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The Structure of Plant Cell Walls

III. A MODEL OF THE WALLS OF SUSPENSION-CULTURED SYCAMORE CELLS BASED ON THE INTERCONNECTIONS OF THE MACROMOLECULAR COMPONENTS[1]

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ABSTRACT

Degradative enzymes have been used to obtain defined fragments of the isolated cell walls of suspension-cultured sycamore cells. These fragments have been purified and structurally characterized. Fragments released from endopolygalacturonase-pretreated cell walls by a purified endoglucanase and the fragments extracted from these walls by urea and alkali provide evidence for a covalent connection between the xyloglucan and pectic polysaccharides. Fragments released by a protease from endopolygalacturonase-endoglucanase-pretreated cell walls provide evidence for a covalent connection between the pectic polysaccharides and the structural protein of the cell wall. Based on these interconnections and the strong binding which occurs between the xyloglucan and cellulose, a tentative structure of the cell wall is proposed.

The polymer composition and partial structures of the pectic and hemicellulosic components of isolated sycamore cell walls are described in the preceding papers (6, 26). There remains the problem of how these components are connected to make a functional wall matrix. It has been suggested that the wall is held together by noncovalent interactions between the various macromolecular components (20). However, the evidence presented in this and in the preceding papers suggests that, with the exception of cellulose, the macromolecules of the wall are covalently cross-linked, and that even the linkage between cellulose and the other cell wall polymers has the strength of a covalent bond.

MATERIALS AND METHODS

All procedures were as described in the preceding two papers (6, 26).

RESULTS AND DISCUSSION

Connection between Xyloglucan and Pectic Polysaccharides. Considerable evidence for the existence of a covalent connection between xyloglucan and the pectic polysaccharides of the wall has been presented in the first two papers of this series (6, 26). This evidence consists of the following results: the presence of small amounts of xyloglucan in the neutral sugar-rich pectic fragments released from the wall by endopolygalacturonase, and the cochromatography of this xyloglucan with these pectic fragments on Bio-Gel P-2 and DEAE-Sephadex (fraction PG-1B of Reference 26); the release of neutral sugar-rich pectic fragments from endopolygalacturonase-pretreated walls by agents which disrupt or weaken the noncovalent binding of xyloglucan to cellulose (fractions U, B, and C of Reference 6); and the cochromatography of endoglucanase-produced xyloglucan oligosaccharide fragments with the neutral sugar-rich pectic fragments on DEAE-Sephadex (Fig. 3 and Table III of Reference 6). Further evidence for the existence of a covalent connection between xyloglucan and the pectic polysaccharides was obtained from the experiments described below.

Fractions U and B (obtained by extraction of endopolygalacturonase-pretreated walls with 8 M urea and 0.5 M base, respectively, see Fig. 1 herein and Reference 6) were chromatographed separately on DEAE-Sephadex equilibrated with 10 mM potassium phosphate, pH 7.0. Approximately one-third of the carbohydrate material from both fractions U and B passed directly through the columns (fractions U-1A and B-1A), whereas the remaining two-thirds of the material in these fractions bound to the column. The ionically bound material (fractions U-1B and B-1B) was eluted from the columns with buffer containing 1 M NaCl. The fractionation and sugar compositions of fractions B-1A and B-1B are shown in Fig. 1 and Table I, respectively. It can be seen from Table I that fraction B-1A (the neutral carbohydrate material extracted by base) consists almost exclusively of xyloglucan polymers. This finding was confirmed by methylation analysis (data not shown). Fraction B-1B, however, contains the sugars characteristic of the neutral sugar-rich pectic fragments (rhamnose, arabinose, galactose, and galacturonic acid, see fraction PG-1 of Reference 26) *and* the sugars characteristic of xyloglucan (fucose, xylose, galactose, and glucose, see Table I of Reference 6). Thus, some of the xyloglucan of fraction B cochromatographs with the neutral sugar-rich pectic fragments on DEAE-Sephadex, indicating a covalent linkage between the two.

The formation of a colored complex with iodine is a characteristic reaction of xyloglucan (6). Fraction U-1B reacted with iodine in aqueous potassium iodide to give darkly colored complexes, indicating that fraction U-1B also contains covalently connected xyloglucan which cochromato-

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graphs with the acidic pectic fragments of fraction U on DEAE-Sephadex.

Since the xyloglucan of fractions U and B should be present as undegraded polymers, in contrast to the endoglucanase-produced xyloglucan oligosaccharides present in the analogous fraction C (6), the xyloglucan of fractions U and B should bind noncovalently to cellulose. If the neutral sugar-rich pectic fragments of fractions U-1B and B-1B are covalently connected to the xyloglucan polymers, then the pectic fragments should also bind to cellulose. Using the cellulose binding assay previously described (6), it was found that more than 75% of the material in fraction B-1A and more than 35% of the material in fraction B-1B bound noncovalently to cellulose. When 500 μg of fraction U-1B were applied to a 20- $\times$ 1-cm column of Whatman CF-11 cellulose and eluted with water, it was found that over 90% of the carbohydrate material in this fraction bound to the cellulose. Thus, the predictions made above are confirmed; it appears that there is a covalent connection between xyloglucan and the neutral sugar-rich pectic fragments found in fractions U-1B and B-1B, as well as in fractions C-2, C-3, and C-4.

The probability that such a covalent interconnection exists raises the important question of whether the reducing ends of the xyloglucan chains are linked to the neutral sugar-rich pectic fragments, or whether the reducing ends of the neutral sugar-rich pectic fragments are linked to the xyloglucan chains. This question was examined by reducing 1 mg of fraction C-2 with [^{3}H]sodium borohydride in 1 N ammonium hydroxide in order to radiolabel the sugar at the reducing ends of the polysaccharide fragments of fraction C-2. If the xyloglucan oligosaccharide fragments of this fraction are linked to the neutral sugar-rich pectic fragments, then the reducing ends of the xyloglucan fragments will be involved in the connecting linkage, and the only reducing sugar available should be the galacturonosyl residues generated by the endopolygalacturonase cleavage of the rhamnogalacturonan. Conversely, if the pectic fragments are linked to the xyloglucan oligosaccharide fragments, then glucosyl residues generated by endoglucanase cleavage of the xyloglucan should be the only available reducing sugars. The [^{3}H]sodium borohydride reduction was stopped by the addition of a few drops of glacial acetic acid, and the reaction mixture was then evaporated to dryness at 45 C by a stream of filtered air. The resulting residue was successively suspended in 1 ml of acidified methanol and evaporated to dryness five times to remove the borate. The dried residue was hydrolyzed in a sealed tube in 0.5 ml of 0.1 or 2.0 N trifluoroacetic acid for 1 hr at 121 C. The hydrolyzed solution was again evaporated at 45 C by a stream of filtered air. The hydrolyzed residue was dissolved in 0.1 ml of water. Aliquots were spotted on Whatman No. 1 paper and chromatographed in two different solvents (ethyl acetate-acetic acid-formic acid-water, 18:3:1:4; and *n*-propanol-ethyl acetate-water, 7:1:2). After hydrolysis in 0.1 N acid, the radioactivity remained at the base line in each solvent. Thus, the radioactivity apparently is in some oligosaccharide which is resistant to 0.1 N acid hydrolysis. The only glycosidic bonds of the primary cell wall polysaccharides which are known not to be hydrolyzed under these conditions are those of cellulose and those of galacturonide. After hydrolysis in 2.0 N acid, the radioactivity chromatographed in each solvent as arabitol. Since neither endoglucanase nor endopolygalacturonase would result in arabinose at the reducing end of polysaccharide fragments, the arabitol was probably formed by acid-catalyzed decarboxylation of galactonic acid. Galactonic acid is the product produced by reduction of galacturonic acid with sodium borohydride.

