BIOCHEMICAL CHANGES IN CELL WALL COMPOSITION ASSOCIATED WITH IN VIVO AND IN VITRO FIBER DEVELOPMENT IN GOSSYPIUM HIRSUTUM

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ABSTRACT

BIOCHEMICAL CHANGES IN CELL WALL COMPOSITION ASSOCIATED WITH *IN VIVO* AND *IN VITRO* FIBER DEVELOPMENT IN *GOSSYPIUM HIRSUTUM*

By

Maureen Christine Meinert

This study represents the first report of analyses of the changes in chemical composition associated with the development of the cell wall of a *single* higher plant cell. The elongating cotton fiber has provided a model in which it was possible to examine the developmental process of *one single cell* throughout the entire period of elongation. Furthermore, because the composition of the cell wall of the elongating fiber can be expressed as grams component per fiber length, only in this type of system was it possible to examine absolute rather than relative changes occurring during primary cell wall elongation. Analyses were performed on cell walls derived from both plant *and* culture grown fibers.

The major components of the cotton fiber cell wall are cellulose, neutral sugars, protein, and a uronic acid containing component. The relative proportions of these components present at any time during development of the cotton fiber continually change. *Hence, the primary cell wall represents a dynamic structure.* The neutral sugar component of the wall is composed of the sugars commonly found

in cell wall polysaccharides: rhamnose, fucose, arabinose, xylose, mannose, galactose, and non-cellulosic glucose. The relative ratio of these sugars in the cell wall varies with age of the fiber, as well. The protein component, which remains constant throughout the development of the fiber, has an amino acid composition that is exceptional only with respect to the presence of hydroxyproline. A significant proportion of this protein consists of discrete polypeptides which are released from the wall on brief treatment at 100°C with an SDS/urea/mercaptoethanol reagent.

The changes in wall composition can be expressed in either of two ways: 1) as a percentage of the weight of the wall, or 2) as an absolute quantity per unit length of the wall. The latter has been found to be a more accurate description of the absolute compositional changes occurring in the elongating fiber.

Expressed as a percentage of the wall, the cellulose content of the primary cell wall amounts to 10 per cent of the weight of the wall through 9 days post-anthesis and 25 per cent through 16 days post-anthesis. In the later stages of development, cellulose, as a secondary wall, amounts to 70 to 95 per cent of the weight of the total cell wall. The neutral sugar component, as a percentage of the cell wall, continually declines throughout primary cell wall elongation. The protein component similarly declines during elongation. The estimated uronic acid-containing component represents the greatest amount of the cell wall and remains, as a percentage of the wall, fairly constant at ca. 40 to 50 per cent through 16 days post-anthesis.

Expressing the data as grams component per fiber length indicates that the cell wall components are all being deposited at

different rates. The protein component per unit length of wall remains fairly constant throughout development, whereas the levels of neutral sugar component steadily increase. Of the neutral sugars, those undergoing the greatest changes are arabinose and glucose. The early rise in total neutral sugars can be primarily attributed to a rise in arabinose content. In later stages of elongation, arabinose content per unit length of wall significantly declines; at the time of this arabinose decline, and just prior to the onset of secondary wall cellulose deposition, a very significant increase in the level of non-cellulosic glucose occurs in the wall. Comparison of the absolute levels of the cellulose and uronic acid components throughout the course of the development of the fiber indicates that the most marked changes occurring toward the end of the elongation period are a sharp decline in uronic acids and the rise in cellulose, the latter being deposited as a secondary wall.

Comparative fractionation studies, in which acetic/nitric reagent was employed as a solubilizing agent, indicate that residual sugars (glucose, mannose and xylose) are associated with cellulose and that comparable ratios of these sugars are observed for cellulose prepared from several primary cell wall sources including cotton fibers and suspension cultured sycamore cells. The existence of a xyloglucan polymer in close association with the cellulose of the cotton fiber cell wall is suggested indicating a comparable interrelationship between the structural components of higher plant cell walls.

The results obtained for compositional analyses of cell walls derived from culture grown fibers indicate that these walls are *remarkably similar* to those derived from fibers grown on the plant

both in terms of composition and in terms of relative changes in composition during development.

Comparing data available in the literature on cell wall compositions of a variety of plant species with the results of compositional and linkage analyses of cotton fiber cell wall suggests that the cell wall of this dicotyledenous angiosperm most closely resembles that of a cultured gymnosperm, Douglas fir, in particular in terms of the neutral sugar composition and in terms of the predominant types of neutral sugar linkages present.

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Maureen Christine Meinert

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"Everything reasonable has been thought before.

We just have to try to think it once anew."

-Goethe

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INTRODUCTION

The biological importance of plant cell walls has long been recognized both in their involvement in the morphogenesis, growth, and differentiation of plant cells and in their involvement in impeding the penetration of pathogens. Cell walls fall into one of two categories: the primary cell wall, possessed by undifferentiated and actively growing cells, and the secondary cell wall, a much thicker wall laid down subsequent to elongation.

The compositional differences between primary and secondary walls are reflected in their functional differences. While both types have the ability to retain turgor forces, only primary walls are able to undergo elongation. Primary walls consist of predominantly transversely oriented cellulose microfibrils embedded in a highly hydrated cell wall matrix composed of hemicelluloses, pectic polysaccharides and, at least in some plant cell walls, a protein rich in hydroxyproline, termed extensin. Secondary walls differ in composition from primary walls in that they lack pectic substances, contain considerably more cellulose microfibrils that are parallel to the axis of growth and, in addition, contain a non-carbohydrate polymer, lignin. Hence, secondary walls are imparted with great strength and rigidity and as such serve a structural role in plants.

In that the primary cell wall's ability to expand limits the size a plant can attain, it is of interest to reveal the mechanism

by which the plant cell wall can undergo an extension or an expansion in surface area. Basic information, as summarized by Cleland (1971) in the following statements, has revealed little as to the actual relationship between the biochemical events and the physiological events occurring in the actively elongating cell wall:

a) cell enlargement involves a stretching of the wall already present as well as the synthesis of new wall
b) the driving force for extension is turgor pressure
c) cell enlargement is an active process that normally requires respiration
d) continuous synthesis of RNA and protein are necessary for cell enlargement
e) the rate of enlargement is regulated in many higher plants by auxin.

In essence, an attempt has been made to describe the functioning of a system under the influence of a variety of inhibitors and stimulators without first having detailed knowledge of the structure of the system under consideration. Determination of the structure of the primary cell wall, then, is the first step in a logical approach designed to relate the biochemical and physiological changes accompanying cell wall elongation.

The major polysaccharide components of primary cell walls are cellulose, accounting for 25-35% of the wall (Setterfield and Bayley, 1961), pectic polysaccharides and hemicelluloses. The pectic fraction is defined as that fraction obtained by treating walls with either hot boiling water, EDTA or dilute acid (Aspinall, 1970), while the hemicellulosic fraction is defined as that fraction obtained by subsequent extraction with alkali (Whistler and Richards, 1970).

The microfibrillar component of the cell wall is made up of cellulose, whose structure is characterized by long chains of $\beta(1\rightarrow4)$ linked glucose residues. These microfibrils (150-250 Å in diameter

rope-like structures) are aggregates of elementary fibrils. Elementary fibrils (35 Å in diameter) are the result of hydrogen bonding between ca. 40 glucan chains (Frey-Wyssling, 1969).

Pectic polysaccharides extracted from walls from a variety of cell types consist of a complex mixture of various neutral (arabinogalactans) and acidic (galacturonorhamnans) polymers (Stoddart *et al.*, 1967). A chain of (1→4)α-D-galacturonopyranosyl units, in which 2-linked rhamnosyl units occur, comprises the basic chemical structure of the acidic polysaccharides (Aspinall, 1970). A variable number of the carboxyl groups are esterified as methyl esters. The neutral polymer, generally an arabinan or galactan, or a combination of these, is usually covalently linked to the acidic polymer (Aspinall, 1970). The side chains may contain L-fucose, D-xylose and/or D-galactose.

The hemicellulosic fraction isolated from a number of different sources and cell types, including cells with secondary walls, varies considerably and includes such polymers as xylans and arabinoxylans, mannans, glucomannans and galactoglucomannans, galactans and arabinogalactans, and glucans (Timell, 1964; Timell, 1965; Whistler *et al.*, 1970; Aspinall, 1959; Whistler and Sanella, 1958). Of the general types of hemicelluloses, xylans form the bulk of the hemicellulosic fraction of the angiosperms (Northcote, 1972), having a main chain of β(1→4) linked D-xylopyranose units with short terminal side chains of 4-O-methyl-D-glucuronic acid attached to the backbone by α(1→2) bonds. The grasses have large amounts of xylan in their hemicellulosic fraction with the main chain being β(1→4) linked, but which contains, in addition to 4-O-methyl-D-glucuronic acid, side units of

L-arabinofuranosyl groups attached by α(1→3) links (Aspinall, 1959). The structure of the xyloglucan found in the cell walls of suspension-cultured sycamore cells consists of a basic unit of 4 residues of β-1-4-linked glucose and 3 residues of terminal xylose (Bauer *et al.*, 1973).

While a variety of polymers isolated from plant cell walls had been extensively characterized, it was not until the elegant work of Albersheim and co-workers (Talmadge *et al.*, 1973; Bauer *et al.*, 1973; Keegstra *et al.*, 1973), in which general components of the primary cell wall were resolved into distinct polymers of definite composition, linkage, size, and sequence with subsequent identification of interconnections of polymers, that the molecular structure of any plant cell wall was presented. In that study, one of the major difficulties in cell wall structure analyses, that of isolating defined fragments from the wall, was partially overcome through use of highly purified hydrolytic enzymes of known specificity. Identification and quantitation of the macromolecular components of sycamore cell walls revealed the composition to consist of 10% arabinan, 2% 3,6-linked arabinogalactan, 23% cellulose, 9% oligoarabinosides (attached to hydroxyproline), 8% 4-linked galactan, 10% hydroxyproline-rich protein, 16% rhamnogalacturonan and 21% xyloglucan. The cell walls of several other suspension-cultured dicots (tomato, Red Kidney bean, soybean) have also been examined and have very similar walls to those found in sycamore (Albersheim *et al.*, 1973; Wilder and Albersheim, 1973). This work was subsequently extended to include a survey of the walls of suspension-cultured monocots, including a comparison of a single suspension-cultured gymnosperm. While the six monocots examined

(only grasses were used in this study) have similar compositions, this composition differs from that of suspension-cultured dicots and from the suspension-cultured gymnosperm. These monocots contain 40% arabinoxylan, 9-14% cellulose, 7-17% protein and 7-18% uronic acids as the principal components. While the monocot primary wall contains cellulose microfibrils embedded in an amorphous matrix, as do the dicots, the monocot primary wall contains an arabinoxylan as the principal matrix component rather than the xyloglucan found in dicot cell walls.

The cell wall composition of the only gymnosperm examined appears surprisingly similar to dicot cell walls. The exact structure of these cell wall polymers, and the linkages relative to one another, remain to be demonstrated in both the monocots and gymnosperm described.

While the structure of the primary cell wall of suspension-cultured sycamore cells has already been at least partially deduced (Talmadge *et al.*, 1973; Keegstra *et al.*, 1973; Bauer *et al.*, 1973), this system has disadvantages from a physiological standpoint. As an experimental system, suspension-cultured sycamore cell walls are useful for developing and perfecting techniques for cell wall structure elucidation (Lamport and Northcote, 1960). As a model system, however, suspension-cultured sycamore cell walls have not been shown to be free of artifacts due to culture, or to be truly representative of plant grown sycamore cells. For example, markedly differing cell wall compositions are obtained for suspension-cultured sycamore cell

walls depending upon the carbon source present in the growth medium (Nevins *et al.*, 1967). For example, the arabinose content varies from 10 to 32% of the walls. However, it has been shown, as previously described, that six suspension-cultured monocots are remarkably similar in their cell wall composition despite the fact that the cell cultures were derived from diverse tissues (Burke *et al.*, 1974). Nevertheless, because suspension-cultured sycamore cells are undergoing proliferation rather than differentiation, they are ill-suited for studies relating physiological aspects of cell wall elongation to biochemical modifications of primary cell wall structure during development or for studies on the mechanism by which the changeover from primary to secondary wall synthesis occurs in plant cells.

Because it grows as a homogeneous cell type, the use of cultured sycamore cells has provided a good system for determining cell wall structure. However, it presents serious disadvantages because of its lack of potential for relating biochemical structure to function in the processes of elongation and differentiation. On the other hand, studies have been made on changes in cell wall composition associated with differentiating systems, but these studies suffer the problem of lacking homogeneous cell type. Systems that are undergoing differentiation, such as the cell walls of various morphological parts (roots, hypocotyls, first internodes, and primary leaves) of bean plants have been found to have characteristic polysaccharide composition that is precisely determined and that appears to be genetically controlled (Nevins *et al.*, 1967). However, the wall composition obtained in this study represents an average of the composition contributed by each of the principal tissue types: dermal,

ground and vascular, each of which is, in turn, composed of many specialized cell types. In addition, these various morphological parts contain both primary and secondary walls.

The use of cotton fibers, however, provides a system uniquely suited for studies of cell elongation from both a biochemical and a physiological standpoint. Fibers occur within the boll as a homogeneous, synchronous cell type that is readily separable from other parts of the seed and fruit. Elongation can be studied free of any complications from cell division that arise when using plant tissues such as stems, roots, leaves, etc., in that the first fiber originates and ends as a single cell type.

Because cells elongate synchronously in time, at any given point in time all cells are at the same stage of development. Depending upon growth conditions, fibers grow in length only, up to the 15th to 20th day after flowering (Roelofson, 1951). Cells with only primary walls can be readily obtained, making cotton an excellent subject for studies on the mechanism of cell enlargement. Since the changeover to secondary wall formation is an abrupt one (Balls, 1915; O'Kelley and Carr, 1953; Marx-Figini, 1969; Meinert and Delmer, 1975), studies on the control of the transition from primary to secondary wall synthesis can also be studied in this system.

In contrast to cotton fibers, which appear to grow by multinet growth (Delmer, unpublished results; O'Kelley and Carr, 1953), root hairs (Haberlandt, 1914) and pollen tubes (Schoch-Bodmer, 1945) often used in the study of elongation, have been shown to grow by tip growth. Thus, cotton cell walls, as do most other plant cell walls (Wardrop,

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1962), elongate over their entire surface and not just in one localized region of the cell.

A further advantage is that fibers can either be allowed to develop on the plant, or the ovules can be dissected from the boll on the day of flowering and, as Beasley (1971) has shown, can be successfully cultured to the point of normal termination of cotton fiber extension with the concomitant deposition of cellulose as the main structural component of the mature fiber. Physiological aspects, involving hormonal interactions, variable conditions of nutrition, temperature, pH, etc., of the process of cell wall differentiation, can conveniently be examined. Radioactive precursors can be readily incorporated and metabolic products determined at any point in the developmental process (Beasley, 1971).

A very significant advantage of the use of cotton fibers over the use of other plant tissues is that the developmental process of one single cell can be examined over the entire period of elongation. This is not possible with other systems currently under investigation.

As previously stated, cotton fibers are single cells which arise by elongation of cells from the outer epidermal layer of cotton ovules. This single cell first grows as a thin-walled tubular structure to its full length before it is thickened by deposition of secondary wall cellulose on its inner surface (Balls, 1915). Elongating cells develop into two distinct fiber types, the long lint fibers used in the textile mills, and the shorter fuzz fibers that make up the bulk of the linters, fibers that remain on the seed after ginning.

The cotton fiber's rate of elongation is not continuous throughout elongation. Elongation proceeds slowly at first, then more

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rapidly for a few days, and again slowly when the fiber is about to reach its full length. Cell elongation and secondary wall formation have been shown (Balls, 1915) to be separate processes with secondary wall formation occurring after cell elongation is complete. However, Anderson and Kerr (1938) subsequently demonstrated that there may be some overlap between the two processes. Parallel layers of cellulose are deposited on the inner surface of the primary wall daily (Balls, 1912). These layers do not follow a direction parallel to the fiber axis, but ascend spirally (Mühlethaler, 1950). Furthermore, their direction of winding is in opposition, giving these fibers a high degree of elasticity (Mühlethaler, 1949). Hence, cellulose and non-cellulose components, embedded between the cellulose fibrils, each form an independent system.

Cotton fiber elongation is essentially the same process as enlargement of other plant cells; however, it does differ somewhat from other growing points, in that relative to its original size the cotton fiber elongates for a longer time and becomes larger than almost any other plant cell (O'Kelley and Carr, 1953).

In view of the commercial value of cotton fibers, it is indeed surprising to find a limited amount of research concerning the composition and structure of the fiber cell wall. Of the numerous chemical studies of cotton, only a few have attempted to determine changes occurring during fiber elongation and then only on greater than 16-day-old fibers, a time when elongation is near completion.

In addition, these studies were directed at changes occurring within the whole fiber rather than the cell wall. Briefly summarized, Levine (1915) has shown that the protein content of developing fibers

undergoes a rapid decrease in crude protein from 13.94% of the dry weight of 16-day-old fibers to 2.35% of those 26 days old, and a slow decrease to 1.13% in 38-day-old fibers. Caskey and Gallup (1931) reported a decrease in percentage of reducing sugars, on a dry weight basis, from 11.34 to 0.41 from 21 days after flowering to 41, with the moisture percentage decreasing at the same time from 70.25 to 12.0%. Compton and Haver (1940) found the per cent reducing sugars to decrease as the fibers mature and the sugars present were determined to be glucose, fructose and pentoses. O'Kelley and Carr (1953) have compared carbohydrates in field- and greenhouse-grown fibers and conclude that (1) reducing sugars are the major constituent of young fibers and cellulose of older fibers (3% in young; 79% in mature) and (2) that the principal differences observed between field and greenhouse samples are the result of a slower rate of elongation and differentiation in the field samples. They furthermore conclude that:

> the constancy of carbohydrates in older elongating fibers and the structural similarity of all the primary wall examined indicate that the carbohydrate translocation and synthesis keep pace with fiber elongation and result in a cell in which there is little change in carbohydrate proportions throughout the elongation period.

Studies on the degree of polymerization of the cellulose of the primary and secondary walls of cotton have been made by Hess *et al.* (1939), Compton (1941) and, more recently, by Marx-Figini (1966). The degree of polymerization of cellulose in young fibers possessing primary walls is of lower and of a more heterogeneous degree than that of secondary cell wall cellulose.

Electron micrographs (O'Kelley and Carr, 1953) show that cellulose exists as strands, or microfibers, of ca. 250 Å in ϕ and lengths of ca. 50,000 Å in the primary wall of cotton fibers. The orientation of microfibers was principally in two directions: the outermost microfibers along the long axis of the cell, and inner ones at approximately right angles to this axis (Mühlethaler, 1950).

In summary, although a considerable amount of information exists on the cellulosic component of the cotton fiber wall, very little information exists on the composition and structure of the other components of the cotton fiber primary cell wall. This is unfortunate because the advantages of the developing cotton fiber system as relates to biosynthesis and structure of plant cell walls are numerous.

The research presented here involves a determination of the composition of the primary cell wall of the cotton fiber at various stages of development and under varying conditions of growth. Such information provides the groundwork for more detailed structural analyses, leading ultimately towards the mechanism by which cells elongate. The composition of the primary cell wall at a stage of development well before secondary wall deposition has occurred, as well as during later stages of development, has been determined. Variance of the components of the primary cell wall during development and some preliminary information concerning variance in carbohydrate linkages with age have also been determined.

The data presented will demonstrate that the developing cotton fiber can be used advantageously as a model system for answering the following questions:

A. Is the composition of the primary cell wall of an elongating *single cell* constant throughout the entire elongation phase?

B. How closely can the cell wall composition and the elongation process be mimicked in an *in vitro* culture system?

METHODS AND MATERIALS

Growth

Gossypium hirsutum ('Acala SJ-1') was germinated and grown as described by Beasley and Ting (1973) with the exception that our plants were maintained in growth chambers under the following light and temperature regime: 11.5 hr light (10 h incandescent and fluorescent followed by 1.5 hours of incandescent only)/12.5 hr dark; 33°C/22°C. Plantings were made at 3-week intervals and plants were discarded after 4 months.

On the morning of anthesis, flowers were labeled with tags and fertilized by gently brushing the anthers to dislodge pollen. At any given time, no more than 3 bolls were allowed to develop on any one plant. Surplus flowers were used for the collection of ovules to be cultured.

Ovule Culture

Unfertilized ovules were taken on the day of anthesis and cultured as described by Beasley (1971). The basal medium described was supplemented with 5 μM indoleacetic acid and 0.5 μM gibberellic acid. Ovules were incubated in darkness at 34°C.

Cell Wall Preparation

Fibers, either plant or culture grown, of a known age were quantitatively removed from the ovule with forceps, immediately

immersed in liquid nitrogen and then lyophilized. Harvested, lyophilized fibers were then finely ground, with dry ice to facilitate grinding, in a mortar and pestle. From the fiber preparation thus obtained as a fine powder, cell walls were prepared essentially by the method of Talmadge *et al.* (1973). The powder was thoroughly homogenized in 10 volumes (v/w) of cold 100 mM potassium phosphate, pH 7.0, centrifuged in a clinical centrifuge for 15 minutes and the supernatant discarded. In this manner, the pellet was successively washed at least 3 times with 10 volumes of: 500 mM potassium phosphate buffer, pH 7.0, distilled water, 1:1 chloroform methanol and, finally, acetone. These washings should remove all cytoplasmic contaminants. The resultant residue was frozen and then lyophilized. Following drying to constant weight at 70°C, the cotton fiber cell wall preparations were stored over phosphorus pentoxide at room temperature in a desiccator.

Extracellular Medium

Twenty-five milliliters of cell-free culture medium was lyophilized, resuspended in 20 ml of 70% ethanol and stored 7 days at 4°C, at which time the supernatant was carefully decanted. The remaining precipitate was resuspended in 70% ethanol, stored at 4°C, centrifuged at 3000 g, 20', pellet lyophilized and analyzed for neutral sugar composition.

α-Amylase Treatment

Cell wall preparations were treated as described by Talmadge *et al.* (1973) with α-amylase (Sigma Chemical Company) to remove any possible starch contaminants present.

Starch Determination

The presence of starch was detected by reaction with an iodine solution, as described by Varner and Mense (1972), to give a blue color, A_{max} 620.

Fractionation Procedures

Urea Extraction of Walls

Three milligrams of cell wall were suspended in 4 ml of a solution, described by Allen and Neuberger (1973), containing 8 M urea, 2% SDS, and 2.5% mercaptoethanol. The suspension was heated at 100°C for 10 minutes, then cooled and centrifuged in a clinical centrifuge for 10 minutes. The supernatant was decanted and saved for subsequent analyses. The pellet was then thoroughly washed with 70% ethanol and analyzed for amino acid content.

Acetic/Nitric Digestion of Walls

Cell walls can be freed of non-cellulosic, organic constituents by digestion with an acetic/nitric reagent according to Kürschner and Hoffer (1930), Crampton and Maynard (1938) and Updegraff (1969). The acetic/nitric reagent, prepared by adding 15 ml concentrated nitric acid to 150 ml 80% acetic acid, was added to the sample and heated to 100°C for at least 20 minutes.

Enzymic Hydrolysis

Crude enzyme preparations of cellulysin (Calbiochem), driselase (Kyowa Hakko Kogyo Co., Ltd.) or mascerozyme (All Japan Biochemicals) were used at concentrations and temperatures which varied depending upon the experiment. Substrate was weighed out and pre-incubated for

30 minutes in 1 volume of water at the specified temperature with either magnetic stirring or shaking, if done in a reciprocal shaking water bath. One volume of an enzyme solution was then added. Aliquots were taken from the reaction mixture at time intervals, boiled in a boiling hot water bath for 10 minutes, centrifuged for 20 minutes in a clinical centrifuge, and the supernatant assayed for uronic acids or reducing sugars. For neutral sugar analyses by gas chromatography, pellets and supernatants were first lyophilized.

