Plant Physiol. (1972) 49, 926–936

A Gas Chromatographic Method for the Determination of Aldose and Uronic Acid Constituents of Plant Cell Wall Polysaccharides[1]

Received for publication October 26, 1971

THOMAS M. JONES AND PETER ALBERSHEIM
Department of Chemistry, University of Colorado, Boulder, Colorado 80302

ABSTRACT

A major problem in determining the composition of plant cell wall polysaccharides has been the lack of a suitable method for accurately determining the amounts of galacturonic and glucuronic acids in such polymers. A gas chromatographic method for aldose analysis has been extended to include uronic acids. Cell wall polysaccharides are depolymerized by acid hydrolysis followed by treatment with a mixture of fungal polysaccharide-degrading enzymes. The aldoses and uronic acids released by this treatment are then reduced with $NaBH_4$ to alditols and aldonic acids, respectively. The aldonic acids are separated from the alditols with Dowex-1 (acetate form) ion exchange resin, which binds the aldonic acids. The alditols, which do not bind, are washed from the resin and then acetylated with acetic anhydride to form the alditol acetate derivatives. The aldonic acids are eluted from the resin with HCl. After the resin has been removed, the HCl solution of the aldonic acids is evaporated to dryness, converting the aldonic acids to aldonolactones. The aldonolactones are reduced with $NaBH_4$ to the corresponding alditols, dried and acetylated. The resulting alditol acetate mixtures produced from the aldoses and those from the uronic acids are analyzed separately by gas chromatography. This technique has been used to determine the changes in composition of Red Kidney bean (*Phaseolus vulgaris*) hypocotyl cell walls during growth, and to compare the cell wall polysaccharide compositions of several parts of bean plants. Galacturonic acid is found to be a major component of all the cell wall polysaccharides examined.

Since polysaccharides are the major constituents of the plant cell wall, (3, 33, 41, 45), verification of hypotheses concerning the physiological and biochemical roles of the cell wall in plant metabolism requires methods for the precise determination of the composition and structure of cell wall polysaccharides. Until recently, methods for determining polysaccharide composition have, in general, relied upon the separation of the monosaccharide components of hydrolytic digests by means of paper chromatography and electrophoresis (51, 64) and identification of these components by a variety of color reactions (20). Such methods are both laborious and subject to errors of considerable magnitude.

[1] This work was supported by Atomic Energy Commission contract No. AT(11-1)-1426.

Two methods for the enzymatic determination of uronic acids have also been described. The first of these methods, employing an NADH-linked enzyme system from *Bacillus polymyxa* for the reduction of D-galacturonic acid, has been used for the quantitative determination of monomeric galacturonic acid in the presence of large amounts of galacturonic acid oligomers (40). An NAD-linked uronate dehydrogenase from *Pseudomonas syringae* has also been used for the determination of D-galacturonic acid (8, 9). In this case both D-galacturonic and D-glucuronic acids are oxidized, and what is determined is the sum of the concentrations of the two acids. Hence, it is impossible, by this method, to distinguish these two compounds, both of which are common constituents of plant polysaccharides.

With the development of gas-liquid chromatography of carbohydrates (11, 23, 24, 61, 67), this powerful method became available for the quantitative analysis of monosaccharide mixtures (54). These techniques have been refined by a number of investigators to the point that the gas chromatographic analysis of the neutral sugar components of plant cell wall polysaccharides is accurate, efficient, and convenient (4, 13–16, 19, 26, 27, 29, 39, 47, 57, 59). Of the various techniques available, those involving the determination of neutral sugars following conversion to the corresponding alditol acetates (4, 13, 16, 59) have proven the most satisfactory for determining cell wall polysaccharide composition.

The gas chromatographic determination of the uronic acids, the other major component of plant cell wall polysaccharides, has received less attention. Tamura and Imanari (28, 62) have investigated a variety of glucuronide derivatives, and have concluded that the trimethylsilyl ethers are satisfactory for gas chromatographic analysis of such compounds. Other investigators have examined the gas chromatographic behavior of the trimethylsilyl ether derivatives of iduronic, glucuronic (34, 35), and galacturonic (56) acids. Separation of the anomeric forms of uronic acids as trimethylsilyl derivative has also been explored (17, 30, 31, 50, 63, 70, 71). In addition, Raymond and Nagel (52) have demonstrated the feasibility of separating the trimethylsilyl derivatives of galacturonic acid oligomers by means of gas chromatography. A major disadvantage in the use of trimethylsilyl derivatives for cell wall polysaccharide analysis lies in the difficulty of achieving satisfactory separations of the derivatives of each of the aldoses and uronic acids found in plant cell walls. No gas chromatographic column material which permits such a separation is available at present.

Another approach to hexuronic acid analysis has been presented by Perry and Hulyalkar (48) in which salts of the uronic acids are converted to aldono-1,4-lactones from which trimethylsilyl derivatives are prepared for gas chromatographic analysis. Similar methods have also been described by other

investigators (37, 49, 58). While procedures of this type obviate some of the difficulties encountered in chromatography of the trimethylsilylated uronic acid monomers, difficulties arising from incomplete derivative formation (12, 13, 48), and from incomplete gas chromatographic separation of the trimethylsilyl aldono-lactones (58) have been reported.

Attempts to convert uronic acids to the corresponding alditols followed by gas chromatography of the hexaacetate derivatives have been described by Blake and Richards (12, 13). However, because of difficulties in obtaining quantitative derivative formation, these authors concluded that such an approach is not feasible.

The present report describes the extension of an earlier method for the analysis of neutral sugar mixtures (4) to encompass gas chromatographic determination of hexuronic acids. This method is based on the quantitative conversion of the uronic acids to alditol hexaacetates by means of a series of reactions similar to those described by Sjöström *et al.* (58). The application of this method to the determination of developmental changes in plant cell wall polysaccharide composition is demonstrated.

MATERIALS AND METHODS

Plant Material and Isolation of Cell Walls. Cell wall material from various parts of the common bean (*Phaseolus vulgaris* L. cv. Red Kidney, Small White, and Pinto) is used in this study. Plants are grown and the cell wall material is isolated as described by English *et al.* (22). Prior to analysis all cell wall preparations are placed in a vacuum oven at 50 C for 72 hr to remove moisture. In some instances, cell wall preparations were found to be contaminated with starch even though plants were kept 30 hr in the dark prior to harvesting to deplete their starch reserves. The starch was removed from such walls by treatment with a purified α-amylase preparation from *Bacillus subtilis* (Sigma Chemical Co.) Cell wall samples of approximately 10 mg were suspended in 1 ml of 100 mM potassium phosphate buffer, pH 7.0. Forty microliters of a 0.1% (w/v) solution of α-amylase were added, and the mixture incubated 24 hr at 25 C. At the end of this period, the cell wall material was pelleted in a clinical centrifuge and the supernatant fluid was removed. The walls were washed twice with 2 ml of water and then analyzed as described below.

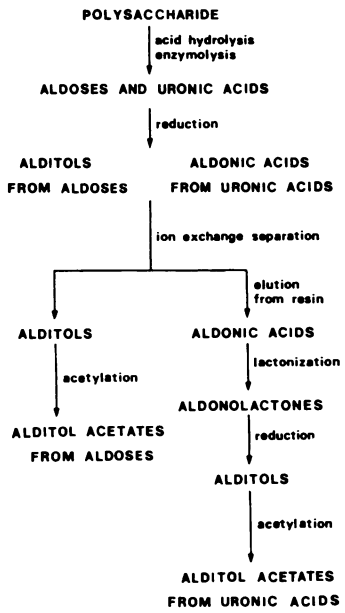

FIG. 1. Preparation of derivatives for gas chromatography. Cell wall polysaccharides are depolymerized by acid hydrolysis followed by treatment with a mixture of polysaccharide-degrading enzymes. The aldoses and hexuronic acids which are liberated are then reduced to alditols and aldonic acids, respectively. These two classes of compounds are then separated by means of Dowex-1 anion exchange resin. The aldonic acids bind to the resin, and the alditols remain in the supernatant solution above the resin. After centrifugation, the supernatant solution is removed, evaporated to dryness, and the alditols are converted to alditol acetates. The aldonic acids are then eluted from the resin with acid and dried to form the aldonolactones. The lactones are then reduced to alditols and acetylated to form the alditol acetates. A detailed description of each step is included under "Materials and Methods."

