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Analysis of cell-wall polymers during cotton fiber development

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Abstract. Although the fibers of cotton (*Gossypium hirsutum* L.) are single cells with a secondary wall composed primarily of cellulose, the cell-wall polymers of the fibers are technically difficult to characterize with respect to molecular weights. This limitation hinders understanding how the fiber wall composition changes during development, particularly with respect to genotypic variations, and how the molecular composition is related to physical properties. We analyzed cell-wall polymers from cotton fibers (cultivar, Texas Marker-1) at several developmental stages (8–60 days post-anthesis; DPA) by gel-permeation chromatography of components soluble in dimethyl acetamide and lithium chloride. This procedure solubilizes fiber cell-wall components directly without prior extraction or derivatization, processes that could lead to degradation of high-molecular-weight components. Cell-wall polymers from fibers at primary cell-wall stages had lower molecular weights than the cellulose from fibers at the secondary wall stages; however, the high-molecular-weight cellulose characteristic of mature cotton was detected as early as 8 DPA. High-molecular-weight material decreased during the period of 10–18 DPA with concomitant increase in lower-molecular-weight wall components, possibly indicating hydrolysis during the later stages of elongation.

Key words: Cellulose – *Gossypium* – Cell wall – Fiber (cotton)

Introduction

In addition to cotton's importance as a commercial commodity, the developing cotton fiber is an attractive system for investigation of plant cell elongation and maturation (Basra and Malik 1984; Ryser 1985; for review, see Delmer 1987). Cotton fibers differentiate from single epidermal cells on the developing cotton seed without complications from cell division. Cotton fiber development is divided into four phases: (i) initiation, (ii) elongation, (iii) secondary wall thickening, and (iv) maturation (Basra and Malik 1984). Initiation starts at anthesis with the elongation stage following immediately. Fiber cells elongate to >2.5 cm in three weeks after anthesis (primary-wall stage). Secondary wall growth (weeks three to eight) is marked by deposition of a thick wall. The approximate timing of these events is summarized in Table 1; some genotypic variability exists.

The secondary cell wall of the cotton fiber is nature's purest form of cellulose which comprises over 95% of the fiber's dry weight. Cellulose is composed of linear molecules of β-D-(1,4)-glucopyranosyl residues that are associated laterally to form microfibrils. In plant cell walls, including those of the cotton seed hairs, these microfibrils are deposited randomly in primary walls and in ordered patterns in secondary walls. The cellulose of secondary cell walls is known to possess a homogeneous and high molecular weight (MW) or degree of polymerization (DP)[1] (Marx-Figini 1982). Primary-cell-wall cellulose, however, has a relatively heterogenous and much lower DP with little information about the development of this broad DP-spectrum (Blaschek et al. 1982). In cotton fibers, the primary cell walls have been reported to contain 35–50% cellulose (Meinert and Delmer 1977; Nevell and Zeronian 1985). Molecular characterization of primary cell walls has been largely by nitration of the cellulose component which means that other polymers (like starch, pectins, β-(1,3)-glucans, or proteins) are not carried through the isolation and nitration procedure (Blaschek et al. 1982).

The composition of the cell wall of the cotton fiber is continuously changing throughout development (Meinert and Delmer 1977). The noncellulosic polymers xyloglucan and β-(1,3)-glucans have been determined in plant

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Abbreviations: DMAC = dimethyl acetamide; DP = degree of polymerization; DPA = days post anthesis; GPC = gel-permeation chromatography; MW = molecular weight; MWD = molecular-weight distribution; TM-1 = Texas Marker 1

[1] Number of monomer units

Table 1. Approximate timing of the phases of cotton fiber development

Phase	DPA	Key polymers	Reference
Initiation	−1 to 2		Basra & Malik 1984
Elongation / Primary wall synthesis	1 to 21	Cellulose (MW≈650000) Xyloglucan (MW≈80000) Pectic fraction	Marx-Figini 1982 Hayashi & Delmer 1988 Meinert & Delmer 1977
Secondary wall deposition	16 to 35	Cellulose (MW≈2200000) β-1,3-Glucans	Marx-Figini 1982 Francy et al. 1989
Maturation	35 to 60	Cellulose β-1,3-Glucans	Rowland & Howley 1985, 1986

cell walls (including cotton fiber) and deposition at specific stages of development identified. Xyloglucan, a polysaccharide found in the primary wall of higher plants, apparently plays key roles in control of cell growth (Fry 1989; Hayashi 1989; Fry et al. 1992). Deposition of xyloglucans appeared restricted to the elongation phase of cotton fiber development (Hayashi and Delmer 1988). The level of xyloglucan synthase increased up to 16 days post anthesis (DPA) in cotton fiber and decreased rapidly at the onset of secondary wall synthesis (Hayashi and Delmer 1988).