Colorimetric Assays

Cellulose

The cellulose content of the cell walls was determined by the method described by Updegraff (1969) with the following exceptions. Basically, the procedure involves a determination, by the anthrone test, of carbohydrate which remains insoluble after an acetic/nitric digestion of cell walls. Subsequent to centrifugation and decantation of the acetic/nitric digest, the resulting pellet was resuspended in *cold* 70% ETOH, rather than in water, in order to minimize excessive swelling of cellulose. This improved pellet formation and minimized cellulose losses. Pellet recoveries were further enhanced by carrying out centrifugations at 0°C. Appropriate dilutions were made and the anthrone assay was reduced to a final volume of 3 ml rather than the cumbersome 15 ml described.

Uronic Acids

Uronic acid concentrations were determined by the method of Blumenkrantz and Asboe-Hansen (1973). Corrections for the contribution

of neutral sugars to the color produced upon addition of meta-hydroxyproline in the last step of the assay were not necessary, as neutral sugars do not interfere with the specificity of this reagent. Corrections were made, however, for the pink chromogen produced by reaction of carbohydrates with sulfuric acid/tetraborate at 100°C in the first reaction of this assay.

Reducing Sugars

The reducing sugar assay of Park and Johnson (1949) was utilized to, independently, quantitate monosaccharide release from cell walls sugjected to trifluoroacetic acid hydrolysis.

Protein

Amino acid analyses were performed using a modified Technicon Autoanalyzer as described by Lamport (1969) subsequent to 6N HCl (redistilled, constant boiling HCl) hydrolysis of 3 mg of cell wall in a 5 ml microvial which was evacuated, sealed under nitrogen, and heated for 18 hours at 105°C. The per cent protein in the wall was calculated, based upon the total nanomoles amino acid recovered, utilizing 120 as the average molecular weight of an amino acid.

Hydroxyproline content was analyzed by a modification of the method of Kivirikko (1963) as described by Lamport and Miller (1971).

SDS molecular weight determinations were performed by the method of Weber and Osborn (1969) as described by Heages (1943), with the exception that vicilin, a storage protein isolated from *Phaseolus aureus*, having subunit molecular weights of 63,000, 50,000, 29,500, and 24,000, was used as a standard for molecular weights.

Neutral Sugar Analyses by Gas Chromatography of Alditol Acetates

Neutral sugar analyses were performed using the gas chromatographic method of Albersheim *et al.* (1967) modified to some extent. To 3 mg of cell wall material, 1 ml of 2N trifluoroacetic acid was added containing 300 nanomoles of inositol as internal standard. Subsequent to autoclaving for 1 hour at 121°C, the hydrolyzed wall was reduced with 0.5 ml sodium borohydride in 3N NH_4OH (4 mg/ml). Upon completion of reduction of aldoses (usually 1.5 hours), excess borohydride was decomposed by dropwise addition of glacial acetic acid. Removal of boric acid was accomplished by a minimum of 6 additions of 100 µl methanol to the reaction mixture, each followed by evaporation to *complete* dryness at 40°C under a stream of nitrogen. To insure complete removal of moisture, samples were stored overnight over phosphorus pentoxide in a vacuum desiccator. Two hundred microliters acetic anhydride were then added and the samples were heated at 121°C for 1 hour.

Gas chromatographic separations, when not combined with mass spectrometry, were carried out on a Varian 2100 gas chromatograph with helium as carrier gas. Acetic anhydride solutions, containing alditol acetate derivatives, were injected directly onto the column. The glass column (180 cm x .2 cm I.D.) was packed with a mixture of 0.2% poly(ethylene glycol adipate), 0.2% poly(ethylene glycol succinate), and 0.4% silicone XE-1150 on Gas Chrom Q (80-100 mesh). Chromatography was performed with temperature programming at 2°/minute from 130°C to 180°C with a helium flow rate of 35 ml/min. Gas chromatographic peaks were integrated with a Hewlett Packard 3370 A integrator, equipped with an internal timer. The peak areas and

retention times were expressed relative to the internal standard added to each sample prior to trifluoroacetic acid hydrolysis. The data so obtained were then corrected for on the basis of the mole per cent recovery of a standard sugars mixture and are expressed as mole per cent of recovered carbohydrate. The per cent of the wall recovered as neutral sugars was calculated as follows:

$$\text{Percent} = \frac{\Sigma\left[\left(\frac{A_S}{A_I}\right)\left(\frac{A_{ISTD}}{A_{SSTD}}\right)(N)\right](W)}{M}(10^{-4})$$

where A_S = area of sugar peak in unknown

A_{SSTD} = area of sugar peak in standard mixture

A_I = area of inositol peak in unknown

A_{ISTD} = area of inositol peak in standard mixture

N = total nanomoles inositol present in unknown

W = molecular weight of sugar

M = total mg cell wall present in unknown

Standard sugars, obtained commercially, were used without further purification. Standard sugar mixtures were run with each set of determinations to minimize error due to minor unavoidable variation during preparations resulting in differential recoveries.

Methylation Analyses

Methylation of unfractionated cell walls was carried out by the method of Hakomori (1964) as described by Sandford and Conrad (1966).

Following methylation of the cell walls, 1 ml of 1:1 chloroform methanol (v/v) was added to the methylation reaction mixture. This solution was then extensively dialyzed against water to remove DMSO,

for ca. 48 hours or until only one phase remained. The dialysate was then dried down by evaporation at 30°C under a stream of pure nitrogen, resuspended in chloroform/methanol and dried again to remove last traces of water. The dry residue was then extracted with chloroform and centrifuged in a clinical centrifuge for 10 minutes. The supernatant containing the permethylated polysaccharides was carefully decanted and saved. The pellet, consisting of chloroform-insoluble polysaccharides, was washed once with chloroform and then saved for remethylation. The methylated polysaccharides present in the combined chloroform soluble fraction were dried at 30°C under a stream of nitrogen.

Permethylated polysaccharide samples were hydrolyzed for 1 hour at 121°C in microvials containing 500 µl of 2.0N trifluoroacetic acid (TFA). Prior to hydrolysis 300 nanomoles of myo-inositol were added as internal standard to each sample. The resulting partially methylated aldoses were then reduced to their corresponding alditols with 500 λ sodium borodeuteride/3N NH_4OH; (4 mg/ml) rather than sodium borohydride to aid in identification of ambiguous mass spectral fragments. The presence of deuterium in the anomeric carbon provides a distinguishable difference between the fragmentation patterns derived from C_3 and C_4 linked hexoses. These partially methylated alditols were then converted to their corresponding alditol acetates as described. Maltose (commercially available) was used without further purification in the synthesis of the two partially methylated alditol acetate standards used:

2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl glucitol (T-GLU) and

2,3,6-tri-O-methyl-1,5-di-O-acetyl glucitol (4-GLU).

When not combined with mass spectrometry, gas chromatographic separations of permethylated sugars were carried out as described for neutral sugar analysis with the following exceptions. Chromatography was performed with temperature programming at 1 degree/minute from 110°C to 190°C with a helium flow rate of 30 ml/min on glass columns (180 cm x .2 cm I.D.) packed with 3% OV-225 on Gas Chrom Q (100-120 mesh).

Combined gas-chromatography-mass spectrometry was performed on an LKB 9000 combined glc-mass spectrometer capable of repetitive scanning as described by Laine *et al.* (1974). A computer data system with a PDP-8/E minicomputer was used as described by Sweeley *et al.* (1970) and modifed by Laine *et al.* (1974).

RESULTS

Growth Parameters Associated with Development

Studies which examine biochemical changes associated with developmental growth require use of a biological system that develops according to an orderly schedule that is reproducible.

In order to quantitatively establish the reproducibility of various growth parameters, under controlled growth chamber conditions, developmental processes associated with the growth of cotton were examined. While it has been shown that these phases can be longer or shorter, depending upon field growth conditions, it was anticipated that cotton plants grown in growth chambers would yield homogeneous samples suitable for cell wall studies during development of the primary cell wall. The parameters measured to establish the synchrony of this system under these growth conditions include boll length, boll weight, dry weight of fibers per boll, and fiber length.

The Boll

Cotton bolls of known age were removed from the plant, and their length and mass measured. These changes with age, as summarized in Figure 1, indicate that the increase in boll length and in mass follow a defined developmental pattern that can be measured reproducibly. However, in collecting these data, it was observed that occasionally under-developed bolls were produced. Thus, the easily

Figure 1. Length and fresh weight of cotton bolls through 35 days post-anthesis.

Standard deviations, where given, are based on an average of five multiple samplings over a two-week period.

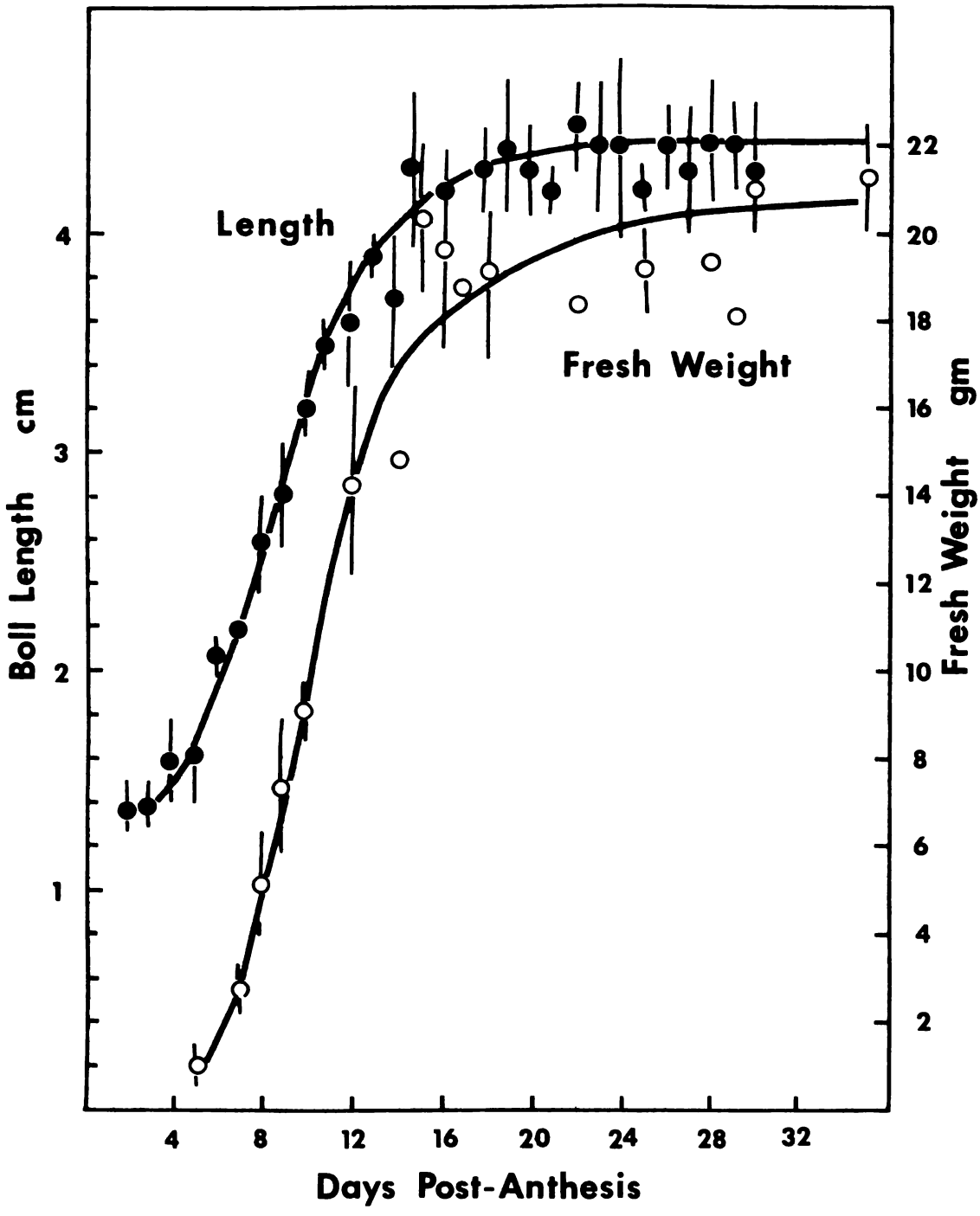

Length
Fresh Weight
Boll Length cm
Fresh Weight gm
Days Post-Anthesis
1
2
3
4
2
4
6
8
10
12
14
16
18
20
22
4
8
12
16
20
24
28
32

Figure 1

measured parameters of boll length and mass can be effectively used to insure use of normally developing bolls rather than the occasional boll that is destined for abortion and/or shows signs of under-development.

The Fiber

Fibers were quantitatively removed from those bolls judged to be normally developed, immediately immersed in liquid nitrogen and lyophilized. The dry weight of fibers per boll with age is shown in Figure 2. In the case of culture grown fibers, the dry weight of fibers harvested is expressed per 24 ovules, the number of ovules found in a normally developing boll. For plant grown fibers an increase in dry weight of fibers per boll is observed until about 32 days post-anthesis (DPA), after which there is no increase. The dry weight of culture grown fibers also undergoes an increase with development, although at a slower rate.

In order to determine the rate of cell elongation, fiber lengths at various stages of development were measured for plant grown fibers and are shown in Figure 3 along with dry weight fibers per boll for comparative purposes. Fiber elongation is complete around 20 days post-anthesis. The increase in fiber length occurs with a short lag period, whereas the increase in fiber dry weight undergoes a much longer lag period. Maximal dry weight is attained around 30 days post-anthesis. The maximum rate of dry weight fiber increase occurs ten days after the maximum rate of fiber elongation.

Figure 2. Dry weight of fibers per boll.

The dry weight of total fibers harvested with age is expressed per boll in the case of plant grown fibers, and in the case of culture grown fibers is expressed per 24 ovules.

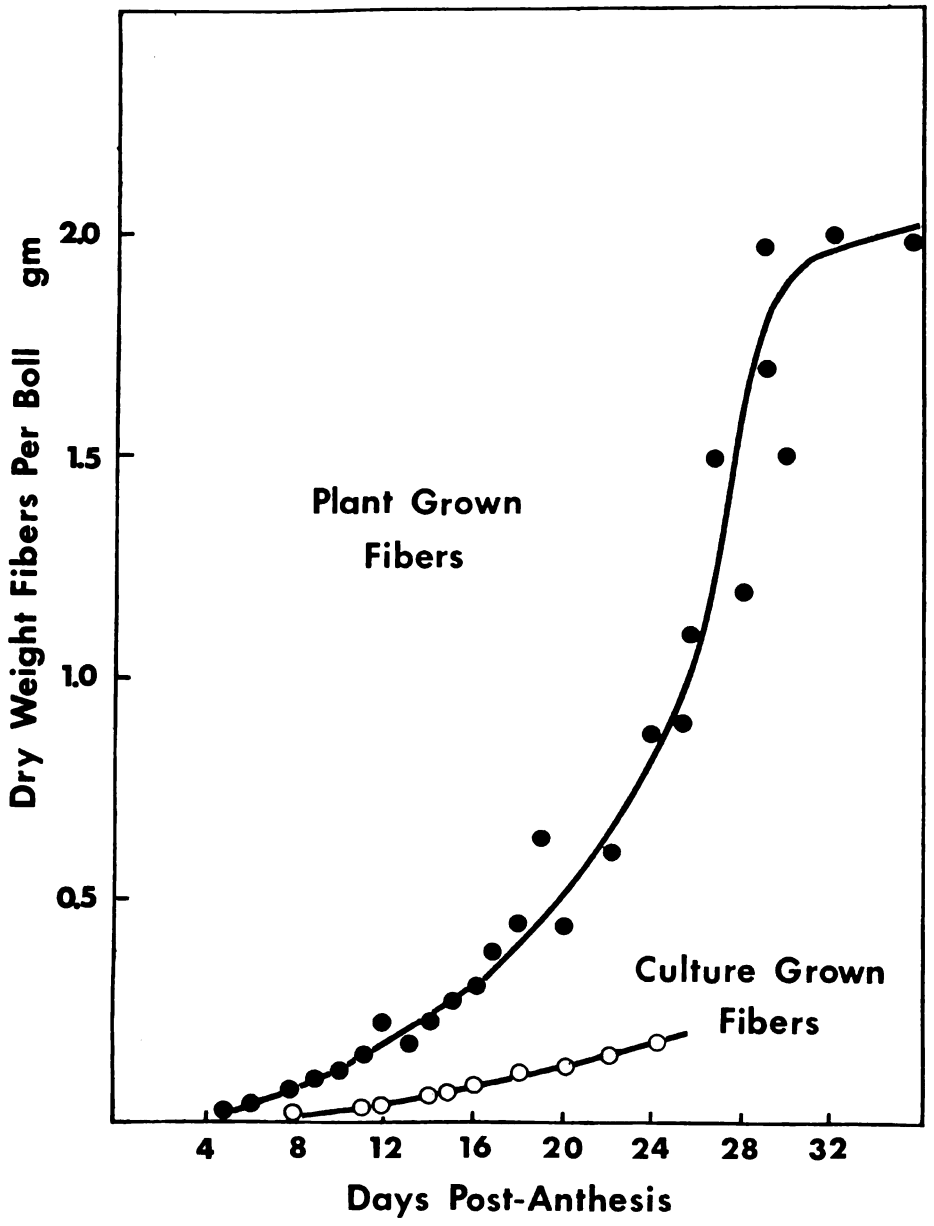

Dry Weight Fibers Per Boll gm
2.0
1.5
1.0
0.5
Plant Grown
Fibers
Culture Grown
Fibers
4
8
12
16
20
24
28
32
Days Post-Anthesis

Figure 2

Figure 3. Fiber length and dry weight through 35 days post-anthesis.

Values given in parentheses are 10^{-6} grams dry weight per fiber and were calculated on the basis of 384,000 fibers per boll.

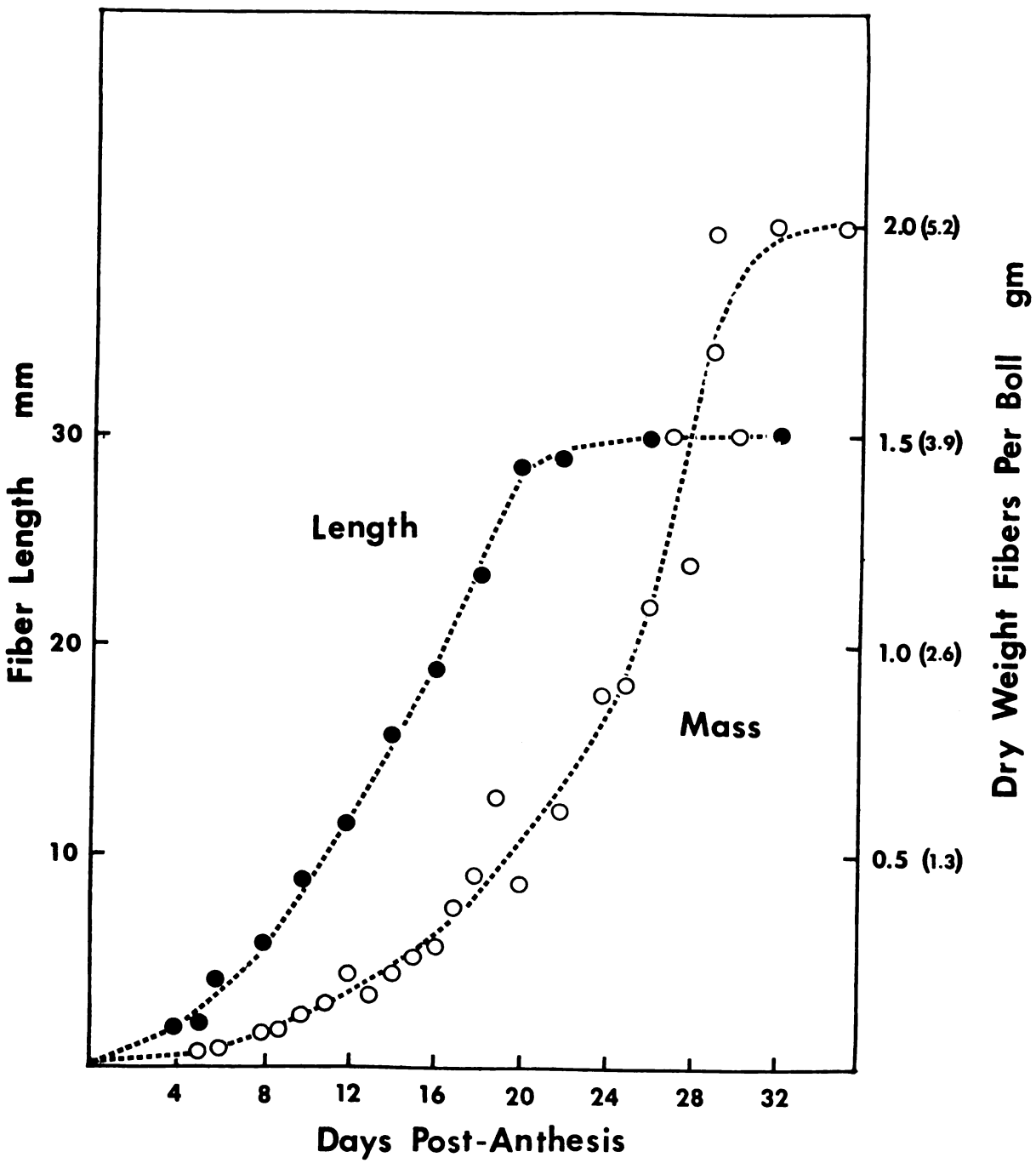

Fiber Length mm
30
20
10
Length
Mass
2.0 (5.2)
1.5 (3.9)
1.0 (2.6)
0.5 (1.3)
Dry Weight Fibers Per Boll gm
4
8
12
16
20
24
28
32
Days Post-Anthesis

Figure 3

The Cell Wall

Cell walls were prepared from lyophilized fibers and the per cent yield of cell wall recovered from fibers was calculated. The per cent yield is defined as the per cent of the total dry weight of the fiber cell that is recovered as cell wall. Cell wall yields for both plant and culture grown fibers (Figure 4) increase with developmental age of the fiber.

Although the increase in the amount of dry weight fibers (whole cells) harvested per boll proceeds at a different rate for plant and culture grown fibers, the data of Figure 4 show that the amount of material isolated as cell walls from fibers is the same for both plant and culture grown fibers at any given age. While the yields increase markedly with age, the per cent yield at any age is reproducible, a fact which suggests we are isolating a defined wall fraction.

The Cell Wall Components

Starch Removal from Cell Wall Preparations

In order to determine the minimal incubation time required for complete removal of starch from cell wall preparations, the time course of the amylase incubation was followed by observing the decrease in absorbance at 260 nm of an aliquot of the incubation mixture to which iodine was added. However, when previously untreated cell walls were assayed, at time zero, the blue color indicative of starch was not obtained. In a separate experiment, whole cotton fibers of eight different ages were boiled to aid solubilization of any starch in the form of granules and, as with purified cell walls,

Figure 4. Cell wall yields during fiber development.

The per cent yield of cell wall is defined as the per cent of the total dry weight of the fiber cell which is recovered as cell wall material and is given for cell wall preparations from both plant and culture grown fibers.