These results clearly demonstrate that the reducing ends of the polymers of fraction C-2 are not glucose. Although galactonic acid was not isolated, the results indicate that the reducing terminus of the C-2 polymers is galacturonic acid. Therefore, these results support a structure which has the reducing ends of the xyloglucan chains attached to the pectic polymers and indicate that the alternative possibility is not true. However, the exact position of the attachment of the xyloglucan to the pectic polysaccharide remains to be determined. A purified enzyme capable of hydrolyzing the internal glycosidic bonds of the galactan chains would be most useful in this regard.

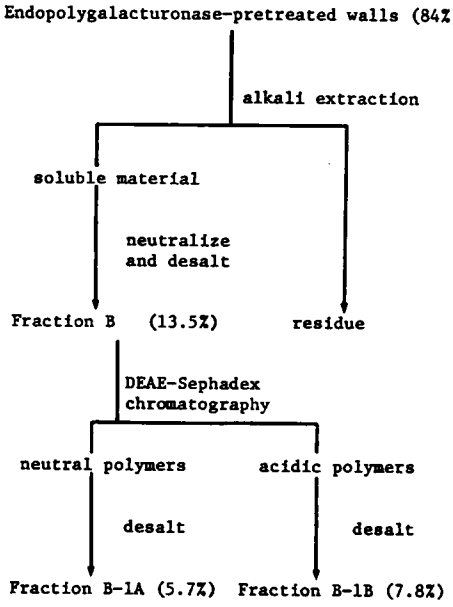


FIG. 1. Fractionation of wall material solubilized by alkali extraction of endopolygalacturonase-pretreated cell walls. Each step was performed as described (6, 26). The values given in parentheses are per cent of the total cell wall accounted for by each fraction.

Table I. *Sugar Composition of Fractions B-1, B-1A, B-1B, and U-1*

Neutral sugar values were determined by gas chromatography (26). Uronic acid values were determined from the anthrone and carbazole test values as described (26).

	Fraction			
	B-1	B-1A	B-1B	U
	mole per cent of recovered carbohydrate			
Rhamnose	5.6	0	8.7	3.5
Fucose	2.1	4.5	2.2	3.0
Arabinose	20.1	6.5	23.6	16.1
Xylose	18.0	36.7	14.0	21.7
Mannose	0	0	0	1.6
Galactose	21.0	10.3	22.0	18.3
Glucose	19.5	36.9	11.7	28.3
Uronic acids	13.5	<5.0	17.8	7.6

Evidence for Covalent Linkage between Polysaccharides and Structural Protein of Cell Wall. In considering possible linkages between wall polysaccharides and structural protein, the most obvious possibility is the attachment of wall polysaccharides to the oligo-arabinosides known to be attached to the hydroxyl groups of almost all of the many hydroxyproline residues of the sycamore cell wall (15). Hydroxyproline accounts for 2% of the weight of the wall and represents over

20% of the amino acid residues of the sycamore cell wall. However, Lamport has established that any carbohydrate which is connected to these arabinosyl residues must be attached by an alkali-labile bond (15). Since glycosidic bonds are stable to the alkali treatment used by Lamport, the glycosidic attachment of wall polysaccharides to arabinosyl tetrasaccharides is ruled out. The galactose, which has been reported to be directly or indirectly attached to these arabinosyl oligosaccharides, may, in fact, be glycosidically attached to the hydroxyl groups of the serine residues of this protein. Serine has been shown to be present in each glycopeptide fragment that contains galactose (15). This possibility is supported by recent results from this laboratory which indicate that most of the serine residues of the cell wall protein contain glycosidically linked sugars (Burke and Albersheim, unpublished results). This was established by treating cell walls with mild alkali. This treatment causes the β-elimination of glycosyl residues linked to serine and results in dehydration of serine to give dehydroalanine. Serine residues with unsubstituted hydroxyl groups are not affected by this treatment. A 70% decrease in the serine content of the cell wall is observed after alkali treatment, while the serine content of a model peptide is not reduced. Thus, a covalent linkage to serine is a likely attachment point between the polysaccharides and protein of plant cell walls.

The glycoprotein of SEPS (sycamore extracellular polysaccharides, see Reference 6) presents an interesting model for the connection between the polysaccharides and the structural protein of the cell wall. The glycoprotein of SEPS binds to DEAE-Sephadex and is eluted in SEPS fraction 2 (Fig. 2). This glycoprotein was isolated from SEPS fraction 2 by exhaustive dialysis against distilled water, titration to pH 2 with 1 N HCl, and chromatography on a column of SE-Sephadex C-50 (Fig. 3). Polysaccharides which have no cationic properties at pH 2 came directly through the column while those which contain positively charged groups (presumably only those polysaccharides covalently bound protein) were absorbed. The column was then eluted with a linear gradient of sodium chloride to remove glycoproteins as well as any contaminating protein. The polymers not sticking to the column constitute SEPS fraction 2A, while those which elute with sodium chloride are designated SEPS fractions 2B-1, 2B-2, and 2B-3, respectively. These four fractions were exhaustively dialyzed against water and lyophilized. A flow diagram summarizing this scheme and giving the yields of each fraction is presented in Figure 4. The sugar composition of each fraction is presented in Table II.