The biosynthesis of callose or β-(1,3)-glucans has been of interest with respect to cotton fiber development because of potential involvement in the synthesis of cellulose (Francy et al. 1989). The content of both soluble and insoluble β-(1,3)-glucans in cotton fiber was low during the period of elongation (primary wall synthesis) and rose abruptly at approximately the time of onset of secondary-wall cellulose synthesis (Maltby et al. 1979; Ryser 1985). In addition to biochemical determinations (Huwyler et al. 1979; Jaquet et al. 1982; Dixon 1985; Pillonel and Meier 1985), the presence of β-(1,3)-glucans was determined cytochemically (Waterkeyn 1981; Ryser 1985). Consistently, it has been observed that the greatest synthesis of callose occurred at the end of elongation of the primary wall and just prior to onset of the secondary wall (Waterkeyn 1981; Ryser 1985). Speculation of the role of the β-(1,3)-glucan polymers has ranged from cell-wall turnover, reserve function or metabolic intermediate. Although definitive functions have not been established, callose is probably not a precursor to secondary-wall cellulose (Pillonel and Meier 1985).

A pectic fraction of protein, glucose, and uronic acids is also present during the elongation phase of cotton fiber development (Meinert and Delmer 1977; Huwyler et al. 1979). Reports indicate that the fiber cell-wall pectins, notably the polygalacturonide fraction, decrease from 10 to 18 DPA (Meinert and Delmer 1977; Huwyler et al. 1979).

Thus, although cotton fibers are single cells whose walls are composed mostly of cellulose, cell-wall polymers have been technically difficult to characterize with respect to MWs, association with other polymers and organization in the wall. Attempts to identify the true MW of native celluloses always leads to difficulties, especially in isolating unchanged celluloses from natural plant products, and in determining the MW of high-MW celluloses by reliable physical methods (Franz and Blaschek 1990). Unfortunately, agents capable of selectively removing the noncellulosic components seldom leave the cellulose unchanged (Beasley 1979; Franz and Blaschek 1990). The methods for obtaining solutions of wall polysaccharides involving derivatization, e.g. nitration, acetylation, or complexing with paraformaldehyde, cupraammonium, can lead to hydrolytic and-or oxidative changes.

Recently introduced solvents for cellulose include dimethyl acetamide and lithium chloride (DMAC-LiCl) (McCormick et al. 1985; Turbak 1983). The DMAC-LiCl system is particularly useful since, in direct contrast to other cellulose solvents that rapidly degrade the macromolecular backbone, there is no degradation of the polymer by the solvent (for review, see Dawsey and McCormick 1990). In our laboratory, methods were developed to determine the MW of cotton-fiber wall polymers by gel-permeation chromatography (GPC) of DMAC-LiCl-soluble components (Timpa 1991). This procedure solubilizes cotton fiber directly without prior extraction or derivatization, processes that can lead to degradation of high-MW species. The DMAC-LiCl solvent system is effective for nondegradative dissolution of a number of naturally-occuring polysaccharides, including cellulose, chitin, dextrans, amylose, amylopectin, proteins and synthetic polyamides (Dawsey and McCormick 1990). Incorporation of a viscometer detector in addition to a concentration detector provides GPC capability for determination of intrinsic viscosity (Haney 1985) and a "universal calibration" (Grubisic et al. 1967). Employing a universal calibration in GPC means that a valid molecular-weight distribution (MWD) for the polymer composition is obtained even if the structural and-or chemical identification is not known (Grubisic et al. 1967; Provder 1987).

It has long been recognized that fiber strength is determined by the structural organization of the cellulose chains (Hessler et al. 1948); agreement about that relationship, particularly with respect to polymeric, supramolecular and morphological structure varies widely (see Nevell and Zeronian 1985). Thus, our objectives are to understand relationships between fiber strength and the formation of cotton fiber structure and the role of the primary wall in determination of that structure. The objectives of this study were twofold: (i) to determine if the solvent system would directly dissolve the cell-wall

polymers of the fiber in the early stages of development to allow GPC analysis; and (ii) to monitor differences in the molecular compositional profiles during the stages of cotton fiber development.

Material and methods

Plant material. American Upland cotton (*Gossypium hirsutum* L.), cultivar Texas Marker 1 (TM-1) was grown in pots under controlled greenhouse conditions. Flowers were tagged on the day of anthesis. Bolls were harvested at various stages of development from 8 to 60 DPA. Cotton bolls were stored under ethanol until ginned. Cotton fiber was detached from seeds and, after drying in a vacuum oven at 40° C, was ground in a Wiley mill to pass a 20-mesh screen. Fibers at 8 to 18 DPA were pooled from several bolls to obtain sufficient material for characterization. For samples from 18 to 60 DPA, fibers from two to three bolls were individually characterized.