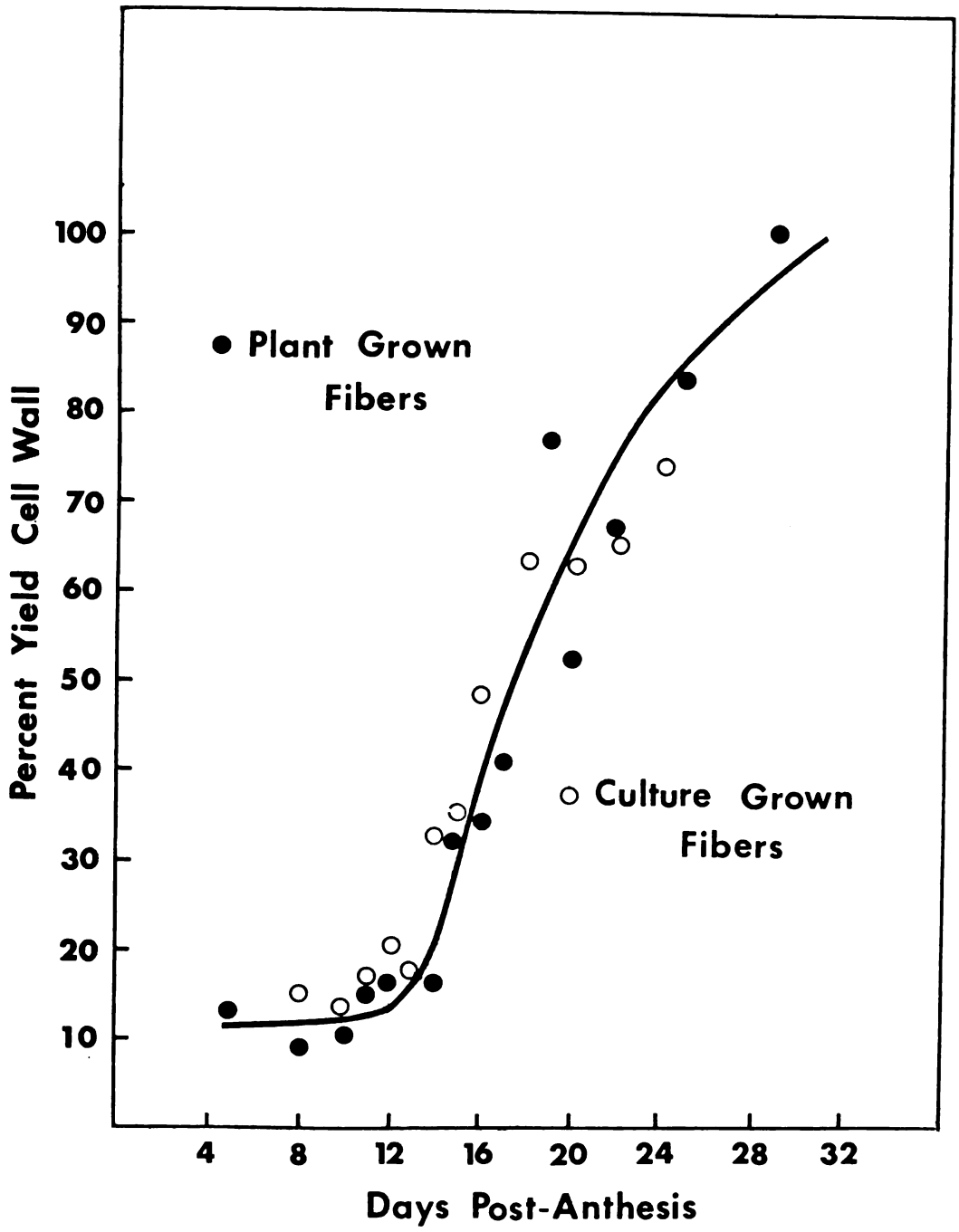

Percent Yield Cell Wall
100
90
80
70
60
50
40
30
20
10
● Plant Grown Fibers
○ Culture Grown Fibers
4
8
12
16
20
24
28
32
Days Post-Anthesis

Figure 4

no starch was detected within the limits of the assay (less than 0.02% of the weight of fibers). Furthermore, a neutral sugar analysis by gas chromatography of 8 days post-anthesis wall treated with α-amylase resulted in a glucose content similar to that of untreated walls. Since the data indicate a negligible starch content in wall preparations, amylase treatment was not employed in subsequent preparations.

Cellulose

The abrupt change from primary to secondary wall synthesis is marked by cellulose deposition as a secondary wall. The per cent of the wall that is cellulose can therefore be used to characterize the stage of wall development at any age. The per cent of the wall material analyzed that is cellulose is given in Figure 5 for both plant and culture grown fibers.

The nature of the timing of the changeover from primary to secondary wall synthesis, under our growth conditions, is very exact in the plant grown fibers, as evidenced by the abrupt increase in cellulose content. Beginning at 16 days post-anthesis, the percentage of the wall that is cellulose increases from 31 to 73% in one day. This transition period in the culture grown fibers occurs earlier and extends over a much longer period of time. In plant grown fibers, there appear to be three distinct phases of cellulose deposition; two phases coincide with the elongation phase when primary wall is synthesized and the third phase corresponds with the end of elongation when secondary wall synthesis occurs. It was surprising to find two phases of cellulose deposition during primary wall synthesis,

Figure 5. Changes in cellulose content of the cell wall during fiber development.

The per cent of the cell wall that is cellulose is given for cell walls derived from both plant and culture grown fibers. Standard deviations are given for multiple samples, representing multiple assays and in most cases multiple fiber harvests and multiple cell wall preparations.

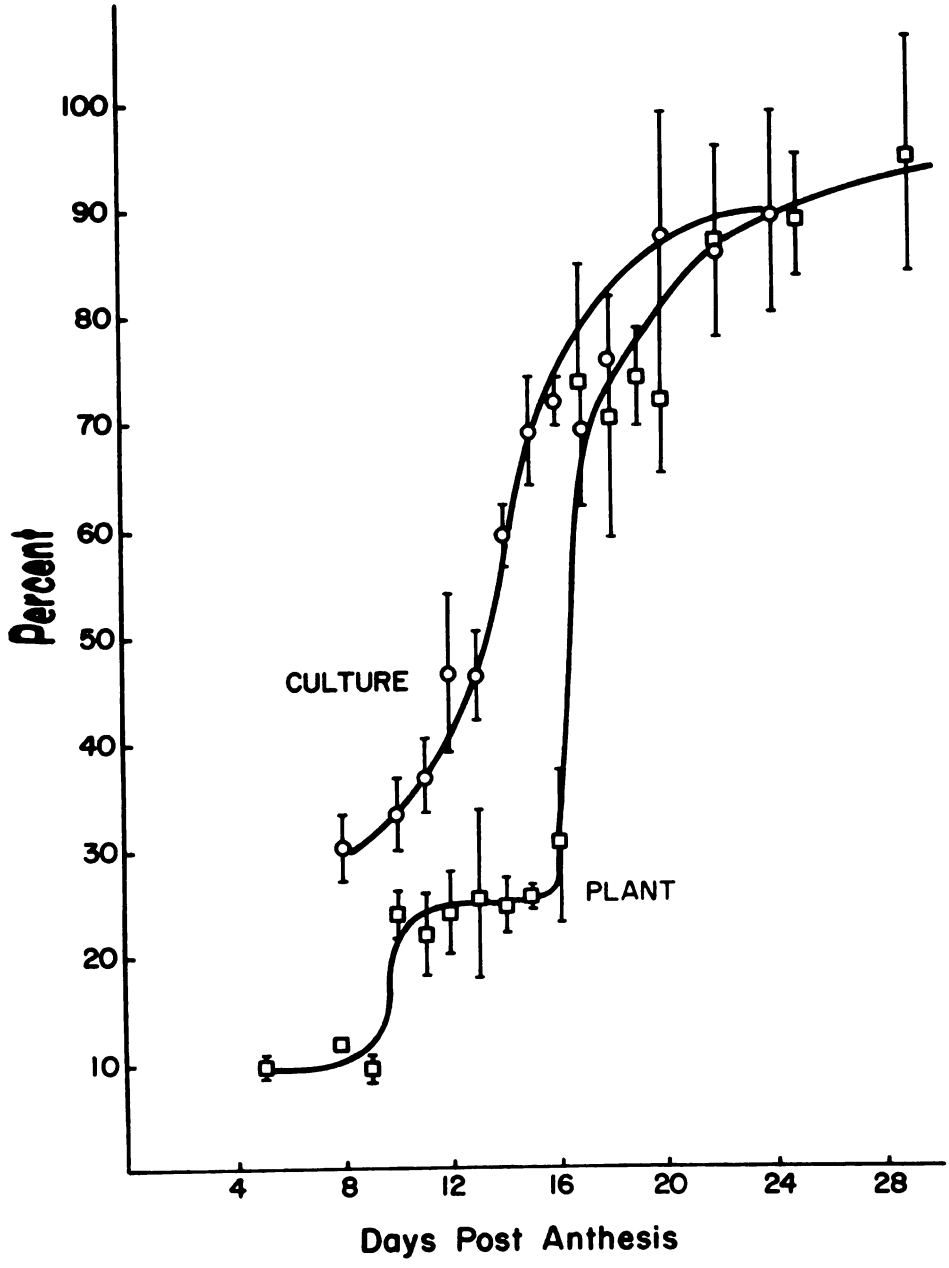

100
90
80
70
60
50
40
30
20
10
Percent
CULTURE
PLANT
4
8
12
16
20
24
28
Days Post Anthesis

Figure 5

especially since the primary cell wall of plants has been considered to be of uniform composition (Albersheim, 1975) throughout the elongation phase.

Neutral Sugars

In view of the distinct changes in cellulose content during the growth of the primary cell wall, we considered it of interest to determine to what extent the neutral sugar components of the wall vary during the various phases of the development of the cotton fiber cell wall.

Purified cell walls of various ages from both plant and culture grown fibers were hydrolyzed with 2N trifluoroacetic acid (TFA) and the neutral sugars determined by gas chromatography as described. The neutral sugars obtained from the wall by this procedure include rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose (non-cellulosic). The quantity of each of the monosaccharides released from the wall upon hydrolysis is expressed as a mole per cent of the total amount of neutral sugars detected (Table 1). The total weight of the wall which could be accounted for by the sugars released in the trifluoroacetic acid hydrolysis treatment decreased from 32 per cent at 5 days post-anthesis to 6 per cent at 29 days post-anthesis. The results of assaying the trifluoroacetic acid hydrolysate supernatant for total reducing sugars by a colorimetric assay are also presented and are in good agreement with the results obtained by gas chromatography.

The most striking observation from these data is that the relative amounts of the individual sugars present in the wall are variable

Table 1. Neutral sugar composition

Purified cell walls were hydrolyzed, and the neutral sugars were determined by gas chromatography of the alditol acetate derivatives as described. Each sugar is expressed as a mole per cent of the total neutral sugars detected. The per cent of the wall released by 2N trifluoroacetic acid hydrolysis as determined by gas chromatography and as determined by colorimetric assay is also presented.

Each value represents an average of at least four alditol acetate preparations, and in many cases multiple cell wall preparations from multiple harvests. Standard deviations are given.

	Cell Wall Age															
Sugar	5	8	9	10	11	12	13	14	14.5	15	16	17	18	22	25	29
Plant Cell Walls																
Rhamnose	$5.9^{.58}$	$7.9^{.42}$	$8.6^{.35}$	$7.4^{.91}$	$8.2^{.13}$	$8.5^{.78}$	$7.8^{1.3}$	$8.4^{.5}$	$6.5^{1.6}$	$6.7^{.4}$	$4.3^{.2}$	$3.5^{.4}$	5.1^{0}	$2.3^{.2}$	3.1	$1.7^{.3}$
Fucose	$.9^{.33}$	$1.2^{.06}$	$.8^{.72}$	$1.2^{.72}$	$1.4^{.59}$	$1.4^{.2}$	$1.1^{.4}$	$1.2^{.3}$	$1.2^{.1}$	$1.0^{.7}$	$.4^{.4}$	$.6^{.2}$	$.5^{.6}$	$.4^{.1}$	.2	$.3^{0}$
Arabinose	$24.4^{2.6}$	$31.8^{3.05}$	$34.7^{.25}$	$32.8^{4.6}$	$28.8^{1.68}$	$22.3^{2.6}$	$20.7^{3.1}$	$22.3^{2.1}$	$18.7^{2.1}$	$13.6^{.2}$	$7.9^{.6}$	$9.1^{1.3}$	$8.6^{1.3}$	$4.1^{.1}$	5.6	$5.5^{.2}$
Xylose	$7.7^{.54}$	$10.0^{.4}$	$10.8^{.55}$	$10.2^{1.4}$	$9.7^{.35}$	$10.8^{1.2}$	$10.4^{.7}$	$10.3^{.4}$	$8.4^{.4}$	$9.0^{.3}$	$6.6^{.4}$	$11.0^{.9}$	$8.5^{.6}$	$4.0^{1.0}$	3.7	$3.5^{.1}$
Mannose	$3.5^{.81}$	$3.9^{.06}$	$4.7^{.87}$	$3.9^{.26}$	$4.0^{.45}$	$4.2^{.3}$	$4.1^{.5}$	$3.9^{.3}$	$3.1^{.2}$	$3.6^{.6}$	$1.7^{.5}$	$1.6^{.6}$	$1.9^{.6}$	$2.5^{1.6}$	1.3	$1.2^{.1}$
Galactose	$12.9^{.73}$	$15.8^{.72}$	$16.7^{.80}$	$17.1^{1.9}$	$16.3^{.9}$	$16.8^{.7}$	$16.2^{1.2}$	$17.8^{.3}$	$12.0^{.4}$	$13.4^{.7}$	$7.4^{.8}$	$6.5^{.6}$	8.4^{0}	$4.5^{1.2}$	5.9	$4.9^{.1}$
Glucose	$45.0^{2.04}$	$29.5^{3.6}$	$23.7^{.69}$	$28.0^{6.3}$	$31.6^{3.11}$	$36.2^{1.6}$	$39.8^{4.6}$	$36.2^{2.5}$	$50.4^{1.0}$	$52.7^{.7}$	$71.7^{.9}$	$67.5^{1.4}$	$67.1^{.9}$	$82.4^{3.6}$	80.4	83.0^{0}
Per cent of cell wall																
Gas chrom.	$32.4^{2.3}$	$25.0^{4.0}$	$8.0^{.35}$	$23.2^{2.1}$	$21.4^{1.3}$	$21.7^{4.4}$	$17.9^{4.9}$	$21.0^{2.2}$	$21.6^{5.9}$	$13.8^{2.3}$	$13.8^{3.5}$	$15.2^{2.5}$	$10.0^{1.1}$	$10.1^{.14}$	10.0	$5.8^{.3}$
Colorimetric	-	-	8.4	25.0	24.0	-	-	24.0	-	21.0	21.0	21.0	11.0	-	-	8.0

	Cell Wall Age							
Sugar	8	10	12	14	16	18	20	24
Culture Cell Walls								
Rhamnose	$8.6^{.6}$	$9.8^{1.1}$	$8.7^{.8}$	$6.1^{.9}$	$4.6^{.8}$	$3.0^{.5}$	$2.8^{.4}$	$2.7^{.1}$
Fucose	$1.1^{.1}$	$1.1^{.2}$	$1.2^{.4}$	$.7^{.1}$	$.5^{.2}$	$.1^{.1}$	$.2^{.1}$	$.2^{.1}$
Arabinose	$29.2^{1.2}$	$29.6^{2.6}$	$26.4^{.3}$	$14.9^{2.2}$	$12.2^{1.0}$	$8.0^{1.9}$	$8.2^{2.2}$	$7.2^{1.9}$
Xylose	$12.3^{1.4}$	$17.4^{1.3}$	$16.7^{1.0}$	$17.5^{1.9}$	$12.5^{1.2}$	$16.2^{.8}$	$9.1^{.6}$	$9.7^{.5}$
Mannose	$3.6^{.1}$	$3.5^{.4}$	$3.0^{.5}$	$2.0^{.5}$	$1.3^{.3}$	$1.2^{.3}$	$1.1^{.5}$	$1.9^{.6}$
Galactose	$18.6^{.4}$	$17.8^{.8}$	$15.9^{.5}$	$9.2^{.9}$	$7.8^{.9}$	$4.3^{.6}$	$4.7^{1.1}$	$5.0^{.3}$
Glucose	$26.9^{.9}$	$20.6^{2.5}$	$27.9^{2.6}$	$49.8^{5.3}$	$61.1^{3.5}$	$67.2^{2.1}$	$73.9^{4.6}$	$73.3^{1.8}$
Per cent of cell wall	$27.3^{3.3}$	$23.3^{4.2}$	$21.1^{1.4}$	$17.3^{4.0}$	$15.7^{2.1}$	$16.4^{3.0}$	$12.3^{.7}$	$11.6^{4.1}$

with age, *and* that quite similar changes are observed in the cell walls derived from culture grown fibers. The sugars that undergo the greatest changes are arabinose and non-cellulosic glucose; the remainder of the sugars are present at a relatively constant mole per cent. These results, shown in Figure 6 for both plant and culture grown fibers, indicate that arabinose undergoes a seven-fold decrease and that glucose undergoes a nearly three and a half-fold increase. For plant grown fibers, the rise in non-cellulosic glucose occurs six days before the deposition of secondary wall cellulose.

Suspension-cultured sycamore cells secrete polysaccharides into the medium in which they are grown. These extracellular polysaccharides are assumed to be either cell wall degradation fragments or cell wall precursors (Becker *et al.*, 1964). Albersheim and coworkers have used this extra-cellular medium as a source of cell wall polysaccharides which can be used successfully as a model for cell wall polymers (Bauer *et al.*, 1973; Keegstra *et al.*, 1973).

In order to see if similar polysaccharides are excreted by culture grown cotton fibers, the ethanol precipitable fraction obtained from cell-free cotton fiber culture medium was analyzed, without further purification, for neutral sugar composition at five different time points. The sugars detected include arabinose, xylose, galactose and glucose. An increase in the per cent of arabinose, xylose and galactose and a decrease in the amount of glucose present during the time course of development examined was observed. These data indicate that polysaccharides may be secreted by culture grown fibers. This information may prove useful for future studies on wall structure, but has not been further pursued.

Figure 6. Changes in non-cellulosic glucose and arabinose content of the neutral sugar component of the cell wall.

Values are expressed as mole percent of the total neutral sugars detected.

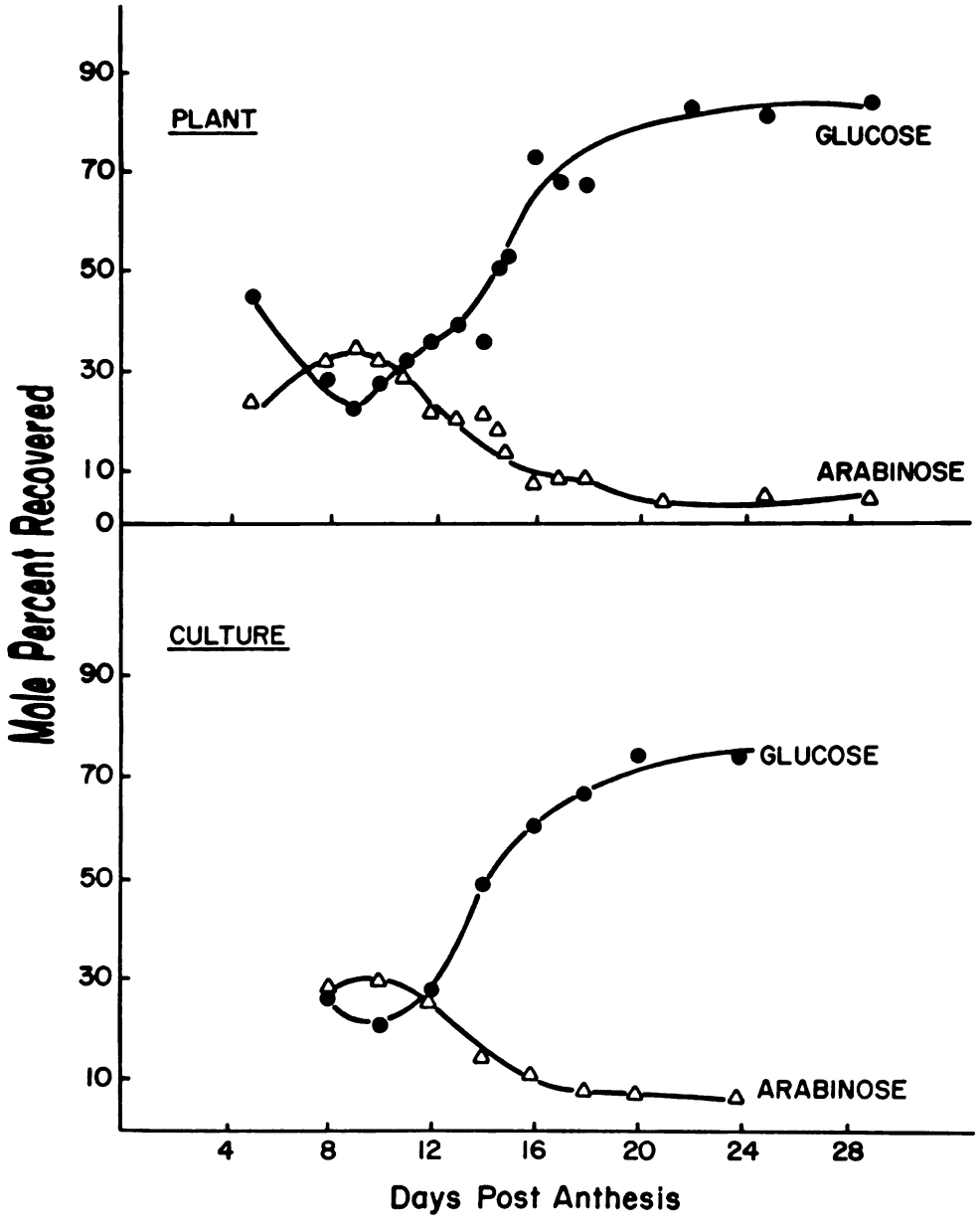

Mole Percent Recovered
PLANT
GLUCOSE
ARABINOSE
CULTURE
GLUCOSE
ARABINOSE
90
70
50
30
10
0
4
8
12
16
20
24
28
Days Post Anthesis

Figure 6

Residual Sugars Associated with Cellulose

Although Talmadge *et al.* (1973) indicate that cellulose is resistant to hydrolysis by 2N trifluoroacetic acid hydrolysis, data were not presented to verify this. Hydrolysis of cellulosic glucose in the cell wall by 2N trifluoroacetic acid would result in an erroneous value for the non-cellulosic glucose component of the cell wall. These corrections, if necessitated, could result in marked changes in the neutral sugar composition of cell walls observed during the development of the cotton fiber.

In order to establish the resistance of cellulose to 2N trifluoroacetic acid hydrolysis, a series of sequential hydrolyses of differentially treated cellulose was performed. The results described below provide strong support for the notion that cellulose is indeed resistant to 2N trifluoroacetic acid.

In an effort to quantitate the susceptibility of cellulose to release by 2N trifluoroacetic acid hydrolysis, it was found that small, but significant, quantities of sugars other than glucose were released from thoroughly washed α-cellulose (Sigma Chemical Co.) during hydrolysis. However, the total amount of sugars released (including glucose) never exceeded ca. 11% of the weight of cellulose originally present (Table 2).

Both ethanol washed and chlorite washed α-cellulose have resulted in a sugar composition indicating removal of xylose. The presence of residual sugars associated with cellulose presented complications in determining the true resistance of cellulose to 2N trifluoroacetic acid because the release of these sugars (including glucose) is not necessarily indicative of cellulose susceptibility. Therefore,

Table 2. Neutral sugar composition of the residual sugars associated with cellulose

α-cellulose was treated with several different reagents in order to remove residual sugars associated with it. These include water (control), ethanol (ethanol), chlorite (chlorite), and acetic/nitric reagent (A/N: a - .5 hour digest, b - 1.0 hour digest, c - 3.0 hour digest). The residue remaining after treatment was hydrolyzed, reduced, acetylated, and analyzed. In addition, the residue for A/N_b and A/N_c after derivitization was washed, lyophilized and re-derivitized and analyzed (TFA-II_b and TFA-II_c). The residue remaining for TFA-II_b and TFA-II_c was washed, lyophilized, re-devitized and analyzed (TFA-III_b and TFA-III_c).

The values for each sugar are expressed as a mole per cent of the total neutral sugars detected, which is, in turn, expressed as a per cent of the cellulose from which derived.

