PARTIAL STRUCTURAL CHARACTERIZATION OF PECTINS FROM COTTON CELL WALLS USING SELECTIVE HYDROGEN FLUORIDE SOLVOLYSIS

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CHAPTER I

INTRODUCTION

The biology and chemistry of plant cell growth are of great interest to scientists but are still not well understood. One way to look at this problem is to study cell wall structure. The cell wall, is an envelope around plant cells that limits cell growth (Cleland, 1971) but must also grow as the cell grows. A better understanding of cell wall chemistry, and the changes that takes place during growth should help to explain cell wall functions. The cell wall gives the cell and thus ultimately the plant, strength and form. It surrounds the plasma membrane that defines the cell's boundary and is permeable to most molecules.

The two types of plant cell walls are referred to as primary and secondary cell walls (Albersheim, 1965). Young cells are enclosed by a primary wall that is thin and enlarges quickly as the cell grows. The primary cell wall defines both the growth rate and final size and shape of plant cells. The secondary wall of a mature, non-growing cell is thicker, and has a shape more distinctive than that of the generally box- like primary wall. It is this cell wall that provides structural support for mature plant parts. The middle lamella is an amorphous intercellular layer between the primary walls of adjacent cells. It is the first layer to be formed when a cell divides and is therefore the initial partition between newly formed daughter cells.

The constituents that make up the walls are complex carbohydrates, proteins, lignin, water and minerals. Complex carbohydrates are the main (80-90%) constituents of cell walls. The carbohydrate composition of cell walls varies both between plant species and between different tissues in a single plant: the same general polysaccharides are found in

very different proportions. Complex carbohydrates of the walls of the different cells in individual organs and tissues of a single plant are also different. As the shape of cells varies, the constituents of the walls also vary. As the cell wall is made up of complex polymers, it is necessary to understand how the different polymers are arranged physically throughout the wall and how these polymers are synthesized during growth. A more complete knowledge of the structure of the cell wall and biosynthesis of plant cell wall polymers could explain the function of cell walls in terms of their structure and may offer opportunities for regulating the growth of crop plants.

The walls of plant cells not only affect the size and shape of the cells that they surround, but also are a barrier to infection by pathogens (Albersheim and Anderson, 1975). Pathogens may have to penetrate the walls to cause infection. Many pathogens secrete enzymes that degrade cell wall: pectinases, hemicellulases and cellulases. Degradation of different cell wall polymers is an important event during infection. The structural complexity of the wall has made workers in this field wonder about the hidden function of cell wall. Recent research by several workers has shown that the fragments of cell wall polymers released during infection by a pathogen act as regulatory molecules (Mc Neil *et al.,* 1984). These fragments can trigger a defensive mechanism, that makes it possible for the plant to protect ifself. Understanding the complete structure of cell walls is important for learning more about host-pathogen interactions in plants.

Pectins are a major component of primary cell walls of dicots and yet the structures, which are heterogeneous and complicated, are little known. A more detailed knowledge of their structure should allow a better understanding of how pectins are involved in cell wall function. Pectins may play a role in cell expansion, cell adhesion, disease responses, and perhaps cell differentiation and recognition. Pectins are complex polysaccharides rich in α1-4 linked galacturonic acid residues. Pectins also contain neutral sugars such as rhamnose, arabinose, xylose, galactose and glucose. Pectins are heterogeneous with respect to both chemical structure and molecular weight.

Sycamore cell wall pectin appears to be composed of at least 3 regions (Mc Neil *et al.*, 1984). 1) A homopolymer of galacturonic acid, partially methyl esterified, called homogalacturonan (HG); 2) a region containing alternately linked D-galacturonic acid and L-rhamnose residues referred to as Rhamnogalacturonan I (RG I); and 3) a complex region containing at least 15 different sugars called Rhamnogalacturonan II (RG II). This project was undertaken to isolate and characterize the RG I region of pectin from suspension cultured cotton cell wall. Suspension cultured cells rather than plant tissues were the source of cell walls for detailed study of structure because of the uniformity of the cell type in cultured cells, and the absence of secondary cell walls. Cotton is a good system for the structural study because of the active research on several aspects of cotton and bacterial blight of cotton at OSU. These studies include the genetic improvement of cotton, role of phytolexins in resistance to the disease, electron microscopy of responses in the plant and differences in responses of tissue cultured cotton cell lines to the bacteria. A complete knowledge of the structure of the pectic polymers from cotton could improve our understanding of the mechanisms involved in the disease resistance in cotton.

The general approach to structure determination is to break complex polysaccharides into smaller, more easily characterizable pieces. Classical methods for extracting pectins from cell walls include hot water extraction, treatment with chelating agents, use of specific enzymes, periodate oxidation and partial acid hydrolysis. All of these methods have advantages and disadvantages. Mort (1983) has shown that anhydrous hydrogen fluoride (HF) can be used to selectively cleave polysaccharides. Experiments with various polysaccharides using a range of temperatures have shown that HF solvolysis is similar to partial acid hydrolysis in some ways, but the labilities of different linkages depend on temperature (Mort and Bauer, 1982; Mort , 1983; Mort *et al.*, 1983; Kuo and Mort, 1986; Kuo *et al.*, 1987). Another great advantage of HF over partial acid hydrolysis is that acyl substituents such as acetate and methyl esters are not cleaved from the sugars. HF is a liquid at pressures less than 1.5 atm and at temperatures between 30°C and -90°C. This

gives a broad range of temperature for treating polysaccharides. Work with bacterial polysaccharides has shown that at -23°C linkages susceptible to HF solvolysis are arabinosyl, xylosyl, rhamnosyl, galactosyl, and fucosyl. Galacturonosyl linkages, cellulose, acetate esters, and methyl esters are resistent to HF solvolysis at -23°C. Susceptibility of rhamnosyl but not galacturonosyl linkages makes this temperature ideal to extract pectic polymers from cell walls. Relative uniformity of the reaction and the availability of the reagent are great advantages of using HF.

In sycamore Rhamnogalacturonan I is a polymer made up of strictly repeating disaccharide galacturonosyl rhamnose (α1--2) (Lau *et al.*, 1985). The length of this polymer is not known. Approximately half of the rhamnose residues have side chains attached to the 4 position. A major objective of this study was to see whether RG I is present in cotton cell walls, and if present, whether it is made up of a strictly repeating disaccharide galacturonosyl rhamnose as in sycamore walls. Since HF at -23°C cleaves rhamnosyl linkages and not galacturonosyl linkages, it should be possible to obtain the disaccharide galacturonosyl rhamnose if significant amounts of RG I are present in the walls. Any side chains on the polymer will be removed, as neutral sugar linkages are susceptible to HF at this temperature. Any acyl substituents present will be retained and can be identifed by n.m.r. spectroscopy.

Results are organized as follows. The apparatus used for solvolysis of cell walls and the sequential extraction procedure for isolating pectins from cell walls are described in detail. Preliminary characterization of the cell wall including compositional analysis of carbohydrates and proteins is discussed. The major portion of the thesis describes the isolation and characterization of the disaccharide galacturonosyl rhamnose. This study showed that cotton as well as tomato, tobacco, and carrot cell walls contain a significant amount of RG I. The disaccharide accounted for ~ 6% of Acala 44 cotton suspension cell walls. About 40% of the disaccharide was acetylated at the 3 position of galacturonic acid. However, HF solvolysis induced an artifactual cyclization of the disaccharide during its

generation involving the 2 postion of galacturonic acid and 1 position of rhamnose. The disaccharide galacturonosyl rhamnose isolated from suspension cultures of two cotton lines, Im 216 and Acala 44 leaf walls, tomato suspension walls, tobacco leaf walls, and carrot root walls all showed the presence of an acetate group on the 3 position of galacturonic acid. This suggests that acetylation of RG I is a basic structural phenomenon found in most plants. The function of RG I acetylation is not known, but some functions are proposed.

Pectic fragments other than RG I isolated by HF solvolysis are discussed, mainly the homogalacturonan region of pectins. This region accounted for about 18% of the cotton suspension cell walls. About 15-20% of the homogalacturonan region was methyl esterified. There were no acetate esters in this region. The fragments isolated from this region had a wide range of molecular weight, mostly above a degree of polymerization of 20.

The third section describes results obtained when cell walls were treated with HF at temperatures other than -23°C. Solvolysis of Acala 44 cotton suspension cell walls at -40°C produced larger fragments of RG I with some side chains attached. Study of these fragments helps in determining the distribution of acetate esters and side chains in the RG I backbone. The preliminary characterization of these fragments is discussed. More research is needed to completely characterize these fragments and to explain the release of these fragments from cell walls at this temperature. Preliminary results obtained during solvolysis of cell walls at 0°C and -73°C are also discussed.

CHAPTER II

LITERATURE REVIEW

Cell Wall Structure

Plant cell walls have several biological roles, which have made knowledge of their structure, biosynthesis, and degradation of great interest. Structural analysis of the cell wall is still at the stage of identifying and elucidating the covalent structure of the macromolecular components of the primary cell wall. Sparse attention has been paid to the secondary, tertiary and quarternary structure of the polysaccharides in the cell wall (Dea *et al.*, 1973, and 1977; Rees, 1972). Other areas, like ultrastructural distribution of polymers within the wall, integration of newly synthesized macromolecules into the wall, and the biochemistry of wall growth are not clearly established (Dey and Brinson, 1983).

Several workers have contributed towards the understanding of the structure of the primary cell wall. Both Northcote (1953; 1963; 1969; 1972) and Lamport (1963a,b; 1964a,b; 1965; 1967,and 1969) are responsible for pioneering work in this regard. Northcote (1972) considered the cell wall to be growing and constantly changing. It consisting of a dispersed phase of microfibrils within a complex, continuous matrix. According to Northcote's model during the growth of the cell, the polymers of the wall interact and change. This results in alteration in the properties of the wall and this can be correlated with a variation in its function. A variation in the environment of the growing cell, brought about by interactions between cells, by gaseous or aqueous changes in the surrounding medium, or by an increase or decrease in the stresses and strains applied to the cell wall, would cause a change in the properties of the wall. This would modify the

properties of the wall; however the fundamental structure, consisting of microfibrils dispersed in a complex matrix, was considered constant.

According to Lamport (1965) the primary cell wall is a single, "bag shaped" macromolecule having a coherent, cross linked structure with bonds present between the hydroxy - L- proline - rich, wall protein "extensin" and wall polysaccharides. Individual polysaccharides are cross linked to each other. The other workers in the structural study of cell wall include Selvedran and coworkers who worked with runner bean hypocotyls (1972; 1975 a,b,c), Monroe and coworkers who worked with lupin hypocotyls (1972; 1974; 1976a,b), Ray and his colleagues worked with oat coleoptiles (1978).

Albersheim and coworkers have investigated the primary wall structure of a number of suspension cultured cells including sycamore, douglas fir, red kidney bean, wheat, rice, oat, sugar cane, and brome grass, rye grass endosperm, and barley aleurone layer (Talmadge *et al.,* 1973; Bauer *et al.,* 1973; Keegstra *et al.,* 1973; Wilder *et al.,* 1973; Valent *et al.,* 1974; Burke *et al.,* 1974; Mc Neil *et al.,* 1975; Darvill *et al.,* 1978; Weinstein and Albersheim, 1979). From their detailed structural study on sycamore cell walls, they have proposed a primary cell wall structure which will be described later.

Classically, wall polysaccharides have been separated into three fractions. The pectic polysaccharides are polymers that can be extracted by hot water, ammonium oxalate solution and weak acids, or chelating agents. The hemicelluloses are polymers that can be extracted by relatively strong alkali, and the residue remaining after the extraction of pectin and hemicelluloses is composed mainly of cellulose. Cellulose is the best known of all plant cell wall polysaccharides which is about 23% of dicot walls (Gardner and Blackwell, 1974; Kolpak and Blackwell, 1976; Preston, 1974; Mc Neil *et al.,* 1979). Cellulose is a linear β 1$\rightarrow$4 linked D-glucan. The D glucan chains of cellulose are aggregated together to form structures called microfibrils. The degree of polymerization of cellulose in secondary cell walls is about 14,000 (Preston, 1974; Marz-Figini and Schulz, 1966), but

primary walls exhibits a biphasic distribution in the degree of polymerization, most of it being either about 500 or between 2,500 and 4,500 (Blaschek *et al.*, 1982).

Hemicelluloses

Hemicelluloses are polymers that are probably covalently linked to pectin, and noncovalently associated with cellulose. They are strongly hydrogen bonded to cellulose (Bauer *et al.*, 1973). Xyloglucan is the most extensively studied hemicellulosic polysaccharide of primary cell wall. It was first isolated by Aspinall and coworkers from the medium of suspension cultured sycamore cell walls (Aspinall *et al.*, 1969) and later by Bauer and coworkers (Bauer *et al.*, 1973) from suspension cultured sycamore cell walls. Xyloglucan is present in dicot as well as monocot walls. Dicot walls contain about 20% of xyloglucan where as monocot wall contain only about 2% of xyloglucan (Darvill *et al.*, 1980). This cell wall polymer consists of a backbone of β 1→4 linked D-glucosyl residues, with D-xylosyl side chains α linked to O-6 of some of the glucosyl residues. Some of the xylosyl side chains have β-linked D-galactosyl residues, and α-linked L fucosyl residues attached to O-2. Occasionally arabinosyl residues are found linked to O-2 of some of the xylosyl residues (Mc Neil *et al.*, 1984). A nonasaccharide and a heptasaccharide were isolated from xyloglucan after treatment with an endo-β 1,4 glucanase. Xyloglucan isolated from the sycamore extracellular polysaccharides has recently been shown to be substituted with O-acetyl groups. The 2-linked β-D-galactosyl residue of the nonasaccharide was the dominant site of O-acetyl substitution. Both mono-O-acetylated and di-O-acetylated β-galactosyl residues were detected (York *et al.*, 1988). Xyloglucan has been isolated from pea-stem cell (Hayashi and Maclachlan, 1983) and from suspension cultured bean cell walls (Wilder and Albersheim, 1973) and has been shown to be composed of nonasaccharides and heptasaccharides with the same structure as above. In monocot cell wall xyloglucan, the glucosyl residues are less frequently substituted with xylosyl residues than in dicot xyloglucan (Kato *et al.*, 1981).