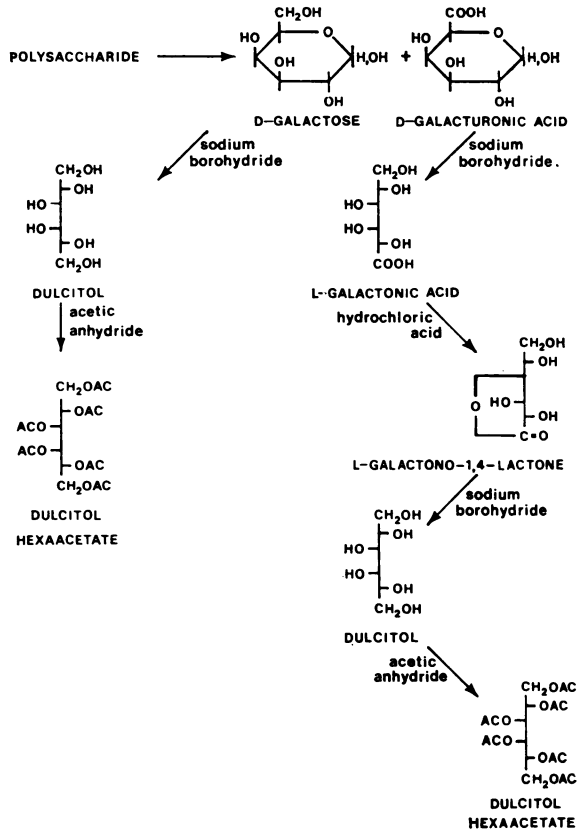

FIG. 2. Reactions involved in the production of alditol acetate derivatives from D-galactose and D-galacturonic acid. After polysaccharide depolymerization, D-galactose, and D-galacturonic acid are reduced with sodium borohydride to dulcitol and L-galactonic acid, respectively. Dulcitol and galactonic acid are then separated using Dowex-1 anion exchange resin. The galactonic acid binds to the resin, and the dulcitol remains in the supernatant solution above the resin. The supernatant solution is removed, evaporated to dryness, and the dulcitol is acetylated with acetic anhydride to yield dulcitol hexaacetate. The galactonic acid is eluted from the resin by treatment with HCl. The acid solution is evaporated to dryness. This converts L-galactonic acid to L-galactono-1,4-lactone. The aldonolactone is then reduced with sodium borohydride to yield dulcitol, which is converted to dulcitol hexaacetate by treatment with acetic anhydride. The reactions are similar for other aldoses and hexuronic acids. See "Materials and Methods" for details of the reaction conditions.

Preparation of Alditol Acetate Derivatives. The procedure employed for the preparation of alditol acetate derivatives suitable for gas chromatography from aldoses and uronic acids is outlined in flow chart form in Figure 1. The reactions involved are illustrated in Figure 2, using D-galactose and D-galacturonic acid as examples.

Hydrolysis of Cell Wall Polysaccharides and Reduction of Liberated Monosaccharides. A portion (5–10 mg) of desiccated cell wall material is weighed into a Pyrex test tube. Two milliliters of 0.2 N trifluoroacetic acid (Eastman Organic Chemicals, reagent grade) containing 1 mg of myoinositol (Calbiochem, three times recrystallized from ethanol and water) are added to each sample. The myoinositol, which serves as an internal standard for the analysis of aldose components of the polysaccharide, is desiccated by 48-hr storage in a vacuum oven at 50 C prior to dissolution in the trifluoroacetic acid solution. The test tube containing the cell wall material, the myoinositol, and the trifluoroacetic acid solution is then sealed, and hydrolysis is carried out for 1 hr at 121 C in a steam autoclave. After hydrolysis, the tube is opened, and the trifluoroacetic acid is removed by evaporation of the solution to dryness at 40 C in a stream of filtered air. The sample is then stored for 12 hr in a vacuum desiccator in the presence of KOH pellets to remove any traces of trifluoroacetic acid remaining in the residue.

The partially hydrolyzed cell wall material is treated with a mixture of extracellular polysaccharide-degrading enzymes secreted by the phytopathogenic fungus (*Sclerotium rolfsii* Sac.) to complete the depolymerization of the polysaccharides. A culture of this organism, which is maintained on potato dextrose agar, was obtained from Dr. D. F. Bateman of Cornell University. The preparation of the *S. rolfsii* enzyme mixture has been described by Van Etten and Bateman (68). Two milliliters of 10 mM sodium acetate, pH 4.5, containing 0.1% (w/v) lyophilized *S. rolfsii* enzyme extract, which has been dialyzed overnight, are added to each sample. This corresponds to approximately 100 μg of protein per sample as determined by the method of Lowry *et al.* (36) using crystalline bovine serum albumin as standard. This amount of enzyme contains insignificant amounts of carbohydrate. The cell wall residue is resuspended, and enzymolysis is permitted to proceed for 6 hr at 30 C. At the end of the enzymolysis period, the aldehyde groups of the liberated neutral sugars and hexuronic acids are reduced. This is accomplished by adding 0.5 ml of 1.0 N NH_4OH containing 3.0 mg of sodium borohydride (Fisher Scientific Co., 98%) and 1.0 mg of L-mannonic acid to each enzymolysis sample (1, 72).

The sodium borohydride reduction of the liberated hemiacetal groups is permitted to continue for 1 hr at room temperature. At this time, the remaining sodium borohydride is decomposed by the dropwise addition of glacial acetic acid until the effervescence has ceased. For complete reduction, excess sodium borohydride should remain at the end of an hour's reduction. Its presence should be evident by vigorous effervescence upon addition of acetic acid. One ml of absolute methanol is then added and the solution mixed. The mixed solutions are centrifuged in a clinical centrifuge for 5 min. The supernatant fluid is transferred with a capillary pipette to a clean test tube, while the residue is washed twice with 0.5 ml of 70% ethanol (v/v). The residue is discarded, and the 70% ethanol washings are added to the supernatant fluid. This solution is then evaporated to dryness at 40 C under a stream of filtered air. Five 1.0-ml portions of 10% (w/v) glacial acetic acid in methanol are added to each sample during evaporation. Treatment with acidic methanol permits the removal of boric acid, which is formed in the decomposition of sodium borohydride, as its volatile trimethyl ester (55). This reduction procedure converts the neutral sugars to the corresponding alditols, and the uronic acids are converted to aldonic acids (Fig. 2).

The L-mannonic acid which is added to each sample at the time of the addition of the sodium borohydride serves as an internal standard for the determination of the uronic acid components of the polysaccharide. L-Mannonic acid is prepared by base hydrolysis of L-mannono-1,4-lactone (Pfanstiehl Laboratories, Inc.). A 1.816-mg per ml solution of mannonolactone in 1.0 N NH_4OH is evaporated to dryness at 45 to 50 C in a rotary evaporator. The residue is taken up in an equal volume of 1.0 N NH_4OH containing 6.0 mg per ml of sodium borohydride. One half milliliter of the resulting solution then contains 1.0 mg of L-mannonic acid and 3.0 mg of sodium borohydride.

Separation of Alditols and Aldonic Acids. The dried residue remaining after the final methanol-acetic acid evaporation is taken up in 3 ml of distilled water, and this solution is transferred to test tubes containing approximately 0.3 g of Dowex-1 anion exchange resin, acetate form, 200 to 400 mesh. The resin-sample suspension is magnetically stirred for 1 hr at room temperature after which the resin is sedimented by centrifugation in a clinical centrifuge. The supernatant fluid is transferred with a capillary pipete to another test tube containing approximately 0.2 g of the Dowex-1 resin. This suspension is stirred for 1 hr at room temperature, after which the resin is sedimented by centrifugation, and the supernatant fluid is transferred to a clean test tube. Each of the two resin pellets is then washed twice with 5-ml portions of distilled water. The washings are combined with the supernatant fluid from the final resin binding, and this solution is evaporated to dryness at 40 C in a stream of filtered air. This fraction contains the alditols formed from the neutral sugars of the polysaccharide. The alditols are further dried by storage for 12 hr in a vacuum desiccator over phosphorous pentoxide prior to acetylation.

Lactonization and Reduction of the Aldonic Acids. The aldonic acids which have been formed on reduction of the uronic acids remain bound to the Dowex-1 resin during the water wash with which the alditols are removed. The aldonic acids are eluted from the washed resin by suspending each washed resin pellet in 2 ml of 1.0 N HCl and stirring for approximately 30 min at room temperature. The resin is then removed by filtration through glass fiber paper (Whatman GFA). The filtrates containing the eluted material from the two resin pellets of each sample are combined and evaporated to dryness at 40 C in a stream of filtered air. This evaporation of the 1.0 N HCl solution serves to convert the aldonic acids to aldonolactones. The dried residue is then stored 12 hr in a vacuum desiccator in the presence of KOH pellets to remove residual HCl.