Sample preparation. The procedure for sample preparation was as previously described (Timpa 1991). Ground fiber (1.5% w/v) was added to 3 mL DMAC (Burdick & Jackson, Muskegon, Mich., USA) in a 10-mL Reacti-Vial (Pierce Chemical Co., Rockford, Ill., USA) in a heating block. The sample was activated by elevating the temperature to 150° C for 60 min and exposing the sample to hot DMAC vapors. The temperature was reduced to 100° C, and when the sample had equilibrated, solid LiCl (8% w/v) was added. Sample temperature was dropped to and maintained at 50° C with gentle stirring until the sample completely dissolved (24–48 h). The dissolved-fiber sample was subsequently diluted in a 50-mL volumetric flask with DMAC. Final sample concentration was 0.9–1.5 $mg \cdot mL^{-1}$ in DMAC with 0.5% LiCl. The sample was filtered through disposable solvent-resistant Millex SR filters (Millipore Corp., Bedford, Mass., USA).

Gel-permeation chromatography. Filtered cotton-fiber solutions were analyzed using a GPC system as previously reported (Timpa 1991) with a viscometer detector (Model 100; Viscotek, Houston, Tex., USA), and a refractive-index detector (Model 410; Waters Associates, Milford, Mass., USA). The mobile phase for GPC was DMAC containing 0.5% (w/v) LiCl at a flow rate of 1.0 $mL \cdot min^{-1}$ pumped through four columns (Ultrastyragel 10^3, 10^4, 10^5, 10^6; Waters) preceded by a guard column (Phenogel, linear; Phenomenex, Rancho Palos Verdes, Calif., USA). Injection volume was 400 µL with a run time of 65 min. Calibration was with polystyrene standards dissolved and run in DMAC–0.5% LiCl. A universal calibration (Grubisic et al. 1967; Haney 1985) was employed with the logarithmic function of the product of the intrinsic viscosity times MW plotted versus retention volume. Data were obtained from two dissolutions per sample with two GPC runs per dissolution. The GPC system and data analysis were all automated and computer-based.

Results and discussion

Direct dissolution of cotton fiber at different stages of development was successful in the DMAC-LiCl solvent. No appreciable amounts of undissolved material were detected. This was expected since DMAC-LiCl has been reported to produce homogeneous solutions of a range of natural polymers and polysaccharides (Dawsey and McCormick 1990). Advantages of this procedure are that the numerous steps in extraction and nitrate derivatization are decreased, appreciable amounts of noncellulosic wall material are not removed, and degradation of wall polymers is avoided. In addition, the universal calibration of the GPC system provides valid MWDs for unknown polymers, leading to definition of all components. Thus, although DP values are more commonly reported in cellulose literature, the following discussion will report MWs because the repeat units of all of the polymers are not known.

Differences in the molecular profiles of cotton fiber during the stages of elongation (primary wall) and secondary wall synthesis were substantial. In the early stages of development (8 to 18 DPA), MWDs of cotton-fiber samples were characterized by the largest peak occurring at low-MW ranges (Log MW = 3–4). As a typical example, Fig. 1 shows the MWD for cotton fiber at 10 DPA. During early stages of development the fiber has only a primary cell wall, therefore these low-MW components are characteristic of the primary cell wall. The differential MWD is the graphical representation of the weight fraction versus the logarithm of MW (Yau et al. 1979). For this particular sample, the weight average molecular weight (MW_w) was 140 000. For comparison, in this series the MW_w was 2 2500 000 for samples of mature cotton fibers from open bolls at >60 DPA (see also Timpa 1991).

The cumulative MW-fraction distributions (Fig. 2) indicate that 10-DPA cotton fiber had 86% of the weight fraction with an MW ≤ 100 000, whereas the 60-DPA sample had 49% of the components with an MW ≥ 1 000 000. The cumulative weight-fraction distribution is obtained by dividing the partially integrated areas by the total area (100%) under the curve (Yau et al. 1979) and provides a convenient means for estimating the percentages of components having MWs of specific levels. This large MW difference between the components of primary and secondary walls of cotton confirms that previously observed for the cellulose fractions. Hessler et al. (1948) determined that the cellulose of immature-fiber samples (primary wall only) had an MW = 960 000, while mature-fiber samples contained cellulose with MW = 1 730 000 (DP = 10 650) with the average MW dependent on the stage of development, the variety and location of growth, and the degree of degradation or damage. Marx-Figini (1982) reported that cellulose from the primary wall of cotton has a heterogeneous MW of 325 000–970 000, while the secondary-wall cellulose has an MW = 2 270 000 (DP = 14 000) and is monodisperse. This substantial MW difference between the broad-spectrum, low-MW chain lengths of the primary wall contrasts with the high and distinct MW of the secondary wall, and has been observed in other plant cell walls (Blaschek et al. 1982). In the previous reports, the cellulose was isolated from noncellulosics and nitrated.