The Pectic Polysaccharides

Pectic polysaccharides make up ~35% of the dicot primary walls of sycamore. Pectic polysaccharides are rich in α-1->4 linked-D-galactosyl uronic acids. Pectins form three recognizable regions (Mc Neil *et al.*, 1984). 1) A region made up of α1→4 linked D-galactosyluronic acid residues is referred to as Homogalacturonan (HG). This region also contains about 10% neutral sugars and is also partially methyl esterified. These polymers form rigid, insoluble gels in the presence of calcium (Rees, 1982).

The second region is referred to as Rhamnogalacturonan I (RG I). This polymer has been purified from sycamore suspension cell walls (Mc Neil *et al.*, 1980). It has a molecular weight of ~200,000 (determined by gel filtration chromatography) and constitutes about 7% of the sycamore cell wall material. The polymer has a backbone made up of a repeating disaccharide unit → 4)-α-D-Gal$_p$A-(1→2)-α-L-Rha$_p$ -(→ (Lau *et al.*, 1985). Half of the rhamnose residues have side chains attached at O-4. Side chains were made up of neutral sugars mainly D-galactosyl and L-arabinosyl residues and were found to range in size from one to fourteen glycosyl residues. There are four distinct families of side chains attached to the backbone of RG I (Lau *et al.*, 1987). RG I like polymers have been isolated from sources other than sycamore, including midrib of tobacco, potato, onion, rice, but these have not been studied in detail (Mc Neil *et al.*, 1984). Studies by Aspinall and his associates (Aspinall *et al.*, 1967, Aspinall *et al.*, 1968 a,b; Aspinall and Jiang, 1974) on rape seed hull, soybean cotyledon, lucerne leaves and stem, and lemon peel, indicated that galacturonic acid residues are linearly linked α-(1→4), and that many L-rhamnose residues present in the chain were linked through O-2 to galacturonic acid. Methylation analysis of the intact polysaccharides from rape seed (Aspinall and Jiang, 1974) and lucerne (Aspinall and Molloy, 1968) showed that ~50% of the rhamnosyl residues to be 2-linked, and the other 50% to be 2,4-linked.

Darvill and coworkers (1978) have isolated the third pectic polysaccharide referred to as Rhamnogalacturonan II (RG II) from suspension cultured sycamore cell walls by

digestion with endo-α-1,4-polygalacturonase. Rhamnogalacturonan II consists of approximately 60 glycosyl residues, which include unusual sugars like 2-O-methyl fucose, 2-O-methyl xylose, apiose, and aceric acid. RG II is rich in rhamnose but rhamnosyl residues are 3-linked, 3,4-linked and 2,3,4-linked, in contrast to 2- and 2,4-linked rhamnose residues in RG I.

Primary cell walls also contain polymers that are almost pure arabinans (Darvill *et al.*, 1980, Steven and Selvendran 1980). These polymers are rich in 5-linked α-L-arabinofuranosyl residues. Primary cell walls also contain polymers that are nearly pure β-4-linked galactans (Eda and Kato, 1978; Meier, 1962). Some of the galactans also contain 6-linked galactosyl residues. There are two types of arabinogalactan in cell wall. The most common contain terminal, 3-, 6-, and 3,6-linked galactosyl residues and 3- or 5-linked arabinofuranosyl residues (Aspinall *et al.*, 1968). Arabinose and galactose are found in other cell wall polymers such as RG I, RG II, extensin, xyloglucan and glucurono arabinoxylans.

Cell wall polysaccharides also contain feruloyl ester substituents (Fry, 1983). Ferulic acid and p-coumaric acid were found esterified with galactopyranose and arabinopyranose residues of pectic polysaccharides from primary walls of spinach suspension culture cells. It is hypothesized that this causes cross linking of pectins in the cell wall by oxidative coupling. This cross linking could have a strengthening effect on the structure of the cell wall, restricting cell expansion.

Cell walls contain various glycoproteins. One of these is extensin, which is rich in hydroxyproline (hyp). This protein is associated with the cell wall via covalent bonds. This protein has been characterized by Lamport and his coworkers (Lamport and Northcote, 1960; Lamport and Miller, 1971; Mort and Lamport, 1971; Lamport *et al.*, 1973). L-arabinosyl residues are attached to the hyp residues and D-galactosyl residues are attached to the serine residues in extensin.

Cell Wall Model

A model of the primary cell wall of suspension cultured sycamore cells showing various interconnections between the constituent polymers was put forward by Keegstra *et al.,* in 1973. In this model, cellulose microfibrils are H-bonded to xyloglucan. Hydrogen bonding is supported by the observation that 8M urea and dilute base are able to extract partially the xyloglucan from endogalacturonase pretreated sycamore walls. The reducing end of the xyloglucan molecules may be covalently bonded to arabinogalactans. Xyloglucan is attached to the Rhamnogalacturonan main chain of the pectic polysaccharide via arabinan and 4-linked galactan side chains of the pectic polymer. From the reducing end of the Rhamnogalacturonan is a 3,6-linked arabinogalactan attached to serine residues of the cell wall protein. It is proposed that each Rhamnogalacturonan molecule is connected to several arabinogalactan chains, each radiating from a different cellulose fibril. Similarly, each cellulose fibril is connected to several rhamnogalacturonans by way of several xyloglucan chains. Cellulose fibrils are thus extensively cross linked. The primary cell wall may be pictured as consisting of cellulose rods embedded in an amorphous matrix of non cellulosic polysaccharides. This model however is not generally accepted. The main concern is that the occurrence of arabinogalactan as a constituent polymer within primary walls is highly tentative (Albersheim, 1975; Keegstra *et al.,* 1973). There are several open questions regarding the exact nature of the interconnections between the constituent polymers in the walls.

The work of Bailey and coworkers on mung bean (Bailey and Kauss, 1974; Monro *et al.,* 1974; 1975; 1976) hypocotyls, have raised objections to the Albersheim model. Their work showed that conditions that remove poly(glycosiduronic acid) from mung bean hypocotyls wall did not remove wall protein, which was unexpected from the early Albersheim model. Ten percent KOH at 20-24°C removed hemicelluloses without extracting poly(glycosiduronic acid) (Bailey and Kauss, 1974). According to the Albersheim model extraction of hemicellulose should be accompanied by release of both

extensin and uronic acid. So poly(glycosiduronic acid) appears not to be a component of a polymer bridge between xyloglucan and extensin. Six M guanidinium thiocyanate, which is a powerful chaotropic agent, removes about one third of the 10% KOH soluble hemicellulose from depectinated lupin hypocotyl. If the only linkage between hemicelluloses and cellulose microfibrils is by hydrogen bonding of xyloglucan, then this reagent should have extracted most of the hemicellulose and protein. Another fraction of the wall is extracted by 10% KOH at 0°C. Release of this fraction may require breaking of very alkali-labile covalent bonds.

From their study with mung bean hypocotyl walls, Bailey and coworkers concluded that poly(glycosiduronic acid) is not likely to be component of a polymer bridge between xyloglucan and extensin. Hemicellulose is associated with cellulose by more than hydrogen bonding and they suggested the involvement of a covalent linkage. They also suggested a more direct cellulose-protein association than one involving arabinogalactan, poly(glycosiduronic acid) or its side chains or xyloglucan.

Bailey and associates (Monro *et al.*, 1976) proposed a model of the primary cell wall of lupin and mung-bean hypocotyls. According to their model there is no covalent linkage between poly(glycosiduronic acid) and extensin. Covalent bonds between hemicelluloses and poly(glycosiduronic acid) are not involved in binding either into the wall structure. O-galactosyl-L-serine links do not play a significant part in binding hemicelluloses to extensin. A large proportion of the hemicellulose is bound into the wall structure by very alkali labile, covalent bonds. Some of the hemicellulose is attached to the wall by more alkali-stable bonds than those broken by 10% KOH at O°C. This hemicellulose fraction may provide a covalently bonded bridge between extensin and the cellulose fibrils. At least part of both the wall glycoprotein and the hemicellulose is strongly bonded to cellulose fibrils. Cellulose fibrils are oriented within the wall of elongating lupin-hypocotyl cells mainly in the transverse directions, that is at right angles to the direction of cell elongation. This model of hypocotyl challenges the existence of some of the linkages

between wall polymers proposed by Albersheim and coworkers (Keegstra *et al.*, 1973) and Lamport (1970) but does not actually indicate the nature of the bonds that are present. So the proposed wall structure depicts the types of network that might occur. It shows covalent extensin-polysaccharide association with unspecified bonding between extensin and the cellulose microfibrils, and the pectin network, not involving extensin, but interconnected at either guanidinium thiocyanate labile or O°-10% KOH-labile junctions. Bailey and coworkers also proposed that their partial model of the cell wall met the requirements for the stresses likely to be imposed on cellulose fibrils and cross-linking matrix polymers, in the walls of elongating cells under turgor pressure, better than the sycamore wall model.

As described by Dey and Brinson (1983) an accurate cell-wall model must eventually take into account stresses on the cell wall, orientation of wall components, detailed structure of wall polymers, and the exact nature of chemical bonds between wall components. However present knowledge of primary cell wall structure is too inadequate to allow any of the models currently proposed to be other than working hypotheses.

Usefulness of Anhydrous HF Solvolysis in Structural Analysis of Polysaccharides

Anhydrous HF has been shown to dissolve many organic and inorganic materials including gum arabic, India rubber and sealing wax (Gore, 1869). Fredenhagen and Cadenbach (1933) showed the dissolution of the polysaccharides of wood. Amino acids and a number of proteins were also shown to be soluble in HF (Katz, 1954).

The mechanism of action of HF on carbohydrates have been elaborated by Knirel *et al.* (1987). Cellulose, amylose, starch, xylan and inulin have been reacted with HF and the nature of the reaction between HF and these polysaccharides has been studied in detail (Mort and Parker, 1982; Defaye *et al.*, 1982 and 1985). These polysaccharides were found to dissolve in HF at temperatures above -20°C and undergo depolymerization to

give the corresponding glycosyl fluorides. These glycosyl fluorides were then converted to other products depending on the reaction conditions.

The ability of HF to dissolve polysaccharides is due to its formation of hydrogen bonds between the polysaccharide molecules (Defaye *et al.*, 1982). Subsequent depolymerization proceeds via protonation of the glycosidic oxygen and cleavage of the aglycone to give the glycosyl-cation (refer to Figure 1) in accordance with the known mechanism of acid hydrolysis of glycosides (Owerend, 1972). Solvolytic cleavage also is enhanced through the stabilization of the resulting glycosyl-cation by HF (Defaye *et al.*, 1982).

Secondary oligomerization can occur by glycosidation of hydroxyl groups of sugars by the glycosyl fluorides when the concentration of sugars is high. At concentrations of sugars less than 1% this is insignificant, but when the concentration becomes high on evaporation of HF from the reaction mixture, secondary oligomerization of the glycosyl fluorides becomes very prominent. This results in the formation of oligosaccharides involving different numbers of monosaccharides linked via different types of linkages (Mort and Parker, 1982; Defaye *et al.*, 1982; Hardt and Lamport, 1982). Solvolysis of inulin was found to proceed differently to give dianhydride (Defaye *et al.*, 1985). However secondary oligomerization was not observed when chitin was cleaved with HF (Mort, 1978; Mort and Lamport, 1977).

Glycosyl fluorides can also alkylate aromatic compounds via the Friedel-Crafts mechanism, where HF acts as an efficient catalyst (Wagner, 1965). Alkylation can be prevented by adding an excess of anisol to the reaction mixture during solvolysis of glycoproteins (Mort and Lamport, 1977). Addition of methanol can also inhibit alkylation, where the methanol reacts with glycosyl fluorides to form methyl glycosides (Mort, 1978; Sanger and Lamport, 1983). The stability of glycosyl fluorides varies greatly. α-Glycosyl fluoride can be obtained from the dissolution of cellulose or glucose in HF followed by quenching the reaction (Mort and Parker, 1982: Hardt and Lamport,

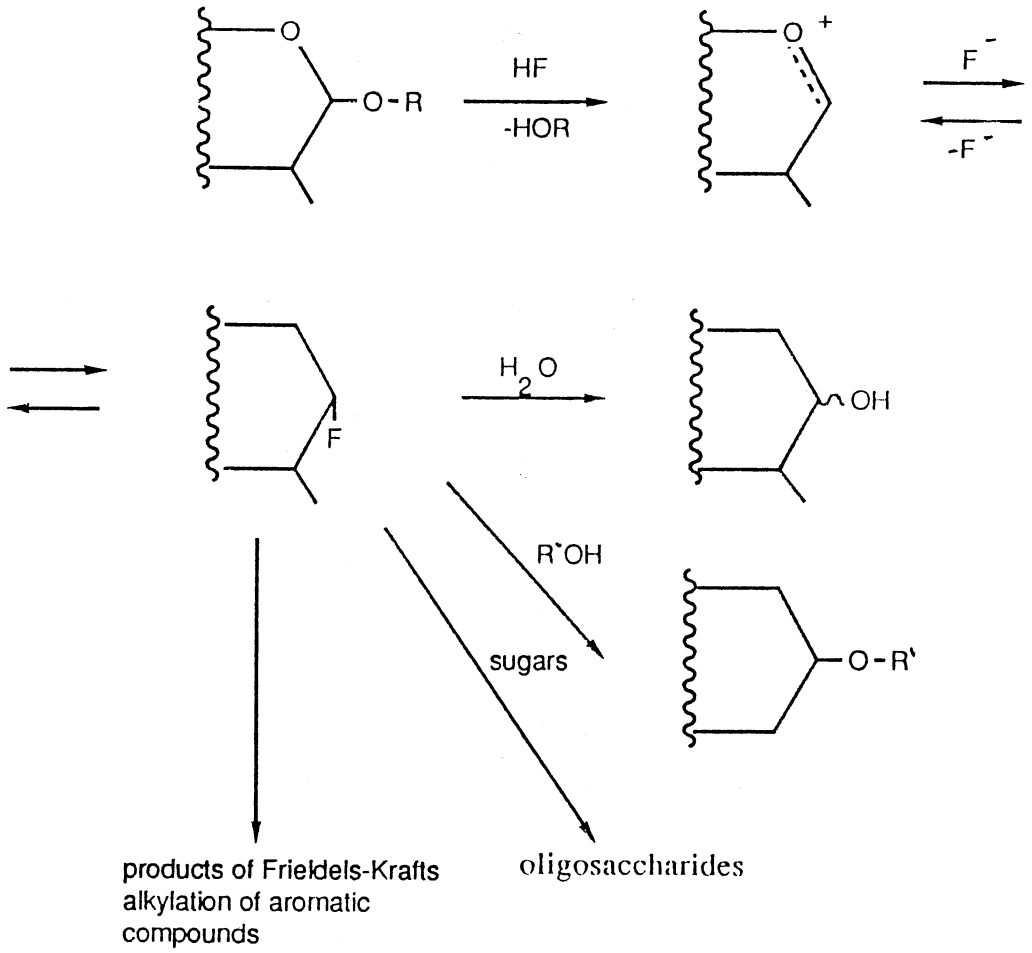

Figure 1. Possible Reactions of Sugars in Anhydrous Liquid HF.