	Treatment									
Sugar	Control	Ethanol	Chlorite	A/N	A/N_b	A/N_c	TFA-II_b	TFA-II_c	TFA-III_b	TFA-III_c
Rhamnose	-	-	-	-	.6	-	-	-	-	-
Fucose	-	-	-	-	.2	.2	-	-	.5	-
Arabinose	-	-	-	-	3.8	2.7	8.1	10.7	.9	1.6
Xylose	46.0	38.0	38.0	27.4	27.9	24.5	14.6	10.4	8.1	6.7
Mannose	7.0	6.1	5.7	6.7	6.7	6.2	6.4	3.9	3.0	2.1
Galactose	-	-	-	-	.5	.4	-	-	-	-
Glucose	47.0	55.5	56.3	65.9	60.4	66.0	70.8	74.8	87.8	89.7
Per cent of cellulose	11.0	12.9	10.6	10.9	6.3	4.7	3.6	3.5	2.4	2.6

water-washed α-cellulose was then treated with acetic/nitric reagent for varying periods of time to attempt to remove the remaining closely associated non-cellulosic sugars. These results indicate that cellulose that has been treated for longer periods of time releases smaller total amounts of sugars when subsequently hydrolyzed with trifluoroacetic acid, although the relative ratios of these sugars present is the same for the three times examined. These ratios of sugars differ from those obtained for cellulose that had been washed with ethanol or washed with chlorite, in that they have, on a percentage basis, relatively less xylose and more arabinose being released. This alditol acetate preparation was centrifuged and the supernatant containing alditol acetates was discarded. The pellet, a more "purified" form of cellulose, was thoroughly washed, lyophilized, and then re-hydrolyzed with 2N trifluoroacetic acid. This treatment resulted in less total carbohydrate released and in a different ratio than previously obtained. The amount of arabinose detected has increased and the amount of xylose has decreased. A third 2N trifluoroacetic acid hydrolysis resulted in only 2.5% sugars released, 90% of which was glucose.

A similar experiment was performed using three different trifluoroacetic acid concentrations for hydrolysis of a three-hour acetic/nitric digest of α-cellulose. The residue remaining after the first hydrolysis was re-hydrolyzed and analyzed for neutral sugars. The residue remaining after this second hydrolysis was then subjected to a third hydrolysis. These results (Table 3A) show an increase in the amount of arabinose released during the second hydrolysis at all concentrations of trifluoroacetic acid. An

increase in trifluoroacetic acid concentration results in a greater percentage of sugars released. However, the second and third hydrolyses release less than the first. The third hydrolysis resulted in 90% of the material released being glucose. The results of these experiments on α-cellulose suggest the possibility that true cellulose is indeed resistant to trifluoroacetic acid hydrolysis because, when the hydrolysis products obtained approached 100% glucose, these products represented less than 3% of the weight of original cellulose present. Whether the residual release of the glucose is due to cellulose hydrolysis or due to hydrolysis of a non-cellulosic polymer has not been shown. Additional hydrolyses of the remaining residue would indicate whether a continual release of a small amount of glucose occurs. If so, the cellulose itself may be sensitive to trifluoroacetic acid hydrolysis but the quantities released are nevertheless minimal.

These results also indicate that a single trifluoroacetic acid hydrolysis may not be sufficient for complete release of neutral sugars from cotton fiber cell walls. For this reason, 12-day-old walls were sequentially hydrolyzed three times with varying strengths of trifluoroacetic acid (Table 3B). A second trifluoroacetic acid hydrolysis yields an additional 2 per cent at most; therefore, the first hydrolysis yields 91-96% of the non-cellulosic neutral sugars present in the wall. While 4N trifluoroacetic acid releases more total carbohydrate, the slight increase in per cent is primarily due to an increase in glucose released as is illustrated when mole per cent sugars are expressed minus glucose. The first hydrolysis, even at concentrations as low as 0.5N trifluroacetic acid, completely

Table 3. Sequential hydrolysis of A) cellulose-3 and B) 12 days post-anthesis cell wall with varying strengths of trifluoroacetic acid.

α-cellulose was treated with acetic/nitric reagent for 3.0 hours (cellulose-3). The residue remaining was washed, lyophilized, hydrolyzed with trifluoroacetic acid concentration as indicated (.5N, 1N, 4N), reduced, acetylated and analyzed for neutral sugars (TFA-I). TFA-II was prepared similarly from TFA-I, as was TFA-III from TFA-II.

Values are expressed as mole per cent of total neutral sugars detected (values in superscript are mole per cent of total neutral sugars minus glucose). The total carbohydrate detected is expressed as per cent of the cellulose/cell wall originally present.

	TFA-I			TFA-II			TFA-III		
Sugar	.5N	1N	4N	.5N	1N	4N	.5N	1N	4N
				A. Cellulose-3					
Rhamnose	-	-	-	-	-	-	-	-	-
Fucose	-	-	-	$.8^{24}$	-	$.6^{1}$	$.4^{3}$	-	$.5^{6}$
Arabinose	4.3^{11}	4.2^{10}	4.2^{13}	11.8^{35}	11.9^{40}	14.0^{53}	$.2^{1}$	-	$.9^{10}$
Xylose	27.0^{70}	30.4^{74}	21.6^{69}	14.8^{44}	12.4^{42}	8.6^{32}	10.5^{77}	7.3^{77}	5.9^{66}
Mannose	5.8^{15}	5.3^{13}	5.0^{16}	5.9^{18}	5.1^{17}	3.7^{14}	2.6^{19}	2.2^{23}	1.7^{19}
Galactose	2.4^{6}	1.3^{3}	1.0^{3}	-	-	-	-	-	-
Glucose	61.6^{0}	58.9^{0}	68.7^{0}	66.4^{0}	70.4^{0}	73.4^{0}	86.3^{0}	90.5^{0}	91.1^{0}
Per cent of cellulose	4.7	6.0	6.5	3.3	4.3	5.0	2.2	2.0	3.2
				B. 12 DPA cell wall					
Rhamnose	7.0^{11}	7.2^{11}	3.7^{11}	-	-	-	-	-	-
Fucose	1.5^{2}	1.6^{2}	$.6^{2}$	$.6^{2}$	1.6^{4}	1.4^{4}	-	-	-
Arabinose	26.8^{40}	28.9^{44}	17.9^{54}	8.0^{21}	8.3^{21}	5.6^{17}	-	-	2.2^{7}
Xylose	12.0^{18}	10.4^{16}	5.8^{18}	11.1^{29}	10.7^{27}	7.9^{25}	4.8^{28}	7.0^{33}	5.1^{24}
Mannose	3.8^{6}	3.6^{6}	1.9^{6}	19.3^{50}	19.2^{48}	17.4^{54}	12.7^{72}	14.3^{67}	14.4^{69}
Galactose	15.6^{24}	14.2^{22}	3.1^{9}	-	-	-	-	-	-
Glucose	33.5^{0}	34.3^{0}	67.0^{0}	61.1^{0}	60.0^{0}	67.7^{0}	82.6^{0}	78.8^{0}	79.1^{0}
Per cent of cell wall	22.0	19.0	25.4	2.0	1.9	1.1	.6	.7	.5

removes rhamnose and galactose. Galactose appears susceptible to degradation at higher concentrations of trifluoroacetic acid. Arabinose is hydrolyzed to a greater extent during the first hydrolysis, *or* more xylose is removed by the second hydrolysis. Mannose is released to a greater extent in the second and third hydrolyses than in the first.

Possibly, with each successive hydrolysis, the polysaccharides immediately adjacent to the cellulose microfibrils are becoming more exposed and susceptible to hydrolysis. Or, polymers composed of sugars analogous to the residual sugars associated with cellulose are slowly being hydrolyzed. Such polymers, however (excluding cellulose), must represent only a small fraction of the wall.

It is not known how the cellulose from cotton fibers compares with that found in sycamore cells; however, it has been shown that celluloses from different sources differ in microfibril width (Balashov and Preston, 1955; Wardrop, 1954), degree of crystallinity (Hermans, 1949), and in the traces of non-glucose residues associated with them (Adams and Bishop, 1955).

Since it was shown that when α-cellulose is treated with acetic/nitric reagent, residual sugars amounting to 5-11% of the cellulose are released upon trifluoroacetic acid hydrolysis, the results of an acetic/nitric digest of wall samples of various ages was compared to that obtained for sycamore cell walls. This enables a comparison of the residual sugars associated with cellulose released by 2N trifluoroacetic acid hydrolysis. Keegstra *et al.* (1973) have proposed a tentative structure of the sycamore cell wall in which xyloglucan chains are in immediate association with cellulose

microfibrils. If the acetic/nitric digest solubilizes all sugars except those closely associated with the cellulose microfibrils, then an analysis of the sugars remaining after such a digest of sycamore cell walls should consist of a large proportion of xylose and glucose. Furthermore, a comparison of these residual sugars with those obtained from cotton fiber cell walls would test the hypothesis of whether the interrelationship between the structural components of the primary cell walls of all higher plants is comparable or not.

Cell walls were treated with acetic/nitric at 100° for 2 hours in one experiment and for 0.5 hours in another. The residue remaining was thoroughly washed, lyophilized, hydrolyzed with 2N trifluoroacetic acid, and analyzed for neutral sugars (Table 4). The predominant sugars present are xylose, mannose and glucose, totaling only 1 to 3% of the wall. On the basis of the association of the xyloglucan with cellulose in sycamore cell walls, the presence of xylose and glucose supports the contention that the polymer(s) resistant to acetic/nitric digest are those most closely associated with cellulose. The relative proportions of these sugars released from *both* cotton cell walls and from sycamore cell walls is the same, with glucose being released in the greatest amount, followed by xylose and mannose. While mannose represents a small mole per cent of the total sycamore cell wall (0.3%), it accounts for ca. 6% of the insoluble acetic/nitric digest of sycamore cell walls. These results could suggest the presence of a mannose-containing polymer which, like cellulose, possesses a structure highly resistant to acetic/nitric reagent but not resistant to trifluroacetic acid.

Table 4. Residual sugars remaining after acetic/nitric digest of cotton fiber cell walls and suspension-cultured sycamore cell walls

Cell walls from plant grown fibers (5, 8, 12, 16 and 20 DPA) and from suspension-cultured sycamore cell walls (syc) were digested with acetic/nitric reagent (.5 hours and 2.0 hours), washed, lyophilized, hydrolyzed with 2N trifluoroacetic acid, reduced, acetylated and analyzed for neutral sugars.

Values are expressed as a mole per cent of the total neutral sugars detected. The total neutral sugars detected is expressed as a per cent of the cell wall.

	Length of Acetic/Nitric Digest										
	2 hours						0.5 hours				
Sugar	5	8	12	16	20	Syc	5	8	12	20	Syc
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Fucose	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	1.0	1.6	.3	1.9	2.4	12.4	3.1	4.3
Xylose	22.1	23.2	20.2	13.0	4.2	16.2	13.4	17.0	16.0	1.8	19.8
Mannose	16.7	20.4	18.8	14.6	4.8	7.0	11.8	16.6	12.2	2.9	5.8
Galactose	-	-	.5	1.3	.6	.3	.7	5.1	-	-	3.3
Glucose	61.2	56.3	60.5	70.1	88.8	76.2	71.6	58.3	59.9	91.4	66.8
Per cent of cell wall	1.0	2.1	1.0	1.9	3.2	1.1	2.6	2.8	1.9	5.1	1.9

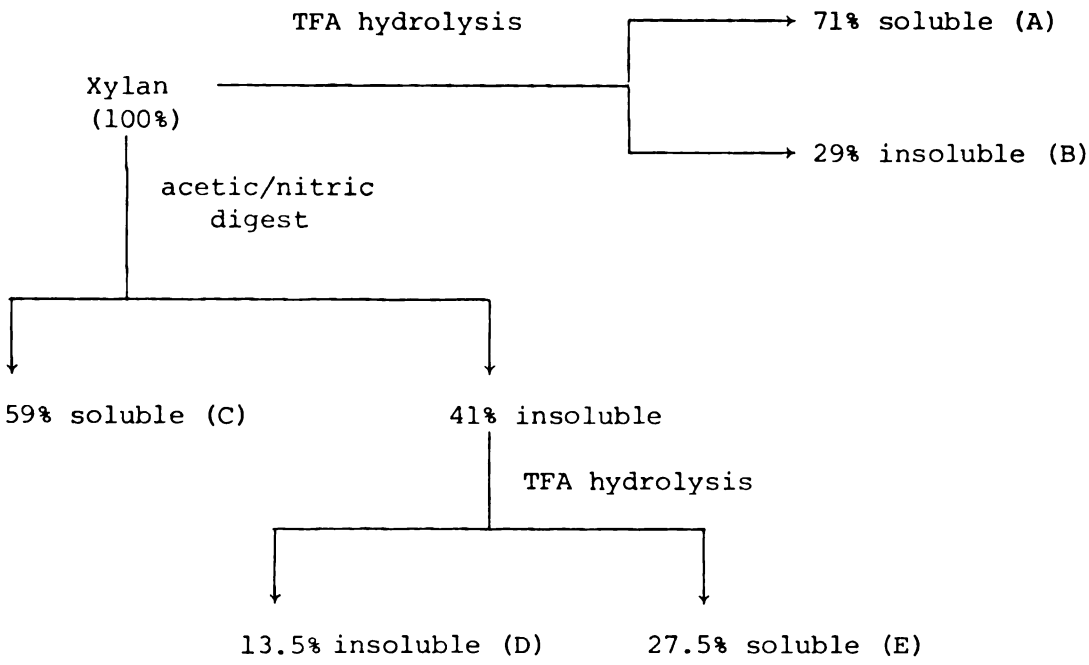
Further evidence for such a polymer is obtained when a xylan, isolated from larchwood, is treated with acetic/nitric reagent for 0.5 hour, and the insoluble residue remaining is thoroughly washed, hydrolyzed with 2N trifluoroacetic acid, and analyzed for neutral sugars present (Table 5). Forty-one per cent of the xylan is insoluble in acetic/nitric reagent. Of this 41%, 67% is trifluoroacetic acid hydrolyzable, which represents 27.5% of the xylan originally present. The acetic/nitric insoluble digest is very high in mannose and very low in xylose compared to the untreated xylan. As before, treatment with acetic/nitric reagent results in a solubilization of the xylan and results in an enrichment for a resistant polymer, possibly a glucomannan. The actual change in ratio of glucose relative to mannose is a result of differential susceptibility to hydrolysis, and appears to indicate that either less mannose or more glucose is hydrolyzed following an acetic/nitric digest by trifluoroacetic acid. However, calculations (see Appendix) show that this is not the case. These calculations show that there must be an increase in the amount of mannose released which is probably accompanied by simultaneous release of glucose. This must be due to an increased susceptibility of acetic/nitric insoluble xylan to subsequent hydrolysis by trifluoroacetic acid. Because, as shown in the diagram below, if treating xylan with acetic/nitric reagent has no bearing on the effectiveness of a subsequent hydrolysis with trifluoroacetic acid, you would expect that since 59% (C) has already been solubilized, only (71-59=) 12% remains to be solubilized from the 41% insoluble material (41% insoluble - 12 soluble = 29 insoluble). However, the results obtained are: 27.5 soluble (E)

Table 5. Neutral sugar composition of larchwood xylan before and after treatment with acetic/nitric reagent

Xylan, untreated (control) and treated with acetic/nitric reagent for 0.5 hours (A/N), was washed, lyophilized, hydrolyzed, reduced, acetylated and analyzed for neutral sugars. Values are expressed as a mole per cent of the total neutral sugars detected. The total neutral sugars detected is expressed as a per cent of the xylan originally present prior to hydrolysis.

Sugar	Treatment	
	Control	A/N
Rhamnose	.02	-
Fucose	-	-
Arabinose	.9	1.0
Xylose	37.5	4.0
Mannose	43.6	59.5
Galactose	-	-
Glucose	18.0	35.9
Percent of original xylan	71.0	27.5*

*This value represents 67.5% of the 41% xylan insoluble in acetic/nitric reagent.



and 13.5 insoluble (D). Thus, treatment of the xylan with acetic/nitric reagent, prior to hydrolysis with trifluoroacetic acid, has not only resulted in solubilizing 59% of the xylan, but it has also made the remaining insoluble 41% more susceptible to hydrolysis (2.3 times more than expected is released).

The actual resultant compositions of these fractions are described in detail in the Appendix. Even though the data of Table 5 indicate that the mannose to glucose ratio is decreasing (hence, less mannose and/or more glucose release), the actual composition of fractions A and E indicate that there is an increase in the amount of mannose released subsequent to trifluoroacetic acid hydrolysis.

It is possible that earlier investigations of sycamore cell walls yielded such low values for mannose because of its resistance to hydrolysis. This resistance to hydrolysis is due to a combination of a number of factors including degree of crystallinity of the

polymers. The results of the acetic/nitric studies may be summarized as follows:

1. Cellulose (>97%) is resistant to trifluoroacetic acid hydrolysis.

2. The existence of similar residual sugars in both commercially prepared cellulose and in cellulose prepared from the walls of both cotton fibers and of cultured sycamore cells has been demonstrated.

3. The existence of a mannose containing polymer which is relatively more resistant to trifluoroacetic acid hydrolysis and to solubilization by acetic/nitric reagent has also been demonstrated in both cell walls from sycamore and cotton and in a xylan from larchwood.

4. Acetic/nitric reagent increases the susceptibility of some polymer to subsequent hydrolysis.

Protein

The protein content of cell walls prepared from both plant and culture grown fibers was measured by a complete amino acid analysis of cell walls of selected ages. Amino acids are expressed as a mole per cent of the total amino acids detected and are shown in Table 6. The amino acid composition, in contrast to the neutral sugar composition, is constant throughout the course of the development of the fiber for both culture and plant grown fibers, and at any age shows no preponderance of any particular amino acid. Approximately 50 per cent of the amino acids are hydrophobic. Table 7 compares the average mole per cent of each amino acid for all ages determined for both plant and culture grown fibers. No differences are observed in amino acid composition for cell walls isolated from the differently grown fibers.

Table 6. The amino acid composition of the protein component of walls as a function of cell wall age from both plant and culture grown fibers

Values given represent the mole per cent of the total amino acids detected. The total amino acids detected are expressed as a weight per cent of the wall (per cent of wall that is protein). Averages and standard deviations are given for multiple samples.

Amino acid	Age										
	5	9	10	11	12	14	15	16	17	18	29
					Plant						
ASP	11.4±1.7	10.1± .57	10.2± .35	10.5± .57	11.0	11.3± .44	10.8±1.0	11.3±1.2	17.2		10.4
THR	6.2± .14	5.4± .36	5.9± .28	6.0± .25	6.0	6.2± .19	6.0± .28	5.7± .49		24.3±2.2	6.6
SER	9.2± .42	8.7± .0	9.1± .0	7.7±1.9	8.9	8.4± .33	8.6± .07	7.5±3.6	7.6		7.8
GLU	12.9± .35	11.6±1.06	11.2± .57	12.1± .84	11.0	11.8± .53	12.4± .78	10.0±1.0	9.6	11.7±1.1	10.4
GLY	11.5± .42	13.1± .78	12.8± .07	12.7± .15	13.8	11.1± .47	20.5±1.3	10.8±1.8	10.0	12.6±2.0	8.9
ALA	9.9±1.1	12.9±4.0	8.9± .07	9.2± .3	8.7	9.8± .56		12.4±1.5	10.0	10.8±1.5	8.9
VAL	7.3± .35	6.8± .64	6.6± .28	7.0± .12	6.7	7.2± .16	6.7± .0	7.9±2.3	7.4	6.9± .36	6.1
MET	-	-	1.1± .07	9.7± .84	1.0	.05± .1	5.5± .2	2.7±2.2	1.9	.5± .6	-
ILU	5.0± .14	4.7± .42	5.0± .49	4.8± .17	4.9	5.1± .32		5.1±1.2	5.1	5.1± .4	4.4
LEU	10.4± .49	9.3± .85	9.8± .0	9.6± .30	8.3	10.0± .30	10.1± .07	10.0± .64	10.0	9.6± .59	9.0
TYR	2.0± .14	2.2± .78	2.3± .07	2.2± .9	2.3	1.9± .64	2.8± .57	2.1± .42	1.8	5.3±1.4	2.3
PHE	4.6± .35	4.4± .35	4.6± .14	4.4± .4	4.7	4.5± .33	4.7± .07	4.4± .28	4.7		4.3
LYS	8.2±1.2	4.3± .85	6.7± .42	6.7± .26	6.6	6.4±2.3	5.9± .35	5.2± .14	7.1	4.8±1.7	6.4
HIS	2.1± .42	2.2± .28	2.3± .14	2.3± .06	2.1	2.4± .34	2.3± .07	2.5± .28	2.1	1.9±1.6	2.2
ARG	4.7± .85	4.3± .35	4.0± .07	4.2± .4	3.9	4.2± .17	4.1± .14	4.2± .42	4.0	3.0±2.6	3.8
Per cent of wall that is protein	18.7± .71	3.3± .42	6.0± .49	7.4±1.4	5.6	11.2±3.6	4.5± .37	2.4± .71	6.0	2.2± .49	1.3

Amino acid	Age			
	10	12	14	16
		Culture		
ASP	9.4	10.1	9.6	9.4
THR	5.4	5.6	5.8	5.3
SER	9.7	8.3	8.2	9.0
GLU	11.6	11.1	8.2	11.0
GLY	15.7	14.1	12.1	12.7
ALA	7.8	8.7	9.5	9.1
VAL	6.6	7.4	7.0	6.9
MET	1.6	-	-	1.4
ILU	4.8	4.9	4.8	4.8
LEU	9.5	9.1	9.4	9.8
TYR	2.4	1.6	1.8	1.7
PHE	4.6	4.2	4.4	4.4
LYS	8.4	8.9	8.1	8.3
HIS	2.6	2.4	2.4	2.5
ARG	-	3.7	3.3	4.0
Per cent of wall that is protein	8.4	5.1	3.7	3.7

Table 7. Comparison of amino acid composition of cell wall protein for cell walls derived from plant and culture grown fibers

Each value represents an average of the mole per cent of each amino acid detected at all ages for both plant and culture grown fibers. Standard deviations are given.

Amino acid	Plant Average	Culture Average
ASP	10.8 ± .74	9.6 ± .33
THR	6.0 ± .36	5.5 ± .22
SER	8.4 ± 1.21	8.8 ± .70
GLU	11.7 ± .98	10.5 ± 1.5
GLY	11.8 ± 1.3	13.7 ± 1.6
ALA	10.0 ± 1.7	8.8 ± .73
VAL	7.0 ± .68	7.0 ± .33
MET	.73± .98	.75± .86
ILU	4.9 ± .43	4.8 ± .05
LEU	9.7 ± .58	9.5 ± .29
TYR	2.0 ± .66	1.9 ± .36
PHE	4.5 ± .27	4.4 ± .16
LYS	6.0 ± 1.6	8.4 ± .34
HIS	2.2 ± .56	2.5 ± .10
ARG	4.2 ± .38	2.8 ± 1.9
Per cent of the cell wall that is protein	7.4 ± 5.2	5.2 ± 2.2

Hydroxyproline, a major constituent of extensin, was determined separately. Expressed as a per cent of the cell wall, these results are shown in Table 8 for both plant and culture grown fibers. These results show a decline in the per cent of the wall that is hydroxyproline. However, in comparison to the 2 per cent hydroxyproline found in suspension-cultured sycamore or tomato cells (Lamport, 1969), the per cent hydroxyproline found in cotton fiber cell walls is indeed small. The importance of these changes in hydroxyproline content with age may not be very significant.

Figure 7 illustrates protein content expressed as a per cent of the wall and shows that this percentage varies with age for both plant and culture grown fibers. Since the protein content is rather small, it is not yet known whether these variations with age are significant.

The variabilities observed could be due to differing amounts of protein contamination from the cytoplasm. To ascertain whether or not this protein is a true structural component of the wall or whether this protein is selectively adsorbed from the cytoplasm during cell wall preparation, 14-day-old walls, having yielded the highest percentage of protein, were extracted with a solution of SDS, urea and mercaptoethanol for five minutes at 100°C prior to hydrolysis and subsequent amino acid analysis. This reagent should remove any protein not covalently bound to cell wall polysaccharides. The protein content in untreated walls at 14 days is 11.2 $\pm$ 3.6 per cent of the dry weight of the wall. After treatment with this reagent, the protein content is reduced to 3.2 $\pm$ 2.2 per cent, indicating that a significant amount of protein is removed by this

Table 8. Per cent of the cell wall that is hydroxyproline

Values given represent the per cent of the wall that is hydroxyproline for both plant and culture grown fibers at different ages.

Age	Plant	Culture
9	.11	-
10	.34	.55
11	.36	-
12	-	.44
13	.20	-
14	.33	.38
15	.25	-
16	.12	.27
17	.29	-
18	.10	-

Figure 7. Changes in protein content of the cell wall during fiber development.