The amount of xylose present in SEPS fraction 2A (Table II) is variable, ranging from 15 to 30 mole per cent in various preparations. Methylation analysis (Table III) shows that the

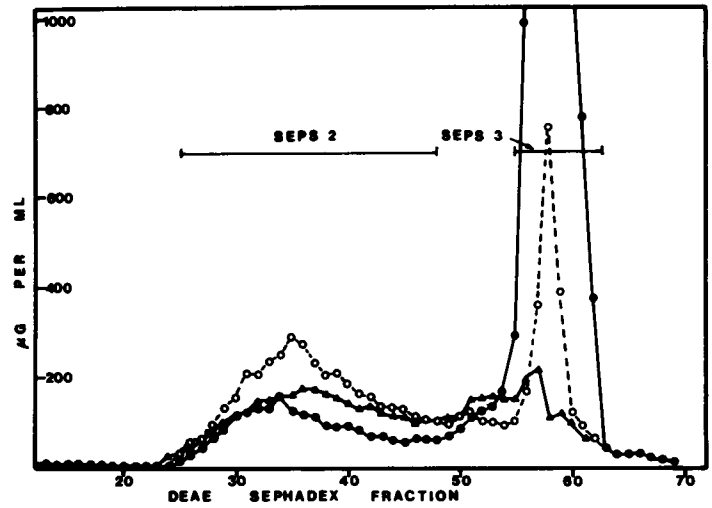


FIG. 2. DEAE-Sephadex A-25 chromatography of SEPS. The dialyzed solution of crude, de-esterified SEPS, obtained as described (6), was applied to a column (19 × 2.6 cm) of DEAE-Sephadex A-25 which had been equilibrated with 10 mM potassium phosphate, pH 6.5. After sample application, the column was washed with 100 ml of the phosphate buffer, and the sample eluate and buffer wash were combined (SEPS fraction 1). The column was then eluted, from fractions 16 through 69, with a linear gradient of sodium chloride (0–600 mM) in buffer. Each fraction was assayed for protein (▲), neutral sugars (○), and uronic acids (●) as described previously (24). Fractions 23 to 48 were combined as SEPS fraction 2, and fractions 55 to 63 were combined as SEPS fraction 3.

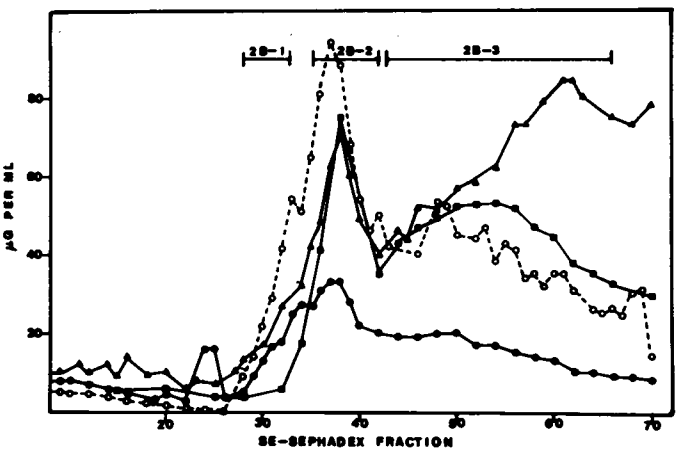


FIG. 3. SE-Sephadex C-50 chromatography of SEPS fraction 2. The solution containing SEPS fraction 2 was titrated to pH 2.0 with 1 N hydrochloric acid and then passed through a column of SE-Sephadex C-50 (2.2 × 20 cm) equilibrated in 10 mM hydrochloric acid. The column was first eluted with 125 ml of 10 mM hydrochloric acid, collecting 6.2 ml fractions. This was followed, from fraction 20 to 72, by a linear 0 to 1.0 M sodium chloride gradient in 10 mM hydrochloric acid. The fractions were assayed for protein (▲), hexose (○), uronic acid (●), and hydroxyproline (■) as described (26). Fractions 28 to 33 were combined as SEPS fraction 2B-1, fractions 35 to 42 were combined as SEPS fraction 2B-2, and fractions 43 to 66 were combined as SEPS fraction 2B-3.

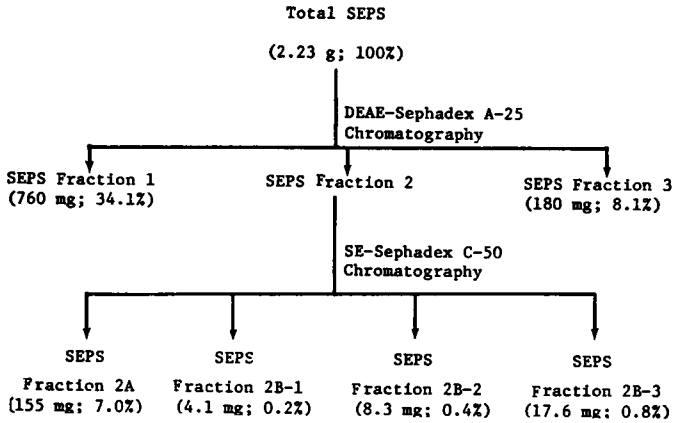


FIG. 4. A summary of the fractionation of SEPS. Each step was performed as described in the text. The values given in parentheses are the weight of each fraction recovered after lyophilization and the percentage of total SEPS which this represents.

Table II. *Sugar Composition of SEPS Fractions*

The compositions presented are for the fractions obtained from the experiment summarized in Figure 4. All neutral sugar values were determined by gas chromatography, uronic acid values were calculated from the carbazole and anthrone determinations, and protein values were measured as described (26). The sugar values presented are expressed as the mole per cent of the recovered carbohydrate. In the glycoprotein containing fractions 2B-1, 2B-2 and 2B-3, the values are adjusted so that carbohydrate plus protein totals 100%.

	Total	SEPS Fraction					
		1	3	2A	2B-1	2B-2	2B-3
	mole per cent of recovered carbohydrate						
Rhamnose	1.7	0.1	1.2	3.0	1.7	4.2	3.8
Fucose	3.1	5.2	0.2	0.4	0.2	0.3	0.5
Arabinose	14.7	1.8	2.9	33.7	31.0	22.4	20.9
Xylose	17.5	34.0	0.6	14.8	2.1	0.7	0.4
Mannose	1.0	1.3	0.1	1.2	1.9	0.9	1.4
Galactose	14.8	7.9	2.7	31.5	29.4	27.2	23.2
Glucose	21.6	48.4	1.1	3.7	4.5	3.7	4.1
Uronic acids	25.6	1.5	91.2	12.0	10.4	14.5	7.6
Protein	<1	0	0	0	19	26	38

Table III. *Sugar Linkages Present in SEPS Fractions 2A and 2B-2*

The methylation analyses were performed as described (26). The values given are expressed as mole per cent of the recovered carbohydrate; uronic acids are not included. The sugars are expressed in terms of the linkages present rather than as their methyl derivatives (26). For example, the value given for the amount of terminal rhamnose actually represents the assayed amount of the alditol acetate of 2,3,4-tri-*O*-methyl rhamnose. All arabinose residues are shown as being in the furanose ring form (26).