The aldonolactones are reduced to the corresponding alditols in the following fashion: the dried samples are dissolved in several drops of 10 mM sodium borate, pH 7.5, and 10 mg of sodium borohydride in 0.5 ml of the borate buffer is added to each sample. The reduction reaction is permitted to proceed for 1 hr at room temperature, after which the remaining sodium borohydride is decomposed by the dropwise addition of glacial acetic acid until effervescence has ceased. The acidified solutions are then evaporated to dryness at 40 C in a stream of filtered air. Five 1-ml portions of 10% (w/v) acetic acid in methanol are added to each sample and evaporated to ensure removal of boric acid. The residue remaining after this drying step contains the alditols formed from the uronic acids of the polysaccharide. Prior to acetylation, the samples are stored 12 hr in a vacuum desiccator to remove any remaining moisture.

Acetylation. The alditols formed from the neutral sugar fraction of the sample and from the uronic acid portion are converted separately to the corresponding peracetyl derivatives. Approximately 0.5 ml of acetic anhydride (Fisher Scientific Co., reagent grade) is added to each sample. The test tubes are sealed, and the reaction mixtures are incubated for 3

hr at 121 C in an autoclave. The sodium acetate remaining after the removal of the boric acid serves as a basic catalyst for the acetylation reaction (4). After the acetylation is complete, the samples are transferred to 1-ml serum vials, and the vials are stoppered with rubber caps which have been thoroughly washed with acetic anhydride. The acetic anhydride solutions of the alditol acetate mixtures are stored at −20 C until they are injected into the gas chromatograph.

Gas Chromatography. Separation of the alditol acetates formed from the components of cell wall polysaccharides can be achieved with 1 μl injections of the derivatives in acetic anhydride solution. The column material and conditions employed are similar to those described by Albersheim *et al.* (4). The column material is a liquid phase consisting of 0.2% (poly)ethylene glycol adipate, 0.2% (poly)ethylene glycol succinate, and 0.4% XF-1150 silicone oil coated on Gas-Chrom P (100–200 mesh) by means of a "Hi-Eff" fluidizer (32). Both the fluidizer and the components of the column material are available from Applied Science Laboratories. Approximately 1.2 g of this material is packed by vibration into each of two 4 foot by ⅛ inch outside diameter copper columns. One column is used for analysis; the other is employed as a balancing column to compensate for "bleeding" of the liquid phase. Columns are conditioned approximately 2 hr at 180 C prior to use. Gas chromatography has been carried out using two F & M Scientific Corp. Model 810 instruments and a Hewlett-Packard Corp. Model 7620 instrument. Electrometer settings are range 10 and attenuation 1. Dual hydrogen flame detectors are operated using either oxygen or compressed air.

For the analysis of the alditol acetates formed from the neutral sugars, the following temperature program is employed: 7 min postinjection hold at 120 C, followed by a linear 1 C per min temperature rise to 180 C. The temperature is then maintained at 180 C for approximately 6 min or until the elution of the final sample component, myoinositol hexaacetate, is completed. For the analysis of the alditol acetates formed from the uronic acids, a linear temperature rise of 1 C per min from 150 to 180 C is employed with an approximately 6 min hold at the upper temperature limit. For both types of analysis, a helium carrier gas flow of 90 ml per min is maintained. The injection port temperature is 190 C, and the dual hydrogen flame ionization detector temperature is 240 C. Traces of oxygen are removed from the carrier gas by passage over copper wire heated to 400 C. This procedure significantly increases the useful life of the column material, permitting up to 150 samples to be analyzed using a single pair of columns. Millivolt electrometer output is recorded on a 5-mv strip chart recorder. In addition, electrometer output is recorded on magnetic tape for subsequent electronic integration of peak areas.

Integration of Peak Areas and Data Reduction. The millivolt output from each gas chromatograph electrometer is converted to an analog signal and recorded on magnetic tape by means of an Infotronics Corp. Model CRS-43R1D recorder. The tape on which the data has been recorded is then played back through an Infotronics Corp. Model CRS-11HS/42 integrator equipped with an interval timer. Area values for 6-second segments of the chromatogram are transferred to punched cards by means of an IBM Corp. Model 029 key punch which is driven by the integrator. The data cards are then processed batchwise on a Control Data Corp. 6400 computer using a simple Fortran IV program (SUGAR) which was prepared for this purpose by University of Colorado Computing Center Staff. SUGAR performs the following operations: a baseline is computed for each 6-sec chromatogram segment and the area below this baseline is subtracted from each segment area. Peak beginnings and endings are defined by changes in the first derivative between adjacent segments of the curve and the area segments across a peak are summed to yield total peak area. Component peaks are identified by their retention times relative to the retention time of an internal standard peak: myoinositol hexaacetate for neutral sugar analysis, mannitol hexaacetate for uronic acids.

The area of each component peak is then divided by the area of the internal standard peak to yield an area expression which is independent of injection size. Subsequent computer calculations reflect experimentally determined recovery factors for each of the components and the weight of the cell wall sample used in each experiment. Output data are presented in tabular form for each sample and include percentage composition of the sample.

Recovery factors for each of the neutral sugars and uronic acids commonly found in cell wall polysaccharides are determined by computing sugar to internal standard area ratios for chromatograms of a large number of samples containing 1 mg of each of the sugars and the appropriate internal standard. Standard compounds were obtained as follows: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, and D-galactose from Sigma Chemical Co. D-Galacturonic acid monohydrate was obtained from Pfanstiel Laboratories, Inc. D-Glucose was purchased from J. T. Baker and Co.; D-glucuronic acid, from Pierce Chemical Co. D-Apiose and 4-O-methyl-D-glucuronic acid were the generous gifts of Dr. David Hart of Michigan State University and Dr. G. O. Aspinall of Trent University, respectively. All standard compounds were stored 72 hr in a vacuum oven at 50 C to remove residual moisture before weighing.

Reducing sugar determinations were carried out using the Nelson-Somogyi procedure (42, 60). Citrus polygalacturonic acid was the generous gift of Sunkist Growers, Ontario, California.

RESULTS

The dual flame ionization detector response for a 1-μl injection of an acetic anhydride solution containing the alditol acetate derivatives prepared from 1 μg each of L-mannonic acid, D-galacturonic acid, and D-glucuronic acid is illustrated in Figure 3. The derivatives are, respectively, mannitol hexaacetate, dulcitol hexaacetate, and sorbitol hexaacetate which have been prepared and separated from the derivatives of the neutral sugars by the procedures described under "Materials and Methods." The chromatogram shown is typical of the separation of the derivatives which is routinely achieved using the chromatographic conditions described. The derivative formed from 4-O-methyl-D-glucuronic acid, 4-O-methyl-sorbitol pentaacetate, which is not present in the sample shown in Figure 3, is eluted from the column between the hexaacetates of mannitol and dulcitol.

The detector response for a 1-μl injection of an acetic anhydride solution containing the alditol acetate derivatives formed from 1 μg each of L-rhamnose, D-fucose, L-arabinose, D-apiose, D-xylose, D-mannose, D-galactose, D-glucose, and myoinositol is illustrated in Figure 4. These derivatives have been prepared and separated from the uronic acid components of a standard mixture by the procedure outlined under "Materials and Methods." The chromatogram shown is typical in terms of the quality of separation achieved using the conditions described. Ribitol pentaacetate, the derivative formed from D-ribose and which is not present in the chromatogram shown in Figure 4, is eluted from the column immediately prior to the arabitol hexaacetate peak.

The relationship between the areas of the derivative peaks and the amounts of D-galacturonic and D-glucuronic acids in

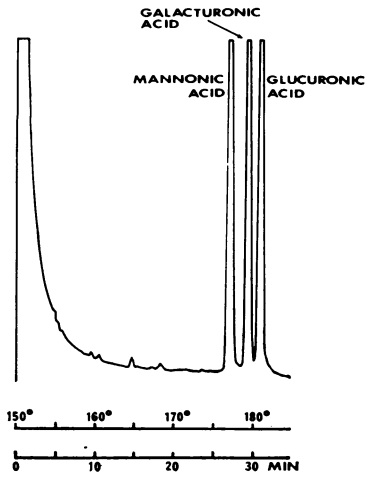

FIG. 3. Detector response for a 1-μl injection of an acetic anhydride solution containing the alditol acetates formed from 1 μg each of L-mannonic acid, D-galacturonic acid, and D-glucuronic acid. These derivatives have been prepared and separated from the aldose derivatives by the procedure described under "Materials and Methods." Gas chromatography has been carried out as described for the analysis of uronic acids. Full scale response on the strip chart recorder represents 5 mv.