Values are expressed as a per cent of the weight of the cell wall.

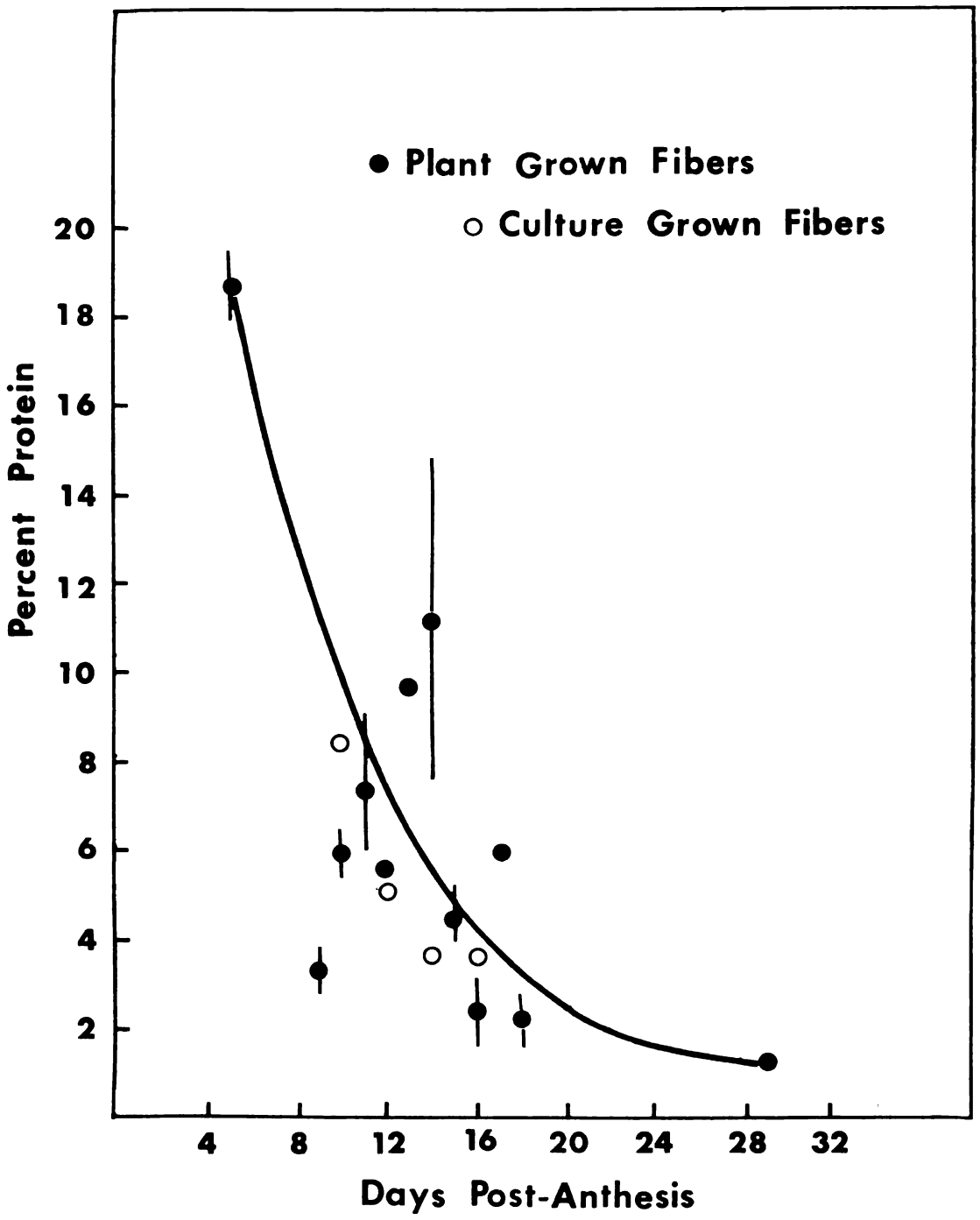

● Plant Grown Fibers
○ Culture Grown Fibers
Percent Protein
20
18
16
14
12
10
8
6
4
2
4
8
12
16
20
24
28
32
Days Post-Anthesis

Figure 7

procedure. The amino acid composition of the residue remaining after extraction is shown in Table 9. Table 10 compares the average mole per cent amino acid composition and the total protein recovered as a per cent of the wall obtained for two separate determinations of extracted wall and for four separate determinations of unextracted wall for 14-day-old walls.

Because the change in amino acid composition of the residue remaining subsequent to extraction was within the standard deviation of all determinations, it was thought that rather than determining the amino acids present in the supernatant to account for what was lost, it would be of greater interest to see to what extent, if any, polypeptides had been released from the wall and if this had led to simultaneous release of significant amounts of carbohydrate material.

Subsequent to dialysis against water for 48 hours, the supernatant of the SDS-urea-mercaptoethanol extraction was analyzed for protein and carbohydrate components. The dialysate was lyophilized and analyzed for polypeptides by SDS gel electrophoresis. As shown in Figure 8, the patterns of protein bands were reproducible. There are at least 8 distinguishable bands and, in addition, there appears to exist high molecular weight component(s) incapable of penetrating the gel. The molecular weights for the bands observed are given in the figure legend.

In a separate experiment the dialyzed, lyophilized urea extract was analyzed for neutral sugars (Table 11). All of the sugars found in unextracted 14-day-old walls were also found in the urea extract, though in different proportions. This extraction procedure resulted in preferential release of xylose and glucose and reduced quantities

Table 9. Amino acid composition of 14 DPA cell walls after treatment with urea reagent

The values given represent the mole per cent of total amino acids detected, and were obtained by averaging multiple runs of duplicate samples (I and II). The average for the two samples (I and II) is given with standard deviations.

Amino acid	I	II	Average
ASP	14.4	14.6	14.4 ± 2.4
THR	6.4	5.4	5.9 ± 1.3
SER	11.2	8.4	9.8 ± 1.7
GLU	13.1	11.1	12.1 ± 1.2
GLY	14.6	11.5	13.1 ± 1.9
ALA	9.4	8.8	9.1 ± .5
VAL	5.6	5.8	5.7 ± .2
MET	-	-	-
ILU	-	3.6	1.8 ± 2.1
LEU	6.4	8.2	7.3 ± 1.1
TYR	4.7	4.7	4.7 ± .3
PHE	-	4.6	2.3 ± 2.7
LYS	7.9	7.6	7.7 ± 2.0
HIS	2.0	.9	1.4 ± 1.9
ARG	4.7	4.8	4.7 ± .5
Per cent of wall that is protein	4.9	1.4	3.2 ± 2.2

Table 10. Comparison of the mole per cent amino acid composition for both urea reagent-treated and untreated 14 DPA cell walls

Values reprsent average mole per cent obtained for two separate preparations of treated wall and four separate preparations of untreated wall.

Amino acid	Treated Average	Untreated Average
ASP	14.4 ± 2.4	11.3 ± .44
THR	5.9 ± 1.3	6.2 ± .19
SER	9.8 ± 1.7	8.4 ± .3
GLU	12.1 ± 1.2	11.8 ± .5
GLY	13.1 ± 1.9	11.0 ± .5
ALA	9.1 ± .5	9.8 ± .6
VAL	5.7 ± .2	7.2 ± .2
MET	-	.1 ± .1
ILU	1.8 ± 2.1	5.1 ± .3
LEU	7.3 ± 1.1	10.0 ± .3
TYR	4.7 ± .3	1.9 ± .6
PHE	2.3 ± 2.7	4.5 ± .3
LYS	7.7 ± 2.0	6.4 ± 2.3
HIS	1.4 ± 1.9	2.4 ± .3
ARG	4.7 ± .5	4.2 ± .2
Per cent protein in the wall	3.2 ± 2.2	11.0 ± 3.6

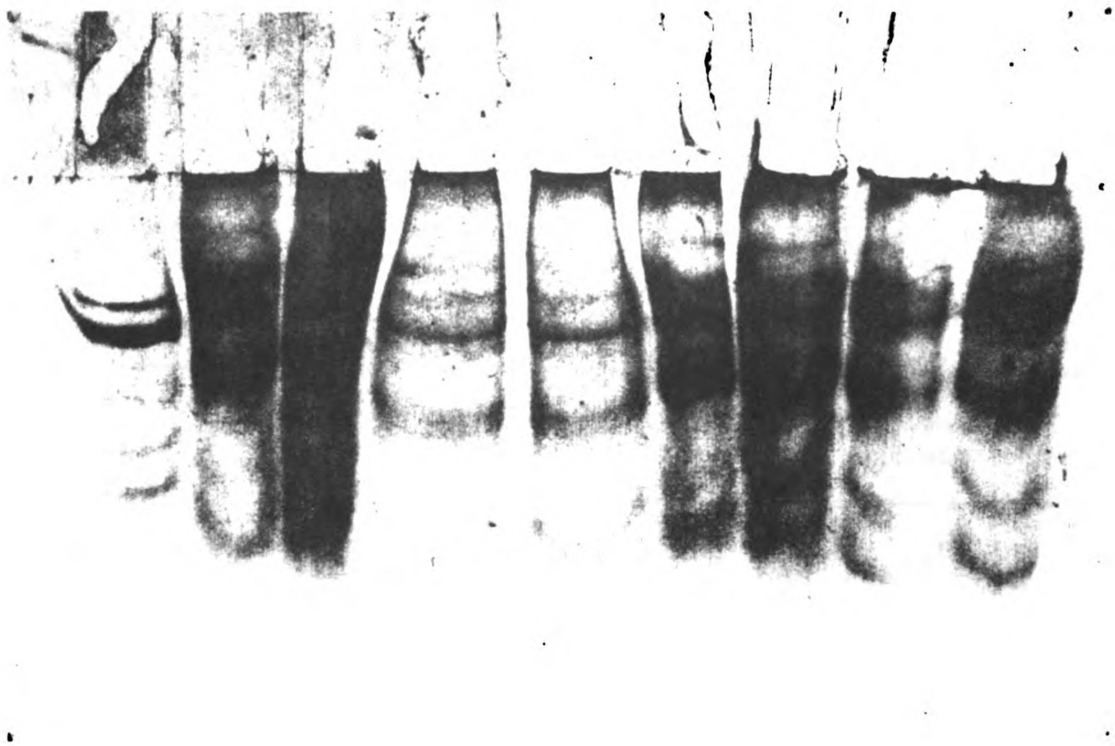


Figure 8. SDS gel electrophoretic separation of protein extracted from cell walls by urea reagent.

The molecular weights of the bands are, from top to bottom:

	MOLECULAR WEIGHTS
1)	92,000
2)	66,000
3)	63,000
4)	50,000
5)	33,000
6)	21,000
7)	15,000

Table 11. Comparison of the neutral sugar composition of unextracted 14 DPA cell wall and urea reagent cell wall extract

Values represent the mole per cent of the total carbohydrate detected. The total carbohydrate detected is expressed as a per cent of the wall.

Sugar	Unextracted cell wall	Cell wall extract
Rhamnose	8.4	1.8
Fucose	1.2	.8
Arabinose	22.3	4.7
Xylose	10.3	13.6
Mannose	3.9	3.8
Galactose	17.8	10.4
Glucose	36.0	65.2
Per cent of wall	21.0	$\geq$ 7.0

of rhamnose and arabinose. With no correction for possible losses during the procedure, the per cent of total carbohydrate released by this treatment amounts to at least 7% of the cell wall. This is a substantial amount of the neutral sugars originally present in the cell wall.

Uronic Acid

The uronic acid component of the cell wall is difficult to quantitate owing to the resistance of the aldobiuronic acid glycosidic linkage to acid hydrolysis (Whistler and Richards, 1958). These sugars are generally degraded before they are hydrolyzed; thus substantial losses occur. For example, a solution of galacturonic acid was subjected to a 2N trifluoroacetic acid hydrolysis and assayed for uronic acids by a specific colorimetric assay. Only 63% of the uronic acids originally present were accounted for. Therefore, a comparative study of the effect of varying strengths of trifluoroacetic acid upon total uronic acid recovery was undertaken. This study utilized 14 days post-anthesis cell walls, a polygalacturonic acid solution and a uronic acid solution as substrates. These results are shown in Table 12A) and indicate that degradation is occurring at all concentrations. Otherwise, the uronic acid solution would have approached 100% yields.

Although a three-fold decrease in uronic acids was observed from walls hydrolyzed in increasing concentrations of trifluoroacetic acid, these degradative effects do not appear to extend to the same degree for hydrolysis of neutral sugars as shown by assaying the

Table 12-A. Per cent uronic acid released at varying TFA concentrations

The uronic acid released upon hydrolysis with four different concentrations of TFA is expressed as a per cent of the substrate hydrolyzed which was recovered as uronic acids.

Substrate	TFA Concentration .5N	1N	2N	4N
14 DPA cell wall	11	9.1	7.0	3.1
polygalacturonic acid solution	12	12.0	11.0	11.0
uronic acid solution	67	82.0	70.0	50.0

Table 12-B. Per cent reducing sugars released at varying TFA concentrations from 14 DPA cell walls

The reducing sugars are expressed as a per cent of the cell wall.

	TFA Concentration .5N	1N	2N	4N
Per cent	26	25	24	23

supernatant of the trifluoroacetic acid hydrolysate of the 14 days post-anthesis cell wall for reducing sugars (Table 12B).

Despite these variabilities, in order to determine a lower limit for the uronic acid concentration present in the wall, cell walls were subjected to 2N trifluoroacetic acid hydrolysis and the supernatant assayed for uronic acids. These results are given in Table 13 for both plant and culture grown fibers. Because a number of factors are involved in non-quantitative release of uronic acids, no attempt has been made to correct these values. The data show that uronic acids are present in the fiber walls and, in view of the fact that these values are probably underestimates of the actual concentrations, the per cent by weight of the walls which is uronic acid could be quite high.

In view of these variabilities obtained, it can be stated that, at best, the effects of trifluoroacetic acid hydrolysis upon release of uronic acid from the wall are not well defined. Hence, another approach to release of uronic acids was used.

Enzymatic hydrolysis of cell walls has been shown to be very effective in releasing defined fragments from the wall. Of the enzymes commercially available to us, cellulysin, driselase and mascerozyme, it was first necessary to determine some of the parameters of optimum activity including temperature optima and reaction rate. However, results of these preliminary experiments were difficult to interpret because of the presence of large quantities of carbohydrate contaminants in the enzyme preparations. Of the three enzymes tested, driselase appeared to be substantially more pure and appeared to be

Table 13. Uronic acids released from cell walls at different ages by 2N trifluoroacetic acid hydrolysis

The uronic acids released are expressed as a per cent by weight of cell walls for both plant and culture grown fibers.

Age	Plant	Culture
5	10	-
8	8	13
9	13	-
10	23	16
11	22	-
12	20	15
13	10	-
14	19	9
15	16	-
16	10	6
17	8	-
18	9	6
20	-	4
29	3	-

considerably more effective in solubilizing cell walls, although the presence of high backgrounds, even with driselase, remained a problem. Nevertheless, a comparative investigation was undertaken to examine the amount of uronic acids released by the action of driselase upon five different ages of cell walls. The incubation was run at 26°C with an enzyme concentration of 1% and a wall concentration of ca. 5 mg/ml. A blank, containing no substrate, was run as a control. Aliquots were removed and assayed at several time points for uronic acids. These results are shown in Table 14.

Table 14. Per cent uronic acids released from cell walls by enzymatic hydrolysis

The uronic acids are expressed as a per cent of the cell wall. Cell walls are from plant grown fibers.

Incubation time (hours)	Cell Wall Age				
	5	8	12	16	20
.25	27	26	23	23	14
2.75	20	20	10	22	8

The data have been corrected for the contribution of the enzyme solution itself to the color produced at each time point. It appears as though optimal release of uronic acids occurs within 15 min, and that longer incubations result in lower yields. Once again, the data indicate the presence of substantial amounts of uronic acids in the walls, but clearly further work is needed to precisely quantitate these values.

Preliminary Linkage Analyses

Despite severe time limitations, preliminary methylation analyses of sic different ages of cell walls were undertaken. Cell walls from plant grown fibers were methylated as described. The methylated cell walls were separated into chloroform-methanol-soluble and -insoluble fractions. The completely methylated products from the chloroform-methanol-soluble fraction were isolated by dialysis and lyophilization. The permethylated wall fraction was hydrolyzed, reduced, and acetylated. The methylated alditol acetates were analyzed by combined gas-chromatography-mass spectrometry by the procedures of Björndahl *et al.* (1970). The gas chromatograms obtained for six ages of cell walls (in addition to duplicates for two of those ages) are presented in Figure 9 to illustrate the similarity of the derivatives obtained. Qualitatively, the derivatives obtained are of the same nature for all ages examined. Quantitatively, however, there are noteworthy differences (Table 15). Most striking is the change in relative areas represented by peaks 5 and 6 (at 5 days post-anthesis the ratio of 5:6 is 2.7:1, whereas at 20 days post-anthesis the ratio of 5:6 is 0.4:1) and the ten-fold increase in the area represented by peak 9. The predominant peak, peak 11, has undergone a nearly 25% decrease from 5 days post-anthesis to 20 days post-anthesis.

Some of the derivatives have only been tentatively identified due to the lack of supportive data. These identifications are listed in Table 16. Unambiguous identification of some of the derivatives awaits preparation of suitable standards and determination of accurate and precise retention times.

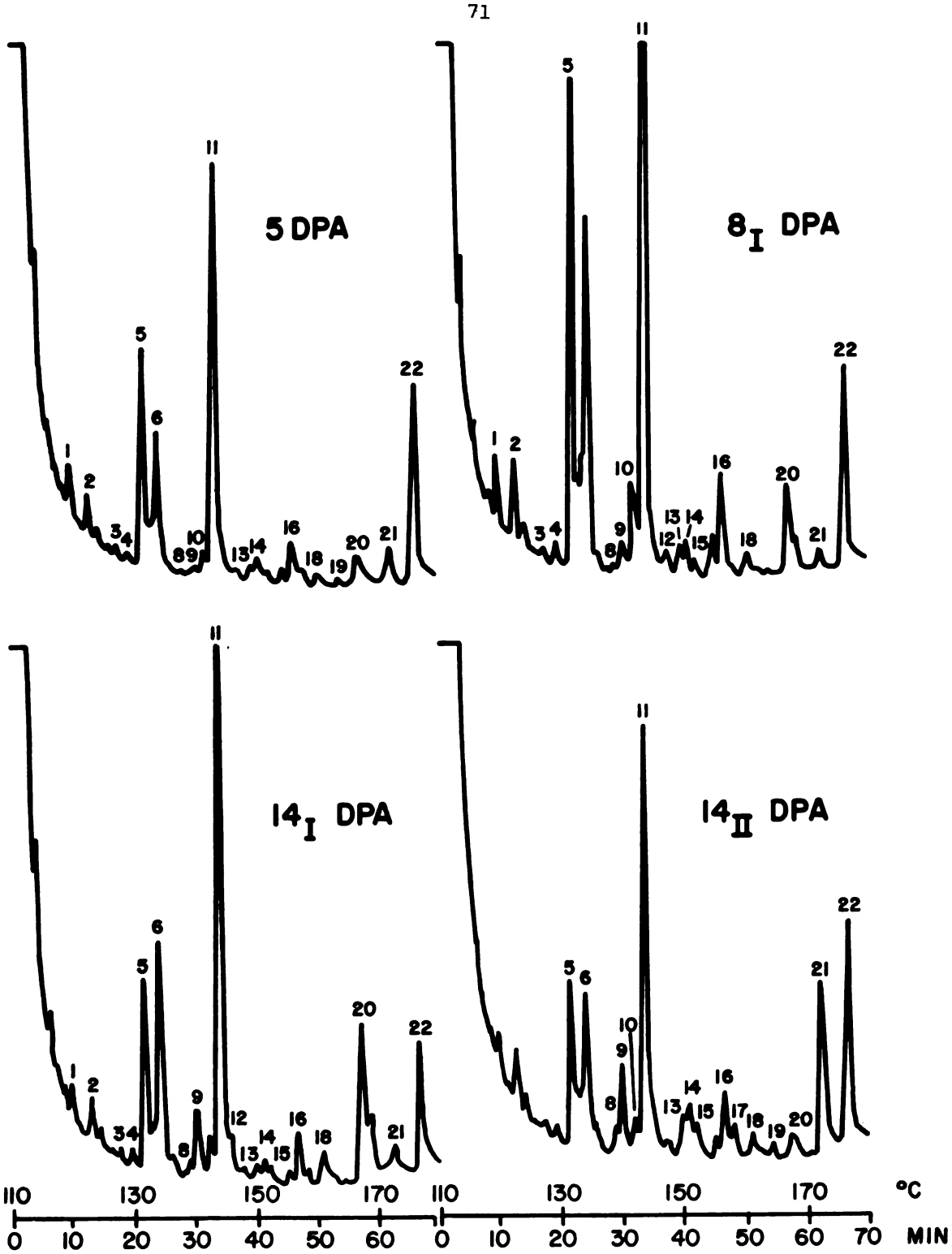
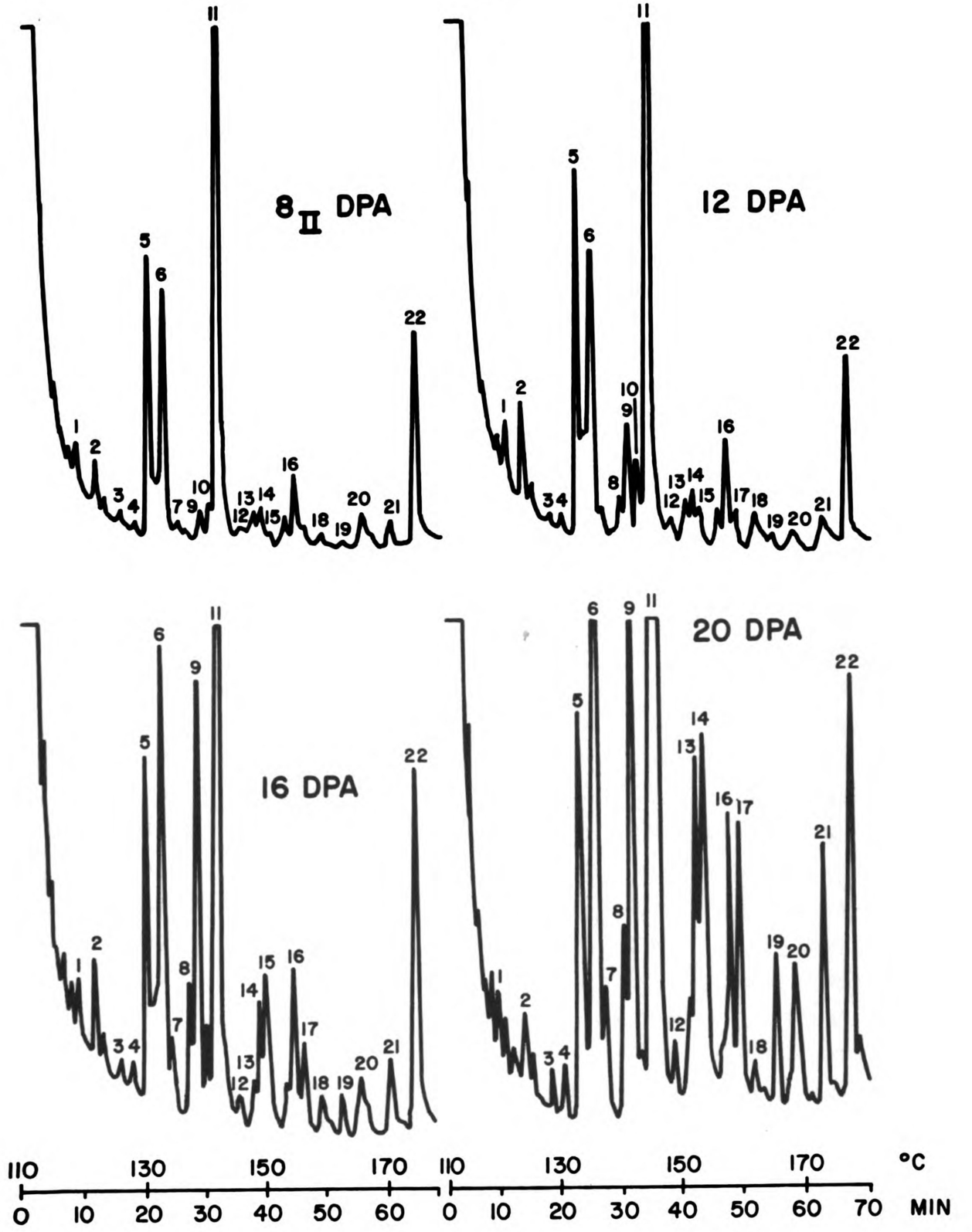


Figure 9. Comparison of the gas chromatographic separations of of various ages.