Sugar Linkage	SEPS Fraction	
	2A	2B-2
Arabinose		
Terminal	24.0	33.0
2	0.6	0.8
3	0.4	0.5
5	1.3	0.9
2,5	3.1	5.9
Galactose		
Terminal	2.3	4.1
3	3.8	9.0
6	5.3	8.8
3,6	21.0	29.8
Rhamnose		
Terminal	1.7	4.4
Xylose		
Terminal	4.3	1.4
4	24.8	0
2,4	6.0	0
Unknown	1.5	1.4

xylose is contained in a 1,4-linked xylan, though the predominantly linear xylan has some branches at carbon 2. Glucuronic acid is frequently found at this position in linear 1,4-xylans (28). This could account for the observation that the SEPS xylan absorbs to DEAE-Sephadex. Thus, it seems likely that this acidic xylan and the acidic arabinogalactan described below are different polymers, although they have not been separated. This xylan is not a major component of sycamore primary cell walls (26), although it is not possible to rule out its presence in small amounts. Thornber and Northcote have reported the presence of an acidic xylan in the secondary cell walls of sycamore cambium cells (27).

The results of the methylation of the arabinogalactan portion of SEPS fraction 2A indicate that it is essentially identical to that found in SEPS fraction 2B-2 (Table III). Thus, the structure discussed below for the carbohydrate portion of SEPS fraction 2B-2 also applies to the arabinogalactan polymer of SEPS fraction 2A. There is, however, one major difference between these two fractions. SEPS fraction 2B-2, which absorbs to the SE-Sephadex column, elutes simultaneously in the linear salt gradient with a hydroxyproline-containing protein. This suggests that the arabinogalactan and the protein are parts of the same glycoprotein molecule.

The carbohydrate portions of fractions 2B-1, 2B-2, and 2B-3 are all very similar (Table II). Thus, only fraction 2B-2 was subjected to methylation analysis. The gas chromatogram of the methylated alditol acetate derivatives obtained from Fraction 2B-2 is presented in Figure 5. The results are summarized in Table III. The results indicate that the arabinogalactan is a highly branched structure containing predominantly 3,6-linked galactosyl residues as branch points with a single arabinosyl residue as the most prevalent side chain.

These results are similar to those reported by Aspinall *et al.* (5) for this arabinogalactan and are also similar to arabinogalactans isolated from coniferous woods (28) and from plant gums (3). The SEPS arabinogalactan differs from those in wood in that it contains rhamnose and a higher percentage of arabinose. An arabinogalactan isolated from maple (*Acer saccharum*) sap is similar to the SEPS arabinogalactan in all respects (1).

One possible structure for the arabinogalactan from SEPS fraction 2B-2 is shown in Figure 6. This same structure, without the protein attached, would apply to the arabinogalactan from SEPS fraction 2A. This structure has two features which suggest that it is an interesting cell wall component. The first is the attachment of this polysaccharide to a hydroxyproline-containing protein, a known component of plant cell walls (5, 13). This suggests that this arabinogalactan may be a wall component and that it may be a connecting point between wall polysaccharides and wall protein. The

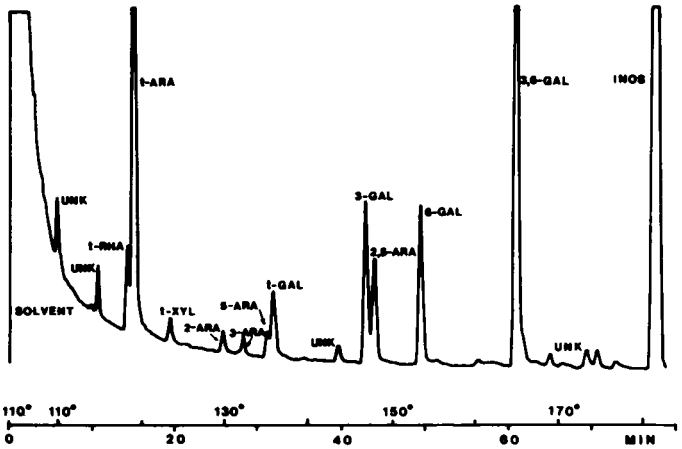


FIG. 5. Gas chromatogram of the methylated alditol acetates obtained from fraction 2B-2. The conditions used for this chromatographic separation and a detailed explanation of this figure are presented in the legend of Figure 1 of Reference 26.

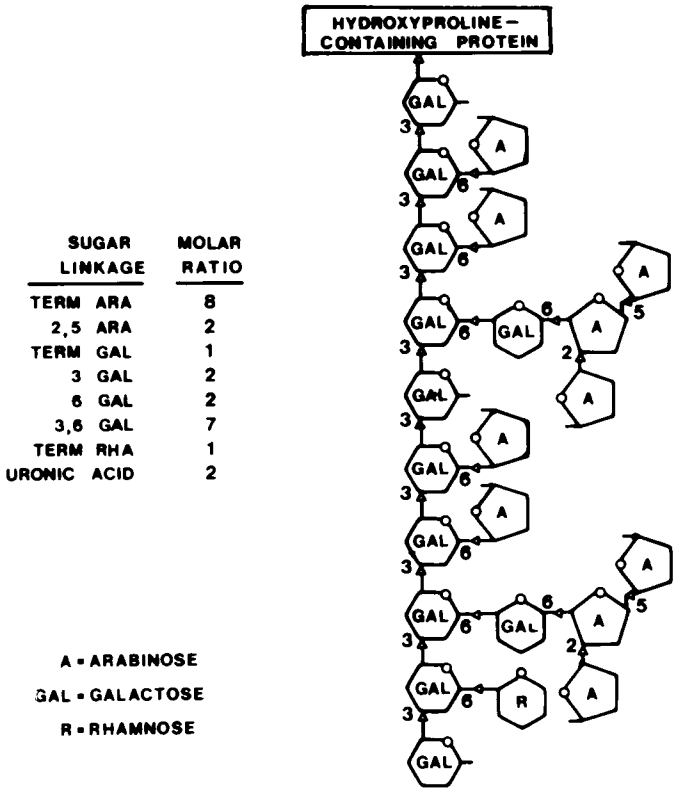


FIG. 6. Molar ratio of the glycosyl derivatives present in SEPS fraction 2B-2 and a proposed structure for a polysaccharide composed of these sugars. The molar ratios were based on the methylation analysis data summarized in Table III. The amount of uronic acid is based on the values given in Table II. Since uronic acids are not recovered from the methylation analysis under the conditions used, it is not possible to tell how these are linked in this structure. The structure shown is not unique to the data but it is consistent with and accounts for the data presented.

second striking feature of this polymer is the presence of a terminal rhamnosyl residue. Since all of the rhamnose in the cell wall is thought to be covalently linked in the rhamnogalacturonan (26), the rhamnosyl residue in this arabinogalactan might act as a primer to which a rhamnogalacturonan can be attached in the cell wall. *This finding leads to the hypothesis that the rhamnogalacturonan is connected to the hydroxyproline-rich wall protein through a highly branched arabinogalactan.* This hypothesis is supported by the ability of the arabinogalactan to act as a cation and thereby bind to SE-Sephadex and also by the simultaneous elution of arabinogalactan and hydroxyproline-rich protein from both DEAE-Sephadex and SE-Sephadex during gradient elution. The fact that SEPS xyloglucan is identical to the xyloglucan of sycamore cell walls makes it reasonable to use the arabinogalactan of SEPS fraction 2B-2 as a model for a similar polymer within the cell wall. Since the arabinogalactan of SEPS fraction 2B-2 has a high proportion of 3,6-linked galactosyl residues, it can be readily distinguished from the arabinan and galactan side chains of the pectic polymers in which the galactosyl residues are predominantly 4-linked and unbranched.