1982). Some glycosyl fluorides have been used as substrates for glycosyl transferases (Figures and Edwards, 1976).

Liquid HF has been used in peptide and protein synthesis in the final deprotection step (Sakakibara and Shimonishi, 1965). Peptides are cleaved from the resin supports and from most of the commonly used protecting groups in a single step. Later, it was found that HF could be used to cleave sugars from glycoproteins, leaving the peptides intact (Mort and Lamport , 1977) and since then has been used for deglycosylation of glycoproteins. The degree of deglycosylation depends on the temperature of the solvolysis. Mort and Lamport (1977) also showed that the rates of cleavage of glycosidic linkages of aminosugars and neutral sugars in HF at 0°C were different. Neutral sugar linkages could be broken in HF at 0°C whereas the amino sugar linkages remained intact. This observation made it clear that HF can be used for selective cleavage of polysaccharides. The selectivity of HF towards sugar linkages made it possible to use this reagent for the cleavage of glycosidic linkages, and with practically no destruction of the sugar. It does not affect the N acyl substituents of amino sugars, and under certain conditions any O-acyl substituents also can be retained. Cleavage of linkages depends on the temperature of the reaction as well as the type of sugar involved. It also depends on the anomeric configuration of the sugar involved and the next sugar residue attached to it.

HF solvolysis has been used in the structural analysis of various bacterial polysaccharides especially in identifying acyl substituents. HF solvolysis at -23°C was used to selectively degrade the extracellular polysaccharides from the bacterium *Rhizobium japonicum* the structure of the repeating pentasaccharide was determined (Mort and Bauer, 1982; Mort *et al.*, 1983) and the acetylation in the O-4 position of galacturonic acid was identified. Acyl substituents were identified on the extracellular polysaccharides of *Rhizobium trifolii* and *Rhizobium leguminosarum* (Kuo and Mort, 1986). An unusual substituent D-3-hydroxy butanoate was also identified. Treatment of gellan gum with HF at -40°C generated oligosaccharides representing the repeating unit of the polymer. This

repeating tetrasaccharide had both acetic and L-glyceric ester attached to the 6 and 2 position, respectively, of the 3-linked β-D-Glc$_p$ residue (Kuo *et al.*, 1986).

CHAPTER III

MATERIALS AND METHODS

Preparation of Cell Walls

Source of Cell Walls

Cell walls were obtained from suspension cultured plant cells and from whole plant tissues. Suspension cultured plant cells from two varieties of cotton (*Gossypium hirsutum* L.) Acala 44 and Im 216, susceptible and resistant, respectively, to the bacterial pathogen *Xanthomonas campestris pv malvacearum*, the causal agent of cotton blight and from tomato (*Lycopersicon esculentum* L.) were used for cell wall preparation. Cell walls were also prepared from Acala 44 cotton leaves, tobacco (*Nicotiana tabacum* L., cv Burley White) leaves, and carrot (*Daucus carota* L.) root.

Preparation of Cell Walls from Suspension Cultured Cells

Acala 44 and Im 216 cotton suspension cells were grown as described by Ruyack *et al.*, 1979. Callus was initiated from stem or leaves of 30-90 day old cotton plants on Schenk and Hildebrandt (SH) solid medium. After 4 weeks callus tissue from the initial explant was excised and subcultured on fresh SH medium. Cultures were maintained at 30°C in continuous light and transferred every 30 days. Suspension cultures were initiated by transferring 0.5 to 1.0 g of callus from SH agar medium to 125 mL culture flasks containing 50 mL of liquid SH medium. The flasks were placed on a gyroshaker at 180 rpm in continuous light at 30°C. Alternately, 10 g of callus was transferred to 500 ml of liquid medium in a Bellco spinner flask. Two to three week old suspension cultured

cells in late log phase were used for cell wall preparation. Cell walls were prepared as described by York *et al.* (1986), except that instead of a pressure bomb, a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) was used to break the cells. Cultured cells were collected on a coarse scintered glass funnel and washed with 100 mM potassium phosphate (pH 7) five times, and with 500 mM potassium phosphate buffer (pH 7) four times to remove extracellular debris. The cells were suspended in one volume of 500 mM phosphate buffer and broken using a polytron for 10 min at high speed, keeping the sample cold (in ice) during the procedure. Preparations were homogenized until microscopic examination indicated that cell breakage was complete. The suspension of broken cells was then centrifuged at 2000 x g for 10 min.

The supernatent was decanted and the pellet was washed twice by suspending in five volumes of the 500 mM phosphate buffer and centrifuging at 2000 x g for 10 min. The washing procedure was repeated five times using distilled water. The washed cell wall pellet was suspended by vigorous stirring in 5 volumes of 1:1 chloroform-methanol and placed in a coarse scintered glass funnel. The organic solvent was removed by applying gentle suction to the funnel, and the cell walls were resuspended in five volumes of acetone. Cell walls were repeatedly washed with and resuspended in acetone before air drying.

Cell walls were tested for the presence of starch by an iodine test. Residual starch was removed by treating the walls with α amylase (*Bacillus* type II-A, from Sigma Chemical Co.) as described by York *et al.* (1986). The cell walls (10 mg/mL) were suspended in potassium phosphate buffer (100 mM, pH 7.0) containing α amylase (50 units/ml). The suspension was stirred for 48 hr at 25°C. The enzyme treated cell walls were centrifuged at 10,000 x g for 10 min. The cell walls were then washed extensively with distilled water followed by acetone and air dried.

Cell Wall Preparation from Plant Tissues

Fully expanded leaves from Acala 44 cotton plants (10-12 week old) and a tobacco plant (10-12 week old) were used to prepare walls. Leaves were rinsed with water and midveins removed before leaf tissue was cut into small pieces and homogenized throughly in one volume of cold 100 mM potassium phosphate buffer (pH 7.0) in a blendor. The mixture was centrifuged at 2000 x g for 10 min and the supernatent discarded. Cell walls were prepared from the residual cell suspension as described above.

Carrot tap roots purchased locally were washed and cut into small pieces, then blended throughly in 100 mM potassium phosphate buffer and centrifuged at 2000 x g for 10 min. The supernatent was discarded, the pellet was washed with phosphate buffer, and cell walls were prepared as described above.

Preparation of Extracellular Polysaccharides

Extracellular polysaccharides were prepared from Acala 44 and Im 216 cotton suspension culture fluid by a modification of the procedure described by York *et al.*, (1986). The suspension culture fluid was passed through a coarse scintered glass funnel. Ethanol (2.33 volume of 95%) was added to the filtrate and left at 4°C overnight. The precipitated polysaccharide was collected as a pellet by centrifugation and redissolved in 1 M imidazole buffer (pH 7.0). Residual salt and sucrose were removed by extensive dialysis: first against distilled water, then against 10 mM imidazole buffer (pH 7), then against 50 mM sodium acetate (pH 5.2), and finally against distilled water. The dialysed polysaccharide was lyophilized.

Cell Wall Composition Analysis

Carbohydrate Composition

Carbohydrate compositions of the whole cell walls and the different cell wall fractions were determined by gas chromatographic analysis of the trimethyl silyl glycosides. Twenty to fifty μg of dry samples were weighed on A Cahn 29 electrobalance and placed in teflon lined screw cap glass vials containing 10 nmol inositol as internal standard. Methanolysis and derivatization were performed by a modification of the method of Chaplin (1982). Two hundred μL of 1.5 M methanolic HCl and 50 μL of methyl acetate were added to the samples. The vials were sealed and placed in a heating block at 80°C for 16 h. After cooling, a few drops of t-butanol were added to each vial and the samples were evaporated to dryness under a stream of nitrogen. Trimethylsilylating reagent was prepared by mixing 1 part of Tri-Sil Concentrate (Pierce Chemical Co) and 3 parts of dry pyridine. Twenty-five μL of the reagent was added to the dried samples and left for 15 min at room temperature. The derivatized samples were evaporated just to dryness under a stream of argon, and redissolved in 90 μL of isooctane. One μL aliquots were injected into the gas chromatograph.

Carbohydrate composition was also determined for whole cell walls treated with hydrogen fluoride (HF) at 0°C for 15 min before methanolysis and derivatization.

Determination of Cell Wall Protein Composition

Protein content and composition of cell walls were determined by amino acid analysis of hydrolysed cell walls. The amino acids were derivatized with phenylisothiocyanate and analyzed by reverse phase chromatography (HPLC) as described by Heinrikson *et al.* (1984).

Hydrolysis of Cell Walls

About 1mg samples of dry cell walls were placed in teflon lined screw cap vials and a few drops of 6 N HCl were added before heating at 110°C for 24 hours. The vials were cooled before HCl was evaporated under nitrogen stream. The amino acids were dissolved in water to give a concentration of 1 μg/μL. Any samples containing solid matter were centrifuged and the supernatent was used for the analysis. Two hundred μL of the hydrolysed sample was dried under argon and 100 μL of coupling buffer (acetonitrile, pyridine, triethylamine and water, 10:5:2:3 ratio) was added along with 5 μl of phenyl isothiocyanate. Contents of the vials were mixed well and allowed to react for 5 min at room temperature. Volatile reagents were evaporated using an argon stream and vials and samples were then lyophilized for 1 h. The amino acid phenyl thiocyanate derivatives were dissolved in 50 μL 0.05 M ammonium acetate pH 6.0 and 5 μL aliquots were analysed by high performance liquid chromatography. Standards were prepared by combining 10 μL of the Pierce standard amino acid mixture (containing 25 nmols each of 17 amino acids) and 25 nmols of hydroxy proline. The mixture was derivatized as described above and dissolved in 250 μL of 0.05 M ammonium acetate buffer. Five μL aliquots were analysed by HPLC.

Determination of Cell Wall Lignin

Lignin in the cell walls was determined by a modification of the method of David *et al.* (1961). Samples of dry cell walls were weighed (~1 mg) and placed in 1 dram glass vials with teflon lined screw caps. Two hundred μL of 25% (by volume) of acetyl bromide in acetic acid was added to each vial, mixed with the sample, and the vials were placed in a 70°C heating block inside the hood for 30 min. The vials were stirred every 10 min to mix the reagent with the sample. After 30 min the vials were cooled to 15°C and 180 μL of sodium hydroxide and 1ml of acetic acid was added and mixed well. Vials were cooled and 20 μL of 7.5 M hydroxyl amine hydrochloride were added, mixed well,

and the A_{280} read. Samples were diluted if desired. Ferrulic acid was used as a standard, and the amount of lignin present was calculated from the standard curve.

Sequential Extraction of Polymers from Cell Walls

The different polymers present in the cotton cell walls were extracted according to Selvendran *et al.* (1985).

Extraction of Pectin

One hundred mg of Acala 44, Im 216 cotton suspension cell walls and Acala 44 cotton leaf cell walls were treated with 10 ml of 0.05 M cyclohexane diamine tetra acetate (CDTA) at room temperature for 6 h. Following centrifugation the residue was washed once with distilled water. The wash and supernatent were combined. The residue was reextracted with CDTA for 2 h at room temperature and centrifuged. The residue was then washed by centrifugation four times with distilled water. All supernatants and the washings were combined, dialysed extensively against distilled water and lyophilized. The resulting solid material was the CDTA extract (pectin).

The residue remaining after CDTA extraction was then extracted with 10 ml of 0.05 M sodium carbonate containing 20mM sodium borohydride for 16 h at 4°C. (This procedure removes pectin left behind in the walls.) It was centrifuged and the residue was washed with water. The residue was reextracted with 0.05 M sodium carbonate for 3 h at room temperature before centrifugation and a water wash. Washings and the supernatants were combined, the pH of supernatants plus washings was adjusted to 5 with acetic acid, the solution was extensively dialysed against water, and lyophilized. The resulting solid material was referred to as sodium carbonate extract. The remaining undissolved residue was referred to as depectinated cell wall. The CDTA and sodium carbonate extracts represent pectin originally present in the cell walls

Extraction of Hemicellulose

The depectinated cell wall material was stirred with 10 ml of oxygen free 1 M KOH containing 10 mM sodium borohydride for 2 h at 4°C and filtered using a scintered funnel. The insoluble residue was reextracted with 1 M KOH for 2 h at room temperature under argon or nitrogen and then filtered as before. The filtrates were combined and the pH of the filtrate was adjusted to 5 with acetic acid before dialysis against distilled water and lyophilization. The resulting solid material was the 1 M KOH extract.

The unsolubilized residue after KOH extraction was treated with oxygen free 100 mM sodium borohydride in 4 M KOH under argon or nitrogen for 2 h at room temperature. This was filtered and the residue was reextracted with 3-4% boric acid in 4 M KOH for 2 h at room temperature. The residue was washed with water. The filtrates and the washings were combined, and the pH was adjusted to 5 with acetic acid before dialysis against water and lyophilization. The resulting solid material was the 4M KOH extract.

The residue left behind after 4 M KOH extraction was then extracted with 4 M KOH containing 3-4% boric acid. The material, solubilized by 3-4% boric acid in 4 M KOH extraction were combined and the pH was adjusted to 5 with acetic acid before dialysis against water and then lyophilized. The final insoluble residue was washed extensively with water and lyophilized.

Extraction of Cell Wall Pectins Using Selective HF Solvolysis

Pectins from cell walls of cotton, tomato, tobacco and carrot were extracted by treating the cell walls with anhydrous hydrogen fluoride at -23°C for 30 min.