The viscometer detector is particularly sensitive to high-MW species (Provder 1987), an advantage for analyzing cotton cellulose (Timpa 1991). Figure 3 displays the viscometric output of the GPC separation of solutions of fibers of different developmental stages (8–30

[2] Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

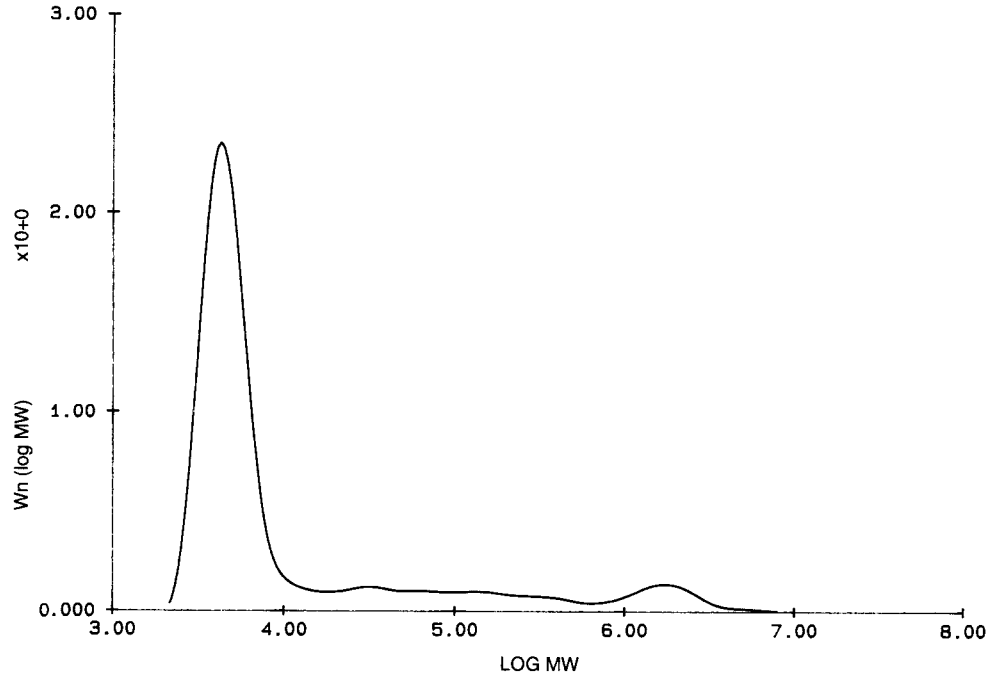


Fig. 1. Molecular-weight distribution of cell-wall polymers of cotton fibers of TM-1 at 10 DPA. Fibers were dissolved in DMAC-LiCl and MWs determined by GPC. Distributions were calculated using refractive-index and viscometer detectors by universal calibration employing polystyrene standards. *Wn(logMW)* = weight fraction of molecular species with a particular MW

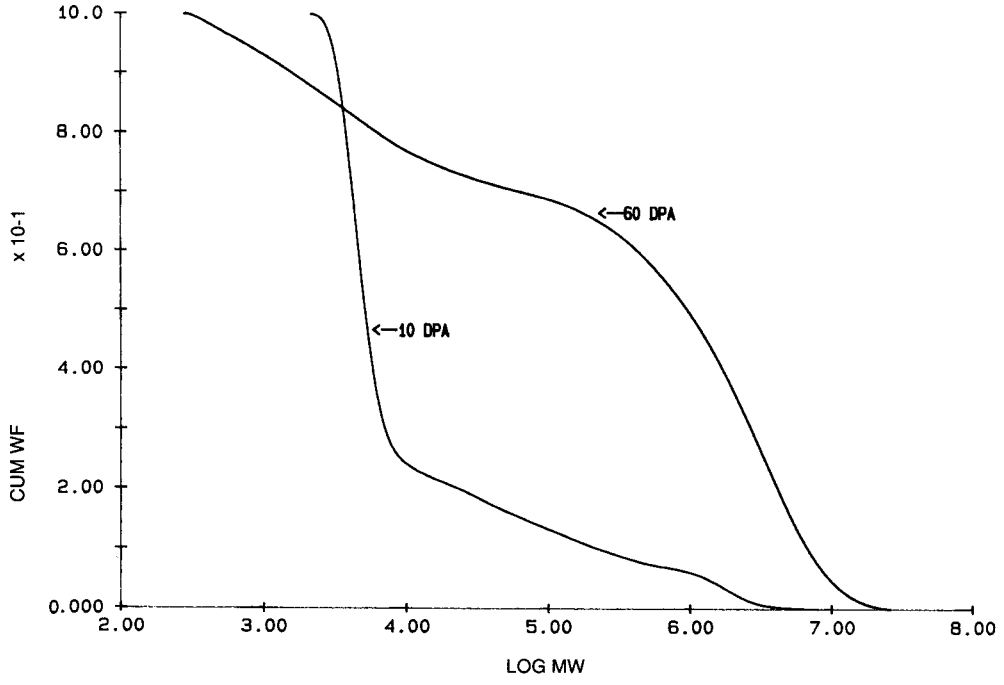


Fig. 2. Plots of cumulative MW fraction (*CUM WF*) for cell-wall polymers of cotton fibers of TM-1 at 10 and 60 DPA. Fibers were dissolved in DMAC-LiCl and MWs determined by GPC. Distributions were calculated using refractive-index and viscometer detectors by universal calibration employing polystyrene standards.