One approach in searching for a connection between the rhamnogalacturonan and the structural protein is to use a protease to hydrolyze the wall protein in order to release glycopeptides for further study. However, if the carbohydrate portion of the glycoprotein is attached through its nonreducing end to other wall polysaccharides, it would be necessary to use walls which have been pretreated with polysaccharide-degrading enzymes before the glycopeptides are released. This possibility was tested in the experiment described below.

Twenty milligrams each of untreated cell walls, endopolygalacturonase-pretreated walls (26), and endopolygalacturonase-cellulase-pretreated walls (6) were suspended in 2.0 ml of 10 mM potassium phosphate, pH 7.0. Two milligrams of pronase (Sigma Chemical Co.) were added to each wall sample, and the reactions were incubated at 30 C for 8 hr. The wall material was removed by centrifugation for 5 min in a clinical centrifuge. The residue was washed one time with 2.0 ml of H_2O, and this wash was added to the supernatant solution of the reaction mixture. The combined solutions were dried, and the residues were analyzed for neutral sugars by gas chromatography as described (26). The results are summarized in Table IV. Less than 0.3% of the wall carbohydrate is released from untreated walls by the action of pronase. On the other hand, when endopolygalacturonase-pretreated walls are used, more than 2% of the wall carbohydrate is solubilized by pronase, and almost 4% of the wall carbohydrate is solubilized if endopolygalacturonase-cellulase-pretreated walls are subsequently treated with pronase. *These results indicate that the carbohydrate of the cell wall glycoprotein is attached to other wall polysaccharides.* These wall polysaccharides must be partially degraded before the protease can solubilize a significant fraction of the wall carbohydrate.

Fractionation of Wall Fragments Liberated from Endopolygalacturonase-cellulase-pretreated Walls by Pronase. The following procedure was used to obtain sufficient pronase-solubilized carbohydrate to allow structural characterization. Five hundred milligrams of endopolygalacturonase-cellulase-pretreated walls were suspended in 100 ml of 10 mM potassium phosphate, pH 7.0, and stored at 2 C for 12 hr. The walls were recovered by centrifugation at 15,000*g* for 10 min and washed one time with the same buffer. This extensive washing was used to ensure that the material solubilized during the pronase reaction is actually released by the action of pronase and is not simply a result of slow extraction by buffer. The walls were then suspended in 100 ml of this buffer, and 50 mg of pronase were added. The reaction was incubated at 30 C for 6 hr. The wall residue was removed by centrifugation at 15,000*g* for 10 min and washed one time with buffer, then one time with buffer containing 1.0 M NaCl and finally

Table IV. *Neutral Sugar Composition of the Wall Carbohydrate Solubilized by Pronase Action*

The reactions were run as described in the text. The neutral sugar composition of the solubilized products was determined by gas chromatography. A control reaction, containing 2 mg of pronase with no cell walls, indicated that the pronase preparation was free of neutral sugars.

	Untreated	Endopolygalacturonase-pretreated	Endopolygalacturonase- and Cellulase-pretreated
	weight per cent of original wall sample		
Rhamnose	0	0.07	0.30
Fucose	0	0	0.01
Arabinose	0.07	1.70	2.50
Xylose	0.05	0.05	0.09
Mannose	0	0	0.05
Galactose	0.10	0.40	0.60
Glucose	0.06	0.07	0.10
Total	0.28	2.29	3.65

three times with water, recovering the residue by centrifugation after each wash. The washed wall residue was suspended in water and lyophilized. The reaction mixture and washes were assayed for carbohydrate material using the anthrone and carbazole tests. The reaction mixture and first buffer wash contained 75% of the carbohydrate released. These solutions were combined for further study. Although the NaCl wash contained 25% of the total carbohydrate released, this material was not included.

The combined reaction mixture and buffer wash were passed through a GF/C glass fiber filter to remove a small amount of residual wall material. The resulting solution was chromatographed on a column of DEAE-Sephadex A-25 which had been equilibrated in 10 mM potassium phosphate, pH 7.0. After application of the sample, the column was eluted with 75 ml of buffer and then with 150 ml of buffer containing 1.0 M NaCl. The 7.8-ml fractions were assayed for carbohydrate by the anthrone and carbazole tests and assayed for protein by the Folin test. Over 95% of the protein and all of the neutral carbohydrate passed directly through the column. The remainder of the protein and all of the acidic polysaccharides bound to the column material and were eluted with the sodium chloride solution. The column fractions containing the material not bound to the column were combined and lyophilized to obtain 246 mg of fraction PR-1. The column fractions containing the acidic polysaccharides were combined, dialyzed exhaustively against distilled water at 2 C, and lyophilized to yield 10.9 mg of fraction PR-2. The fractionation scheme for the pronase products is summarized in Figure 7. The sugar compositions of fractions PR-1 and PR-2 are given in Table V. The results of a methylation analysis of fraction PR-2 are summarized in Table VI.

Based on gas chromatographic analysis, fraction PR-1 is only 5.4% carbohydrate. This amount of carbohydrate represents 2.1% of the total cell wall. The remainder of fraction PR-1 consists of potassium phosphate from the buffer solution and protein. A large portion of the protein in fraction PR-1 originates from the 50 mg of pronase used to degrade the wall protein. However, a portion of the protein in fraction PR-1 originates from the cell wall. This is demonstrated by the presence in fraction PR-1 of hydroxyprolyl residues; hydroxyproline is not present in the pronase preparation. The

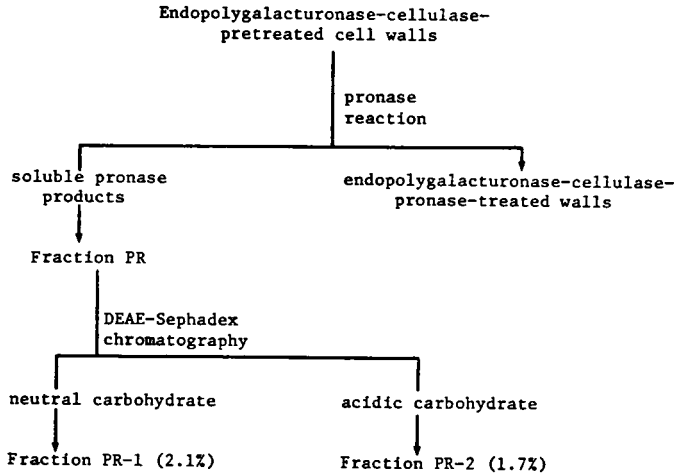


FIG. 7. Fractionation of the material solubilized from endopolygalacturonase-cellulase-pretreated cell walls by pronase. Each step was carried out as described in the text. The values given in the parentheses are the per cent of the total cell wall accounted for by the carbohydrate of that fraction. The amount of wall protein in each fraction could not be determined because of interference from the protein of the pronase preparation.