Hydrogen fluoride is a fuming volatile liquid at room temperature, the fumes are very reactive: toxic to living things, corrosive to glass and many metals. In order to safely handle this material, it is necessary to use an apparatus that is closed to the atmosphere and constructed of material with which the HF does not react. These requirements are met

with an apparatus constructed entirely of teflon and includes a manometer (Mort, 1983). Details of the stepwise operation of the apparatus are contained in Appendix A.

The basic components of the apparatus are shown in Figure 2. It consists of (1) a HF container attached to (2) a teflon main line divided into segments with (3) stopcocks allowing sections of the apparatus to be isolated and/or connected to teflon vessels. The main line is also connected to a (4) vacuum pump through a calcium oxide trap to prevent escape of HF into the pump or atmosphere at that end of the apparatus. The main line is connected to a manometer filled with mercury and silicon oil that allows determination of pressures in the main line or any of the individual vessels for leak detection.

The major steps in opertion of the apparatus are first the transfer of liquid HF from the steel storage HF tank 18 into the smaller teflon reservoir 11. This is done by evacuating the main line and the reservoir 11 with the vacuum pump 23, and then with only the HF reservoir 11 open onto the main line, the valve on the storage tank is opened. Movement of the HF is by cold distillation: the reservoir 11 is cooled with liquid nitrogen, and HF vapors are condensed there from the main line. When enough HF has been moved, the valve on the HF tank is closed, the stopcock above the reservoir is closed 1, and the main line is evacuated to draw remaining HF vapors into the calcium oxide trap 22.

The apparatus set up for solvolysis of cell walls is shown in Figure 2 (for detailed description of the reaction set up, see Appendix A). Dry cell walls (500 mg) were placed in reaction vessel 15 along with a stirrer bar. The whole apparatus was evacuated and leaks in any part of the system were checked and corrected. HF was transferred from the reservoir 11 to the vessel 12. This was done by cooling the vessel 12 with dry ice and acetone and allowing the HF to distill from the reservoir 11. About 20 mL of HF was required for 500 mg cell walls. Once HF was transferred to the vessel 12, it was allowed to reach temperature equilibrium. Vessel 12 and vessel 15 were immersed in the cooling bath of 95% ethanol, the temperature of which was maintained at -23°C using an

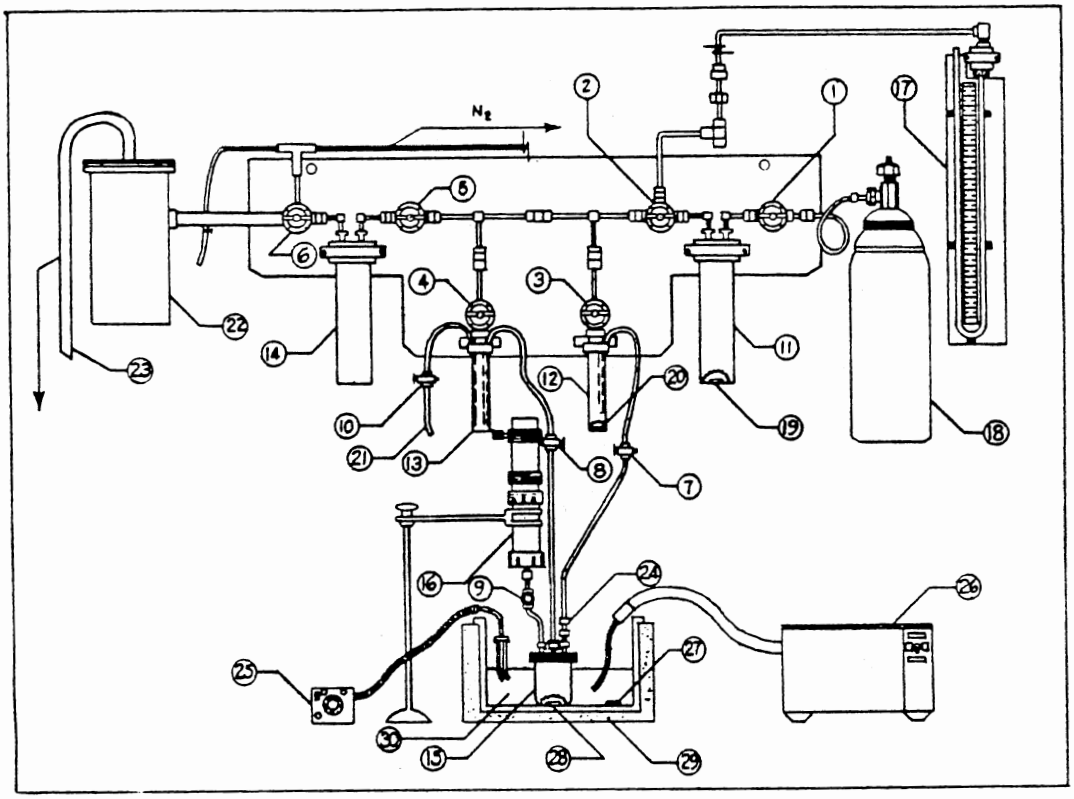

Figure 2. Schematic Representation of the Hydrogen Fluoride Solvolysis Apparatus. 1-8, 10, stopcocks; 9, teflon needle valve; 11-16, teflon and Kel-F vessels; 17, manometer; 18, hydrogen fluoride tank; 19, 20, stirrer bars; 21, exit to the sink for pressure release, if necessary; 22, calcium oxide trap; 23, connection to the vacuum pump; 24, 3 mm to 6 mm adaptor; 25, heater/regulator; 26, immersion cooler; 27, 28, stirrer bars; 29, insulated container; 30, 95% ethanol.

immersion cooler offset by a regulated immersion heater. Once the temperature equilibrium was reached, HF was transferred from vessel 12 to the reaction vessel 15. This was achieved by using slight nitrogen pressure. The reaction vessel 15 was stirred and the reaction was allowed to continue for 30 min. After 30 min, the reaction was stopped by adding cold (cooled by adding dry ice) ether (300 mL) from vessel 16. The cooling bath was removed and the reaction mixture was allowed to stir for 30 min and warm to room temperature. After 30 min the reaction mixture was filtered using a Teflon filter (50 mm diameter, fine grade, Savillex Corporation).

Sugars from the filtrate (HF/ether/sugar mixture) were recovered by evaporation and the sugars were dissolved in water and freeze dried. The residue in the filter unit was extracted with water thrice and the water extract and the water insoluble residue were freeze dried (refer to Schemes 1 and 2 in Figures 5 and 6).

Hydrogen Fluoride Solvolysis of Cell Walls at Different Temperatures

Acala 44 cotton suspension culture cell walls were also treated with anhydrous liquid hydrogen fluoride (HF) at temperatures other than -23°C by altering the temperature of the cooling bath. Three different temperatures selected for the reaction were 0°C, -40°C, and -73°C. For the 0°C reaction, the cooling bath was ice in water, and for the -73°C reaction, the bath was dry ice and acetone. All reactions were carried out for 30 min and the subsequent treatment of the walls was similar to that of the -23°C reaction.

Imidazole Extraction

The insoluble cell wall residue remaining after HF reaction and water extraction was extracted with 0.5 M imidazole buffer pH 7. Imidazole buffer (10 mg wall/ml of buffer) was added to the residue and stirred overnight at room temperature. The residue was suspended by sonication if necessary. The mixture was filtered using a scintered glass

funnel, washed twice with distilled water and filtered as above. The residue was then extracted with 0.5 M imidazole buffer by stirring overnight at 4°C. Alternately, the extract could be concentrated using an Armcon concentrator and then dialysed. In some cases 1 M instead of 0.5 M imidazole buffer was used for the second extraction. The residue was filtered and extensively washed (at least 10 times) with distilled water. All filtrates and washings were combined and extensively dialysed against distilled water (minimum 4 days with 3 changes a day) using a dialysis membrane (3500 MW or 6500 MW cutoff) before lyophilization. The solid material remaining after lyophylization was referred to as the imidazole extract.

Ethylenediamine Tetraacetic Acid (EDTA) Extraction

Pectin, if left behind in the HF treated walls after imidazole extraction was removed by using 0.1 M EDTA solution. The residue was stirred with 0.1 M EDTA solution (10mg/ml) overnight at room temperature. The mixture was filtered and the residue was washed extensively with distilled water. The washings were combined with the filtrate and dialysed extensively against distilled water before lyophilization. The solid material obtained was referred to as the EDTA extract.

Methylation Analysis

Various fractions obtained after HF treatment of cell walls were methylated using the method of Prehm (1980). This procedure does not remove acetic esters on sugars. Samples (0.5-1 mg) of disaccharide, oligosaccharide or polysaccharide were weighed and dried over phosphorous pentoxide in a vacum oven. These were then suspended in trimethyl phosphate (100 μL, Aldrich Chemical Co., Milwaukee, WI) by sonication. The methylating mixture: 15 μL of 2,6 Di-(tert. butyl) pyridine and 10 μL methyl trifluoromethanesulfonate (both from Aldrich Chemical Co.) were added to the suspension under nitrogen, and the mixture was allowed to react for 2 h at 50°C. Purified water was

added to the methylated samples and they were then loaded onto preconditioned Sep-Pak C_{18} Cartridges (Waters Associates, Inc., Milford, MA). The methylated sugars were eluted with methanol by a modification of the method described by Mort *et al.* (1983). The Sep-Pak cartridges were preconditioned with (i) 5 ml of methanol, and then (ii) 10 ml of water before the sample was applied. The samples were diluted with 5 ml of water to ensure a high enough solvent polarity for the sugars to be adsorbed and then applied slowly to the Sep-Pak with a 10 ml glass syringe. The Sep-Pak was next washed with 10 ml of water and the syringe was removed. The Sep-Pak was dried by pumping air through it using a dry glass syringe. Adsorbed sugars were eluted using 4 ml of methanol in two steps. Sep-Paks were then washed with more methanol and water as before and reused.

The methylated sugars eluted with methanol were divided into two equal portions. One portion was directly converted into partially methylated alditol acetates, while the other portion was reduced in order to convert uronic acids into hexoses before conversion to alditol acetates.

Reduction of Uronic Acid to Alcohol

A simplified version of the procedure described by Brown *et al.* (1982) was used. The methylated sample (~0.3 mg) was dried over phosphorous pentoxide in a vial, 100 µL of 2 M lithium borohydride in tetrahydrofuran (Aldrich Chemical Co.) was added, and the mixture was allowed to react for 2 h at 50°C. Excess lithium borohydride was decomposed by adding 10 µL of glacial acetic acid. The mixture was diluted with 5 ml water and purified using a Sep-Pak as described above. The eluted sugars were dried and stored in a desiccator.

Hydrolysis of Methylated Sugars

The dry methylated sugars and the methylated, reduced sugars were hydrolysed by adding 100 µL of 88% formic acid and reacting for 1 h at 100°C. After evaporating the acid with a nitrogen stream, 100 µL of 2 M trifluoroacetic acid was added, and hydrolysis was continued for 1.5 h at 121°C. The vials were cooled, the acid was evaporated, and the internal standard, 1000 nmols of inositol, were added to each sample.

Reduction of Samples

The procedure of Blakeney *et al.* (1983) was used for reduction of monosaccharides. The methylated, hydrolysed samples, and the methylated, reduced and hydrolysed samples were dried. Ten µL of 1 M ammonium hydroxide was then added, followed by 100 µL of 0.3 M potassium borohydride in dimethyl sulfoxide (Aldrich Chemical Co.). The mixture was allowed to react for 1.5 h at 40°C. After the reduction, excess potassium borohydride was decomposed by adding glacial acetic acid (10 µL).

Acetylation

The reduced samples from the previous step were acetylated using the procedure of Blakeney *et al.* (1983). Twenty µL of 1-methyl imidazole and 200 µL of acetic anhydride were added to each reduced sample. The reaction was allowed to proceed for 10 min at room temperature. Excess acetic anhydride was decomposed by addition of 5 ml of distilled water. The alditol acetates were purified using Sep-Pak C_{18} cartridges. Alditol acetates were eluted in 4 ml of methylene chloride in two steps. A pinch of anhydrous sodium sulfate was added to absorb any water present. The samples in methylene chloride were transferred into vials, and the methylene chloride was evaporated using a nitrogen stream. Samples were analyzed by gas chromatography.

Methylation Analysis Using a Modified Hakomori Method

Methylation was also performed using the Darvill *et al.* (1978) modification of the Hakamori (1964) method. This method removes esters present on sugars. The ionizing reagent was prepared as follows. A small teflon coated stirring bar and approximately 2 ml potassium hydride in mineral oil (stored in oil) were placed in a small flat bottomed bottle (a hypo vial). The bottle was sealed with a silicon stopper that had an inlet for nitrogen, and an outlet. A steady flow of nitrogen under low pressure was maintained and the bottle was stirred mechanically. The mineral oil was washed out using hexane by repeated addition and removal, until all the mineral oil was removed. Any remaining hexane was evaporated in a nitrogen stream. Small amounts of (0.25 μL) of dimethyl sulfoxide (DMSO) were added to the vial. Additions of DMSO was continued until a clear liquid formed in the bottle and no white solid and/or bubbles remained after shaking. Samples in 0.5 -1.0 mg quantity were placed in test tubes with rubber stoppers, and 0.5 ml of DMSO was added. The samples were sonicated if necessary. The samples were left in the nitrogen atmosphere for 15 min, and then 8-10 drops of the KH-DMSO reagent were added to each sample. The samples were mixed well and kept in a nitrogen atmosphere for 5 min. The treated samples were left at room temperature overnight.

Twenty five μL of iodomethane was added to each sample and mixed well. The addition of iodomethane was repeated every 5 min until the liquid in the test tube did not change color and no heat was generated after the addition of iodomethane. After 1h samples were separated on C_{18} Sep-Pak as described in the previous methylation method. The methylated samples were reduced, hydrolysed and converted to alditol acetates as described above.

Reducing End Detection of the Disaccharide

The disaccharide galacturonosyl-rhamnose, isolated from the cell walls after HF solvolysis at -23°C, was tested for the presence of a reducing end. The disaccharide was

dissolved in potassium borohydride solution (10 mg/ml in 1 M ammonia) at a concentration of 10 mg/mL. The reduction proceeded for 4 h at room temperature, and then for 1 hr at 50°C. The reaction was terminated by dropwise addition of acetic acid (glacial) until efferverscence ceased. Ammonia and water and acid were evaporated from the sample with a nitrogen stream. Borate was removed as its trimethyl ester by addition and evaporation of 0.5 ml 10% acetic acid in methanol, repeated 4 times, followed by addition and evaporation of pure methanol, 4 times. Internal standard, inositol, was added prior to the reduction proceedure. The reduced sample was methanolysed, derivatized and analysed by gas chromatography.