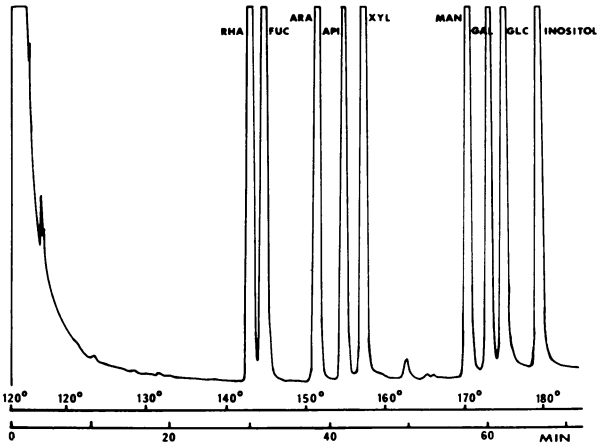

FIG. 4. Detector response for a 1-μl injection of an acetic anhydride solution containing the alditol acetate derivatives formed from 1 μg each of L-rhamnose (Rha), D-fucose (Fuc), L-arabinose (Ara), D-apiose (Api), D-xylose (Xyl), D-mannose (Man), D-galactose (Gal), D-glucose (Glc), and myoinositol. The aldose derivatives have been prepared and separated from the uronic acid derivatives using the procedure described under "Materials and Methods." Gas chromatography has been carried out as described for the analysis of aldoses. Full scale response on the strip chart recorder represents 5.0 mv.

a series of samples is illustrated in Figure 5. The alditol acetate derivatives were prepared as described. The amounts of each of the two hexuronic acids in the samples ranged from 0.3 to 3.0 mg. In addition, each sample contained 3.0 mg of L-mannonic acid as an internal standard. Normalized peak area (uronic acid peak area divided by mannonic acid peak area) is plotted *versus* the amount of the uronic acid in the sample. The detector response for the derivatives of D-galacturonic acid and D-glucuronic acid is observed to be a linear function of the amount of each uronic acid in the sample over the range investigated.

The differing slopes of the lines obtained for the two uronic acids reflect the different efficiencies of derivatization process for the two compounds. It has been shown, by following the disappearance of free reducing groups from the sample solution, that the reduction of both D-galacturonic acid and D-glucuronic acid to the corresponding aldonic acids is complete in less than 1 hr when the reaction conditions are those described under "Materials and Methods." It has also been observed that no detectable reduction of the uronic acids to the alditols occurs during the first sodium borohydride treatment. The efficiency of the binding procedure was tested using D-galacturonic and D-glucuronic acids because of the ease with which reducing group equivalents remaining in solution could be determined. It is possible to demonstrate that more than 95% of each of the two uronic acids is removed from sample solutions containing up to 50 μmoles of total uronic acid in the two Dowex-1 binding steps under the conditions described under "Materials and Methods." Likewise, it has been observed that the removal of the acids from the resin with 1 N HCl is complete.

In view of these observations, the difference between the observed recovery factors for the two uronic acids, which leads to different slopes of the two lines in Figure 5, must arise from differences in the efficiency of the lactonization and lactone reduction reactions. This possibility has been investigated by comparing the recoveries of the alditol acetates formed from the two uronic acids with the recoveries of equimolar amounts of the corresponding alditols. By this means, it has been determined that the recoveries of D-galacturonic acid and D-glucuronic acid as the corresponding alditol acetates are 84% and 92%, respectively. The failure to achieve 100% recoveries is believed to reflect opening of the aldonolactone ring prior to reduction. These recovery figures have been observed to remain constant, however, provided that the derivatization procedure is carried out in a similar manner each time samples are prepared.

The precision of the analytical method is illustrated by the data presented in Table I. The recovery factor values obtained for six repetitive injections of the aldose and uronic acid portions of a single standard sample containing each of the nine components and carried through the derivatization procedure are presented together with the mean for each value. These recovery factors represent, in the case of the seven aldoses, the derivative peak area for 1 μg of each sugar divided by the derivative peak area for 1 μg of myoinositol. In the case of

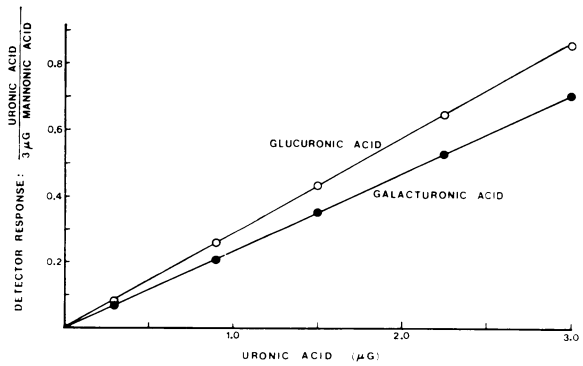

FIG. 5. Detector response for the derivatives of D-galacturonic acid and D-glucuronic acid as a function of the amount of each uronic acid in the sample. The alditol acetate derivatives have been prepared as described under "Materials and Methods." The amounts of each of the two uronic acids in the 1-ml samples ranged from 0.3 to 3.0 mg. In addition, each sample contained 3 mg of L-mannonic acid as an internal standard. One microliter of each sample was injected. The peak area for each of the uronic acid peaks divided by the area of the mannonic acid peak is plotted *versus* the amounts of each uronic acid in the sample.

Table I. *Recovery Factors for the Individual Aldoses and Hexuronic Acids*

The precision of the analytical method is illustrated by the recovery factor values obtained for six repetitive injections of the aldose and uronic acid portions of a single standard sample containing each of the nine components and carried through the derivatization and separation procedure. These recovery factors represent, in the case of the aldoses, the derivative peak area for 1 μg of each sugar divided by the derivative peak area for 1 μg of myoinositol. In the case of the uronic acids, the derivative peak area for 1 μg of each of the acids is divided by the derivative peak area for 1 μg of L-mannonic acid to yield the recovery factor. Standard recovery factors for each aldose and uronic acid are also presented along with standard deviation for each of the standard recovery factors. The standard recovery factors represent the mean values for each component in 18 samples injected twice each. The components are designated as follows: Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; Gal A: galacturonic acid; and Glc A: glucuronic acid.

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A	Glc A
Injection No.									
1	0.738	0.691	0.751	0.836	0.779	0.847	0.860	0.835	0.952
2	0.722	0.679	0.733	0.823	0.763	0.836	0.879	0.840	0.947
3	0.738	0.696	0.745	0.834	0.784	0.859	0.864	0.836	0.962
4	0.738	0.686	0.722	0.856	0.773	0.865	0.853	0.841	0.962
5	0.741	0.679	0.739	0.827	0.771	0.836	0.877	0.828	0.952
6	0.747	0.695	0.745	0.833	0.777	0.844	0.844	0.840	0.951
Mean of above	0.737	0.687	0.739	0.834	0.774	0.847	0.862	0.862	0.954
Standard value	0.739	0.675	0.740	0.839	0.766	0.841	0.857	0.829	0.953
Standard deviation	0.012	0.014	0.012	0.013	0.011	0.012	0.014	0.010	0.014

the uronic acids, the derivative peak area for 1 μg of each acid is divided by the derivative peak area for 1 μg of L-mannonic acid. Standard recovery factor values for each neutral sugar and uronic acid are also presented along with the standard deviation for each of the recovery factors. The standard recovery factors represent the mean values obtained for each component in each of 18 samples injected twice each.

In determining these recovery factors, an effort has been made to approximate as closely as possible the conditions of polymer hydrolysis. While it is impossible to determine exactly the recovery factors for glycosidically-linked sugars through the use of monomeric standards, the recovery factors shown are believed to reflect quite well the proportion of each of the component carbohydrates lost in the course of hydrolysis and derivatization. The recovery factors for the neutral sugars have been determined by carrying a series of samples containing each of the nine components through the entire procedure described under "Materials and Methods." In the case of the hexuronic acids, recovery factors have been determined after completion of the derivatization procedure from which the acid hydrolysis has been omitted. This was done because the aldobiouronic acid linkage is resistant to acid hydrolysis and, as a result, the hexuronic acid components of a polymer would not be exposed, as monomers, to the acid hydrolysis conditions. This approach appears justified in that 95 to 105% of citrus polygalacturonic acid is accounted for in analysis using the method described here and the recovery factors presented in Table I.

Recovery factors for both the aldoses and the hexuronic acids have been observed to vary slightly from one preparation of Dowex-1 resin to another. In addition, some of the sugars may be differentially lost in the course of the drying procedures used (K. W. Talmadge, unpublished results). For these reasons, it is necessary to include standard samples in each series of analyses.