Table V. *Sugar Composition of Fractions PR-1 and PR-2*

The neutral sugar values were determined by gas chromatography. The uronic acid values were determined from the anthrone and carbazole test results by the procedure described (26).

	Wall fraction	
	PR-1	PR-2
	mole per cent of recovered carbohydrate	
Rhamnose	0	14.4
Fucose	0	1.2
Arabinose	93.0	40.6
Xylose	0	2.2
Mannose	0	0
Galactose	3.5	19.2
Glucose	3.5	2.9
Uronic acids	0	20.4
Percentage of total wall	2.1	1.7

Table VI. *Glycosyl Linkages Present in Fraction PR-2*

The assignment of the glycosyl linkages was deduced from the partially methylated alditol acetates by the procedures described (26).

	Sugar Linkage	Fraction PR-2
		mole per cent of recovered carbohydrate
Arabinose	Terminal	20.8
	2	3.8
	3	3.7
	5	15.5
	2,5	3.2
	3,5	10.0
	2,3,5	3.0
Rhamnose	2	3.8
	2,4	4.6
Galactose	Terminal	5.4
	3	3.5
	4	13.2
	6	3.0
	3,6	2.6
Fucose	Terminal	0.8
Xylose	Terminal	1.5
	2 or 4	0.5
Glucose	4	<0.5
	4,6	1.0

carbohydrate of fraction PR-1 is predominantly arabinose (Table V). The arabinose probably arises from the oligoarabinosides that are glycosidically attached to the hydroxyproline residues of the cell wall protein. Because of the large amount of contaminating protein and potassium phosphate present in this fraction, an attempted methylation analysis was not successful. No further characterization of this fraction was performed.

Fraction PR-2 is 95% carbohydrate; this carbohydrate represents 1.7% of the total cell wall. The remaining 5% of this fraction is protein. Approximately 12% of this protein is hydroxyproline, indicating that about 60% of the protein of

this fraction comes from the cell wall. The carbohydrate of fraction PR-2 (Table VI) is very similar both to the pectic fragments in fraction PG-1B of the endopolygalacturonase products (26) and to the pectic fragments in fraction C-2 of the cellulase products (6). This indicates that fraction PR-2 consists predominantly of pectic fragments. *Since the pectic fragments of Fraction PR-2 were solubilized by the action of a protease, this finding constitutes evidence for a linkage between the pectic polysaccharides and the protein.*

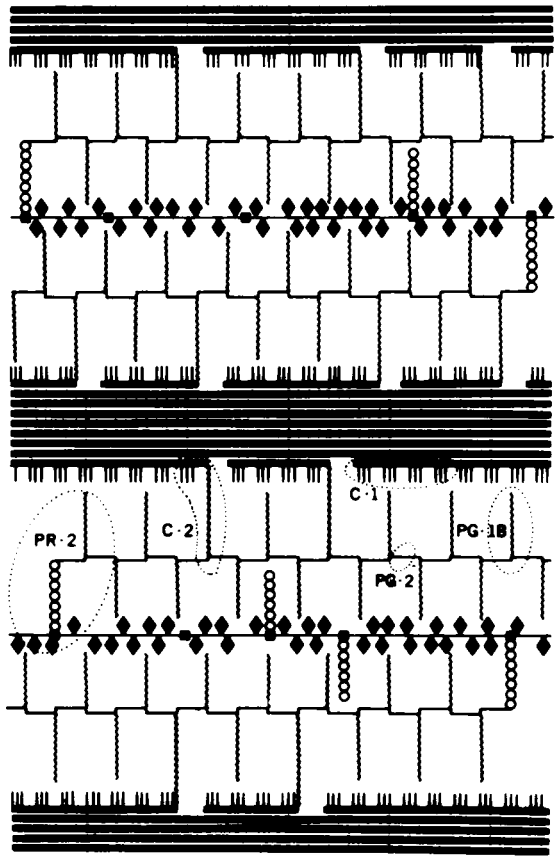
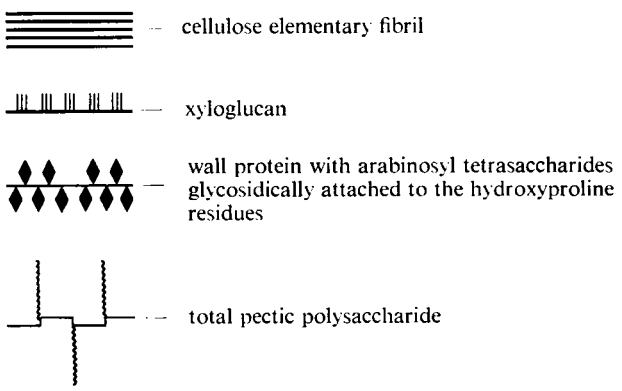
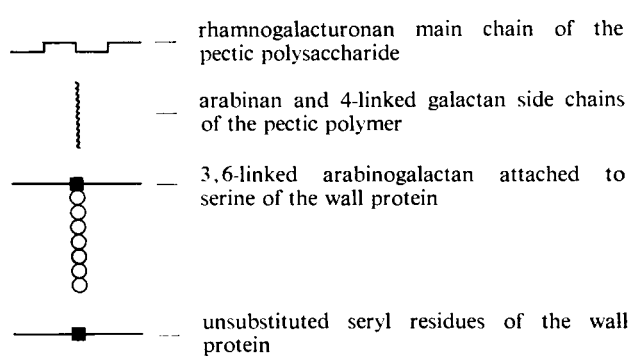


FIG. 8. Tentative structure of sycamore cell walls. The structure presented is based on the data described in the text. This model is not intended to be quantitative, but an effort was made to present the wall components in approximately proper proportions. The distance between cellulose elementary fibrils is expanded to allow room to present the interconnecting structure. A detailed discussion of this model is included in the text. The circled areas are representative wall fractions released by the degradative enzymes. They are fractions PG-1B and PG-2 released by endopolygalacturonase (26), fractions C-1 and C-2 released by endoglucanase (6), and fraction PR-2 released by pronase (Fig. 6). The following symbols are used to represent the various components of the cell wall:





Further evidence that the pectic polysaccharide is connected to the protein comes from chromatography of fraction PR-2 on SE-Sephadex at pH 2. About 75% of the pectic polysaccharides absorb to the column (data not presented), indicating that these polysaccharides contain positively charged groups, a property which can only be derived from covalently attached peptides.