Time Course Study of Hydrolysis of the Disaccharide with DCl Followed by NMR Analysis

The disacccharide, galacturonosyl rhamnose, isolated from cell walls was hydrolysed with DCl (deuterium chloride) and the time course of hydrolysis was followed by n.m.r analysis. About 5 mg of the disaccharide was placed in an n.m.r tube and 0.7 ml 1 M DCl in 100% D_20 was added. The n.m.r tube was heated to 95°C for 1 h. The ^1H n.m.r spectrum of the sample was recorded at 25°C and 70°C. The tube was returned to the heating block and hydrolysis was continued. ^1H n.m.r spectra were recorded at intervals of 2, 4, 9, 12 and 26 h. After 26 h, the chloride from the sample was removed by precipitation with solid silver carbonate. The mixture was centrifuged and the supernatant was lyophilized. Pure D_20 was then added and the sample's ^1H n.m.r spectrum was recorded. The sample was reduced by adding sodium borodeuteride directly to the n.m.r tube and heated at 70°C until effervescence stopped. A final ^1H n.m.r spectrum was then recorded.

Analytical Methods

Gas liquid chromatography: The trimethyl silyl derivatives of sugars were separated on a fused silica capillary column (30 m X 0.25 mm i.d, Durabond - 1 liquid phase, J & W Scientific, Inc., Rancho Cordova, CA) installed in a Tracor 560 gas-liquid chromatograph equipped with an on column injector and helium carrier gas. One μL aliquots of the samples were injected at 105°C and the temperature was immediately raised to 140°C and held for 4 min, then raised 1°C min^{-1} to 200°C. Peak integration and data system analysis were performed using an Apple IIe micro computer and a peak integration program developed by Dr. Jerry Merz. In addition, a Varian 3300 gas chromatograph fitted with a DB-1 capillary column and an on column injector was also used for the sugar analysis. In this machine, the sample was injected at 105°C and the temperature was immediately raised to 160°C and held for 4 min, and then raised 2°C min^{-1} to 200°C. Peaks were integrated using a Varian 4290 integrator.

The methylated sugars were separated on DB-210 capillary column (30 m X 0.25 mm i.d) installed in a Tracor 560 gas chromatograph. One μL aliquots of the methylated samples in isooctane were injected at 105°C and the temperature was raised to 160°C and held for 4 min, then raised 2°C min^{-1} to 240°C. Peak integration and data system analysis were performed using the Apple IIe micro computer and integration program.

Liquid Chromatography

Gel Filtration. The solubilized fractions from the cell walls after HF treatment at -23°C were separated on a column (62 x 2.5cm) of Bio-gel P-2 (Bio-Rad Laboratories, Richmond California). The samples were applied to the column in 0.05 M sodium acetate pH 5.2, eluted with 0.05 M sodium acetate pH 5.2, and collected in fractions of 2.5 ml. The oligosaccharides isolated from cell walls after HF reaction at -40°C were separated on

a column (50 x 2.5 cm) of Bio-gel P-6 (Bio-Rad Laboratories, Richmond, CA) and eluted with 0.3 M sodium acetate buffer pH 5.2 in fractions of 3 ml.

Sugars in the fractions were detected by the phenol-sulfuric acid test (Ashwell, 1966). Portions (50 µL) of the fractions were mixed with 450 µL of water and 12.5 µL of phenol reagent (80% by weight in distilled water) followed by 1.25 ml of concentrated sulfuric acid. Samples were vortexed and color was measured at 485 nm after 30 min. Sodium ions in the pooled fractions were removed by passage through a small column of AG 50W X-8 (H^+) cation exchange resin (Bio-Rad Laboratories) prior to lyophilization.

Ion Exchange Chromatography. Fractions from the Bio-gel P-2 column were further separated on a column (5 x 1 cm) of QAE Sephadex anion exchanger (A-25-120, 40-120 m, Sigma Chemical Co., St. Louis, Missouri). Samples were applied in 0.025 M sodium acetate pH 5.2 and eluted with 0.05 M sodium acetate, pH 5.2. In some cases 0.3 M, 0.5 M, and 1 M sodium acetate buffer at pH 5.2 were also used for elution. Oligosaccharides isolated after HF treatment were also separated on a column (20 x 2cm) of DEAE Sephadex (A-25-120, 40-120 m, Sigma Chemical Co., St. Louis, Missouri). Weak (0.05 M) sodium acetate buffer pH 5.2 was used to load the column and the acidic sugars were eluted using 0.3 M, 0.5 M and 1 M sodium acetate buffer.

High Performance Liquid Chromatography (HPLC)

The oligosaccharides obtained from cell walls after HF solvolysis were fractionated using a Bio-Rad TSK 400 and a Beckman TSK 2000SW column (7.5 mm x 30 cm) in series and a Beckman TSk 2000 SW alone in some cases, on a Waters Associates pump system. The elution buffer was 0.3 M sodium acetate, pH 5.2, the flow rate was 1 ml min^{-1}. Sugars were detected by refractive index monitor. The molecular weight standards used were pullulan (Polymer Laboratories Technical Centre, Armherst Field Research

Park, 160 Old Farm Road, Amherst, MA 01002). Pullulan or polymaltotriose, is a linear macromolecule polysaccharide consisting of links of maltotriose.

The amino acid phenyl thiocyanate derivatives were separated using a C_{18} reverse phase Ultrapore ODS column (7.5 cm x 4.6 mm ID, Altex) fitted on a Beckman 334 gradient liquid chromatograph consisting of a 421 microprocessor system controller, two model 110A single-piston reciprocating pumps, a dynamically stirred gradient mixing chamber, a model 210 sample injection valve, and a model 153 detector with an 8 μL analytical flow cell for UV detection at 254nm. A Varian 4290 integrator was used for peak integration. Solvent A, the equilibrating buffer, was 0.05 M ammonium acetate, pH 6 with 0.2 g l^{-1} sodium azide and solvent B was 0.1 M ammonium acetate in acetonitrile, methanol and water (44:10:46) containing 0.2 g l^{-1} sodium azide. The columns were pre-equilibrated with 90% A and 10% B. Five μL aliquots of samples were injected and separated by a gradient made up of 10-100% B in 51 min with a gradient program, time, 0 min-10% B-duration, 0 min; time 1 min-20% B-duration, 15 min; time 16 min-40% B-duration, 15 min; time 31 min-70% B-duration, 10 min; time 41 min-80% B-duration, 10 min; time-51 min-100% B-duration, 0 min.

NMR Spectroscopy

^{1}H and ^{13}C n.m.r spectra were recorded with a Varian (Palo Alto, CA) XL-300 n.m.r spectrometer (300 MHz). ^{1}H spectra were recorded at both 25°C and 70°C. O-linked hydrogen atoms were exchanged for deuterium atoms in the following way. Samples (4-8 mg) were weighed and dissolved in 98% D_20 (Aldrich Chemical Co.) before lyophilization. The samples were then dissolved in 100% D_20 and lyophilized again to exchange the hydrogen with deuterium and then dissolved in 650 μL of 100% D_20. Sodium 2,2,3,3-tetra deuterio-4,4-dimethyl-4 silapentanoate (TSP) was used as the internal standard (0.00 ppm).

Two dimensional n.m.r spectroscopy was performed as described by Gray (1983) using 30-40 mg samples. O-linked hydrogen atoms were exchanged for deuterium as described above, and both ^{1}H-^{1}H 2D homonuclear spectrum and ^{13}C-^{1}H heteronuclear spectrum were recorded with the Varian XL-300 n.m.r spectrometer (300 MHz) at 25°C.

Mass Spectroscopy

Mass spectrum of the disaccharide galacturonosyl rhamnose isolated from Acala 44 cotton suspension cell walls was recorded using a laser desorption fourier transform mass spectrometry-FTMS-2000 mass spectrometer in Nicolet, Analytical Division/Madison, Wisconsin. Mass spectra of the partially methylated alditol acetates of disaccharides and oligosaccharides were recorded by Dr. Kenneth Gross.

CHAPTER IV

RESULTS AND DISCUSSION

Preliminary Characterization of Cell Walls

Preliminary characterization of cell walls provides information on the amount and the nature of polymers in the walls. Determination of chemical composition of the samples is part of the overall characterization of molecules. The gas chromatogram of the trimethyl silyl derivatives of the methyl glycosides of sugars from Acala 44 cotton suspension cell wall is shown in Figure 3. It shows that these walls have ara, rha, xyl, galA, gal and glc along with some unidentified compounds (unidentified signals in the chromatogram). The mole percents of sugars in cell walls from cotton, tomato, tobacco and carrot are shown in Table I and the total weight percents of sugars in these walls are shown in Table II. The total weight percent of sugar accounted for in the different walls does not include cellulose. All the walls tested are rich in pectin. Mole percents and total weight percents of sugars in different cotton cell walls after HF treatment at 0°C are shown in Table III. Hydrogen fluoride treatment at 0°C breaks cellulose to glucose. So the glucose composition after hydrogen fluoride treatment represents both cellulosic and non-cellulosic glucose, while plain methanolysis of walls gives non cellulosic glucose composition. Without any HF treatment 48% of the dry weight of Acala 44 cotton suspension cell walls, 53% of the dry weight of Im 216 cotton suspension walls, and 30% of dry weight of the Acala 44 cotton leaf cell walls were accounted for by sugars. After HF treatment 74% of the dry weight of Acala 44 walls, 79% of the dry weight of the Im 216 walls, and 57% of the dry weight of the Acala 44 leaf walls were accounted for by sugars (Table III). Mole percent and total weight percent of sugars given in all the tables listed are an average

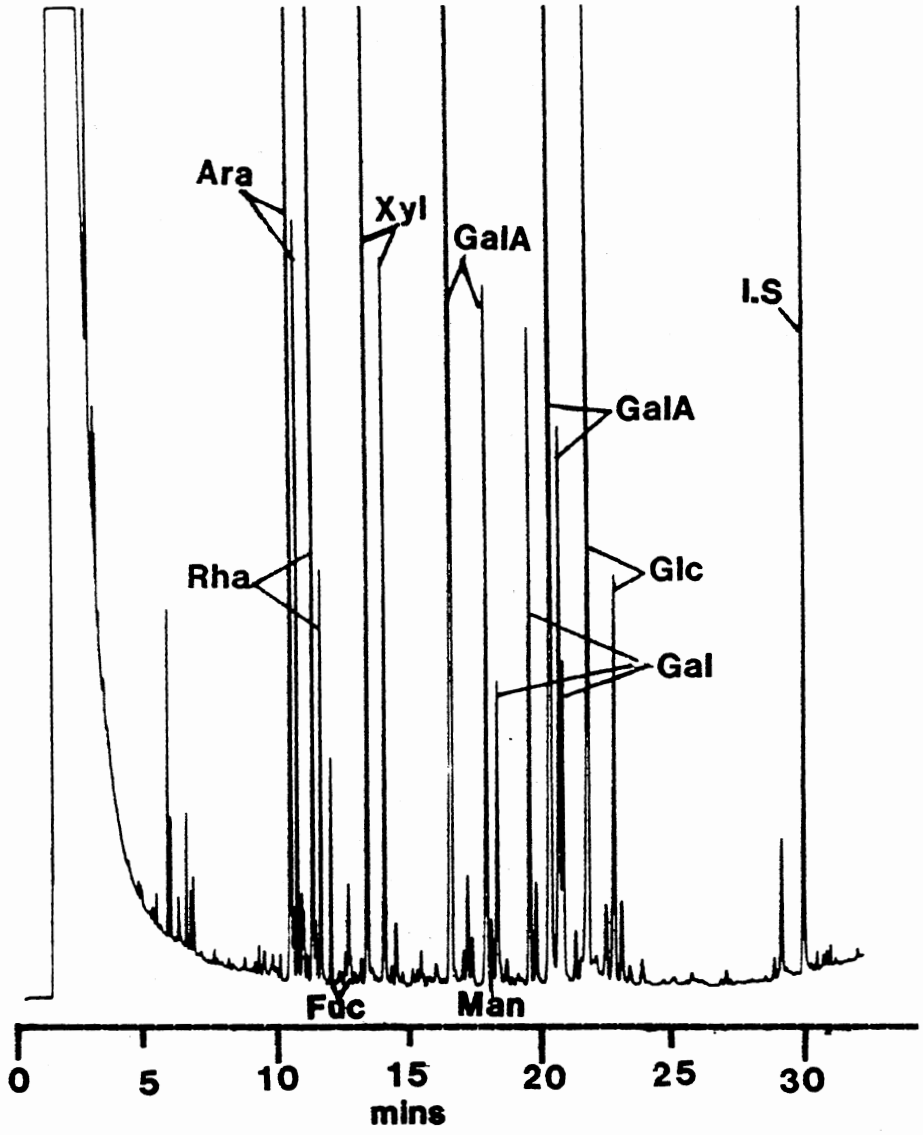

Figure 3. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides from Acala 44 Cotton Suspension Cell Walls. Peaks are identified as follows: Rha, Rhamnose; Ara, Arabinose; Fuc, Fucose; Xyl, Xylose; GalA, Galacturonic acid; Man, Mannose; Gal, Galactose; Glc, Glucose; IS, Inositol, the internal standard.