DPA). It is not always necessary to calculate the MW averages or MWDs to obtain interesting information about a sample from the GPC profile (Yau et al. 1979); simple inspection of chromatograms often reveals important information. High-MW species (retention volume $\approx$26–27 ml) were present in cotton fiber even at 8 DPA. In GPC separations, the material of highest MW elutes from the columns first (lower retention volume). Comparisons presented in Fig. 3 are all scaled to a common range of the electrical signal output from the viscosity detector and represent approximately the same concentration of sample. What is also striking is that, as the cotton fiber elongated in the primary cell-wall stages (10–14 DPA), the amount of high-MW material decreased. At 16 and 18 DPA, most of the wall components were of lower MW. The results presented in Fig. 3 could arise either by degradation of existing high-MW wall polymers, or by rapid synthesis of some other cell-wall

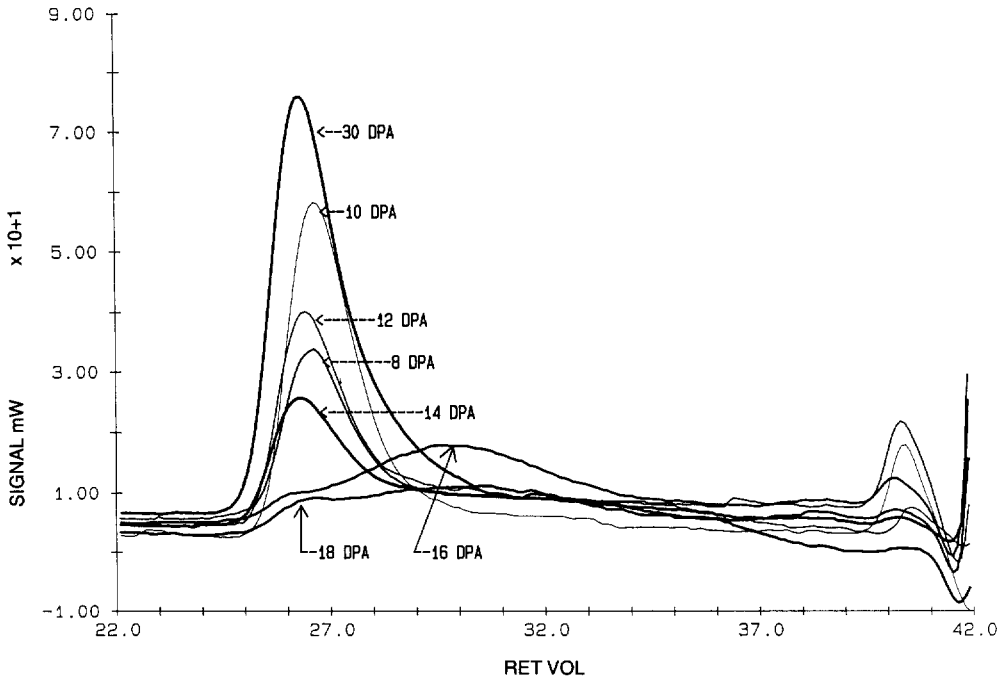


Fig. 3. Gel-permeation chromatography of cell-wall polymers of cotton fibers of TM-1 at 8, 10, 12, 14, 16, 18, and 30 DPA. Chromatograms were analyzed using the viscometer detector. *RET VOL* = retention volume in mL