The accuracy of the analytical procedure is illustrated by the data of Table II. Several neutral sugar and uronic acid mixtures of unknown composition have been prepared by one author and their compositions determined by the other. The unknown samples have been carried through the analytical procedure in duplicate. The results obtained for each of the duplicate analyses, and the actual composition of the mixtures are presented in Table II. In all but six cases, the mean of the determined composition is within ±5% of the actual values for each component. In five of the six cases in which errors greater than 5% are observed, the amount of the component in question corresponds to no more than 2% of the usual 10-mg cell wall sample. In the sixth case, the disparity may be due to an error in weighing the unknown sample.

Among the applications for which the method described here seems likely to be useful is in assessing the changes in the composition of plant cell wall polysaccharides during growth and development. Results from such applications of the method are illustrated in Figures 6, 7, and 8. The changes in the composition of the cell wall polysaccharides from hypocotyl tissue of Red Kidney bean (*Phaseolus vulgaris*) as a function of age are shown in Figure 6. Samples, 10 mg, of dried cell wall material prepared from hypocotyl tissue of each age were analyzed as described under "Materials and Methods." Analysis were carried out in duplicate and the values plotted in Figure 6 are the means of the two determinations. Cell walls prepared from hypocotyl tissue older than 11 days were found to be contaminated with starch. These walls were treated with α-amylase as described in "Materials and Methods" to remove the starch. The amounts of starch removed by this treatment from 12-, 14-, 17-, and 20-day-old cell walls were 0.6, 1.6, 2.9, and 10.9%, respectively. The data in Figure 6 reflect the glucose remaining after α-amylase treatment.

It is apparent from these data that dramatic changes in the cell wall polysaccharide composition of Red Kidney bean hypocotyl tissue occur during the early phases of growth. In addition to changes in the relative amounts of the various polysaccharide constituents, significant changes in the total amount of the cell wall polysaccharide recovered as monomeric constituents are also observed as the tissue ages. In the case of Red Kidney bean hypocotyl tissue, the proportion of the cell wall material recovered ranges from 59% for 3-day-old tissue to 27% for 20-day-old tissue.

An interesting pattern of change in polysaccharide composition is apparent as the hypocotyl tissue ages. Early in development, the major constituents of the cell wall are galactose, galacturonic acid, and arabinose. As the hypocotyl

Table II. *Analysis of Carbohydrate Mixtures of Unknown Composition*

Neutral sugar and uronic acid mixtures were prepared by one author and analyzed by the other who was unaware of their composition. The unknown samples were carried through the analytical procedure in duplicate with each sample injected once. The results obtained for each of the duplicate analyses, and the actual composition of the mixtures are indicated. The components of the mixtures are indicated as follows: Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; Gal A: galacturonic acid; and Glc A: glucuronic acid. Values given are in micrograms per sample.

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A	Glc A
Mixture I									
1	119	1390	56	484	655	2316	124	115	1971
2	120	1390	54	485	660	2256	126	115	1976
Actual	120	1424	54	486	650	2310	102[1]	115	1933
Mixture II									
1	584	847	291	1415	105	1279	833	1004	3025
2	585	851	293	1422	107	1283	838	1005	3053
Actual	582	844	289	1421	103	1288	828	1001	2992
Mixture III									
1	929	627	128	129	524	557	230	507	890
2	938	628	124	132	541	558	232	505	916
Actual	891	584[1]	126	129	532	561	206[1]	520	869
Mixture IV									
1	1377	243	213	284	675	288	850	111	670
2	1379	247	216	284	675	289	854	110	657
Actual	1318	216[1]	208	280	660	281	827	97[1]	644
Mixture V									
1	1588	161	241	995	1024	858	536	302	147
2	1612	161	242	986	1024	863	549	320	139
Actual	1538	122[1]	230	971	989	862	500	320	140

[1] Determinations in which the mean of the duplicates varies by more than 5% from the actual value.

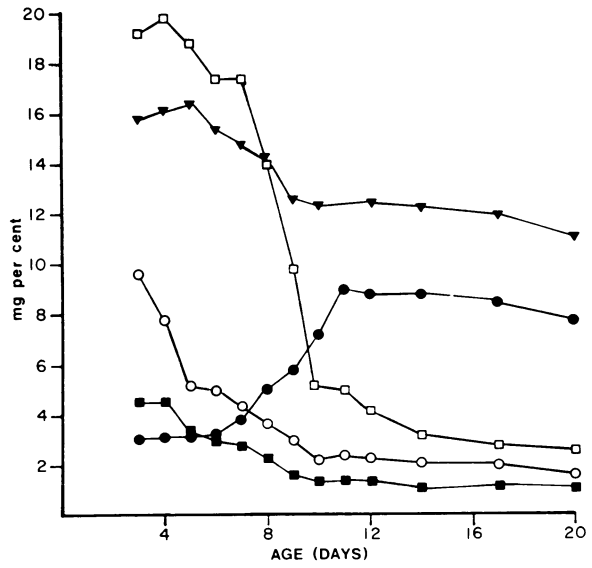

FIG. 6. Changes in the composition of cell wall polysaccharides from Red Kidney bean hypocotyls as a function of age. In each case, approximately 10 mg of dried cell wall material was analyzed as described under "Materials and Methods." The percentage yields (milligram per 100 mg of wall material) are indicated on the ordinate axis. The various components are designated as follows: arabinose, ○; xylose, ●; galactose, □; glucose, ■; and galacturonic acid, ▼.

ages and elongates, the relative proportion of the cell wall represented by each of these components declines. The decline is much more dramatic for galactose and arabinose than for galacturonic acid. Xylose, which represents a relatively minor portion of the cell wall in young hypocotyl tissue, increases to become a major compositional feature of the older hypocotyl cell wall. Glucose, which accounts for approximately 4.5% of the hypocotyl cell wall on day 3, also declines substantially as the hypocotyl ages. Certain minor constituents of the Red Kidney hypocotyl cell wall, which are not shown in Figure 6, also change as the tissue ages. Rhamnose and fucose, which represent 1.3 and 0.8%, respectively, of the hypocotyl cell wall polysaccharide on day 3, decline to approximately 0.5 and 0.2% of the cell wall, respectively, by day 20. Detectable mannose and glucuronic acid in the hypocotyl cell wall remain at approximately 1% of the cell wall from day 3 to day 20.

The cell wall polysaccharide composition of hypocotyl tissue from three varieties of bean (*Phaseolus vulgaris*) plants at 8 and 20 days after planting are shown in Figure 7. Duplicate 10-mg samples of cell wall material from hypocotyl tissue of Small White, Red Kidney, and Pinto bean plants were analyzed as described under "Materials and Methods." The percentage yields (milligrams per 100 mg of wall material) for each of the polysaccharide constituents are given on the ordinate axis. Cell wall preparations from the 20-day-old hypocotyls were found to be contaminated with starch. These walls were treated with α-amylase as described in "Materials and Methods" to remove the starch. The amounts of starch

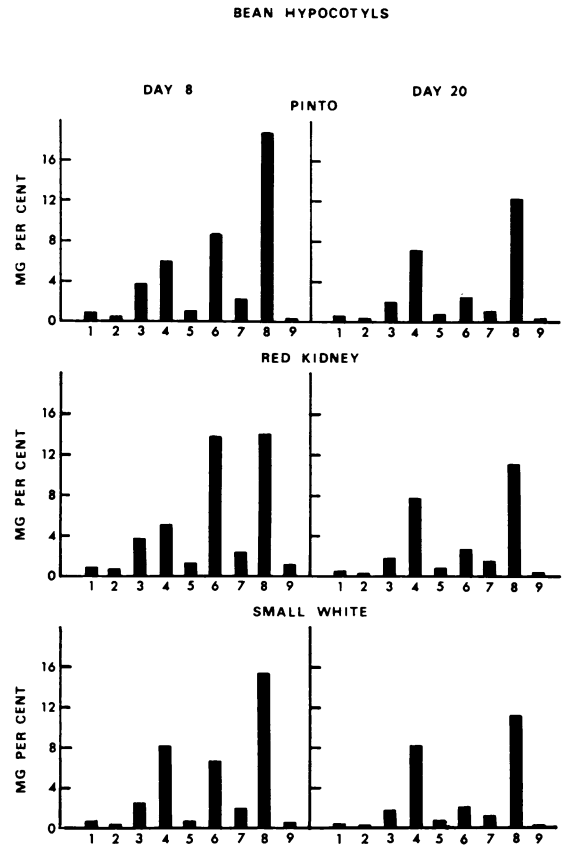

FIG. 7. Cell wall polysaccharide composition of hypocotyls of three bean varieties at 8 and 20 days after planting. In each instance, duplicate 10-mg samples of cell wall material were analyzed as described under "Materials and Methods." The percentage yields of each component (milligrams per 100 mg of wall material) are given on the ordinate axis. The numerical designations on the abscissa represent the following polysaccharide components: 1: rhamnose; 2: fucose; 3: arabinose; 4: xylose; 5: mannose; 6: galactose; 7: glucose; 8: galacturonic acid; and 9: glucuronic acid.

detected in the cell wall preparations from 20-day-old Pinto, Red Kidney, and Small White beans were 6.2, 10.9 and 5.2%, respectively. The data of Figure 7 reflect the glucose remaining after α-amylase treatment. These data demonstrate that, while substantial differences in hypocotyl cell wall composition between the three varieties of *Phaseolus vulgaris* are evident at day 8, the cell wall compositions for the three varieties are very similar by day 20. The galacturonic acid content of such tissue, which has not previously been determined accurately, is observed to represent a major portion of the cell wall of the 3 bean varieties at both 8 and 20 days of age.