TABLE I

MOLE PERCENTAGE OF SUGARS IN DIFFERENT TYPES OF CELL WALLS

Sugar	Acala 44 cotton suspension cell wall	Im 216 cotton suspension cell wall	Acala 44 cotton leaf cell wall	Tomato suspension cell wall	Tobacco leaf cell wall	Carrot root cell wall
Ara	17.5	10.5	16.8	14.2	6.5	13.9
Rha	10.6	5.6	8.9	8.6	8.1	8.3
Fuc	2.5	2.1	Trace	-	Trace	-
Xyl	14.1	12.2	10.6	14.8	13.2	4.7
GalA	37.6	28.5	35.8	39.9	41.7	45.2
Man	0.7	-	2.9	3.2	3.6	2.8
Gal	8.3	9.3	11.1	9.9	8.6	19.3
Glc (non-cellulosic)	8.7	31.8	13.9	9.4	18.3	5.8

TABLE II

TOTAL WEIGHT PERCENT OF SUGARS IN DIFFERENT TYPES OF CELL WALLS

Sugar	Acala44 cotton suspension cell wall	Im 216 cotton suspension cell wall	Acala 44 cotton leaf cell wall	Tomato suspension cell wall	Tobacco leaf cell wall	Carrot root cell wall
Ara	7.5	4.6	4.3	6.1	1.8	4.3
Rha	3.9	2.7	2.5	4.3	2.4	2.8
Fuc	1.3	1.1	Trace	-	Trace	-
Xyl	5.2	5.4	2.7	6.4	3.6	1.5
GalA	21.7	16.7	12.1	23.1	15.1	18.7
Man	0.5	-	0.9	1.7	1.2	1.1
Gal	3.2	5.1	3.4	5.3	2.9	7.3
Glc (non cellulosic)	4.8	17.3	4.3	4.8	6.1	2.2
Total	48.1	52.9	30.2	51.7	33.1	37.9

TABLE III

MOLE PERCENT AND TOTAL WEIGHT PERCENT OF SUGARS IN COTTON CELL WALLS AFTER HF TREATMENT AT 0°C

Sugar	Acala 44 cotton suspension cell wall		Im 216 cotton suspension cell wall		Acala 44 cotto n leaf cell wall	
	Mole%	Total wt%	Mole%	Total wt%	Mole%	Total wt%
Ara	8.8	5.5	9.2	6.2	10.7	5.1
Rha	6.7	4.6	4.9	3.7	5.1	2.7
Fuc	1.3	0.9	1.8	1.3	0.9	0.5
Xyl	13.3	8.2	14.2	9.6	11.5	5.4
GalA	23.7	19.5	13.4	9.4	19.5	12.4
Man	0.5	0.4	0.4	0.3	2.7	1.5
Gal	6.5	4.9	7.4	6.1	8.5	5.1
Glc cellulosic& non cellulosic	39.2	29.9	48.7	42.7	41.1	24.1
Total	100	73.9	100	79.3	100	56.8

of 2 to 3 determinations. Unless otherwise stated sugar composition of the samples were determined by plain methanolysis and derivatization without any prior HF treatment. Polysaccharides are polydisperse, so that information on both molecular size and composition can be obtained only as averages. As a polysaccharide contains repetitive features, compositional analysis gives essential information on the relative proportions of sugar constituents. The absolute composition cannot be determined as it varies.

Protein contents of the walls were determined by amino acid analysis. The HPLC profile of the standard amino acid derivatives is shown in Figure 4. The mole percent and total weight percent of amino acids in different cotton cell walls are shown in Table IV. The amounts of protein in Acala 44 and Im 216 cotton suspension cell walls were found to be 7.2 and 7.5% of the dry weight of the walls, respectively, while Acala 44 leaf cell walls had 8.9% protein. Hydroxyproline was 0.6% in Acala 44 suspension walls and 0.5% in Im 216 cotton suspension walls, while Acala 44 cotton leaf walls had only 0.1% of hydroxyproline. Cell walls from all the three samples were rich in valine, leucine, serine and glycine.

Sequential Extraction of Polymers from Cotton Cell Walls

The pectins from the cell walls were isolated by CDTA extraction followed by sodium carbonate extraction of the walls and hemicellulose fraction was isolated by extraction with 1M and 4 M potassium hydroxide containing sodium borohydride. The final residue left behind was cellulose. The sugar composition of the different extracts from Acala 44 suspension walls, Im 216 suspension walls and from Acala 44 leaf walls are shown in Tables V, VI and VII, respectively. Total pectin accounted for ~42 weight % of Acala 44 suspension walls by weight, ~50% of Im 216 walls and ~38% of Acala 44 leaf walls. The hemicellulose fraction accounted for ~20% by weight of Acala 44 suspension walls, ~18% of Im 216 suspension walls and ~28% of Acala 44 leaf walls. The final residue accounted for ~10% of the original weight in Acala 44 suspension walls, 9% in Im 216

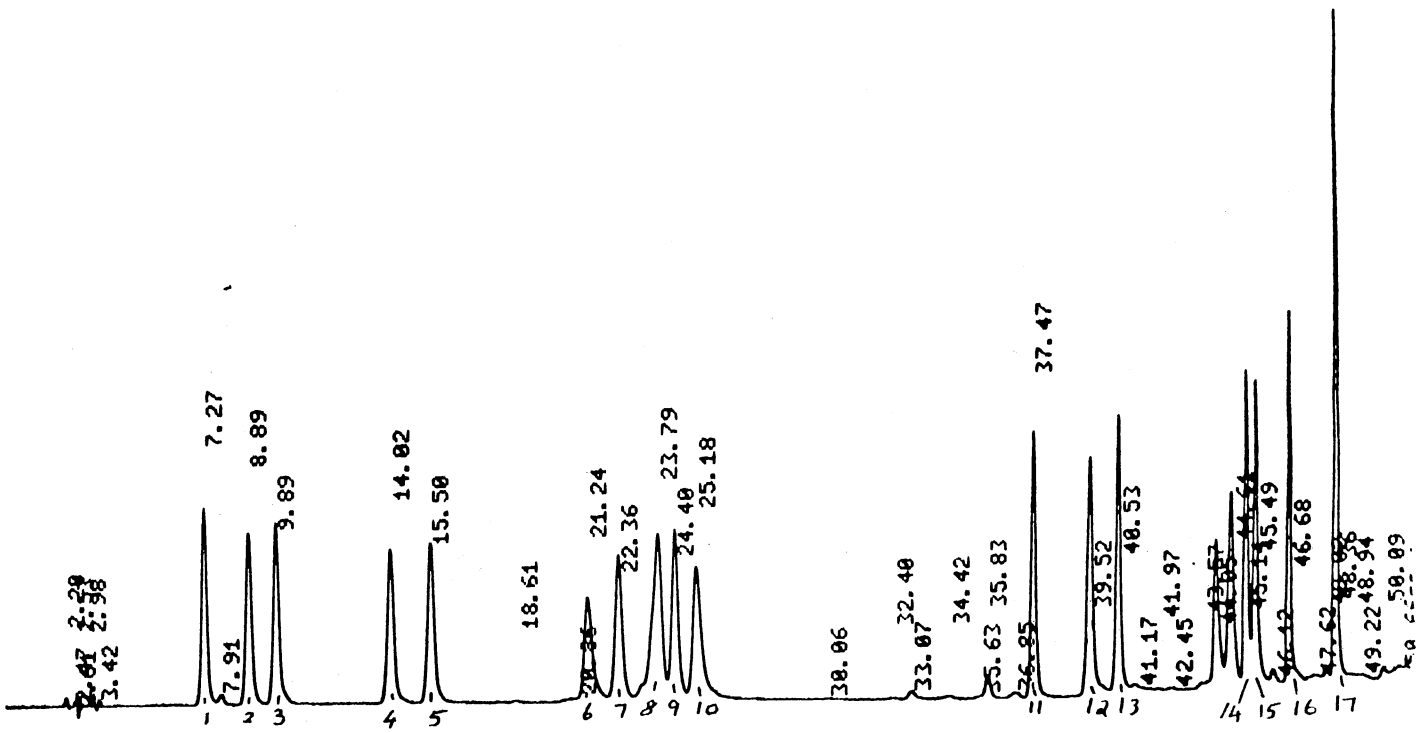

Figure 4. HPLC Trace of the Standard Amino Acid Derivatives. 500 pmols of standard amino acid phenyl thiocyanate derivatives were injected on to a C_{18} column and eluted with a gradient of 10-100% B, buffer B was 0.1 M ammonium acetate in acetonitrile:methanol:water (44:10:46). Buffer A was 0.05 M ammonium acetate. Retention time in min are printed on the peaks. Flow rate was 1 ml/min, chart speed was 0.5 cm/min, detection was by UV absorbance at 254 nm, sensitivity was 0.2. Peaks are identified as follows: 1, Asp; 2, Glu; 3, Hyp; 4, Ser; 5, Gly; 6, His; 7, Thr; 8, Ala; 9, Arg; 10, Pro; 11, Val; 12, Tyr; 13, Meth; 14, Leu; 15, Ileu; 16, Phe; 17, Lys.

TABLE IV

MOLE PERCENTAGE AND TOTAL WEIGHT PERCENTAGE OF AMINO ACIDS IN COTTON CELL WALLS

Amino acid	Acala 44 suspension cotton cell walls		Im216 cotton suspension cell walls		Acala 44 leaf cell walls	
	Mole %	Total wt%	Mole %	Total wt%	Mole %	Total wt%
Asp	3.6	0.3	2.4	0.2	0.4	0.1
Glu	4.5	0.4	4.6	0.4	2.2	0.2
Hyp	7.6	0.6	5.8	0.5	0.6	0.1
Ser	8.1	0.5	8.9	0.7	4.9	0.4
Gly	8.6	0.3	9.6	0.4	11.6	0.6
His	1.7	0.2	0.9	0.1	0.3	0.1
Thr	7.1	0.5	7.8	0.6	7.9	0.7
Ala	7.2	0.3	7.7	0.4	9.4	0.6
Arg	4.4	0.4	4.7	0.5	5.7	0.7
Pro	7.3	0.5	7.6	0.5	9.9	0.8
Tyr	2.3	0.3	2.1	0.2	2.4	0.3
Val	9.7	0.6	10.2	0.7	11.3	0.9
Met	1.1	0.1	1.4	0.1	1.1	0.1
Ileu	6.5	0.5	6.4	0.5	8.1	0.8
Leu	10.8	0.8	11.5	0.9	13.8	1.3
Phe	4.9	0.5	4.6	0.4	7.3	0.9
Lys	4.6	0.4	3.8	0.4	3.1	0.3
Total	100	7.2	100	7.5	100	8.9

TABLE V

MOLE PERCENT OF SUGARS IN DIFFERENT EXTRACTS OF ACALA 44 COTTON SUSPENSION CELL WALLS

Extracts from Acala44 suspension cell walls	Ara	Rha	Fuc	Xyl	GalA	Man	Gal	Glc(non-cellulosic)
CDTA extract	11.4	6.5	Trace	4.9	54.1	Trace	6.6	16.2
Sodium carbonate extract	10.8	5.9	-	6.1	46.1	6.8	5.1	19.1
1MKOH+sodium boro hydride extract	19.4	5.1	-	29.6	Trace	Trace	Trace	45.9
4MKOH+sodium boro hydride extract	26.7	4.1	-	28.1	-	6.1	12.8	22.2
4MKOH+boric acid extract	35.5	8.7	-	11.6	21.8	2.5	7.2	12.7
Final residue	20.3	11.9	-	20.9	23.7	-	10.6	12.6

TABLE VI

MOLE PERCENT OF SUGARS IN DIFFERENT EXTRACTS OF IM 216 COTTON SUSPENSION CELL WALLS

Materials from Im216 suspension walls	Ara	Rha	Fuc	Xyl	GalA	Man	Gal	Glc(non-cellulosic)
CDTA extract	8.9	7.7	Trace	6.3	60.6	-	6.3	10.3
Sodium carbonate extract	9.6	8.1	-	8.4	36.7	5.1	8.1	23.9
1MKOH+sodium boro hydride extract	14.5	5.6	2.5	25.1	14.7	1.2	10.8	25.6
4MKOH+sodium boro hydride extract	18.4	5.7	3.9	23.2	13.9	1.6	14.5	18.8
4MKOH+boric acid extract	26.5	9.1	1.5	17.2	18.2	2.2	10.4	14.9
Final residue	15.1	8.8	2.9	21.7	21.8	0.5	11.3	17.9

TABLE VII

MOLE PERCENT OF SUGARS IN DIFFERENT EXTRACTS OF ACALA 44 LEAF CELL WALLS

Materials from Acala 44 leaf walls	Ara	Rha	Fuc	Xyl	GalA	Man	Gal	Glc(non-cellulosic)
CDTA extract	16.5	10.4	-	3.1	59.3	-	5.8	4.9
Sodium carbonate extract	27.1	13.2	-	4.1	37.4	1.4	9.1	7.7
1MKOH+sodium boro-hydride extract	16.2	5.5	1.3	31.5	11.1	1.1	9.9	23.4
4MKOH+sodium boro-hydride extract	18.8	6.2	2.7	19.9	11.1	7.5	18.6	15.3
4MKOH+boric acid extract	30.8	10.4	-	10.3	22.5	3.1	14.8	8.1
Final residue	22.4	7.2	-	11.4	13.4	2.7	10.8	32.1

suspension walls and ~12% in Acala 44 leaf walls. Some pectin was also extracted along with the hemicelluloses and some remained with the cellulose residue. The different extracts obtained by this method of extraction were found to be very heterogeneous with respect to size and composition. The total weight percents of sugars in the different extracts were very low. One reason is the residual reagents present in the extracts and the other in the case of pectin is the difficulty in breaking uronic acids by methanolysis. This problem can be overcome partly by treating pectin samples with HF at 0°C for 15 min before methanolysis and derivatization.

Analysis of Extracellular Polysaccharides (EPS) from Cotton Suspension Culture Medium

The mole percentage of sugars in extracellular polysaccharides isolated from Acala 44 and Im 216 cotton suspension culture medium is shown in Table VIII. Both Acala 44 and Im 216 cotton EPS were rich in xylose, glucose and galactose. Galacturonic acid and rhamnose were present in small amounts showing that the EPS is richer in hemicelluloses and not pectin.

Lignin Determination

Lignin was found to be ~1% of Acala 44 and Im 216 suspension cell walls and ~5% of Acala 44 cotton leaf cell walls.

Extraction of Pectins by Hydrogen Fluoride Solvolysis at -23°C

250 mg of Acala 44 cotton suspension cell walls were treated with HF and fractionated as shown in schemes 1 and 2 (refer to Figures 5 and 6). The sugar composition of different fractions of cell walls is given in Table IX. At -23°C HF solvolysis of cell walls breaks ara, rha, fuc, xyl, man, gal, glc (non cellulosic) linkages.