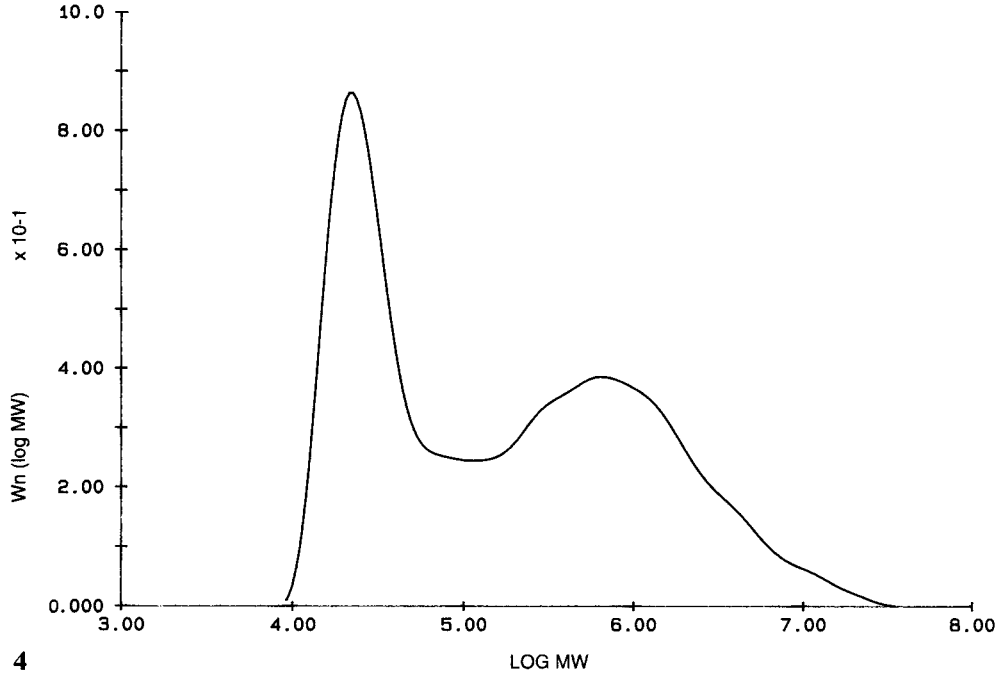
constituents. The large difference in the peak size for 18 DPA versus 30 DPA substantiates the tremendous increase in high-MW secondary-wall cellulose during this period. The dip in the chromatogram between retention volumes of 41 and 42 ml is the location of the solvent-air peak, typical of GPC separations. Very low-MW species (MW $\approx$ 1000) which eluted past this time were impossible to measure.

The MWDs calculated for some of the above samples can be compared in Figs. 4–7 for samples at 16, 20, 25, and 40 DPA, respectively. The MWDs are plotted as a function of increasing Log MW, the reverse of the GPC elution profile shown in Fig. 3. Differences can be readily discerned between 10 DPA (Fig. 1) and 16 DPA (Fig. 4). Note that the primary-wall peak has shifted from MW $\approx$ 6300 for solutions of cotton fiber at 10 DPA to MW $\approx$ 20 000 for 16-DPA fibers. This observation supports Marx-Figini's reports of an increase of chain length of primary-wall cellulose in cotton fiber as a function of time after anthesis.

The MWD of wall polymers changed during the period of transition from the elongation stage (16 DPA) to the stages when secondary cell-wall deposition increases (20–25 DPA). A broad peak of molecular components (MW $\approx$ 630 000) is evident for the 16-DPA sample (Fig. 4) whereas few peaks are distinct other than that representing the primary wall in the MWD for the 20-DPA sample, although a wide range of polymeric material is present. At 25 DPA, the high-MW peak characteristic of the secondary-wall cellulose is obvious. The MW difference between primary wall and distinct secondary-wall cellulose was maintained with no evidence of chain-length increase for the secondary wall as a function of time of synthesis. At 40 DPA, the distribution is similar but not identical to that of mature fibers (Timpa 1991). The most distinctive difference lies in a larger primary-wall peak and more mid-range molecular components than seen in mature-fiber samples, supporting previous reports of changes occurring in cotton fibers during maturation (Rowland and Howley 1985, 1986).

Although we have not chemically identified the molecular species in the DMAC-LiCl-soluble mixture, it is likely that noncellulosic components of the fiber cell wall have MWs in the mid or lower ranges. The 16-DPA fiber sample had wall components with a large broad peak at MW $\approx$ 630 000 that was not evident at earlier stages of development (Fig. 4 vs. Fig. 1) and that decreased in later stages of development (20 and 25 DPA, Figs. 5 and 6, respectively). A potential candidate for this noncellulosic component could be xyloglucan (Francy et al. 1989: Fry 1989; see Hayashi 1989). Prior to 16 DPA, xyloglucan synthesis in developing cotton fibers is active (Hayashi and Delmer 1988). At 16 DPA, nearly equal amounts of noncellulosic polysaccharides and cellulosic β-(1,4)-glucans are present in the cell walls of cotton fibers (Huwyler et al. 1979). While the size of the mid-range component (Fig. 4) is larger than the MW range previously reported for cotton-fiber xyloglucan (Hayashi and Delmer 1988), the method used for prior fractionation is subject to criticism. Potassium hydroxide and extraction procedures with alkali lower the yield and MW of cellulose (Franz and Blaschek 1990).