The cell wall polysaccharide compositions of four morphological parts of 8-day-old Red Kidney bean plants are shown in Figure 8. Duplicate 10-mg samples of cell wall material from each of the four parts of the bean plant were analyzed as described in "Materials and Methods." The percentage yields (milligrams per 100 mg of wall material) for each of the polysaccharide constituents are given on the ordinate axis. From the data presented in Figure 8, it is evident that the composition of the cell wall polysaccharides, as determined by the method described here, differs from one morphological part of the plant to another. Cell walls from leaf and epicotyl tissue of 8-day-old plants are quite similar, with galacturonic acid, galactose, and arabinose being the major components.

Cell wall polysaccharides from hypocotyl tissue are also similar in composition to those from leaves and epicotyl tissue, but in this tissue the proportions of galacturonic acid and of galactose are aproximately equal, and the arabinose to xylose ratio has shifted, making xylose the third largest component of the cell wall polysaccharide. The composition of root cell wall polysaccharides differs considerably from the composition of walls from other parts of the bean plant. In root tissue, the major constituents are arabinose and galacturonic acid, which are present in approximately equal amounts, and a smaller amount of xylose. In addition to these compositional differences, the total yield of monomeric polysaccharide constituents is lower for root cell walls than for walls from the other morphological regions. Approximately 30% of the cell wall from 8-day-old Red Kidney bean roots is hydrolyzed to monomers by the procedure used. For 8-day-old Red Kidney bean hypocotyls, the monomer recovery is approximately 43%.

DISCUSSION

Perhaps the most difficult aspect of cell wall polysaccharide analysis is the hydrolysis of such polymers to their constituent sugars. While the various methods of acid hydrolysis (4, 53) are useful for releasing the neutral sugar constituents of wall

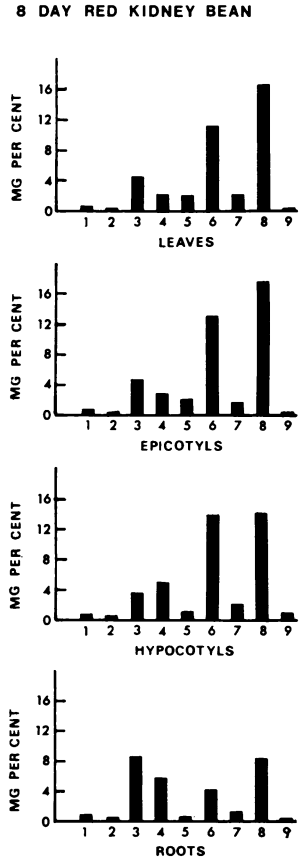

FIG. 8. Cell wall polysaccharide composition of four morphological parts of 8-day-old Red Kidney bean plants. In each instance, duplicate 10-mg samples of wall material were analyzed as described under "Materials and Methods." The percentage yields of each component (milligram per 100 mg of wall material) are given on the ordinate axis. The numerical designations on the abscissa represent the following polysaccharide components: 1: rhamnose; 2: fucose; 3: arabinose; 4: xylose; 5: mannose; 6: galactose; 7: glucose; 8: galacturonic acid; and 9: glucuronic acid.

polysaccharides, these methods are less than adequate for use in uronic acid analysis. The difficulties in hydrolysis of uronide-containing polysaccharides for analytical purposes stem from the chemical properties of this class of compounds. Among these is the unusual resistance of aldobiouronic acids to acid hydrolysis. This phenomenon, which has yet to be completely explained, is believed to result from steric factors which render difficult the formation of the partially planar carbonium ion intermediate in the hydrolysis reaction (10). For this reason, relatively strong acids, high temperatures and/or extended treatments are necessary for the hydrolysis of uronide-containing polysaccharides (53). Such extreme conditions, however, lead to various side reactions involving the hexuronic acids once they are liberated from the polymer. Decarboxylation (65) and lactonization (12) are known to occur under such conditions, and can lead to difficulties in estimating the uronic acid composition of hydrolytic digests. Degradation of neutral sugar polysaccharide components is also a problem under such conditions (4). When hydrolysis conditions sufficiently mild to minimize such side reactions are employed, incomplete hydrolysis of aldobiouronic acids and such crystallne polysaccharides as cellulose often results.

For these reasons, it was decided to investigate enzymatic methods of degrading cell wall polysaccharides. The extracellular enzyme mixture secreted by *Sclerotium rolfsii* Sac. has been found to provide a partial solution to the problems of cell wall polysaccharide hydrolysis. This fungus, which attacks a wide variety of hosts, produces a broad spectrum of polysaccharide-degrading enzymes (68) including a very active polygalacturonase which liberates monomeric galacturonic acid (7). Furthermore, all of the *S. rolfsii* polysaccharide-degrading enzymes which have been described have similar pH optima. No pectic lyase activity is detectable in *S. rolfsii* extracts. This represents an obvious advantage in using this enzyme source for analytical purposes in that only monomeric D-galacturonic acid is liberated in the hydrolysis of galacturonide bonds and not the $\Delta 4,5$ unsaturated product of the lyase reaction. In addition the fungus is easily maintained in culture, and preparation of the enzyme mixture is quite simple.

The combination of mild acid hydrolysis followed by enzymatic degradation of the wall polysaccharides has been found to be most useful for the analysis of the hemicellulosic and pectic components of the cell wall. It appears that the weak acid hydrolysis degrades many of the glycosidic linkages in the wall polymers and serves to make the pectic polysaccharides more accessible to enzymatic action since enzymatic release of galacturonic acid is facilitated by such pretreatment.

Depolymerization of cell wall polysaccharides by this method is not without disadvantages, however. Chief among these is incomplete hydrolysis. Yields of some polysaccharide components, *e.g.*, L-rhamnose, are increased by stronger acid hydrolysis, while such treatment reduces the recovery of others such as L-arabinose. Such relationships between yields necessitate a compromise on hydrolysis conditions to achieve reasonable yields of all components. Were all of the enzymes necessary to degrade plant cell walls to their monomeric constituents available in the *S. rolfsii* extract, such compromise solutions would not be necessary.

Furthermore, the cellulosic portions of cell wall polysaccharides are not hydrolyzed in this procedure since treatment with 0.2 N trifluoracetic acid does not hydrolyze such polymers and the *S. rolfsii* enzyme mixture does not contain the enzymatic components necessary to degrade crystalline cellulose to D-glucose. While this is convenient if one is focusing his analysis on the pectic and hemicellulosic portions of the wall, the analysis of the cellulosic portions of cell wall polysaccharides requires alterations of the hydrolysis procedure described here (66).

Notwithstanding the difficulties involved in achieving complete hydrolysis of plant cell wall polysaccharides under conditions which permit recovery of the monomeric carbohydrate constituents, the data on cell wall polysaccharide composition presented in Figures 6, 7, and 8 demonstrate the usefulness of the method described here in investigations of plant cell wall polysaccharide composition.

The data presented in Figure 6 for the composition of Red Kidney bean hypocotyl cell walls from 3- to 20-day-old plants are, in most respects, very similar to the neutral sugar compositions reported by Nevins *et al.* (48) for the cell walls from the same tissues. The most dramatic changes in the levels of each of the cell wall constituents occur between 5 and 10 days after planting, the period during which hypocotyl elongation occurs (48). During this period, the amounts of the polysaccharide components which are characteristic of the primary cell wall (44), galacturonic acid, galactose, arabinose, and rhamnose, decline sharply. The proportion of xylose, generally considered to be a secondary wall component, increases during this period.