TABLE VIII

MOLE PERCENTAGE OF SUGARS IN EPS FROM COTTON CULTURE FLUID

Sugar	EPS from Acala 44 cotton culture fluid	EPS from Im 216 cotton culture fluid
Ara	8.8	3.9
Rha	2.4	1.1
Fuc	3.1	4.1
Xyl	21.2	26.4
GalA	1.1	5.1
Man	1.5	1.5
Gal	28.4	13.5
Glc	33.5	44.4

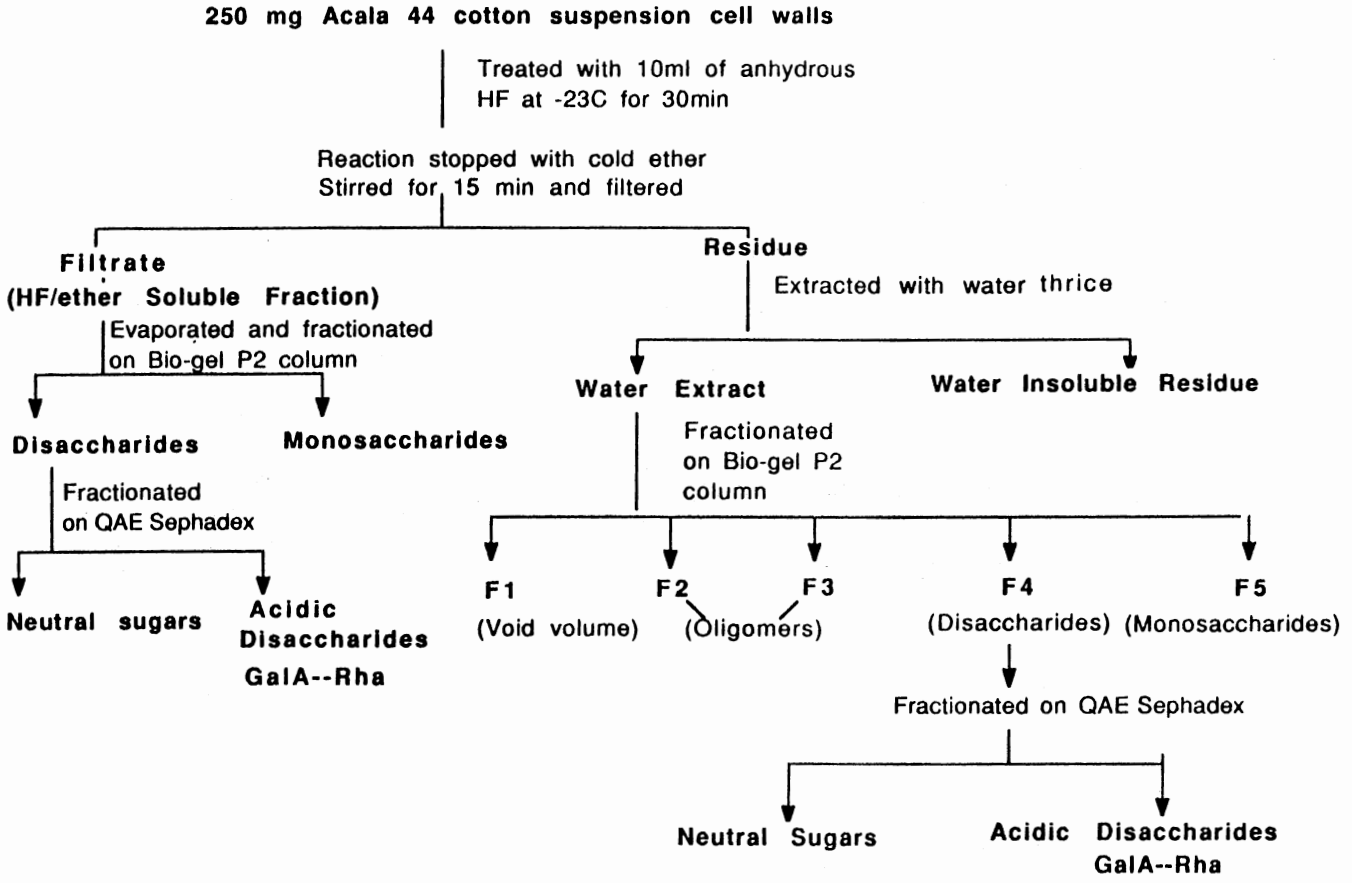

Figure 5. Scheme 1 for HF Solvolysis of Cell Walls at -23°C.

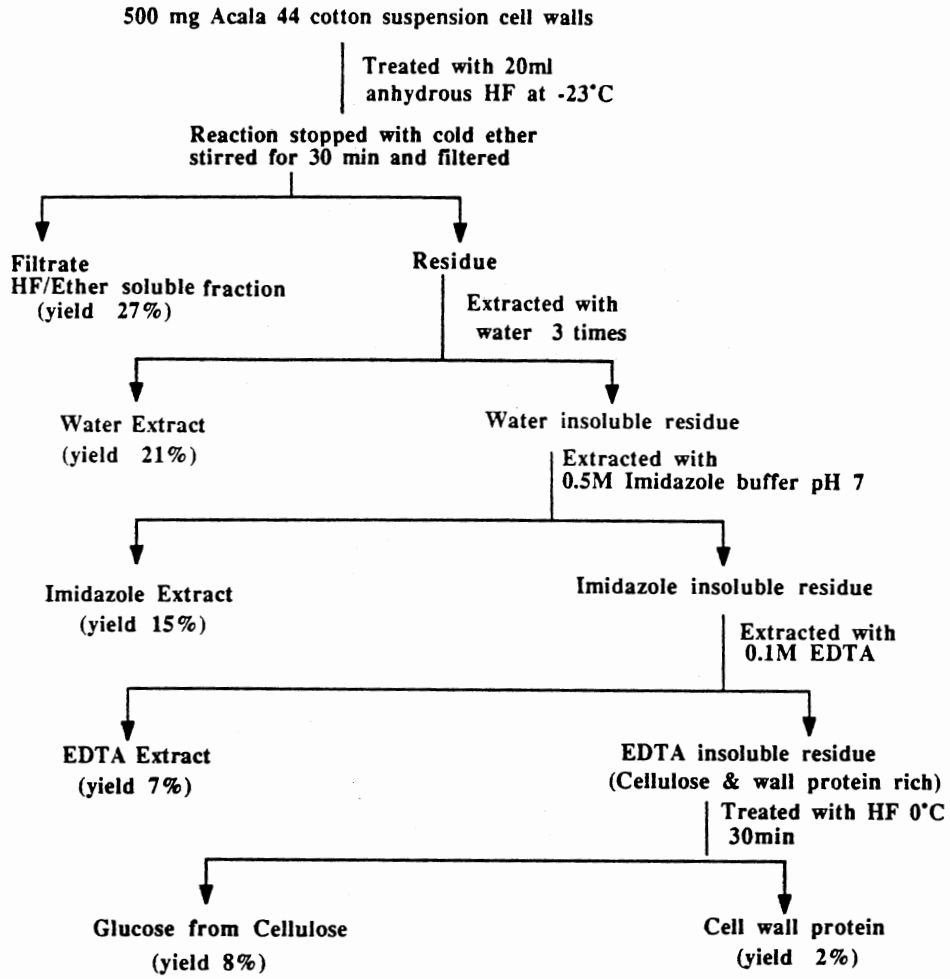

Figure 6. Scheme 2 for HF Solvolysis and Fractionation of Cell Walls at -23°C.

TABLE IX

MOLE PERCENTAGE OF SUGARS IN DIFFERENT FRACTIONS OF CELL WALLS AFTER HF SOLVOLYSIS AT -23°C

Material	Percent of Starting Weight Recovered	Ara	Rha	Fuc	Xyl	GalA	Man	Gal	Glc
Whole cell wall	100	17.5	10.6	2.5	14.2	37.7	0.7	8.3	8.7
Cell Wall*	74% as sugar	8.8	6.7	1.3	13.3	23.7	0.5	6.5	39.3
Cell Wall HF/ether -23°Residue	65	4.8	11.6	-	7.3	40.19	1.5	11.5	22.5
Ether-soluble	27	22.2	11.0	3.4	31.9	8.1	0.5	11.8	11.1
Water Extract	21	4.9	6.78	-	7.5	29.9	0.8	6.8	43.3
Residue after water extraction	44	1.8	1.1	-	2.4	65.2	-	1.5	28.0
Imidazole Extract	15	1.1	1.1	-	3.5	90.4	-	-	3.9
Residue after Imidazole extraction	26	2.2	1.3	-	3.2	53.7	-	2.3	37.3
EDTA Extract (0.1 M disodium salt)	7	0.5	0.7	-	1.5	93.5	-	-	3.9
Residue after EDTA extraction	12	Trace	Trace	-	6.6	21.7	-	3.7	68.1
Residue after EDTA extraction*	8	-	0.6	-	1.8	5.4	-	1.5	90.7
HF 0° insoluble Residue (Protein)	2	-	-	-	3.6	87.6	-	-	8.8

Sugar compositions of each fraction were determined after methanolysis and trimethylsilylation (Chaplin 1982) except in the cases marked with an * in which the sample was given a 30 minute treatment in HF at 0°C to solubilize cellulose and any other sugar polymers resistant to methanolysis. Thus, most of the compositions shown do not reflect the cellulose content of the sample in the values for glucose.

So the polymers like arabinogalactan, arabinan, galactan, xyloglucan are broken down to the corresponding sugars. Galacturonosyl linkages are not labile at this temperature. Rhamnose rich portion of pectin (RG I) was converted into disaccharides of galacturonic acid and rhamnose. Side chains on this portion of pectin were removed. The galacturonic acid rich portion of pectin (Homogalacturonan) was not broken down and was extracted using chelators and imidazole buffer. The acyl substituents on the sugars were retained on the sugars. Cellulose and cell wall protein remained insoluble after HF treatment at -23°C. Cellulose seems to undergo some structural changes during solvolysis making it susceptible to methanolysis. This causes an increase in the glucose content in water insoluble residue samples obtained from walls after HF treatment at -23C (Table IX).

Analysis of HF/Ether Soluble Sugars

Isolation of the Disaccharide Galacturonosyl Rhamnose from RG I Region of Pectins

About 28% of the dry weight of walls was soluble in the HF/ether mixture when ether was added to quench the reaction. The HF/ether soluble fraction contained mainly monosaccharides and some disaccharides due to their solubility (Kuo and Mort, 1986). The amount of monosaccharides in HF/ether fraction to some extent depends on the amount of ether added to quench the reaction. HF/ether sugars were separated into mono and disaccharides by Bio-gel P-2 column chromatography (Figure 7). The monosaccharides were found to be ara-35%, rha- 5.1%, fuc- 4%, xyl- 31%, man- 0.2%, gal- 11.1%, and glc- 14%. The disaccharide mixture obtained from the P-2 column contained both acidic and neutral disaccharides. Neutral disaccharides were separated by ion exchange chromatography on a QAE Sephadex column (Figure 8). The sugars retained on the column were rich in the disaccharide galacturonosyl rhamnose the structure of which was determined by n.m.r. spectroscopy which will be described later.

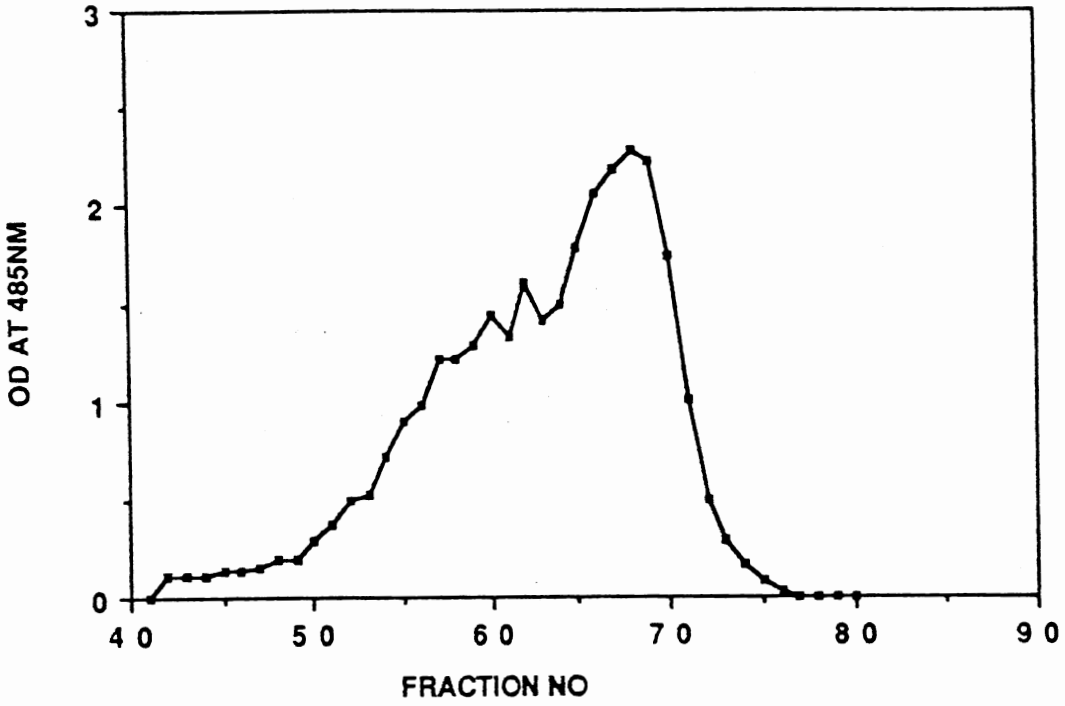

Figure 7. Chromatography on a Bio-gel P-2 Column (62 x 2.5 cm) of the HF/ether Soluble Fraction (refer to scheme 1, Fig. 5) from Acala 44 Cotton Suspension Cell Walls After HF Solvolysis at -23°C, in 0.05 M Sodium Acetate Buffer pH 5.2. 2.5 ml fractions were collected. 50 μL of the fractions were tested for sugar by phenol sulfuric test. Tubes 40-65 were pooled into fraction 1 and tubes 66-75 were pooled into fraction 2.