Peak numbers refer to those in Tables 15 and 16.



the alditol acetate derivatives of permethylated cotton fiber cell walls

Table 15. Changes in relative percentages of partially methylated alditol acetates from cell wall isolated from plant grown fibers of various ages

The area of each peak is expressed as a per cent of the total area represented by all 21 peaks. Peak numbers refer to peaks in Figure 9 and Table 16.

I and II refer to duplicate derivitizations of the same cell wall preparation.

Peak number	Age (days post-anthesis)							
	5	8_I	8_{II}	12	14_I	14_{II}	16	20
1	1.87	.88	1.33	1.20	.92	.42	7.24	2.17
2	2.57	1.70	2.43	3.35	2.57	1.50	1.75	.73
3	.26	.03	.21	.19	-	.03	.33	.39
4	.17	.32	.64	.22	-	.34	.36	.42
5	20.65	16.68	17.84	15.69	11.91	10.91	7.56	5.96
6	7.79	17.08	17.67	15.28	12.20	16.71	12.98	14.19
7	-	.09	-	-	-	-	1.24	1.58
8	.06	-	.05	1.63	1.04	.45	2.60	2.05
9	.92	2.01	.70	6.20	6.64	3.47	11.02	8.16
10	1.93	1.96	3.06	3.17	1.99	1.93	1.99	.79
11	51.65	47.55	43.03	37.72	37.40	46.73	31.40	38.99
12	-	.37	.31	.53	-	.03	.27	.67
13	1.43	1.55	.88	1.66	2.71	1.03	1.12	1.00
14	.78	1.43	.45	2.07	1.61	.39	2.85	3.77
15	-	.03	.21	1.00	-	.06	4.65	4.87
16	3.44	3.73	4.48	5.56	3.86	-	4.68	3.49
17	-	-	-	.30	.47	.06	2.21	2.81
18	.26	.23	1.08	1.37	.72	2.01	.86	.29
19	.13	.08	-	.26	.05	-	.86	1.63
20	2.20	2.62	4.96	1.04	1.29	12.83	1.84	2.83
21	1.95	1.65	.33	1.55	14.62	1.11	2.20	3.20

Table 16. Sugar linkages present in cell walls from plant grown cotton fibers

The glycosidic linkages to each sugar derivative are indicated by numerical prefixes: thus 4,6-Glucose indicates that the sugars are glycosidically linked in the polysaccharides to the 4- and the 6-carbons of these glucosyl residues. Terminal residues are indicated by T- (e.g., T-Xylose).

Peak number	Deduced sugar with glucosidic linkage	Determined position of O-methyl group(s)
1	T-Arabinose	2,3,5
*2	T-Xylose	2,3,4
3	2-Arabinose	3,4
4	T-Glucose	2,3,4,6
*5	5-Arabinose	2,3
*6	T-Galactose and	2,3,4,6 and
*7	4-Xylose	2,3
8	2-Hexose	3,4,6
*9	3-Glucose or 3-Mannose	2,4,6
10	3-Galactose	2,4,6
*11	4-Glucose	2,3,6
12	4- and/or 6-Hexose	2,3,6 and/or 2,3,4
13	6-Galactose	2,3,4
14	(di-methyl hexose)	
15	(di-methyl hexose)	
*16	4,6-Glucose	2,3
17	(mono-methyl hexose)	
18	3,6-Galactose	
19	(mono-methyl hexose)	
20	(mono-methyl hexose)	
21	(mono-methyl hexose)	

*Represent sugars for which identification is supported by *both* mass spectral data *and* relative retention times. Other identifications are somewhat less conclusive and, in some cases, lack sufficient data for definitive identification.

Although only a preliminary one, this investigation yields data from which at least two important generalizations can be made:

1. In view of the large number of derivatives possible, the limited number of different derivatives obtained suggests that the polymers present in the wall are of limited heterogeneity.

2. The variance of the ratios of the major components appears to be age dependent.

DISCUSSION

Compositional analysis of the primary cell wall of cotton fibers has led to several intriguing conclusions. First, and foremost, the developing primary cell wall is *not* a static structure. Compositional analyses have clearly demonstrated that throughout the course of development, the per cent of the wall that is represented by any component examined (neutral sugars, protein, or uronic acids) is continually changing. Second, the monomeric composition of the primary cell wall components themselves has been demonstrated to be continually changing only in the case of the neutral sugar component. The amino acid composition of the protein component is constant throughout development. Third, these changes in primary cell wall composition observed in plant grown fibers are also observed in culture grown fibers with a striking similarity. Quite remarkably, these changes occur relative to each other in precisely the same developmental sequence. However, although the sequence is the same, these developmental changes are displaced in time occurring earlier in culture.

To present a composite picture of the compositional changes occurring in an elongating primary cell wall and to examine the relationship between these observed changes necessitates first a detailing of some of the initial implications and conclusions drawn from an examination of each individual wall component.

Growth

Since the changes in boll length and mass associated with development of the cotton boll are easily and reproducibly measured, these parameters can be effectively used in judging whether or not a boll has developed normally and will yield fibers characteristic of that developmental age. That the yield of fibers proceeds at a decreased rate for the culture grown fibers can be attributed to growth conditions that are less than ideal. The medium in which the cotton ovules are cultured provides an environment that is static in terms of hormonal levels present and in terms of the carbon source and other nutrients present. It is likely that the precise hormonal concentrations necessary for optimal development of the fiber are not present during growth of these fibers. It is not known whether other nutritional factors change during development of the fiber on the plant.

Beasley (1973) has shown that *fertilized* ovules can be cultured and require no supplemental hormones. It is quite possible that fertilized ovules have the optimum balance of hormonal levels and might therefore produce higher yields of fibers.

The results obtained for fiber elongation for plant grown fibers compare well with the results obtained by previous workers (Benedict *et al.*, 1973; Tharp, 1965), with the exceptions that our growth rates are greater and maximal length is obtained at an earlier stage of development, both of which can be attributed primarily to differences in growth conditions. Not only do the growth conditions employed result in a greater rate of growth but also, when normally developing bolls are chosen, fibers are obtained which elongate synchronously and

exceedingly reproducibly with time. This cell system, therefore, provides a source of homogeneous cell wall material suitable for studies on changes in cell wall composition associated with growth.

Although the maximum rate of fiber dry weight increase occurs nearly ten days after the maximum rate of fiber elongation, that the cessation of elongation may not be the immediate result of secondary wall thickening, is not precluded since deposition of secondary wall cellulose begins *before* the elongation rate begins to decline. A similar result was also noted by Benedict *et al.* (1973).

Despite the fact that the increase in fiber dry weight proceeds at a different rate for plant and culture grown fibers, the per cent cell wall yield is comparable for both plant and culture grown fibers. This suggests that the lowered rate of fiber production in culture grown fibers has not affected the rate at which cell wall material is being deposited under these growth conditions.

The observed differences in cell wall yield with age are probably due to a combination of factors that vary with age, including: the fragility of the wall, the actual amount of wall present, and the actual amount of the dry weight of fiber that can be accounted for by cytoplasmic components.

It is certainly possible that the properties of cell walls, in terms of fragility, are different at different stages of development and that the increased yields observed with age are a reflection of this difference. In fact, we do observe that the younger fibers are more easily pulverized than older fibers. The wall preparations for younger ages are therefore more difficult to handle quantitatively, which could result in decreased yields from younger fibers. The

differing fragilities could be due to differences in cellulose content of the wall. This polymer is highly resistant to removal by the reagents and methods employed in isolating cell walls and may play a role in maintaining structural integrity of the cell wall during preparation. This line of reasoning is not completely supported, however, since there is a period of time during primary wall growth (9-10 days post-anthesis) in plant grown fibers when cellulose content increases two-fold but the per cent yield of wall remains relatively constant. Also, from 10-14 days post-anthesis, cellulose content remains constant in the wall, but cell wall yields increase two- to three-fold during this period. Regardless of the cause of change in yield of cell wall with age, if there were a difference in fragility, earlier ages, being more susceptible to higher losses, would yield lower recoveries.

Another more likely possibility which could explain variations in wall yields with age is that there is an actual change in the ratio of cell wall to cytoplasmic components. Increase in thickness of the wall or decrease in cytoplasmic components (or both) with age would account for the results observed. In order to circumvent the unknown weight contribution of the cytoplasmic component to fiber dry weight, the following calculation was made:

$$\frac{\text{grams cell wall}}{\text{fiber length}} = \frac{\left(\frac{\text{grams cell wall}}{\text{grams fiber}}\right)\left(\frac{\text{grams fiber}}{\text{boll}}\right)\left(\frac{\text{boll}}{\text{number of fibers}}\right)}{\text{fiber length}}$$

which assumes that the number of fibers per boll is relatively constant and is equal to 16,000 per ovule or 384,000 per boll (Beasley, personal communication). Division by the length of the fiber is

necessary in order to compensate for the effect upon the thickness of the cell wall of a cell which is continually elongating. This measure of the yield of cell wall per unit length of fiber (Figure 10) leads to some very interesting conclusions. First, cell wall yields are *constant* throughout most of primary cell wall elongation. This is in support of reports that the thickness of the primary cell wall is constant throughout elongation. Second, a positive correlation between wall synthesis and elongation does not imply a causal relationship, but may indicate that the two are affected in a parallel manner. This is so, because if the rate of elongation were greater than the rate at which cell wall material is deposited, cell wall yields would not appear constant throughout elongation; they would appear to decline. Third, the per cent of the dry weight of the cell wall at earlier ages accounted for by cytoplasmic components must be quite high. In agreement with these results, it has been noted (Levine, 1915; Caskey and Gallup, 1931; Delmer, personal communication) that various components of the cotton fiber cytoplasm, such as proteins and reducing sugars, undergo dramatic declines during development.

The "thickness" of the wall (grams per length) during primary cell wall elongation is constant but undergoes rapid thickening beginning at 14 days post-anthesis. However, increase in cellulose deposition as a secondary wall is not measurable until 16 to 17 days post-anthesis. To account for the earlier increase in cell wall per fiber length, it is possible to propose either an increase in synthesis of the primary cell wall in its entirety or, more likely, an increase in the synthesis of a specific carbohydrate polymer. These relationships will be discussed in greater detail later.

Figure 10. Comparison of the grams cellulose per fiber length, the grams cell wall per fiber length, and the change in fiber length during the development of the cotton fiber.

Values given are expressed as grams per millimeter fiber length. The derivative of the grams per fiber length is expressed by a factor of ten greater than indicated on the ordinate scale.

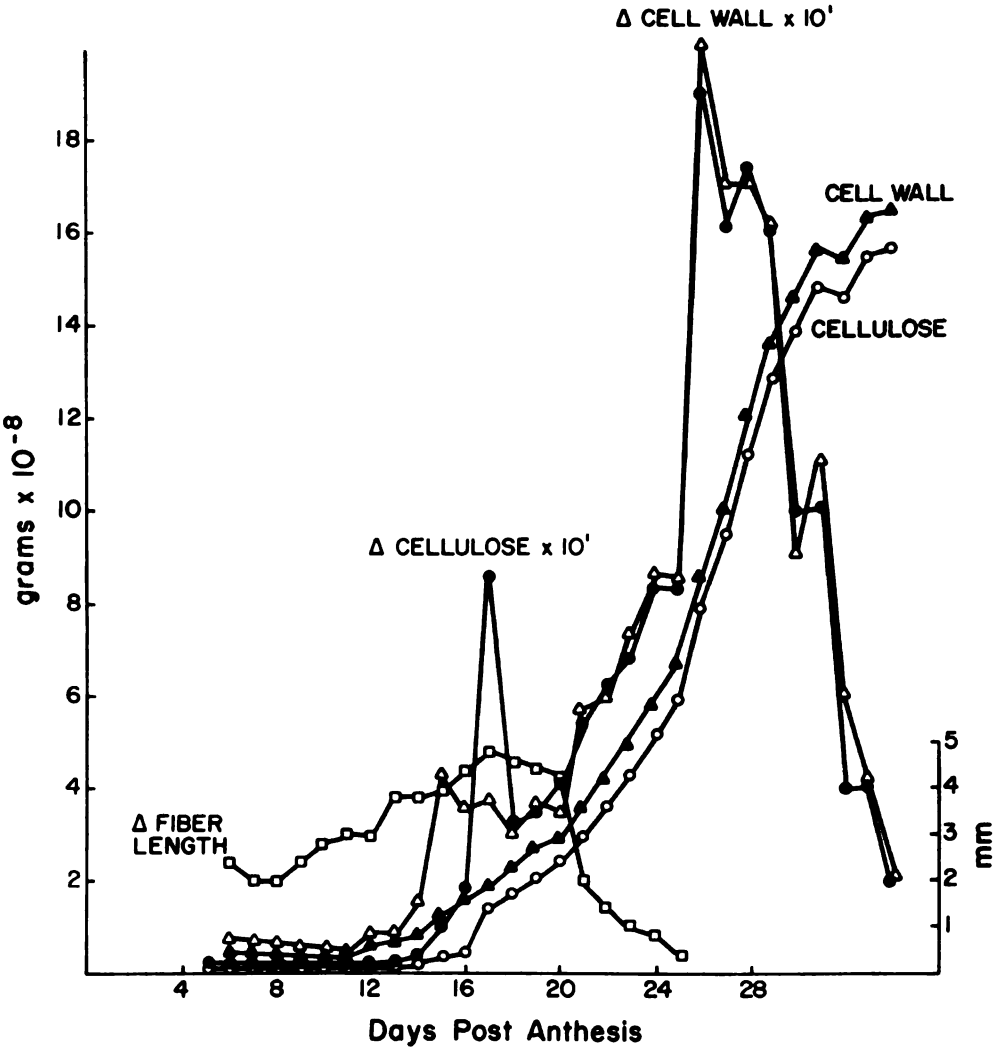

Δ CELL WALL x 10'
CELL WALL
CELLULOSE
Δ CELLULOSE x 10'
Δ FIBER LENGTH
grams x 10⁻⁸
mm
Days Post Anthesis
2
4
6
8
10
12
14
16
18
1
2
3
4
5
4
8
12
16
20
24
28

Figure 10

Cellulose

It was surprising to find what appeared to be three distinct phases of cellulose deposition in plant grown fibers (see Figure 5). Culture grown fibers may exhibit similar phases of cellulose deposition but it appears that while developmental changes are duplicated in culture grown fibers, these changes occur somewhat sooner. Therefore, the earliest phase of lowest cellulose content could be displaced to earlier ages. This is a time when sampling is very difficult and, therefore, no data are available to support this conjecture.

The two phases of differing cellulose content which correspond to primary cell wall synthesis in plant grown fibers may be the result of a nascent cellulose, present through 9 days post-anthesis, which is susceptible to acetic/nitric digestion. If in addition to susceptibility to acetic/nitric digestion, such a nascent cellulose was also susceptible to 2N trifluoroacetic acid hydrolysis, one would expect to see higher yields of glucose from these ages in the neutral sugar enalyses. However, this occurs only in the case of 5 days post-anthesis cell walls and, therefore, assuming susceptibility to hydrolysis by 2N trifluoroacetic acid, the data do not really support the existence of such a polymer.

In fact, arguments about changes in the rate of cellulose deposition based on values of components expressed as a percentage by weight of the wall can lead to erroneous conclusions. For example, because the cellulose is expressed as a per cent of the wall, even if the actual rate of cellulose synthesis had remained constant, expressed as a per cent of the wall, it would appear to increase if the rate of synthesis of other wall components had

declined. Per cent is clearly not necessarily an indicator of rate of deposition and is therefore not particularly useful for comparing rate of component change in the cell wall. It was found that the rate at which cell wall material is being deposited was obtained by calculating the grams cell wall per fiber length (Figure 10).* Similarly, converting the per cent of the wall that a particular component represents to *grams* per fiber length more clearly illustrates the changes in composition occurring in an elongating fiber. Figure 11 illustrates the grams per fiber length with age for three components of the wall: cellulose, neutral sugars, and protein, as well as a curve for the content of the remaining unaccounted for components including uronic acids. From these curves we can now reexamine the question of changes in rate of cellulose deposition. Recall that the data of Figure 5 showed that the largest differences in cellulose content as a percentage of the wall occurred between 9 and 10 days post-anthesis and between 16 and 17 days post-anthesis. The rate of deposition of non-cellulosic components is, in the case of protein and neutral sugars, not as great as that observed for cellulose deposition between 16 and 17 days post-anthesis. Therefore, the observed differences in per cent cellulose at this time are the result of the increased rate in cellulose deposition combined with the slightly decreased rate in deposition of neutral sugars and protein and a *large* decrease in the rate of deposition of the "other" components. However, analysis of the situation between 9 and 10 days

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*This ignores the possibility of turnover of cell wall components--an assumption which is probably valid at least for the cellulose, protein and neutral sugar components.

Figure 11. Comparison of the grams per fiber length of cellulose, neutral sugar, protein, and "other" component during development.

Values are expressed as grams per millimeter fiber length. "Other component" represents that fraction of the wall not accounted for by cellulose, neutral sugars, and protein.

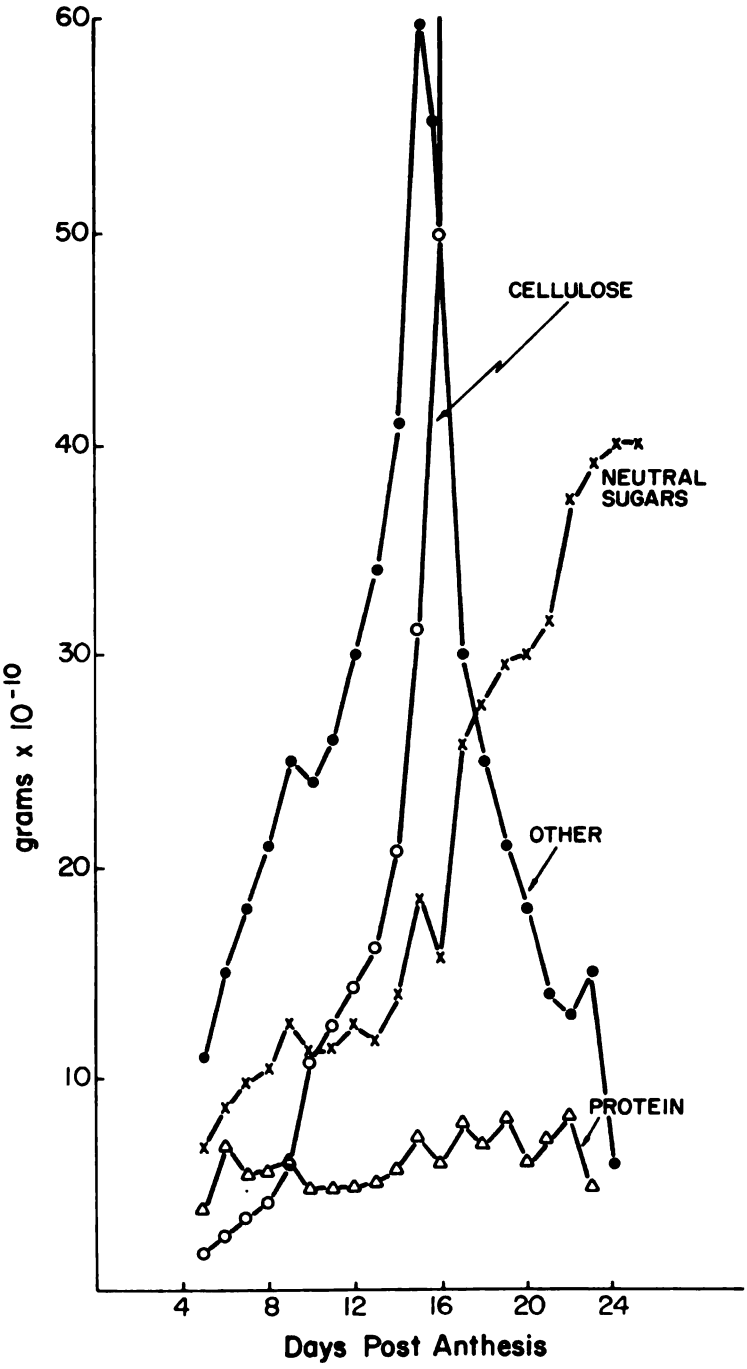
60
50
40
30
20
10
grams x 10-10
CELLULOSE
NEUTRAL SUGARS
OTHER
PROTEIN
4
8
12
16
20
24
Days Post Anthesis

Figure 11

post-anthesis is more complex due to the large rate of deposition of the "other" components. Although the rate of deposition of this fraction is quite high, it can still be observed that it is approximately constant between 9 and 10 days post-anthesis, whereas the rate of cellulose deposition shows a sharp increase. Coupled with the fact that protein and neutral sugar content remains constant, the increase in per cent cellulose observed between 9 and 10 days post-anthesis can be explained.

While it is possible that at earlier ages culture-grown fibers may show similar distinct phases, due to the extreme difficulty in harvesting fibers less than 8 days post-anthesis, data are not available concerning the per cent cellulose present at those times. However, since the observed differences in per cent cellulose in walls from plant grown fibers is probably the result of the differential rates at which cellulosic and non-cellulosic components are being deposited, the per cent cellulose observed in cell walls from culture grown fibers is a result of similar differences in deposition of cell wall components.

The actual rate of cellulose deposition is obtained from the derivative of the grams cellulose per fiber length (Figure 10). Expressed as a differential over one-day intervals, the rate of cellulose deposition appears to be fairly constant throughout most of primary cell wall elongation. (Because the ordinates of Figures 10 and 11 differ by a factor of 100, the increase in rate of cellulose deposition that occurs between 9 and 10 days post-anthesis is not apparent in Figure 10). At 14 days post-anthesis the rate begins to increase through 16 days post-anthesis at which time there is a dramatic five-fold increase in the rate of cellulose deposition.

It is at this time that the rate of elongation begins to decrease. Surprisingly, the rate of cellulose deposition then decreases, before resuming an even greater increase in rate. The maximal rate occurs at 28 days post-anthesis after which the rate drops precipitously. The increase in cellulose deposition at 20 days post-anthesis is accompanied by a sharp decline in the rate of fiber elongation.

These data indicate that there is still some elongation occurring at the time of secondary wall cellulose deposition. It is not clear whether secondary wall thickening brings about the cessation of elongation. If cellulose deposition leads to cessation of elongation, then possibly administration of an inhibitor of cellulose synthesis, such as coumarin (Hara *et al.*, 1973), could be employed to examine this hypothesis. This assumes that coumarin has no effect on the process of elongation. A better approach might be to search for ways to delay the onset of secondary wall deposition and observe the resultant effect on elongation.

Protein

The original aim of the amino acid analyses of the cell wall protein component was to determine to what extent, if any, the hydroxyproline concentration of the cotton fiber cell wall varies with age. Lamport (1969) has described a cell wall matrix glycoprotein, extensin, the protein moiety of which is rich (at least 25% of the residues) in hydroxyproline. Most of the hydroxyproline residues are glycosylated to arabinose via O-glycosidic bonds (Lamport, 1967). Lamport (1965) has hypothesized that the extensin acts as a strengthening agent by cross-linking the wall. Because of the resistance of the cell wall, the plant cell may be rendered incapable of further growth. After

cell elongation in pea hypotocyls ceases (Cleland and Karlsnes, 1967) or is induced to cease (Ridge and Osborne, 1970), an increase in cell wall hydroxyproline as a percentage of the *fresh weight* tissue has been observed. Sadava *et al.* (1973) have also shown a positive correlation between the synthesis and accumulation of cell wall hydroxyproline and the cessation of elongation in pea epicotyls. The hydroxyproline content was expressed as a percentage of the total protein present. However, Klis and Lamport (Plant Physiol., in press), also working with peas, have recently found that when hydroxyproline is expressed as *dry weight* of the wall, hydroxyproline content increases during cell elongation, there being no further increase once the cells have attained their final size. Thus, quite necessarily, the maximum amount of hydroxyproline is attained when the cells have stopped elongating. It is important to point out that previous work indicated only an *apparent* increase in hydroxyproline after cell elongation had stopped. This discrepancy is merely a result of the manner in which the data were expressed.