The question of how the pectic polysaccharides are attached to the wall protein cannot be determined from the data presented. However, the data are consistent with the hypothesis that the pectic polysaccharides are connected to wall protein by short arabinogalactan chains similar to those in SEPS fraction 2B-2 (Fig. 6). The fragments of wall fraction PR-2 (Table VI), like those in SEPS fraction 2B-2, include hydroxyproline-containing peptides and have a higher proportion of 3- and 3,6-linked galactosyl residues than do the pectic fragments isolated from the wall by treatment with either endopolygalacturonase (fraction PG-1B, see Table V of Reference 26) or endoglucanase (fraction C-2, see Table II of Reference 6). These results are consistent with the idea that the pronase fragments are rich in the arabinogalactan chains which may be associated with the reducing ends of the pectic polysaccharides, that is, chains which may connect the rhamnogalacturonan and the hydroxyproline-rich protein. The presence of the very small amounts of 3- and 3,6-linked galactosyl residues detected in the endopolygalacturonase and endoglucanase pectic fragments can be explained by a failure of some of the 3,6-linked arabinogalactan chains to be covalently linked to the hydroxyproline-rich protein.

The evidence that at least a portion of the pectic polysaccharides are connected in some manner to the hydroxyproline-rich protein is rather conclusive, but our evidence that this connection involves the 3,6-linked arabinogalactan and attachment to the serine residues of this protein is, as yet, not convincing. An alternative possibility is that the pectic polysaccharides are attached to the wall protein via the tetra-arabinosides that are glycosidically attached to the hydroxyproline residues. The presence of these tetra-arabinosides in fraction PR-2 is demonstrated by the presence of 2-linked arabinosyl residues, residues which have been found in the wall only in this tetrasaccharide. As pointed out previously, if pectic polysaccharides are attached to the arabinosyl tetrasaccharides, they must be linked by an alkali-labile bond. One such linkage would be an ester bond between a hydroxyl group of an arabinosyl residue of the tetrasaccharide and the carboxyl group of a galacturonosyl residue of the rhamnogalacturonan. From the available evidence, it is not possible to distinguish between such an ester linkage and a glycosidic linkage to serine. It is also possible that the pectic polymers

may be attached to the protein in some manner not yet considered. Further investigation will be necessary to determine the nature of the connection between the pectic polysaccharides and the protein.

A Tentative Molecular Structure of Sycamore Cell Walls. The results presented here can be summarized most readily in terms of a model of the sycamore cell well (Fig. 8). Although other structures are possible, the one presented is consistent with all of the data obtained. The model utilizes the fact that xyloglucan has been shown to bind tightly to purified cellulose (5) as well as to the cellulose of the cell wall (6). Several lines of experimental evidence discussed earlier in this paper indicate that the reducing ends of the xyloglucans are attached to the galactan side chains of the rhamnogalacturonan (Fig. 8). In contrast to the model presented in Figure 8, it is possible to construct a coherent, cross-linked structure of the cell wall which utilizes only the linkages between cellulose and xyloglucan, and between xyloglucan and the pectic polysaccharides. In such a model, a single pectic polysaccharide is attached through xyloglucan chains to more than one cellulose fibril; and a single cellulose fibril is attached through xyloglucan chains to more than one pectic polysaccharide. This arrangement would result in cross-linking of the cellulose fibrils and is consistent with all of the data obtained except that which indicates a connection between protein and polysaccharide. The hypothetical structure presented in Figure 8 suggests that the pectic polysaccharides are attached to the serine residues of the wall protein through a 3,6-linked arabinogalactan chain similar to that present in SEPS fraction 2B-2 (Fig. 6). In reality, the structure of the wall is likely to contain the cross-linking features of both models.

The structure presented in Figure 8 allows an understanding of the wall fragments that each enzyme releases. An example to that portion of the wall solubilized by each enzyme is circled and labeled. These fractions are summarized as follows. The endopolygalacturonase attacks the galacturonosyl linkages of the main pectic chain releasing tri-, di-, and monogalacturonic acid (fraction PG-2 of Reference 26) as well as arabinan and galactan chains attached to acidic fragments of the main chain (fraction PG-1B of Reference 26). After the pectic polysaccharide has been partially degraded by the endopolygalacturonase, endoglucanase more readily degrades xyloglucan, releasing neutral oligosaccharides (fraction C-1 of Reference 6) as well as pectic fragments that had been held insoluble by their connection with xyloglucan (fractions C-2, C-3, and C-4 of Reference 6). Pronase, which cannot release carbohydrate from untreated walls, is able to release pectic fragments after endopolygalacturonase pretreatment, and larger amounts of carbohydrate are released by pronase after a combination of endopolygalacturonase and endoglucanase treatment (fraction PR-2, Tables IV, V, and VI).

The primary cell wall of sycamore cells can be considered as a single macromolecule (Fig. 8). The components of the cell wall are, with the exception of the connection between xyloglucan and cellulose, interconnected by covalent bonds; and the many hydrogen bonds which interconnect cellulose and xyloglucan make this connection as strong as a covalent bond. It has been suggested (16) that the plant cell wall contains a protein-glycan network analogous to the peptido-glycan network of bacterial cell walls (10). Our results support this analogy as we find that the structural component of the sycamore cell wall is composed of well defined interconnected polymers in the form of a large "bag-shaped" molecule (30).

Cell Wall Model as Basis for Further Experimentation. One important aspect of the model presented is that it provides a basis for designing additional experiments to characterize the structure of cell walls. For example, knowledge of the existence of the 3,6-linked arabinogalactan and its association with the hydroxyproline-rich protein will facilitate studies of the connection between the pectic polysaccharides and this structural protein; this is a portion of the wall structure which still remains obscure.

The model also provides a basis for investigating wall biosynthesis. Stoddart and Northcote (25) have provided evidence that in sycamore cells a neutral arabinogalactan fraction is converted to a weakly acidic pectic fraction. This evidence is consistent with our model and suggests that the arabinan and galactan polymers are synthesized as separate entities and then are connected into the wall matrix. The model suggests, too, that other wall polymers, such as xyloglucan and rhamnogalacturonan, are also synthesized separately and then incorporated into the wall, perhaps by transglycosidation. Since the xyloglucan and rhamnogalacturonan polysaccharides appear to be composed of repeating units (6, 26), the biosynthesis of plant cell walls, like that already demonstrated for bacterial cell walls (22), is probably achieved by polymerization of the repeated unit. Studies of the biosynthesis of the individual polysaccharides are likely to be advanced by looking for lipid or lipoprotein intermediates (12, 29) which contain an oligosaccharide equivalent to a polysaccharide subunit.