Several reports indicate that the pectins in fiber cell walls, notably the polygalacturonide fraction, decrease from 10 to 18 DPA (Meinert and Delmer 1977; Huwyler et al. 1979). The later stages of this period (16–18 DPA) coincide exactly with the developmental phase when the rate of cellulose synthesis increases fourfold for a limited time (Meinert and Delmer 1977). It is equally possible that rapid synthesis of β-(1,4)-glucan during this period



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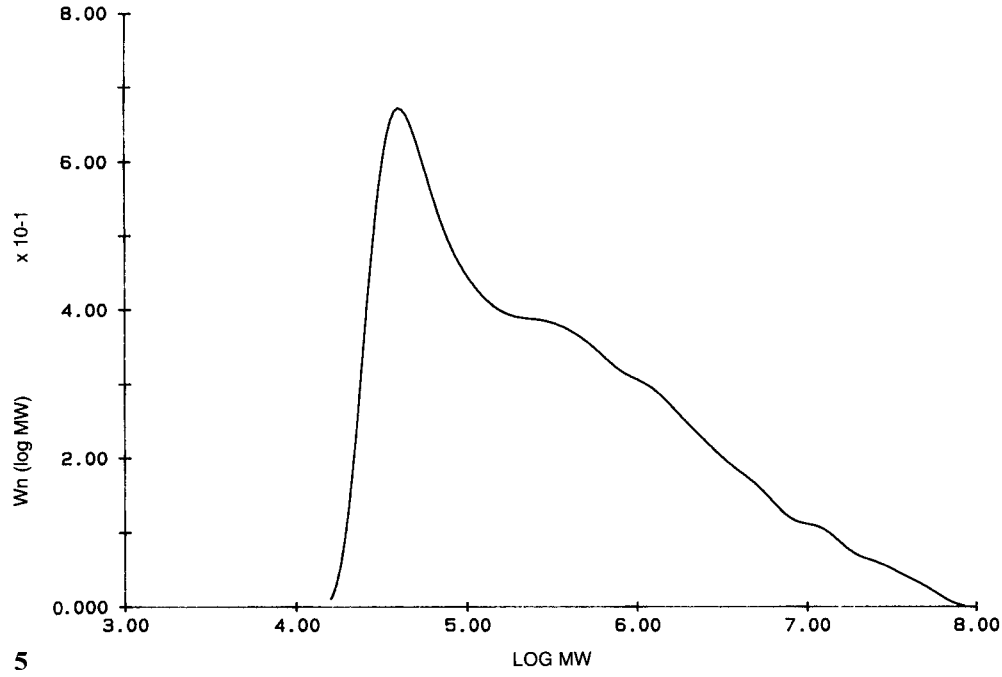

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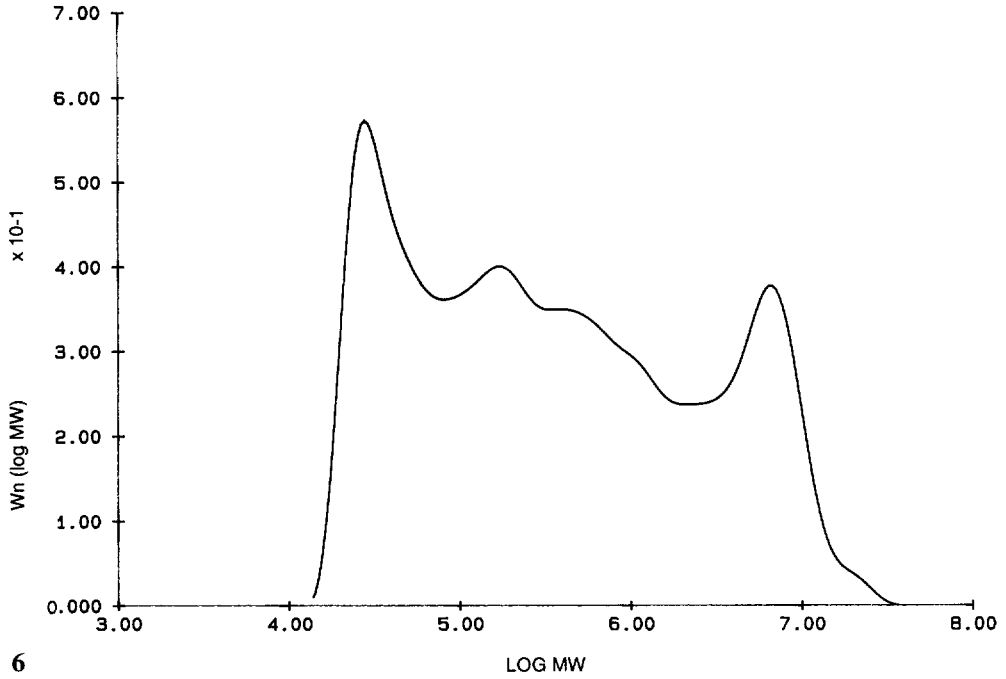
Fig. 4–7. Molecular-weight distribution of cell-wall polymers of cotton fibers of TM–1 at 16 DPA **(Fig. 4)**, 20 DPA **(Fig. 5.)**, 25 DPA **(Fig. 6.)** And 40 DPA **(Fig. 7.)**. Distributions were calculated using refractive-index and viscometer detectors by universal calibration employing polystyrene standards. *Wn(logMW)* = weight fraction of molecular species with a particular MW.

yields the major wall component shown in Fig. 3 for the 16- and 18-DPA samples. It is unlikely that the component is β-(1,3)-glucan (callose) since this material is a minor component at 16–18 DPA and synthesis is not detected until much later in development at 25–35 DPA (Ryser 1985).