In order to quantitate the hydroxyproline present during the development of the cotton fiber primary cell wall, a complete amino acid analysis was employed. However, this method of assaying for hydroxyproline proved to be much less sensitive than assaying separately for hydroxyproline. The results obtained by the specific assay for hydroxyproline indicate that it comprises less than 0.6% of the weight of the wall at any age, and that the percentage declines rather than increases coincident with the cessation of elongation in both plant and culture grown fibers. Culture grown fibers, however, may have a higher concentration than plant grown fibers at all ages.

While these results do not appear to be in agreement with those obtained in other elongating systems, it is possible that a) this mechanism for cessation of elongation is not operative in cotton fibers, b) the concentrations of hydroxyproline are so low that it would require extensive determinations in order to observe a reproducible increase in this small percentage, or c) once again, the use of percentage values has led to erroneous conclusions. If the actual values obtained for the per cent of the wall that is hydroxyproline are plotted, the grams hydroxyproline per length can be calculated utilizing values derived from this curve. These calculations reveal that the grams hydroxyproline per fiber length steadily increase through 16 days post-anthesis when they then drop to nearly half the maximum value. This value is maintained through 20 days post-anthesis. It therefore appears that these results, at least with regard to the early stages of elongation, are in agreement with those of Klis and Lamport, indicating that hydroxyproline is continually incorporated during cell elongation.

In contrast to the neutral sugar composition, the amino acid composition of the protein component of cotton fiber cell walls is constant throughout the course of the fiber's development. Furthermore, this compositional integrity is maintained in cell walls derived from culture grown fibers. Comparison of the amino acid composition obtained for cell walls from other sources reveals that there are approximately 40% hydrophobic and 60% hydrophilic residues in cell wall protein. This balance between hydrophobic and hydrophilic residues is also observed in cell walls from cotton fibers. Lamport (1965) made a comparison of the amino acid composition obtained from ten cell wall

sources and concluded that the range of amino acid compositions was sufficiently wide so as to suggest the possibility that there is perhaps other bulk protein firmly bound to the wall in addition to the hydroxyproline-rich protein.

Indeed, this analysis of the cotton fiber cell wall protein represents the average composition of possibly a number of proteins as evidenced by the urea reagent extraction. This reagent was employed in an effort to ascertain whether or not the cell wall protein is a true structural component of the wall or whether this protein is selectively adsorbed from the cytoplasm during cell wall preparation. If treating cell walls with urea reagent removed any non-covalently linked protein, the amino acid composition of the non-extractable protein might be different with possibly an enrichment for hydroxyproline-rich protein component.

Although this reagent resulted in a decreased protein concentration for walls that had been treated, the amino acid composition of the non-extractable protein was quite similar to that obtained for the protein of non-extracted walls and showed no enrichment for hydroxyproline. Furthermore, the action of this reagent also resulted in substantial release of carbohydrate material suggesting that the effect of this reagent upon the cell wall structure may have resulted in the release of more than just non-covalently bound material. Thus, information concerning how the protein(s) is bound to the cell wall was not obtained. Such information could be obtained from a study in which milder conditions are employed. These studies might also be directed towards determining whether the role of the protein is structural, enzymatic and/or both.

This study does, however, reveal several interesting aspects of the wall protein. First, gel electrophoresis of the protein extracted from the cell wall reveals that discrete polypeptides, having molecular weights ranging upwards from 15,000, were released from the wall. Not only because of the discrete bands observed, but also because duplicate preparations yielded identical bands, the action of the urea reagent upon the wall appears to be limited to release of defined polypeptides rather than random release of individual amino acids. Duplicate preparations also yielded identical carbohydrate compositions. Again, the action of this reagent is limited to releasing 60% of the glucose originally present, 40% of the xylose, 32% mannose, 20% galactose, 7% rhamnose, 7% arabinose and 2.4% fucose.

These data do not answer the question of whether the action of urea reagent upon the wall is selective for removal of *specific types* of proteins. If this reagent were selective for specific types of proteins, it is possible that the average amino acid composition of those polypeptides removed from the wall and those not removed from the wall would yield dissimilar amino acid compositions. The amino acid compositions obtained, however, are quite similar.

In addition, the data obtained do not indicate whether or not this reagent is selective for removal of specific carbohydrate polymers from the wall. The carbohydrate composition obtained for the urea extract suggests that this treatment resulted in selective solubilization of a polymer(s) rich in glucose, xylose and/or mannose.

It is clear, however, that this extraction procedure can be successfully employed to release polypeptides from the cell wall.

The polypeptides thus obtained could be further analyzed in order to arrive at a more clear picture of the structure and ultimately function of the cell wall protein. Furthermore, this extraction procedure can be employed to fractionate the cell wall into discrete polymers useful for cell wall structural analyses. In addition, the products of this extraction procedure may include glycopeptides, the structure of which may provide helpful insights into the interconnections between the major polymers of the wall.

The aim of this study was not to determine the structure and/or function of the protein component of the cell wall, merely to quantitate its existence. The data obtained can be interpreted to indicate that as a per cent of the wall, the protein content declines from 19% at 5 days post-anthesis to <2% at 30 days post-anthesis. At later ages, this decline in percentage is simply a function of dilution by the cellulosic component of the wall. These findings, however, require further substantiation. Interestingly enough, when the protein component of the cell wall is expressed as grams per fiber length (Figure 11), the protein content, relative to the neutral sugar and cellulosic components, remains relatively constant. The implications of this observation are that, while the rate of synthesis of protein component is considerably lower than that of neutral sugars or cellulose, some synthesis of protein is continually occurring and occurring at a rate which matches that of fiber elongation.

Uronic Acids

Despite several problems with the approaches employed to quantitate the uronic acid component of the cell wall, this conclusion can

be made. The uronic acid component probably accounts for at least one-third of the primary cell wall and, at younger ages, the percentage of the wall that is uronic acids may be higher. The enzyme of the three employed that shows the greatest potential is driselase. This is so for a number of reasons, including: 1) greatest and most complete solubilization of cell walls, 2) greatest purity and therefore least amount of interfering substances in terms of the colorimetric assays employed. Even though there are other known enzymes of greater specificity and hence better suited for quantitative release of uronic acids from cell walls, driselase, if further purified, may be effectively used to obtain fractions suitable for structural studies of the wall.

There are some other methods available which, once perfected, may be the preferred approach. One alternative approach to quantitating the uronic acid component of the cell wall involves quantitatively reducing the uronic acid residues to their respective aldoses. The increase in hexose observed upon hydrolysis can then be attributed to hexuronic acid. Attempts to convert free uronic acids to the corresponding alditols followed by gas chromatography of the hexaacetate derivatives have been described by Blake and Richards (1968, 1970). Reduction is carried out under acidic conditions which are necessary to prevent hydrolysis of the lactone to the resistant carboxylate ion. Problems of pH and temperature control are the major concerns. Because of difficulties in obtaining quantitative derivative formation, these authors concluded that such an approach is probably not feasible.

The uronic acid residues of a uronic acid-containing polymer can be reduced to hexose units prior to hydrolysis. There are a number of procedures which can be employed. Reduction can be done with diborane or by producing the methyl ester with diazomethane and reducing this with borohydride (Aspinall, 1965).

Resistance of the glycosidic linkage in the uronic acid can be used to advantage. Because acid-catalyzed hydrolysis leads to cleavage of all glycosidic bonds except those involved in an aldobiuronic linkage, this leads to an accumulation of oligosaccharide fragments that can be separated and quantitated. The nature of the contained glycosidic linkage can be identified. Knowing which sugar(s) is attached to uronic acids could potentially be used to quantitate the uronic acid component. This, of course, assumes a) that the sugar residue attached to the uronic acid is in some way unique and can therefore be distinguished from similar sugar residues involved in other linkages of other polymers, b) that this sugar can be quantitated, and c) that the ratio of the uronic acid residue(s) to this sugar residue can be established. For example, in sycamore cell walls, the rhamnose to galacturonic acid ratio has been approximated as being ca. 1:10. It has also been established that rhamnose is found exclusively in the rhamnogalacturonan polymer of sycamore cell walls. The wall concentration of galacturonic acid can therefore be readily calculated.

Knowing the percentage of any neutral sugars present in the cotton fiber cell wall, it only remains to establish whether a similar uronic acid-containing polymer is present in the cotton fiber cell wall.

Preliminary investigations have indicated that the extracellular medium of the culture grown fibers may be an excellent source of cell wall polymers. Furthermore, Burke *et al.* (1974) have shown that 49% of the material precipitated by ethanol from the extracellular culture medium of Douglas fir cells consists of uronic acids. Another dicot, sycamore, secretes 25% uronic acids into the medium. It is therefore plausible that the extracellular medium may readily provide uronic acid-containing polymers that are suitable for preliminary investigations concerning the nature of this polymer.

The results of this study, together with the results obtained for the other components of the wall, including cellulose, neutral sugars and protein, indicate that the uronic acid component accounts for 30 to 50 per cent of the wall. Assuming that uronic acids comprise the remainder of the wall not accounted for by cellulose, neutral sugars, and protein, and converting this percentage to grams uronic acid per fiber length yields a surprising result (see Figure 11). Not only does the increase in grams of uronic acid per fiber length exceed that of neutral sugars and protein, but also, through 16 days post-anthesis, it exceeds that of cellulose. Furthermore, the highest content of uronic acid occurs just prior to the maximum rate of fiber elongation (see Figure 10). The decline in uronic acid concentration occurs just as the fiber attains its maximum length. These conclusions are based upon the assumption that the uronic acid comprises the remainder of the wall not accounted for by other components. In contrast to the changes observed when uronic acids are expressed as grams per fiber length, the remainder of the wall not accounted for, when expressed as a *percentage* by weight of

the wall is constant through 16 days post-anthesis (Table 17). The data available from direct measurement of uronic acids also indicate that the percentage of the wall represented by uronic acids is constant through 15 days post-anthesis. The uncorrected value obtained for uronic acids through 15 days post-anthesis is ca. 30%. Using an estimate of the minimum correction factor required to account for degradation and incomplete hydrolysis estimated from the data of Table 12A, the per cent of the wall that is uronic acids approaches 40%. Utilizing either the percentage values *actually* obtained for uronic acids, or *corrected* values, in calculating grams per fiber length, would still yield an *identically* shaped curve as that which resulted from employing estimated values (see Figure 11). As before, the rate of deposition also exceeds that of cellulose. Therefore, the conclusions based on the assumption that the total unaccounted-for components of the wall are largely represented by uronic acids are also supported by direct measurements of the uronic acid content.

Regardless of the actual concentrations present, the uronic acid component undoubtedly represents a major portion of the cotton fiber cell wall and, as such, should not be overlooked in terms of the role it plays in the mechanism of fiber elongation. The pectic component of the wall, which has the ability to form reversible gels and viscous solutions with water (Rees, 1969), may provide a means of controlling elongation. In particular, if, as suggested by Northcote (1972), pectins can influence the water distribution within the wall and also the water relationship between the polysaccharides of the matrix and between the matrix and the microfibrils, the hydrogen bonding between matrix polysaccharides and microfibrils

Table 17. Per cent of the cotton fiber cell wall accounted for

Values are derived from graphs of the results obtained for determination of each of the three components listed. The values listed for "other" represent the remainder of the wall not accounted for by cellulose, protein and/or neutral sugars.

Component	Age (days post-anthesis)																	
	5	6	8	9	10	11	12	13	14	15	16	17	18	19	20	22	25	29
Cellulose	9	9	10	12	23	24	24	24	25	25	31	69	74	77	82	87	90	94
Protein	19	17	13	12	10	9	8	8	7	6	5	4	3	3	2	2	2	1
Neutral sugars	33	30	25	25	24	22	20	18	17	15	14	13	12	11	10	9	7	4
Other	39	44	52	51	43	45	48	56	51	54	50	14	11	6	6	2	1	1

will be weakened by the presence of a pectic component so that the wall will be less rigid. Because of the considerably decreased mechanical interactions between the two phases, under stress, the microfibrils will slip over one another in the more fluid matrix. The decline in uronic acid concentration occurs just as the fiber attains its maximum length. Because the fiber is no longer increasing in length, the decline in grams uronic acid per length is not the result of simple dilution, nor is it the result of a leveling off of the rate at which it is synthesized. Rather, this observed decline in uronic acids is indicative of an example of actual polymer removal from the cell wall. It is tempting to speculate that the removal of these uronic acids and the resulting changes in water activity of the wall may be an important factor which leads to the cessation of elongation. It would be of interest to search for an increase in pectinase activity coincident with the decline in uronic acid content of the wall. Furthermore, development of better techniques for quantitating the uronic acids present in wall polysaccharides will undoubtedly prove useful in establishing the role(s) of the uronic acid-containing polymers in the structure and functioning of the plant cell wall.

Residual Sugars Associated with Cellulose

The results of the acetic/nitric studies indicate that up to one-fourth of commercially prepared α-cellulose consists of residual sugars that can be removed by treating the cellulose with a series of reagents including acetic/nitric reagent and 2N trifluoroacetic acid. The relative ratios of the sugars released is dependent upon the

sequence of treatments employed. Xylose, glucose and mannose are continually removed throughout these treatments. The release of arabinose is only detected after an acetic/nitric digest followed by two hydrolyses with 2N trifluoroacetic acid. When the hydrolysis products obtained approached 100% glucose, these products represented less than 3% of the weight of the original cellulose present. Additional hydrolyses of the remaining residue would indicate whether a continual release of a small amount of glucose occurs. Under these circumstances, the quantities released would be minimal. It can be concluded, therefore, that >97% of purified cellulose is resistant to trifluoroacetic acid hydrolysis. This conclusion is further substantiated by the results of treating α-cellulose with acetic/nitric reagent for 3.0 hours and then sequentially hydrolyzing the residual material with varying strengths of trifluoroacetic acid. These results also indicate that complete removal of arabinose is only achieved after a substantial amount of xylose has been released. This release of arabinose is then followed by release of additional amounts of xylose, mannose and glucose. The fact that the ratios of sugars (particularly xylose:mannose) are changing during sequential hydrolysis suggests that it is not one polymer of consistent repeating structure that is being slowly hydrolyzed. Because, if such a polymer existed, the ratio of released sugars would either remain constant or at least change in a continuous direction; such is not the case (see Table 2).

Sequentially hydrolyzing 12 days post-anthesis cell walls with varying strengths of trifluoroacetic acid yields similar results. An important difference, however, is that for commercial cellulose the amount of xylose released always exceeds the amount of mannose

released. For cell walls, the first hydrolysis yields three times as much xylose as mannose; however, the second and third hydrolyses result in twice as much mannose as xylose released. Again, a resistant mannose-containing polymer is suggested.

The residual sugars associated with sycamore cell walls subsequent to an acetic/nitric digest are xylose and glucose. This finding supports the contention that the polymer(s) resistant to acetic/nitric digest are those most closely associated with cellulose in that a tentative structure for sycamore cell walls has been proposed in which xyloglucan chains are in immediate association with cellulose (Keegstra *et al.*, 1973).

Comparison of these residual sugars with those obtained from cotton fiber cell walls (see Table 4) indicates a comparable interrelationship between the structural components of the cell wall, in that these same three sugars are released, with glucose being released in the greatest amount followed by xylose and mannose. However, the ratio of xylose to mannose is substantially greater in sycamore cell walls. The existence of a mannose-containing polymer, relatively more resistant to trifluoroacetic acid and to solubilization by acetic/nitric reagent has also been demonstrated in a xylan from larchwood.

It is clear that acetic/nitric reagent not only selectively solubilizes specific cell wall polymers, but that this reagent also increases the susceptibility of certain polymer(s) to subsequent hydrolysis. The use of this reagent has provided evidence which suggests a similarity between the composition of the polymer postulated to be closely associated with cellulose in sycamore cell walls

and in cotton fiber cell walls. This reagent will, undoubtedly, be very useful as a selective fractionating procedure.

The products obtained after an acetic/nitric digest can be further characterized by methylation studies. If the linkage studies do indeed confirm the residual sugars to be the xyloglucan in association with cellulose in sycamore cell walls, then it will also be possible to identify the polymer in close association with the cellulose microfibrils of cotton fiber cell walls.

Neutral Sugars

That the development of the primary cell wall is a regulated developmental phenomenon is clearly demonstrated by the changes in the neutral sugar composition repeatedly observed throughout the development of the cotton fiber cell wall.

The results of the cellulose studies suggested that the development of the cotton fiber in culture might closely resemble plant grown fibers. However, it was not known how closely culture grown fibers truly resemble plant grown in the actual composition of the neutral sugar component of the wall. The culture grown fibers provide for a more convenient system for studying physiological aspects of the process of cell wall differentiation; however, culture grown fibers have not been clearly demonstrated to be an accurate representation of an elongating primary cell wall. For example, Nevins *et al.* (1967) have found marked differences in suspension-cultured sycamore cell wall composition as a result of being grown on different media. It was therefore a significant finding to demonstrate that the changes observed in the cell wall neutral sugar composition of

plant grown fibers is clearly mimicked in culture grown fibers.

The actual neutral sugar composition of the cell wall varies markedly with age and, at any age, is not comparable to the neutral sugar composition observed for suspension-cultured sycamore cell walls (see Tables 1 and 19). This is primarily due to a much higher non-cellulosic glucose content in cotton fiber cell walls. If the neutral sugar composition is expressed as a per cent of the total *minus* glucose, a comparison between suspension-cultured sycamore cell walls and cotton fiber cell walls indicates (Table 18) that the mole per cent composition is most similar to sycamore cell walls at early ages (5 to 9 days post-anthesis). The cotton fiber cell wall, however, has relatively more rhamnose. The composition of Douglas fir is also shown (Table 19) and indicates that walls from this species are more similar to young cotton fiber walls than that of sycamore. In this case the compositions are very similar even when non-cellulosic glucose is included in the comparison. There is more mannose in the cotton fiber cell wall, however. The composition of a monocot, wheat, is also shown in Table 19 and indicates that this wall is very dissimilar to that obtained for cotton fiber cell walls. The most marked difference is the large amount of xylose present in the wheat cell wall. Burke *et al.* (1974) have shown that the most profound difference between monocot and dicot primary cell wall matrix is that the monocots contain an arabinoxylan as the principal component whereas a xyloglucan is the major component of dicot cell walls.

These compositional comparisons indicate that, at earlier ages, the cotton fiber cell wall composition more closely resembles that

Table 18. Comparison of the neutral sugar composition for cotton fiber cell walls and for sycamore cell walls

Values are expressed as a mole per cent of the total neutral sugars detected minus glucose.

Sugar	Sycamore* cell wall	Cotton Fiber Cell Wall Age (days post-anthesis) 5	8	9	10	11	12	13	14	15	16	17	18	22	25	29
Rhamnose	6.6	10.7	11.2	11.3	10.3	12.0	13.3	13.0	13.2	14.2	15.2	10.8	16.0	13.1	15.8	10.0
Fucose	2.9	1.6	1.7	1.0	1.7	2.0	2.2	1.8	1.9	2.1	1.4	1.8	1.5	2.3	1.0	1.8
Arabinose	48.0	44.4	45.1	45.5	45.6	42.1	35.0	34.4	35.0	28.8	27.9	28.0	26.1	23.3	28.6	32.4
Xylose	17.4	14.0	14.2	14.2	13.5	14.2	16.9	17.3	16.0	19.1	23.0	34.0	26.0	22.8	18.9	20.6
Mannose	5.4	6.4	5.5	6.2	5.4	5.8	6.6	6.8	6.1	7.6	6.0	4.9	5.8	14.2	6.6	7.1
Galactose	24.7	23.0	22.4	21.9	23.7	23.8	26.3	26.9	27.9	28.3	26.1	20.0	26.0	25.6	30.1	28.8

*Calculated from data presented by Talmadge *et al.* (1973).

Table 19. Comparison of neutral sugar composition of cell walls from wheat, sycamore, Douglas fir and cotton

Values are expressed as a per cent of the total neutral sugars detected (I) and as a per cent of the total neutral sugars detected minus glucose (II).

Sugar	Wheat[1]		Sycamore[1]		Douglas fir[1]		Cotton[2]	
	I	II	I	II	I	II	I	II
Rhamnose	1.0	1.1	6.2	6.6	6.8	8.7	8.6	11.3
Fucose	.2	.2	2.6	2.9	2.7	3.4	.8	1.0
Arabinose	32.9	35.0	42.0	48.0	34.5	43.8	34.7	45.5
Xylose	39.5	42.0	15.0	17.4	13.6	17.3	10.8	14.2
Mannose	1.6	1.7	.6	.5	1.4	1.8	4.7	6.2
Galactose	19.1	20.4	25.6	24.7	20.7	26.3	16.7	21.9
Glucose	6.2	0	7.4	0	21.4	0	23.7	0

[1]Calculated from data presented by Burke *et al.* (1974).

[2]Cell wall from 9 days post-anthesis.

of a gymnosperm and that of a dicot (angiosperm) rather than that of a monocot (angiosperm). These results are therefore evolutionarily consistent since cotton is a decotyledonous angiosperm. Although these comparisons do not prove or disprove the existence of similar polymers in the cotton fiber cell wall, there is supportive evidence from a linkage analysis (see Table 16) which would suggest it.

The data presented in Tables 1 and 18 indicate that not only is there more glucose at earlier ages in cotton fiber cell walls than in sycamore (and possibly even in Douglas fir), but also that the percentage of glucose steadily increases after 9 days post-anthesis through 29 days post-anthesis. Also, the percentage of arabinose steadily declines throughout this period and there is an indication, from Table 18 at least, that the percentage of xylose may be increasing during this period. The grams per fiber length of total neutral sugars, glucose, galactose, xylose, arabinose, and mannose, was calculated in order to compare the rates of deposition of the various sugars. These data are presented in Figure 12. Since the growing wall maintains its thickness throughout cell elongation (Figure 10), new material must be continually added to the wall. The proportions in which this material is added to the wall must be reflected in the altering chemical composition of the wall. Clearly, the content of total neutral sugars is the sum of the content of each individual sugar. The grams of mannose per fiber length appears to be fairly constant through 21 days post-anthesis. However, the amount of the other sugars is not constant. In particular, through 8 days post-anthesis, arabinose has the greatest increase in grams per fiber length. The rate of change of xylose and galactose content is also

Figure 12. Comparison of the grams per fiber length of total and individual neutral sugars during development of the cotton fiber cell wall.

Values are expressed as grams per millimeter of fiber length.