The data presented in Figures 7 and 8 are also in agreement with the neutral sugar compositions of cell walls from 8- and 20-day-old hypocotyl tissue from the three bean varieties and from the various morphological parts of the 8-day-old Red Kidney bean plant which have previously been reported (43). The data presented in this report for the galacturonic acid content of the cell walls of these tissues extends the results of the previous study to a new class of compounds and also permits the characterization of another major portion of the cell wall of these tissues.

A major difference between the compositions of Red Kidney bean hypocotyl cell walls reported here and those reported by Nevins *et al.* (43) is the failure to detect the large amounts of glucose observed in the earlier study to be present in the cell wall polysaccharides of 4- to 9-day-old bean hypocotyls. The results of the present study indicate a glucose content of approximately 4% for Red Kidney hypocotyl cell walls 4 days after planting. Nevins *et al.* (4) reported a glucose content for these walls of approximately 26%. In an effort to resolve this difference, the cell wall preparation from day 4 Red Kidney bean hypocotyls used in the present study was analyzed using the technique employed by Nevins *et al.* (43). This analysis indicated a glucose content of approximately 5% for these walls.

In view of this result, one can conclude that the large amounts of glucose detected in the study by Nevins *et al.* (43) are not present in the cell wall preparations employed in the present study. It should be noted that the experimental plants in the study by Nevins *et al.* (43) were kept in the dark for only 10 hr to deplete their starch reserves prior to harvesting and wall preparation. In the present study, all plants were kept 30 hr in the dark prior to harvesting and preparation of the cell wall material. Even when this treatment was used, starch was detected in some wall preparations. In addition, the α-amylase treatment used by Nevins *et al.* (43) (10 units of enzyme for 10 min at 25 C) appears insufficient to conclude that no starch is present in the cell wall preparations. In the course of the present study, it has been observed that 18 to 24 hr treatment with 56 units of *Bacillus subtilis* α-amylase is necessary to remove completely the starch from hypocotyl cell wall preparations. No starch is observed to have been released after 2 hr treatment. In addition, had Nevins *et al.* (43) used the I_2-KI test to check their cell wall polysaccharide preparations for starch con-

tamination, these results would also have been misleading if the cell walls had not been heated in water before testing for starch. It has been observed that cell wall preparations which are contaminated with starch do not give a positive I_2-KI test unless they have been heated at 60 C in water for 10 min (K. W. Talmadge, unpublished results). When these facts are considered, it appears likely that the large amounts of glucose reported in the earlier study as part of the cell wall of 4- to 9-day-old Red Kidney bean hypocotyls were the result of contamination of the cell wall preparations with starch.

The method of uronic acid analysis described in this report represents an important improvement over color reactions, titrimetry, and enzymatic assays in that each of the uronic acid components which can be liberated from higher plant polysaccharides can be resolved and determined quantitatively. The ability to determine accurately such minor acidic components as glucuronic acid and 4-O-methyl-glucuronic acid becomes increasingly important as one turns from the analysis of total cell wall polysaccharide to the analysis of the individual polymers which comprise the wall. In various xylans, for example, D-glucuronic acid is a major constituent (69).

Gas chromatographic analysis of the neutral sugar components of plant cell wall polysaccharides as the alditol acetate derivatives has become a routine technique in a number of laboratories (6, 13, 14, 16, 21, 25, 27, 68). The method described here, which extends this technique to encompass uronic acid analysis, retains the features which make gas chromatography of the alditol acetate derivatives attractive for neutral sugar analysis. The preparation of the derivatives remains straightforward, and only a single derivative is formed from each polysaccharide component. In addition, gas chromatographic column material is available which permits resolution of the peracetyl derivatives of all of the common polysaccharide constituents.

In view of these advantages, and of the modest additional effort required to include the uronic acids in the analysis of wall polysaccharides, it is hoped that the technique described here will be useful in establishing the roles of the uronide-containing polymers in the structure and function of the plant cell wall. The analysis of cell wall polysaccharide composition may, for example, prove a fruitful method for investigating such questions of plant morphogenesis as the mechanisms of abscission (2, 38) and of auxin action (18). Such phenomena as the role of the cell wall and of cell wall-degrading enzymes in plant pathogenesis (5) also seem amenable to clarification using the method described here.

LITERATURE CITED

1. Abdel-ahker, M., J. K. Hamilton and F. Smith. 1954. The reduction of sugars with sodium borohydride. J. Amer. Chem. Soc. 73: 4691-4692.
2. Abeles, F. B. 1969. Abscission: role of cellulase. Plant Physiol. 44: 447-452.
3. Albersheim, P. 1965. The substructure and function of the cell wall. *In:* J. Bonner and J. E. Varner, eds. Plant Biochemistry. New York, Academic Press. pp. 151-186.
4. Albersheim, P., D. J. Nevins, P. D. English, and A. Karr. 1967. A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohyd. Res. 5: 340-345.
5. Albersheim, P., T. M. Jones, and P. D. English. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7: 171-194.
6. Auriol, P., and A. Touzé. 1970. Sur les polyosides des parois cellulaires d'organes végétaux sains et parasites. Physiol. Vég. 8: 263-275.
7. Bateman, D. F. and S. V. Beer. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. Phytopathology 55: 204-211.
8. Bateman, D. F., T. Kosuge, and W. W. Kilgore. 1970. Purification and properties of uronate dehydrogenase from *Pseudomonas syringae*. Arch. Biochem. Biophys. 136: 97-105.
9. Bateman, D. F. 1970. Depletion of galacturonic acid content in bean hypocotyl cell walls during pathogenesis by *Rhizoctonia solani* and *Sclerotium rolfsii*. Phytopathology 60: 1846-1847.
10. BeMiller, J. N. 1967. Acid-catalyzed hydrolysis of glycosides. Advan. Carbohyd. Chem. 22: 25-108.
11. Bishop, C. T. 1964. Gas-liquid chromatography of carbohydrate derivatives. Advan. Carbohyd. Chem. 19: 95-147.
12. Blake, J. D. and G. N. Richards. 1968. Problems of lactonisation in the analysis of uronic acids. Carbohyd. Res. 8: 275-281.
13. Blake, J. D. and G. N. Richards. 1970. A critical reexamination of problems inherent in compositional analysis of hemicelluloses by gas-liquid chromatography. Carbohyd. Res. 14: 375-387.
14. Borchardt, L. G. and C. V. Piper. 1970. A gas chromatographic method for carbohydrates as alditol-acetates. Tappi 53: 257-260.
15. Brower, H. E., J. E. Jeffery, and M. W. Folsom. 1966. Gas chromatographic sugar analysis in hydrolyzates of wood constituents. Anal. Chem. 38: 362-364.
16. Buchala, A. J., C. G. Fraser, and K. C. B. Wilkie. 1971. Quantitative studies on the polysaccharides in the nonendospermic tissues of the oat plant in relation to growth. Phytochemistry 10: 1285-1291.
17. Cheminat, A. and M. Brini. 1966. Separation par chromatographic en phase gazeuse de quelques monosaccharides. Bull. Soc. Chim. France 80-83.
18. Cleland, R. 1971. Cell wall extension. Annu. Rev. Plant Physiol. 22: 197-223.
19. Crowell, E. P. and B. B. Burnett. 1967. Determination of the carbohydrate composition of wood pulps by gas chromatography of the alditol acetates. Anal. Chem. 39: 121-124.
20. Dische, A. 1962. Color reactions of carbohydrates. *In:* R. C. Whistler and M. L. Wolfrom, eds. Methods in Carbohydrate Chemistry, Vol. 1. Academic Press, New York. pp. 477-515.
21. English. P. D. and P. Albersheim. 1969. Host-pathogen interactions. I. A correlation between α-galactosidase production and virulence. Plant Physiol. 44: 217-224.
22. English. P. D., J. B. Jurale, and P. Albersheim. 1971. Host-pathogen interactions. II. Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. Plant Physiol. 47: 1-6.
23. Gunner, S. W., J. K. N. Jones, and M. B. Perry. 1961. The gas-liquid partition chromatography of carbohydrate derivatives. I. The separation of glycitol and glycose acetates. Can. J. Chem. 39: 1892-1899.
24. Gunner, S. W., J. K. N. Jones, and M. B. Perry. 1961. Analysis of sugar mixtures by gas-liquid partition chromatography. Chem. Ind. (London). 255-256.
25. Hoff, J. E. and M. D. Castro. 1969. Chemical composition of potato cell wall. J. Agr. Food Chem. 17: 1328-1331.
26. Holligan, P. M. 1971. Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. I. A review of techniques used for the separation, identification and estimation of carbohydrates by gas-liquid chromatography. New Phytol. 70: 239-269.
27. Holliagn, P. M. and E. A. Drew. 1971. Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. II. Quantitative analysis of standard carbohydrates, and the separation and estimation of soluble sugars and polyols from a variety of plant tissues. New Phytol. 70: 271-297.
28. Imanari, T. and Z. Tamura. 1967. Gas chromatography of glucuronides. Chem. Pharm. Bull. 15: 1677-1681.
29. Imanari, T., Y. Arakawa, and Z. Tamura. 1969. Gas chromatographic analysis of aldoses. Chem. Pharm. Bull. 17: 1967-1969.
30. Jaakonmaki, P. I., K. L. Knox, E. C. Horning, and M. G. Horning. 1967. The characterization by gas-liquid chromatography of ethyl-β-D-glucosiduronic acid as a metabolite of ethanol in rat and man. Eur. J. Pharmacol. 1: 63-70.
31. Knaak, J. B., J. M. Eldridge, and L. J. Sullivan. 1967. Systematic approach to preparation and identification of glucuronic acid conjugates. J. Agr. Food Chem. 15: 605-609.
32. Kruppa, R. F., R. S. Henly, and D. L. Smead. 1967. Improved gas-chromatography packings with fluidized drying. Anal. Chem. 39: 851-853.
33. Lamport, D. T. A. 1970. Cell wall metabolism. Annu. Rev. Plant Physiol. 21: 235-270.
34. Lehtonen, A. A., J. K. Karkkainen, and E. O. Haahti. 1966. Detection of iduronic acid in mucopolysaccharide mixtures. Anal. Biochem. 16: 526-530.
35. Lehtonen, A., J. Karkkainen, and E. Haahti. 1966. Gas-chromatographic characterization of the electrophoretically separated fractions of acid mucopolysaccharides. J. Chromatog. 24: 179-182.
36. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
37. Matsui, M., M. Okada, T. Imanari, and Z. Tamura. 1968. Gas chromatography of trifluoroacetyl derivatives of alditols and trimethylsilyl derivatives of aldonolactones. Chem. Pharm. Bull. 16: 1383-1387.
38. Morré, D. J. 1968. Cell wall dissolution and enzyme secretion during leaf abscisson. Plant Physiol. 43: 1545-1559.
39. Morrison, I. M. and M. B. Perry. 1966. The analysis of neutral glycoses in biological materials by gas-liquid partition chromatography. Can. J. Biochem. 44: 1115-1126.
40. Nagel, C. W. and S. Hasegawa. 1967. The enzymic determination of galacturonic acid. Anal. Biochem. 21: 411-415.
41. Neish, A. C. 1965. Coumarins, phenylpropanes, and lignin. *In:* J. Bonner and