Cell Wall Model and Extension Growth. Many, if not most, of the workers in cell wall research have as a goal an elucidation of the mechanism underlying control of cell wall extension. Cleland (8) has summarized the current thinking about wall extension. It is generally agreed that addition of auxin to tissues deficient in this hormone quickly causes the primary cell walls of the tissue to be loosened or weakened, such that the rate of cell extension is increased. Perhaps not as widely held, but nevertheless accepted by us, is the view that auxin initiates wall loosening so quickly that *de novo* protein synthesis and *de novo* polysaccharide synthesis cannot participate in this *initiation*. Thus, we have examined our structural model of the primary cell wall with the idea that *initiation* of wall extension probably results from a rearrangement or alteration of the existing wall structure. Regardless of the mechanism involved, since walls grow throughout their length, in order for a wall to elongate, the cellulose fibrils within the wall must be able to slide along their length relative to each other. It is also important to recognize that walls that have extended are essentially as strong as those that have not. Therefore, the number of cross-links and the amount of material in an extended cell wall are unlikely to be significantly different from those in an equivalent area of the wall before it grows. In other words, if the wall extends as a result of the cleavage of polymer cross-links, then a mechanism must exist for the synthesis of new cross-links.

In the walls of suspension-cultured sycamore cells (Fig. 8), there are several polymer connections whose cleavage could result in the weakening of the wall. For example, hydrolysis of the connection between the rhamnogalacturonan and the hydroxyproline-rich protein might effectively loosen the wall. On the other hand, enzymic hydrolysis of cellulose, xyloglucan, the 4-linked galactan, or the rhamnogalacturonan would have to be quite extensive in order to loosen the wall significantly.

Further examination of the wall structure (Fig. 8) suggests an attractive alternative to a hydrolytic cleavage mechanism. The only noncovalent cross-link between the structural polymers of the wall is the hydrogen bond-mediated connection between the xyloglucan chains and the cellulose fibers. Extension of the wall would result if the xyloglucan chains and the cellulose fibers moved relative to one another. Movement of the xyloglucan chains along the cellulose fibers could be accomplished by a nonenzymatically catalyzed creep, that is,

by the xyloglucan moving like an inchworm along the cellulose fiber. Such a mechanism would only require the simultaneous breaking of about four consecutive hydrogen bonds. This possibility is highly feasible as is demonstrated by the relatively weak binding of small xyloglucan fragments to cellulose (see Table IV in Reference 6).

The rate at which the xyloglucan chains move along the surface of the cellulose fibrils should be increased by conditions which weaken hydrogen bonds, conditions such as high hydrogen ion concentrations and elevated temperatures. The enhanced rate of creep resulting from low pH or high temperatures would be apparent when dead as well as live tissues are placed under tension. The enhanced rate of creep in such tissues would be completely and immediately reversed by raising the pH or lowering the temperature. In addition, it is easy to conceive of unidirectional creep in walls that are under tension, for the xyloglucan chains are covalently linked at their reducing ends to the noncellulosic wall matrix; therefore, when neighboring cellulose chains are "pulled" in opposite directions, the xyloglucans on each would creep in only one direction relative to the cellulose fiber to which it is attached. *Auxin's ability to weaken the wall and, thereby, to stimulate growth could be explained by suggesting that auxin activates a hydrogen ion pump in the cell membrane; that is, auxin acts to lower the pH of the cell wall and thereby enhances the rate of xyloglucan creep.* The conditions which would control the rate of xyloglucan creep are exactly those that Cleland (8) concludes are able to regulate the rate-limiting step of cell wall extension. Growth by hydrogen bond creep has the wonderful advantage that a source of high energy phosphates within the wall is not required to reform cross-links and thereby maintain the strength of the wall.

Are the Features of the Sycamore Cell Wall Structure Found Universally in the Primary Cell Walls of All Plants? Another important aspect of the model presented is that it provides a framework for interpreting results already obtained. It is rather difficult to compare the structures of the wall components described here with the data in the literature. The comparison is difficult because of the wide variety of wall preparatory procedures used as well as the heterogeneity of chemically extracted fractions (9, 19, 24). Most of the preparatory procedures that have been used result in the presence of water-soluble polymers in the wall preparations. While these polymers may be interesting in their own right, their presence confuses the study of the structural portion of the wall. In addition, the chemical extraction procedures which have been used to solubilize classical wall fractions cause a wide variety of effects. For example, the acid solutions that generally have been used to extract the pectic polymers (16) result in the hydrolysis of such bonds as arabinosyl or rhamnosyl glycosides (26). On the other hand, the strong alkali used to extract hemicellulose simultaneously results in transelimination of uronic acids (2, 18) and β-elimination of serine glycosides (23).

Despite the difficulties described above, there are important findings that have been reported in the literature which are consistent with the results reported here. Lamport (14) has used chemical extraction procedures to determine that cell walls isolated from suspension-cultured sycamore cells contain 36% pectin, 34% hemicellulose, and 27% cellulose. He reported that most of the wall protein is in the hemicellulose fraction. Using the values presented earlier (26) and including both protein and xyloglucan in the hemicellulose fraction, our corresponding values would be 34% pectin, 38% hemicellulose, and 26% cellulose. These components of the sycamore cell wall are found in all primary cell walls. Roelofsen (21) has noted that primary cell walls are typically one-third cellulose, one-third hemicellulose, and one-third pectin plus protein. Thus, the values reported here are in good agreement with the typical primary wall values given by Roelofsen.

Many of the specific features of the sycamore cell wall are found in cell walls of other plants. The hydroxyproline-rich protein and its associated oligo-arabinosides are widespread in the plant kingdom (16, 17). Kooiman (13) has demonstrated that xyloglucan is found in the cell walls of the cotyledons or endosperm of a wide variety of plants. There is even a report which provides some evidence of a connection between the xyloglucan and pectic polysaccharides of the cell walls of mustard cotyledons; the report describes a pectic polysaccharide which has been purified to a state that "if not homogeneous, consists of a family of related species" (20). Methylation analysis was used to demonstrate that their preparation contained xyloglucan as well as the pectic polymers. Although they considered the xyloglucan to be a contaminant, we interpret their data as evidence in support of a covalent linkage between these wall components.

There are variations in the polysaccharides which constitute the structural portion of the cell wall. For example, it is known that several hemicelluloses other than xyloglucan bind tightly to cellulose (7). These hemicelluloses may substitute for xyloglucans as a connector between cellulose and the pectic polysaccharides in some plants. Variation has been observed in the arabinose and galactose composition of the side chains of the pectic polysaccharides (4). Although the side chains containing these sugars may vary in composition and detailed structure, these polysaccharides can still serve to bridge covalently the hemicellulose and polyuronide chains.

An interesting observation concerning the structure of plant cell walls has been reported by Grant *et al.* (11). They have isolated a soluble mucilage particle from mustard seedlings and have speculated that this particle may represent a structural unit of the cell wall. The particle consists of a cellulose elementary fibril encapsulated by other polysaccharides. The composition of the encapsulating polysaccharides suggests that they are xyloglucan and pectic polymers. Thus, the "cell wall unit" of mustard seedlings may be similar to the structure of the cell walls of the distantly related sycamore tree.

The evidence available in the literature and that reported here strongly sustain the hypothesis that the interrelationship between the structural components of the primary cell walls of all higher plants is comparable.

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