Cell-wall hydrolytic activity in developing fibers has been measured for a limited number of enzymes. Total β-(1,3)-glucanase activity is low during the elongation phases of fiber growth, but increases throughout the period of secondary wall synthesis. Activity of β-(1,4)-glucanase is negligible throughout fiber development when carboxymethylcellulose is used as a substrate (Bucheli et al. 1985). It is possible, however, that enzymatic turnover of β-glucans is taking place, but the enzyme has a very specific substrate specificity. Reports indicate that autolysis is slight when the level of endogenous substrate of β-(1,3)-glucans is low, and the



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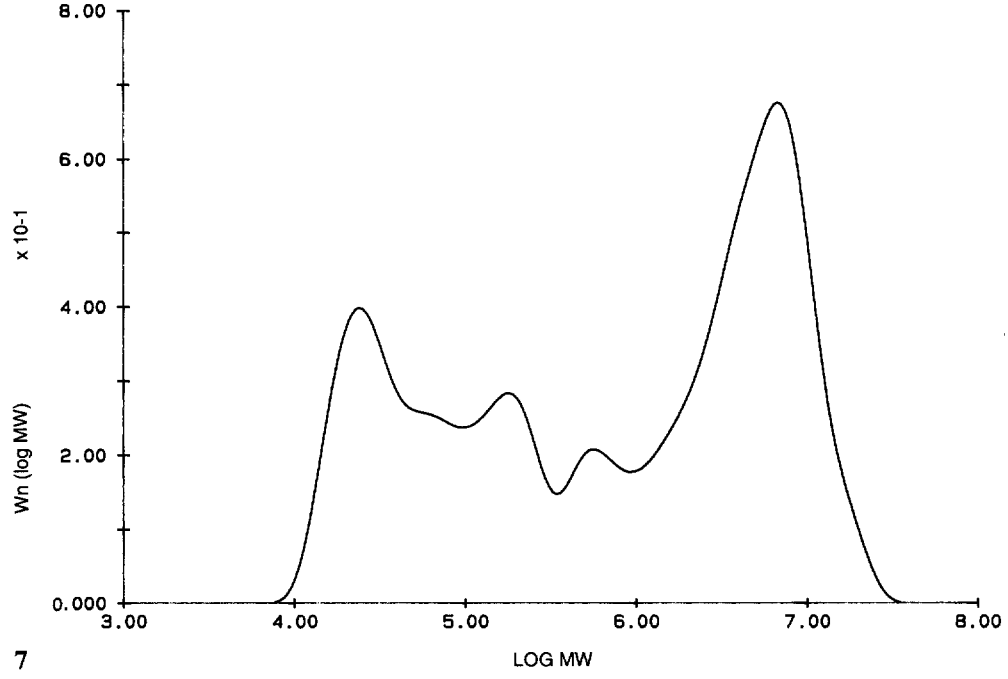

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onset of the marked increase in autolysis corresponds to the maximum in the callose content of cotton fibers at 25 DPA (Bucheli et al. 1987). On the other hand, hydrolytic enzymes (glucosidases, galactosides, acid invertase and acid phophatase) were positively related to the rate of fiber elongation but the biochemical functions were not identified (Richa et al. 1986; Kumar et al. 1987).

Our results differ from previous reports because fiber samples have not been subjected to isolation, fractionation, or derivatization which can cause degradation of polymers. Previous MW determinations have been by viscometry, light-scattering or ultracentrifugation (Franz and Blaschek 1990). The LiCl-DMAC solvent system completely solubilizes wall polymers without extraction or derivatization for GPC separation. This approach provides an opportunity for the characterization of the entire array of cell-wall polymers not possible before and represents in important step toward solution of the major

problem of precise product analysis identified by Franz and Blaschek (1990). These results confirm the developing cotton-fiber cell wall to be a dynamic structure. Analysis of the MWD of the wall polymers has enabled us to identify key developmental time periods at which chemical analysis of wall fractions should be performed. Collection of fractions from these GPC separations is hampered by the presence of LiCl in the mobile phase although chemical identification of major components will be the subject of future investigations. The use of GPC techniques for assessment of the presence of branched versus linear polymers is under evaluation.

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