J. E. Varner, eds. Plant Biochemistry. Academic Press, New York. pp. 581–614.

42. NELSON, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375–380.
43. NEVINS, D. J., P. D. ENGLISH, AND P. ALBERSHEIM. 1968. Changes in cell wall polysaccharides associated with growth. Plant Physiol. 43: 914–922.
44. NORTHCOTE, D. H. 1963. Changes in the cell walls of plants during differentiation. *In:* Symposia of The Society for Experimental Biology. Number XVII, Cell Differentiation. pp. 157–174.
45. NORTHCOTE, D. H. 1969. The synthesis and metabolic control of polysaccharides and lignin during the differentiation of plant cells. *In:* P. N. Campbell and G. D. Greville, eds. Essays in Biochemistry, vol. 5. Academic Press, New York. pp. 89–137.
46. OADES, J. M. 1967. Gas-liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates. J. Chromatogr. 28: 246–252.
47. OVODOV, Y. S. AND E. V. EVTUSHENKO. 1967. Analysis of sugar mixtures by gas-liquid chromatography. J. Chromatogr. 31: 527–530.
48. PERRY, M. B. AND R. K. HULYALKAR. 1965. The analysis of hexuronic acids in biological materials by gas-liquid chromatography. Can. J. Biochem. 43: 573–584.
49. PETERSSON, G., O. SAMUELSON, AND H. RIEDL. 1967. Gas chromatographic separation of aldonic acids as trimethylsilylated derivatives. Svensk Papperstidning. 70: 371–375.
50. RAUNHARDT, VON O., H. W. H. SCHMIDT, AND H. NEUKOM. 1967. Gas-chromatographische Untersuchungen an Uronsäuren und Uronsäurederivaten. Helv. Chim. Acta 50: 1267–1274.
51. RAY, P. M. 1963. Sugar composition of oat-coleoptile cell walls. Biochem. J. 89: 144–150.
52. RAYMOND, W. R. AND C. W. NAGEL. 1969. Gas-liquid chromatographic determination of oligogalacturonic acids. Anal. Chem. 41: 1700–1703.
53. SAEMAN, J. F., W. E. MOORE, R .L. MITCHELL, AND M. A. MILLETT. 1954. Techniques for the determination of pulp constituents by quantitative paper chromatography. Tappi 37: 336–342.
54. SAWARDEKER, J. S., J. H. SLONEKER, AND A. JEANES. 1965. Quantitative determination of monosaccharides as their alditol acetates by gas-liquid chromatography. Anal. Chem. 37: 1602–1604.
55. SCHLESINGER, H. E., H. C. BROWN, D. L. MAYFIELD AND J. R. GILBREATH. 1953. Procedures for the preparation of methyl borate. J. Amer. Chem. Soc. 75: 213–215.
56. SCHMIDT, H. W. H. AND H. NEUKOM. 1966. Zur Säurekatalysierten Reaktion von Galakturonsäure mit Methanol. II. Isomerverteilung und Isomerisierung der Galakturonoside. Helv. Chim. Acta 49: 510–516.
57. SHAPIRA, J. 1969. Identification of sugars as their trifluoroacetyl polyol derivatives. Nature 222: 792–793.
58. SJÖSTRÖM, E., P. HAGLUND AND J. JANSON. 1966. Separation and quantitative determination of aldonic acids by gas-liquid chromatography. Acta Chem. Scand. 20: 1718–1719.
59. SJÖSTRÖM, E., P. HAGLUND AND J. JANSON. 1966. Quantitative determination of carbohydrates in cellulosic materials by gas-liquid chromatography. Svensk Papperstidning. 69: 381–385.
60. SOMOGYI, M. 1952. Notes on sugar determination. J. Biol. Chem. 195: 19–23.
61. SWEELEY, C. C., R. BENTLY, M. MAKITA, AND W. W. WELLS. 1963. Gas-liquid chromatography of trimethysilyl derivatives of sugars and related substances. J. Amer. Chem. Soc. 85: 2497–2507.
62. TAMURA, Z. AND E. IMANARI. 1964. Gas chromatography of D-glucuronides. Chem. Pharm. Bull. 12: 1386–1388.
63. TAVAKOLI, M. AND R. C. WILEY. 1968. Gas chromatographic analysis of fruit tissue polysaccharides. Proc. Amer. Soc. Hort. Sci. 92: 772–779.
64. THORNBER, J. P., AND D. H. NORTHCOTE. 1961. Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissue in trees. 2. Carbohydrate constituents of each main component. Biochem. J. 81: 455–464.
65. TRACEY, M. V. 1948. A manometric method for the estimation of milligram quantities of uronic acids. Biochem. J. 43: 185–189.
66. UPDEGROFF, D. M. 1969. Semimicro determination of cellulose in biological materials. Anal. Biochem. 32: 420–424.
67. VANDENHEUVEL, W. J. A., AND E. C. HORNING. 1961. Gas chromatographic separations of sugars and related compounds as acetyl derivatives. Biochem. Biophys. Res. Comm. 4: 399–403.
68. VAN ETTEN, H. D. AND D. F. BATEMAN. 1969. Enzymatic degradation of galactan, galactomannan, and xylan by *Sclerotium rolfsii*. Photopathology 59: 968–972.
69. WHISTLER, R. L. AND E. C. RICHARDS. 1970. Hemicelluloses. *In:* W. Pigman and D. Horton, eds. The Carbohydrates: Chemistry and Biochemistry, 2nd Edition, vol. 5, II A, chap. 37. Academic Press, New York. pp. 447–469.
70. WILEY, R. C., M. TAVAKOLI, AND M. D. MOORE. 1966. Determination of hexuronic acids in fruit by gas chromatography. Proc. Amer. Soc. Hort. Sci. 89: 34–39.
71. WILEY, R. C. AND M. TAVAKOLI. 1969. Trimethylsilyl derivatives of commercial pectins. Food Technol. 23: 167–168.
72. WOLFROM, M. L., H. B. WOOD, AND K. ANNO. 1951. Sodium borohydride as a reducing agent for acids, lactones and esters. Abs. Papers Amer. Chem. Soc. 119: 8Q.