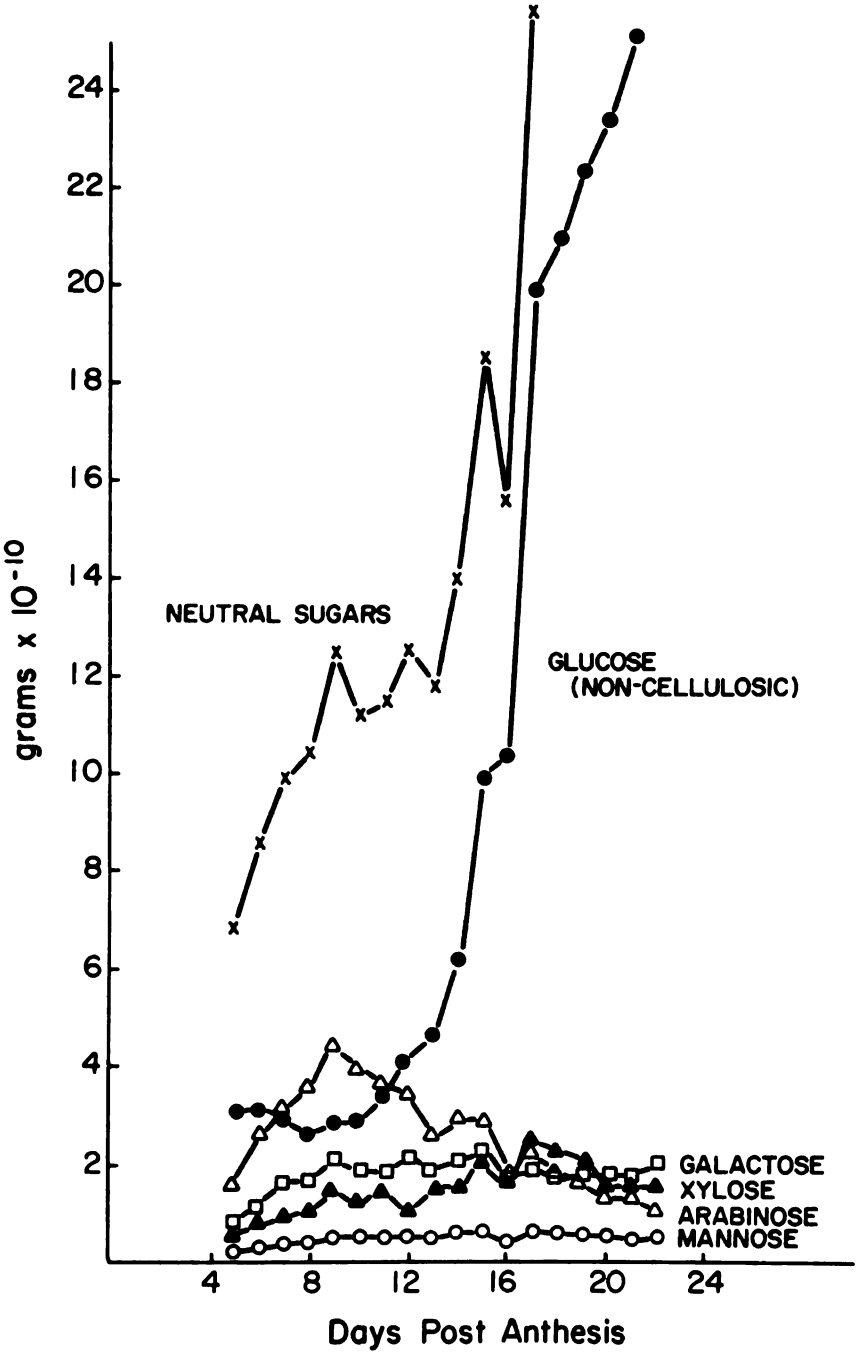
grams x 10⁻¹⁰
24
22
20
18
16
14
12
10
8
6
4
2
NEUTRAL SUGARS
GLUCOSE
(NON-CELLULOSIC)
GALACTOSE
XYLOSE
ARABINOSE
MANNOSE
4
8
12
16
20
24
Days Post Anthesis

Figure 12

increasing during this period; however, the rate of change of glucose content is not. It can therefore be concluded that the increase in total grams neutral sugars per fiber length is predominantly due to an increase in the grams per fiber length of sugars other than glucose during the period through 9 days post-anthesis. At this time the grams arabinose per fiber length begins to decline and the grams glucose per fiber length begins to increase. And, although there continues to be an increase in the grams of other sugars per fiber length, the increase in grams neutral sugar per length is primarily due to the increase in grams glucose per fiber length. The results of the methylation analysis suggest that this increase in glucose content is due in large part to an increase in a 3-linked glucose-containing polymer. Further evidence for such a polymer has also been obtained by other studies in our laboratory which have demonstrated the presence of a β-(1→3)-glucan synthetase in cotton fibers which is very active during the time of increase in non-cellulosic glucan (Heiniger and Delmer, unpublished).

The results of the methylation analysis also suggest that the increase in grams arabinose per fiber length is the result of an increase in the amount of a 5-linked arabinose-containing polymer.

Regardless of the actual nature of the polymers present, it is clear that the rate of deposition of these polymers is changing throughout the elongation of the cotton fiber cell. It is not known what the altering rate of deposition of these various polymers means in terms of the actual mechanism by which cells elongate. Nevins *et al.* (1968) have noted a glucose-containing polymer to be the predominant polysaccharide of cell walls in tissues prior to

elongation. Ray (1963) has also observed a large proportion of glucan in the non-cellulosic polysaccharides of oat coleoptiles prior to elongation. It is possible that this glucose content from 5-8 days post-anthesis may exist as a glucan which replaces cellulose in a functional sense although it does not exhibit the acid resistance of cellulose in more mature cell walls.

Following the auxin-induced elongation of oat coleptiles (Ray, 1963), a decrease in the glucose content of the "hemicellulose" fraction has been noted. A similar decline in the percentage of glucose present in the cell wall during the early stages (5 to 8 days post-anthesis) of elongation of the cotton fiber has been observed in this study.

Comparisons of neutral sugar compositional changes observed in other cell systems are not very meaningful for a number of reasons. First, fractionation procedures employed widely differ. Second, changes observed were in systems employing whole tissues. Third, these changes are expressed as a percentage of the cell wall which makes it exceedingly difficult to detect absolute changes in amounts of sugars present.

The aim of the neutral sugars studies was two-fold: 1) to determine whether the neutral sugar composition varies with age of the wall, and 2) to determine whether a similar neutral sugar composition is observed in culture grown fibers. The results of these studies show that the neutral sugar composition of the elongating primary wall continually changes. This result is particularly noteworthy in view of the recent notion expressed by Albersheim (1975) "that the chemical composition of the walls is not changed by

elongation." Furthermore, similar changes in neutral sugar composition are observed in cell walls from culture grown fibers.

Linkage Analyses

Following methylation of the cell wall, fractionation was effected by differential solubility. Methylated cell walls were separated into chloroform-methanol-soluble and -insoluble fractions. This methylation study represents the chloroform-soluble fraction. Talmadge *et al.* (1973) have shown that for sycamore cell walls, the chloroform-methanol-soluble polymers were the non-cellulosic cell wall polymers. The chloroform-methanol-insoluble polymers were primarily incompletely methylated cellulose and methylated oligo-arabinosides attached to the hydroxyproline-rich protein. In the case of cotton fiber cell walls, it is not known what percentage of the wall is represented by the chloroform-methanol-soluble fraction or whether this percentage is in any way age dependent. A complete methylation study would require re-methylation of the chloroform-methanol-insoluble fraction followed by re-extraction with chloroform-methanol and analysis of this chloroform-methanol-soluble fraction. From the results of those data it could then be determined how complete the first methylation was and whether a third methylation is required. The results of the first methylation, both qualitative and quantitative, should therefore be interpreted with this in mind.

Of the 21 peaks observed, peak 11 (4-linked-glucose) represents the largest percentage at all ages. At 5 days post-anthesis, the per cent of the total area detected represents 52 per cent. This

percentage steadily decreases to 39 per cent at 20 days post-anthesis. The total amount of glucose detected in this analysis is predominantly in the form of 4-linked glucose. However, comparison of the variance in percentage of this peak with the results of the neutral sugar analyses reveals that the per cent glucose varies in a manner markedly different. Because cellulose is resistant to trifluoroacetic acid hydrolysis, and also because the per cent of 4-linked glucose detected declines when the per cent of the wall that is cellulose is abruptly increasing, the 4-linked glucose is not derived from cellulose. Nor is this 4-glucose derived from starch in that walls were shown to be devoid of starch. The discrepancy in relative amount of glucose detected by neutral sugar analyses and by methylation analyses may simply be the result of fractionating the methylated wall into chloroform-methanol-soluble and -insoluble fractions. Comparison of the other methylated sugar derivatives obtained, with the total neutral sugar compositional analyses, indicates that the fraction represented by the methylation analyses is in some respects quite different in composition.

Assuming that the fraction of the wall analyzed at any age represents a constant proportion of the wall, the area represented by a majority of the peaks appears to be age dependent. The most marked changes are observed for peak 5.

Peak 5 (5-linked arabinose)* undergoes a decline in percentage from 21 per cent at 5 days post-anthesis to 6 per cent at 20 days post-anthesis. The neutral sugar analysis results in a decline of 24 to 6 per cent from 5 days post-anthesis to 20 days post-anthesis. This is in excellent agreement and may suggest that the fractionation employed extracted 100 per cent of the arabinose present in the wall.

Peaks 6 and 7 (T-linked galactose and 4-linked xylose) undergo an increase at 8 days post-anthesis followed by a slight decrease at 12 days post-anthesis. The neutral sugar analysis, however, indicates that the sum of galactose plus xylose undergoes an increase at 12 days post-anthesis and a decrease at 16 days post-anthesis. The summation of all xylose and galactose derivatives detected (peaks 2, 6, 7, 10, 13 and 18) is also not in agreement with the neutral sugar analysis.

Another interesting result of this methylation analysis is the greater than eleven-fold increase observed in the amount of peak 9 (3-glucose or 3-mannose) from 5 days post-anthesis to 16 days post-anthesis. The increase in amount of this derivative observed is supported by the demonstration of the presence of an active β-(1→3)-

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*The identification of 4-linked and 5-linked glycosyl residues is uncertain since the data obtained do not distinguish between 4-linked glycosyl residues in the pyranose form and 5-linked glycosyl residues in the furanose forms. In these ambiguous cases, Talmadge *et al.* (1973) arbitrarily considered each sugar to have been in the same ring form as the other residues of that sugar for which it was possible to determine the ring form. This results in all the arabinosyl residues being assigned the furanose ring form and all the other sugars being assigned the pyranose ring form. Until sufficient data become available, it is assumed that the arbitrary sugar ring forms assigned in the analysis of sycamore cell walls may appropriately be used in the analysis of the methylation data obtained.

glucan synthetase activity at this stage of fiber development (Heiniger and Delmer, unpublished). We now have available in our laboratory a purified β-(1→3)-glucanase which could be used to quantitate what amount of the rise in non-cellulosic glucose is due to the appearance of a 3-linked glucan.

In addition to the fractionation procedure employed, these observed discrepancies between quantitations derived from methylation and neutral sugar analyses could also be due to incomplete derivitization. While the variances in the amount of minor peaks may prove to be significant, it may be possible that these peaks represent incomplete methylation products. Indeed, mass-spectral data would indicate that some of these peaks are mono- and di-methylated hexoses. The mass-spectral data, however, do not provide unambiguous information concerning the substitution pattern for these hexoses. This suggests that other components are present giving rise to m/e values which lead to ambiguous conclusions. A good indication of complete derivitization is obtained by comparing the amount of terminal derivatives obtained with the amount of branched derivatives (Table 20). Incomplete methylation is indicated when the amount of branched sugars exceeds the amount of terminal sugars obtained. This was only observed for 14_I and for 20. The data of Table 20 also indicate that the greatest proportion of polymers present in this analysis are linear polymers rather than branched polymers.

The exact nature of the polymers present in the wall cannot be determined from a methylation analysis of un-fractionated cell walls.

Table 20. Summation of the per cent terminal, linear, and branched partially methylated alditol acetates derived from various ages of cotton fiber cell wall

This summation assumes that peak 6 is comprised predominantly of T-galactose.

Sugar derivative	Age (DPA)							
	5	8_I	8_{II}	12	14_I	14_{II}	16	20
Terminal	12	20	22	21	16	19	22	18
Linear	77	70	66	66	62	64	58	60
Branched	10	10	12	13	22	17	20	22

However, this analysis is important because it yields a summary of most of the sugar linkages present in the wall. When compared with results obtained by previous workers, these sugar linkages provide an indication of the likelihood of the presence or absence of certain polymers in the cotton fiber cell wall.

Burke *et al.* (1974) have presented data indicating that the cell walls of the suspension-cultured monocots are similar to each other yet quite different from the suspension-cultured dicots examined (Albersheim *et al.*, 1973; Whistler and Richards, 1970; Wilder and Albersheim, 1973) to date. The polymers comprising the amorphous matrix in which the cellulose microfibrils are embedded is fundamentally different. The most profound difference is that the monocot primary cell wall matrix contains an arabinoxylan as the principal component. No such polymer is present in the primary cell walls of any dicots examined to date (Albersheim *et al.*, 1973; Whistler and

Richards, 1970; Wilder and Albersheim, 1973). A xyloglucan containing mostly 4,6-linked glucosyl and terminal residues is the major polymer of dicot walls. The data obtained are in agreement with the existence of such a polymer in cotton fiber cell walls as well.

At earlier stages of cotton fiber cell wall development, arabinose is the most abundant of the neutral sugars present. The forms of this sugar detected include T-arabinose, 2-linked arabinose and 5-linked arabinose. The preponderance of 5-linked arabinose may indicate the presence of a 1,5 arabinan. Indeed, polymers with an α-1,5 arabinan backbone have been isolated from a number of plant tissues (Aspinall, 1970). Burke *et al.* (1974) have demonstrated preponderance of 5-linked and the occurrence of 3,5- and 2,3,5-linked arabinosyl residues and have suggested the possibility of a highly branched arabinan in Douglas fir cells.

Hydroxyproline arabinosides have been previously reported in the walls of three other cultured gymnosperms (Lamport and Miller, 1971). Although the amount of this distinct amino acid is less in cotton cell walls than in some other walls, the presence of 2-linked arabinosyl residues could suggest such arabinosides. Unlike monocot cell walls, branched xylosyl residues are not observed for cotton fiber cell walls. The presence of 4,6-linked glucosyl, terminal and 4-linked xylosyl, and terminal galactosyl residues may indicate the presence of a galactoxyloglucan similar to the one postulated for Douglas fir cells (Burke *et al.*, 1974) and similar to the xyloglucan of sycamore (Bauer *et al.*, 1973).

From the structural model proposed for sycamore cell walls (Bauer *et al.*, 1973), it has been shown that the hemicellulose of

sycamore cell wall, a xyloglucan, functions as an important cross-link in the structure of the cell wall. This xyloglucan is hydrogen bonded to cellulose and covalently attached to the pectic polymers. Wilder and Albersheim (1973) have further shown that the cell wall of the distantly related angiosperm, Red kidney bean (*Phaseolus vulgaris*) possesses a similar hemicellulose and have determined that the basic structural features of this polymer have been conserved during evolution. Conclusions of a comparison of the residual sugars associated with cellulose from sycamore cell wall and from cotton fiber cell wall and conclusions of compositional and linkage analyses suggest that a similar xyloglucan with a similar cell wall structure and function is also found in cotton fiber cell walls.

The results of this methylation analysis indicate that the polymers present in cotton fiber cell walls may be very similar to those observed in other angiosperm dicot cell walls or in particular in Douglas fir cell walls, a gymnosperm. Clearly, more studies are needed to determine whether cotton fiber cell walls are as similar to other dicot cell walls or to gymnosperm cell walls as the composition and methylation data presented suggest. Additional studies may reveal that the wall structure of cotton fiber cell walls is indeed representative of dicots and of higher plants, and as such elongating cotton fibers are even more suited as a model system for elongation studies. In addition, more detailed information concerning the linkages of the polymers present can be advantageously used toward directed studies on polymer biosynthesis. Specifically, continued research in this area should examine the linkages present in fractionated cell walls.

This study represents the first report of a compositional analysis of the cell wall of a single elongating higher plant cell.

The most significant advantage of the use of cotton fibers over the use of other plant tissues is that the developmental process of one single cell can be examined over the entire period of elongation. This is not possible with other systems under investigation. Examination of various growth parameters of the cotton boll and of the cotton fiber have indicated that cotton fibers which elongate synchronously can be readily obtained. This cell system therefore provides a source of homogeneous cell wall material which is suitable for studies on changes in cell wall composition associated with growth.

The changes in composition occurring in an elongating fiber are more clearly illustrated when the per cent of the wall that a particular component represents is expressed as grams component per fiber length. Although not immediately obvious, this manner of expressing cell wall changes represents a definite advantage over any other cell system currently employed, because such calculations allow one to examine *absolute* rather than *relative* changes occurring during primary cell wall elongation. For other multicellular systems under investigation, a part of the observed decline in the percentage of a component during growth may result from simple dilution of these components by other components deposited during secondary wall formation such as cellulose and from dilution as a result of elongation. For these systems it is not possible to compare the true rates at which cell wall materials are being deposited. The use of cotton fibers, however, allows one to calculate the actual

grams of any component per fiber length throughout the entire process of elongation and secondary wall formation.

Having discussed measurements of the individual components of the wall, it is now possible to present an overall picture of the changes in cell wall composition as the cotton fiber develops.

The relationship between the rate of fiber elongation and cell wall deposition indicates that the rate of cell wall deposition is constant throughout most of primary cell wall elongation and that therefore the rate of fiber elongation never exceeds the rate of cell wall deposition. The rate at which cell wall material is deposited represents the summation of the individual rates of deposition of cell wall components. During the time when cell wall deposition is most constant, through 12 days post-anthesis, the increase in grams per fiber length is greatest for the neutral sugar component and for the "other" component. In fact, through 9 days post-anthesis, the increase in grams per fiber length for the neutral sugar component exceeds that for the cellulose component and through 14 days post-anthesis the increase in "other" component exceeds that for cellulose as well. The initial increase in the total grams neutral sugars per fiber length has been attributed to an increase in the 5-linked arabinose, whereas the second increase in total grams neutral sugars beginning at 13 days post-anthesis has been attributed to increased synthesis of a glucan, possibly 3-linked. A decline in amount of 5-linked arabinose is observed at 9 days post-anthesis.

It has been postulated that the "other" component represents primarily the uronic acids of the cell wall. The deposition of

this component is very great through 15 days post-anthesis, as is the rate of deposition of cell wall material. Sixteen days post-anthesis marks the maximum rate of cell elongation. Although the rate of deposition of cell wall material remains fairly constant at this time, this is in reality a reflection of the rapid decline in uronic acid content accompanied by a rapid increase in cellulose. During this period from 16 to 20 days post-anthesis the increase in grams neutral sugar component is beginning to level off and the protein content still remains fairly constant. The period from 20 to 24 days post-anthesis is marked by increased cellulose synthesis and cessation of elongation. The greatest rate of cell wall deposition occurs at 26 days post-anthesis. This is, of course, accompanied by the greatest rate of cellulose synthesis as secondary wall cellulose.

Whether these changes observed in the specific composition of the cell wall are a cause and/or a result of physiological processes associated with the elongation of the cell wall poses an interesting question. Kinetic differences in the rate of deposition of certain wall components correlate with the rate of elongation for plant grown fibers; however, clear cause and effect relationships between wall composition and the physiological process of elongation remain to be established. Although culture grown fibers yield cell walls that are remarkably similar in terms of composition and in terms of relative changes in composition, culture grown fibers do not attain the lengths that plant grown fibers do. This difference could potentially be used to advantage. Because the rate at which fibers elongate under these two growth conditions differ, it is possible

that the differences in the timing of the developmental sequences involved in the incorporation of wall polysaccharides between culture and plant grown fibers may be significant enough to account for this difference in elongation rate. For example, secondary wall cellulose deposition is not as abrupt in culture grown fibers and begins ca. 2 days earlier. The decreased rate of elongation may be a result of the premature deposition of secondary wall cellulose.

Furthermore, relative changes in the other components examined (arabinose, non-cellulosic glucose, and uronic acids) also occur earlier in culture grown fibers, a fact which supports the contention that all of the developmental sequences are compressed in time. This may in fact be the primary cause of a decreased rate of elongation in culture. Manipulation of hormonal levels may result in selective changes in content of certain components of the cell wall. It may then be possible to establish cause and effect relationships between relative changes in wall composition and elongation.

Determination of the structure of the primary cell wall is the first step in a logical approach designed to relate the biochemical and physiological changes accompanying cell wall elongation. The compositional analyses of the cotton fiber cell wall reveal that it is a dynamic structure. This is true for cell walls derived from culture grown fibers as well. These studies have been valuable in terms of correlating physiological changes with biochemical changes.

The most marked changes occurring at the time the cell attains its maximal length are the decline in pectic component and the rise in the cellulosic component. Research directed towards defining the controls upon synthesis, turnover, and degradation of these

polysaccharides could prove fruitful for elucidating factors which control elongation.

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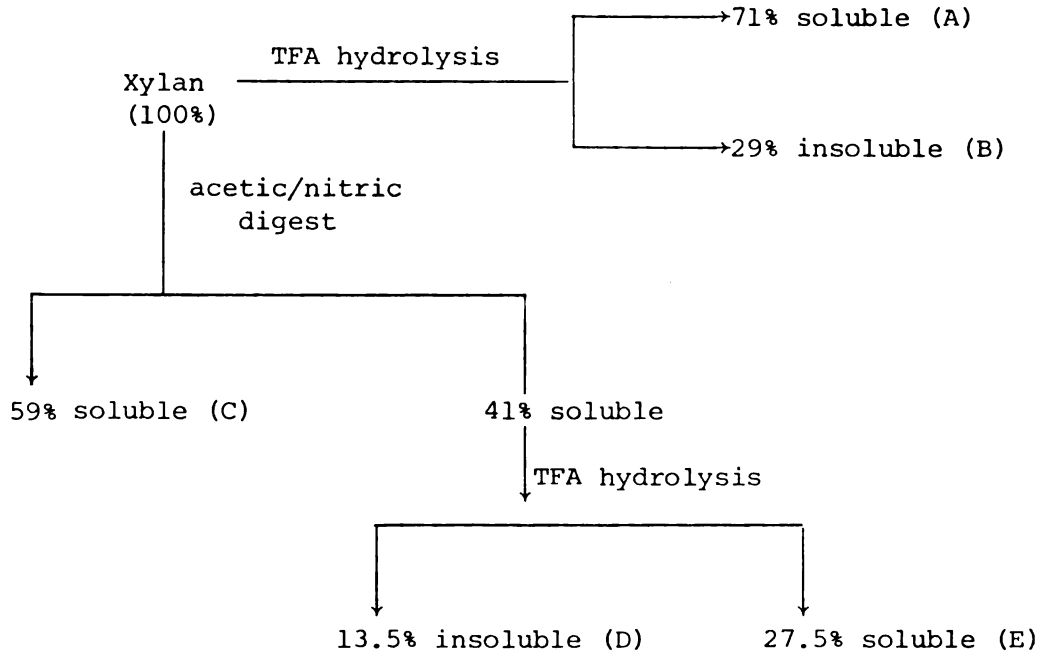
APPENDIX

APPENDIX

HYDROLYSIS PRODUCTS OF LARCHWOOD XYLAN

Treatment of larchwood xylan with acetic/nitric reagent and/or trifluoroacetic acid gave the following results:



The neutral sugar composition of A and of E is known and is given in Table 5. Converting each sugar present from mole per cent to weight per cent of the xylan originally present results in:

Sugar	A	E
Arabinose	.6	.3
Xylose	26.6	1.1
Mannose	31.0	16.4
Glucose	12.8	9.9

Clearly, A + B is equivalent to the sum of C + D + E. Further, the sum of E + C (86.5) is greater than A (71). Therefore, the sum of E + C is equivalent to A *plus* some portion of B. If C is completely derived from A, then A' = A-C. The sum of the components of C has to equal 59. Assume that the source of arabinose and xylose is solely from A. Then the two extreme cases for the composition of C are when (I) the mannose is derived totally from A and when (II) the glucose is derived totally from A. The following hypothetical compositions result for C and A'.

(I)

	C	A'
Arabinose	.3	.3
Xylose	25.5	1.1
Mannose	31.0	0
Glucose	2.1	10.7

(II)

	C	A'
Arabinose	.3	.3
Xylose	25.5	1.1
Mannose	20.3	10.7
Glucose	12.8	0

Now, in either case I or II, there is sufficient arabinose and xylose in A' to go to E. However, the following deficiencies result for mannose and glucose.

	Case I	Case II
Arabinose	-	-
Xylose	-	-
Mannose	16.4	5.7
Glucose	-	9.9

In either of these extreme cases, additional hydrolysis of mannose from B is required and, in case II, hydrolysis of glucose is required in order to arrive at the composition observed for E. In reality the true situation is possibly a combination of these extremes presented. These calculations reveal that there has to be an increase in the amount of mannose that is released upon hydrolysis with trifluoro-acetic acid from the xylan that is pre-treated with acetic/nitric reagent.