40-min lag of this experiment with that of Fig. 4 where the incubation temperature was 34 C instead of 23 C).

Such a lag would be consistent with the interpretation that an intermediate polymer such as β-1,3-glucan could be on the pathway of cellulose biosynthesis. However, in repeated pulse-chase experiments (one example is shown in Fig. 4), we found no evidence for any significant turnover of β-1,3-glucan in these cells.

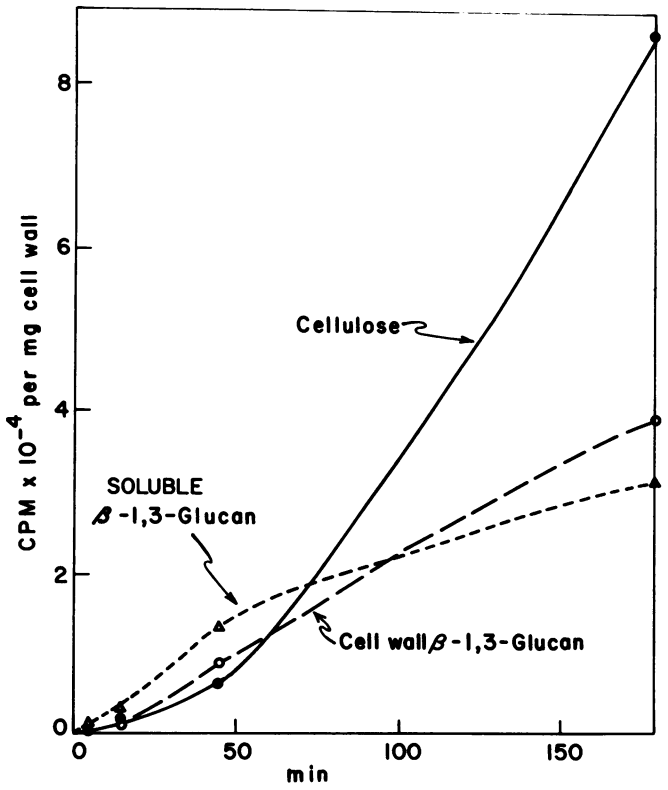


FIG. 3. Short term kinetics of labeling of cellulose and β-1,3-glucan. Fibers, cultured *in vitro* for 15 DPA with their associated ovules, were pulsed at 23 C for the times indicated in a medium containing [$^{14}$C]glucose (10 mM, 4.0 μCi/μmol) and 90 mM pentaerythritol. β-1,3-Glucan was quantitated by β-1,3-glucanase digestion as described under "Materials and Methods."

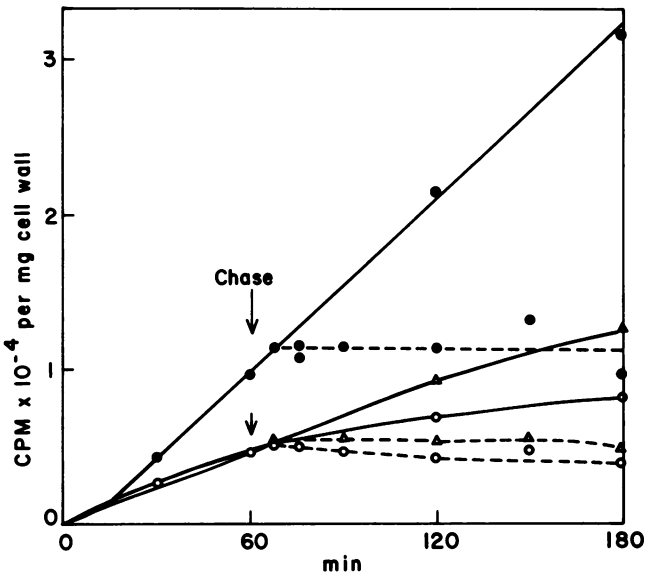


FIG. 4. Pulse chase experiment; kinetics of labeling of cellulose and β-1,3-glucan. Procedures were performed as for the experiment in Figure 3 with the following modifications: temperature of incubation was 34 C; [$^{14}$C]glucose concentration was 20 mM (0.08 μCi/μmol) and pentaerythritol was 80 mM. At the time of the chase, one-half of the remaining ovules with associated fibers were rinsed briefly and then incubated in medium containing unlabeled glucose (100 mM) and lacking pentaerythritol. (——): pulse; (---): chase. (●): cellulose; (○): soluble β-1,3-glucan; (△): insoluble β-1,3-glucan.

One puzzling observation does remain unexplained. At this stage of development (15 DPA), the content of β-1,3-glucan in the fibers is less than 10% that of cellulose. Yet in the early stages of labeling (Fig. 3) the rate of incorporation of label into total β-1,3-glucan exceeds that of cellulose, and even at later times the rate is about 50% that of cellulose. It seemed possible that the fibers, even though handled very gently during the labeling procedures, were damaged slightly and that this stimulated an additional wound synthesis of β-1,3-glucan. However, this possibility appeared to be unlikely in face of results of an experiment in which one set of ovules was pulsed without washing or handling in any way other than a gentle mixing of medium when label was added. The kinetics of labeling of cellulose and insoluble β-1,3-glucan were nearly identical to those obtained for ovules which were washed and handled in the standard fashion, while that of the soluble β-1,3-glucan was stimulated no more than 30% by the washing procedure.

## DISCUSSION AND CONCLUSIONS

The secondary wall of the cotton fiber is commonly referred to as a pure cellulosic cell wall. Indeed, at maturity, the cotton fiber represents one of the purest natural sources of cellulose known to man. However, these studies show that the fiber also contains, in addition to cellulose, a β-1,3-glucan. The fact that this polymer, like cellulose, is a β-glucan and occurs at considerably lower levels than cellulose undoubtedly accounts for the fact it was not discovered previously. This glucan is not unique to Acala SJ-1, but also exists in a variety of other cultivars of *G. hirsutum*. We have also demonstrated its presence in *G. barbadensis*, and a recent report (11) describes a similar β-1,3-glucan in fibers of *G. arboreum* which also showed varying solubility in aqueous ammonium oxalate and alkali.

With regard to structure, the polymer appears to be essentially a linear β-1,3-glucan with a limited degree of branching. With regard to localization, we propose that all of the β-1,3-glucan, including the soluble fraction, is located extracellularly. The fact that at least a portion of the insoluble glucan can be removed by enzyme digestion of intact fibers argues strongly for an extracellular location for this fraction. Support for this localization for the soluble glucan comes from the fact that less than 2% of this glucan is associated with membranous organelles involved in secretion or with the soluble fraction but requires rigorous homogenization to release it from cell walls. We cannot exclude the possibility that this fraction is associated with so-called callose deposits in the cytoplasm. Although such deposits have been observed cytologically in pollen tubes (15), they have not been discovered in cotton fibers (20). Furthermore, the kinetic data of labeling and turnover of the soluble β-1,3-glucan are not entirely consistent with its role as an intracellular precursor to the insoluble glucan found in the cell wall fraction. An extracellular localization is also consistent with the finding that β-1,3-glucan synthetase is localized at the cell surface in *Pisum sativum* (2).

At present, we can only speculate about the role of the β-1,3-glucan in developing cotton fibers. In higher plants, β-1,3-glucan, commonly referred to as callose, is often associated with wound responses. In the developing cotton fibers, this glucan is deposited naturally during a specific stage of fiber development. Another example of β-1,3-glucan being found as a cell wall component is in the pollen tube wall of *Lilium longiflorum* which, along with cellulose, contains alkali-insoluble β-1,3-glucan fibrils as a major structural component (7). In the cotton fiber, the timing of the onset of β-1,3-glucan synthesis coincides with the earliest stage of secondary wall cellulose deposition. This first layer of secondary wall has been observed microscopically and has been referred to as the winding layer in cotton (12), and therefore it seems likely that most of the β-1,3-glucan is associated with this layer. Of particular interest is the observation made by us and others (13, 17, 18) that fiber elongation continues during the period of deposition of the winding layer (from 16 to 22 DPA in our system) even though, the fibers appear to elongate, not by tip growth, but by over-all extension, and cellulose is deposited uniformly throughout the length of the fiber (16, and Delmer, unpublished results). Since this period of development coincides with the time of maximal β-1,3-glucan deposition, it is possible that the glucan may play some role in determining the plasticity of the wall at this stage of development. There seem to be differences among cotton varieties in the extent of elongation which occurs after the onset of secondary wall formation (18), and currently, we are examining the pattern of β-1,3-glucan deposition in varieties which differ significantly in this respect.

The pulse-chase experiments performed here seem to rule out a role for the glucan as a storage polymer or precursor to cellulose. The kinetics of labeling of the β-1,3-glucan and cellulose does leave some questions unanswered. First, it is unclear why the rate of incorporation of carbon from [$^{14}$C]glucose into β-1,3-glucan is so high compared to that of cellulose. The high rate of synthesis of β-1,3-glucan under conditions when the steady-state is low would seem to support the idea of turnover of this glucan, yet turnover has not been demonstrable. The relatively high rate of synthesis cannot be accounted for by a stimulation due to fiber damage. Second, why the lag observed before incorporation of label from [$^{14}$C]glucose into cellulose (but not β-1,3-glucan) becomes linear with respect to time deserves further study. These data may offer some important clues regarding the as yet undetermined path of carbon into cellulose in cotton fibers. We know that UDP-glucose is the precursor to β-1,3-glucan in cotton (4, 6); UDP-glucose is also considered to be the most likely candidate for precursor to cellulose (3). However, if the lag were due simply to the time required to equilibrate nucleotide sugar pools, then the kinetics of labeling of cellulose should parallel that of β-1,3-glucan. The fact that it does not implies either that UDP-glucose is not on the pathway to cellulose or that there is some large

intermediate pool beyond UDP-glucose on the pathway which must be exhausted before incorporation into cellulose becomes linear with time. In this regard, recent experiments of Hopp *et al.* (9) with the alga *Prototheca* indicated that lipid oligosaccharides and protein-linked oligosaccharides might be on the pathway to cellulose in this organism. Current work in our laboratory is directed at using specific inhibitors in order to perturb the flow of carbon into cellulose with the hope of observing the increased accumulation of potential precursors to cellulose.

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