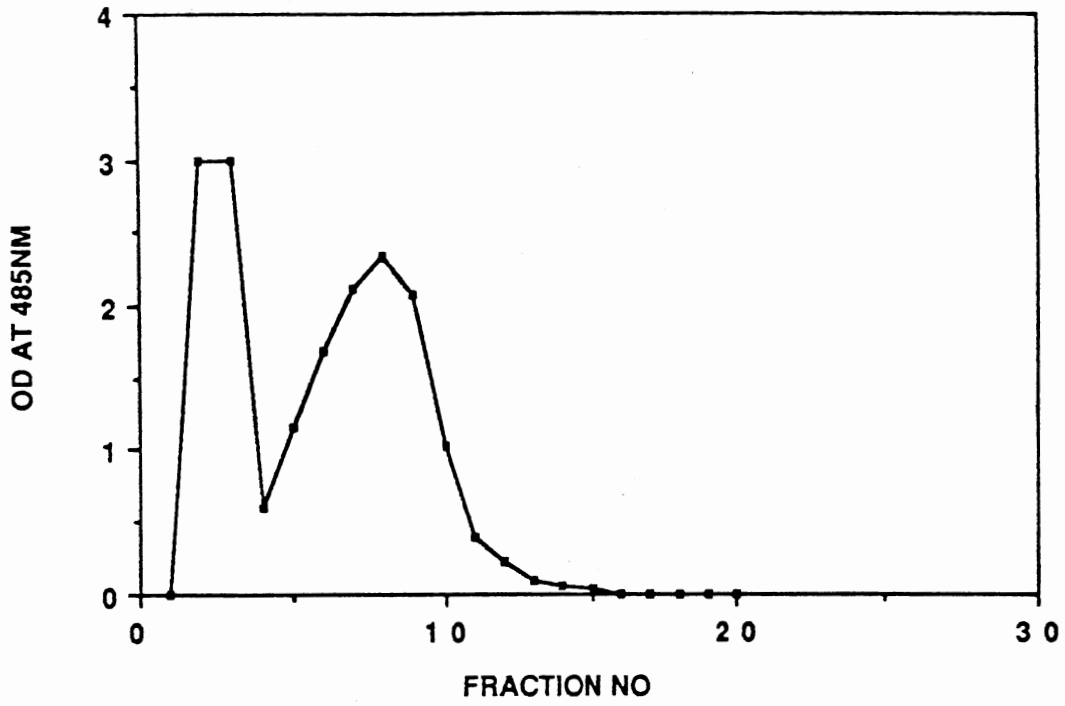

Figure 8. Chromatography on a QAE Sephadex Anion Exchange Column (5 x 1 cm) of the Fraction 1 of the Bio-gel P-2 Column of the HF/ether Soluble Fraction from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. Sample was applied in 0.025 M sodium acetate buffer pH 5.2 and eluted with 0.05 M sodium acetate buffer pH 5.2. 2.5 ml fractions were collected. Fractions were assayed for sugar by phenol sulfuric acid test. Tubes 1-4 were pooled into fraction 1 (neutral sugars) and tubes 5-15 were pooled into fraction 2 (acidic sugars).

Isolation of the Disaccharide Galacturonosyl Rhamnose from the Water Extract

The water extract from the residue after HF solvolysis at -23°C was found to be about 21% of the total cell walls. The total weight percent of sugar in this fraction was 80%. Fractionation of the water extract on a Bio-gel P-2 column is shown in Figure 9. Five fractions were made. The sugar compositions of the different fractions are shown in Table XII. The amino acid analysis of the water extract showed that the water extract did not have any significant amount protein. Fraction 4 contained disaccharides. This was further purified using a QAE Sephadex column (Figure 10). The sugars retained on the column were rich in the disaccharide galacturonosyl rhamnose. The composition of the disaccharide mixture isolated from the HF/ether mixture and from the water extract is shown in Table X. The mole percent of sugars in the disaccharide mixture shows that both the disaccharide fractions were rich in the disaccharide galacturonosyl rhamnose.

Structure Determination of the Disaccharide

N.M.R. Analysis

The ^{1}H n.m.r. spectra of the disaccharide isolated from the water extract and from HF/ether soluble sugars are shown in Figures 11 and 12. The anomeric proton signals of each sugar are separated from the bulk of the ring protons by having a higher chemical shift and this helps to identify the different types of sugars present in the sample. From the splitting pattern of the anomeric signals it is possible to identify the configuration of the sugars. The signals were identified using a 2D homocorrelation n.m.r. spectrum of the disaccharide (Figure 13). The spectrum could be interpreted as that of a disaccharide of galacturonic acid and rhamnose. There were some unusual chemical shifts for some signals. The spectrum of the disaccharide from HF/ether soluble sugars (Figure 12) was more complex than the spectrum of the disaccharide from the water extract (Figure 11).

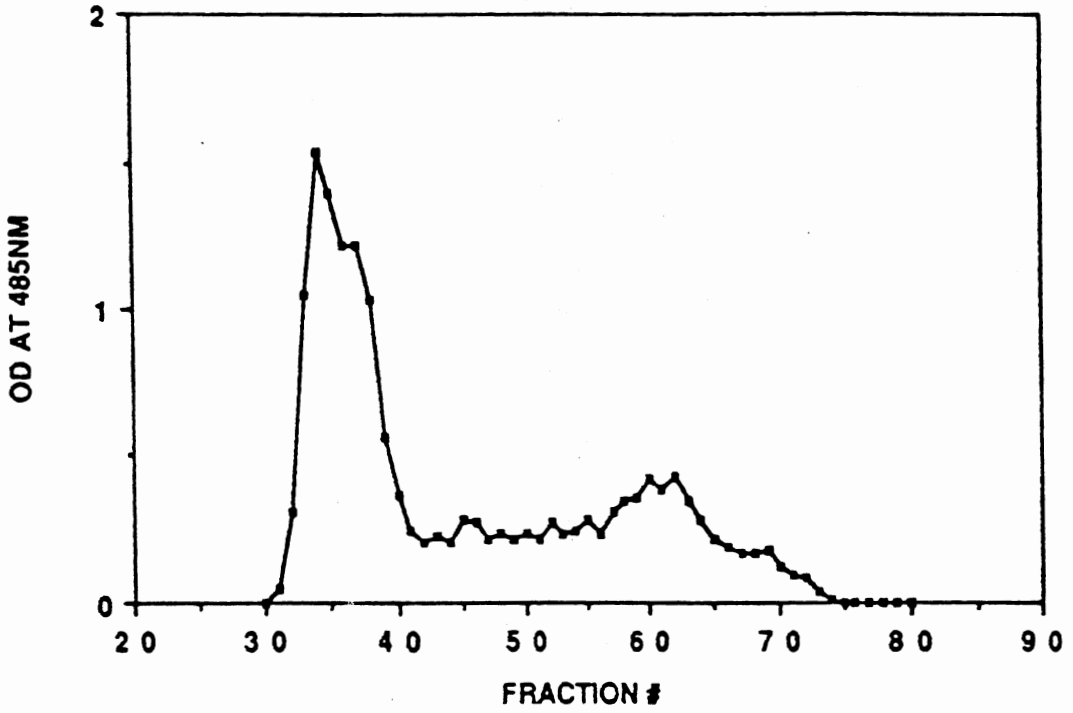

Figure 9. Chromatography on a Bio-gel P-2 (62 x 2.5 cm) of the Water Extract (refer to scheme 1, Fig. 5) from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C, in 0.05 M Sodium Acetate pH 5.2. 2.5 ml fractions were collected. Fractions were assayed for sugar by phenol sulfuric test. Tubes 30-40 were pooled into fraction 1, tubes 41-50 were pooled into fraction 2, tubes 51-58 into fraction 3, tubes 59-66 into fraction 4 and tubes 67-75 were pooled into fraction 5.

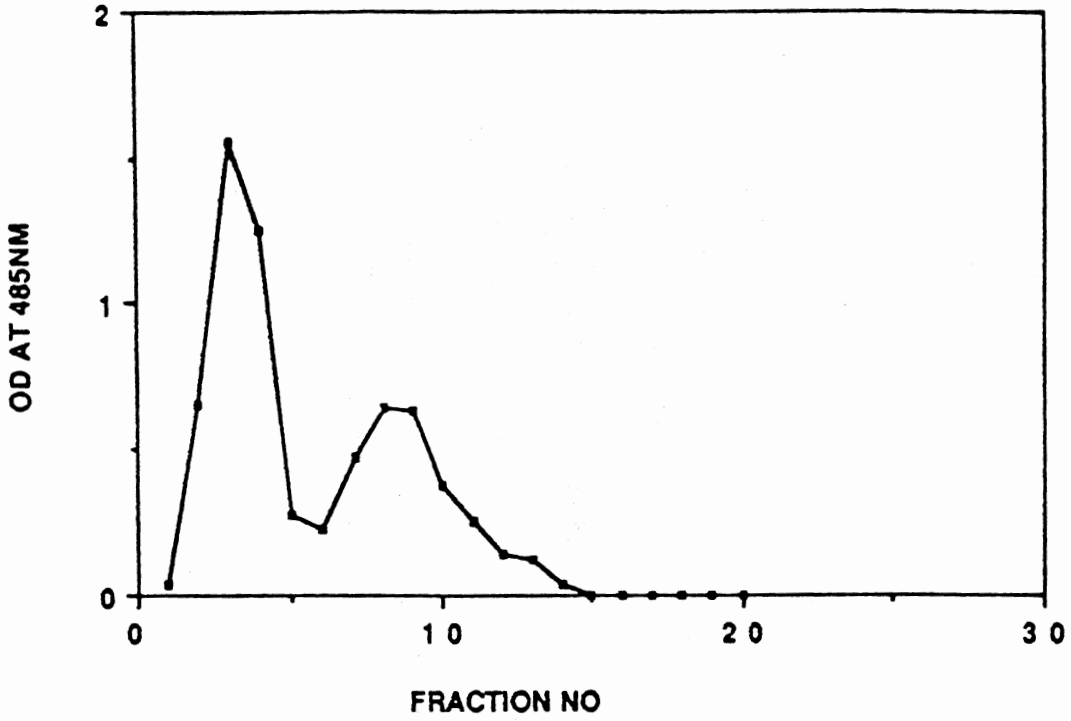

Figure 10. Chromatography on a QAE Sephadex Anion Exchange Column (5 x 1 cm) of the Fraction 1 of the Bio-gel P-2 Column of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. Sample was applied in 0.025 M sodium acetate buffer pH 5.2 and eluted with 0.05 M sodium acetate pH 5.2. 2.5 ml fractions were collected and assayed for sugar by phenol sulfuric acid test. Tubes 1-5 were pooled into fraction 1 (neutral sugars) and tubes 6-15 were pooled into fraction 2 (acidic sugars).

TABLE X

MOLE PERCENTAGE OF SUGARS IN THE DISACCHARIDE FRACTIONS OBTAINED AFTER HF SOLVOLYSIS AT -23°C

Sugar	HF/ether soluble fraction	Disaccharide isolated from HF/ether soluble fraction	Water Extract	Disaccharide isolated from Water Extract
Ara	22.2	5.5	4.8	4.4
Rha	11.1	39.3	6.8	34.9
Fuc	3.4	-	-	-
Xyl	31.9	8.3	6.4	5.2
GlcA	Trace	-	1.3	8.4
4(o) methyl GlcA	Present	Present	-	-
GalA	8.1	40.4	29.9	35.7
Man	0.5	-	0.8	-
Gal	11.7	4.9	6.8	9.7
Glc	11.1	1.6	43.2	1.7

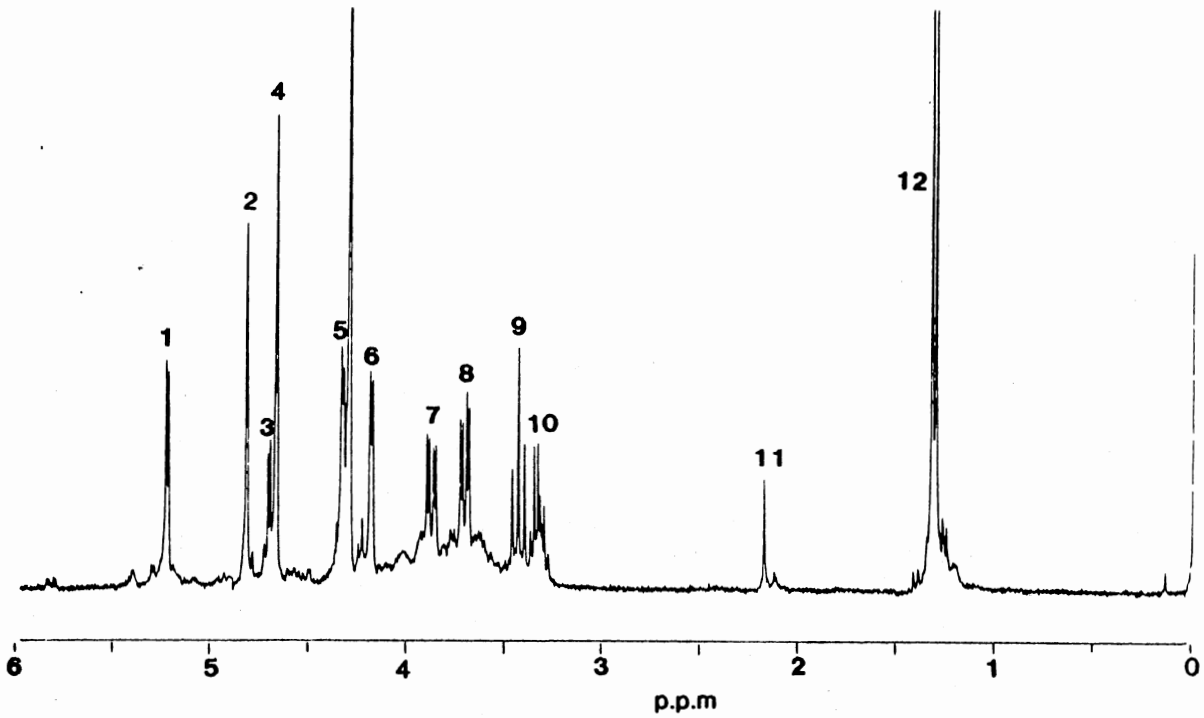

Figure 11. ^{1}H n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose (GalA-Rha) Isolated from the Water Extract of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-1 of GalA; 2) H-1 of Rha; 3) H-3 of GalA; 4) H-5 of GalA; 5) H-4 of GalA; 6) H-2 of Rha; 7) H-2 of GalA; 8) H-3 of Rha; 9) H-4 of Rha; 10) H-5 of Rha; 11) CH_3 of acetate; 12) CH_3 of Rha.

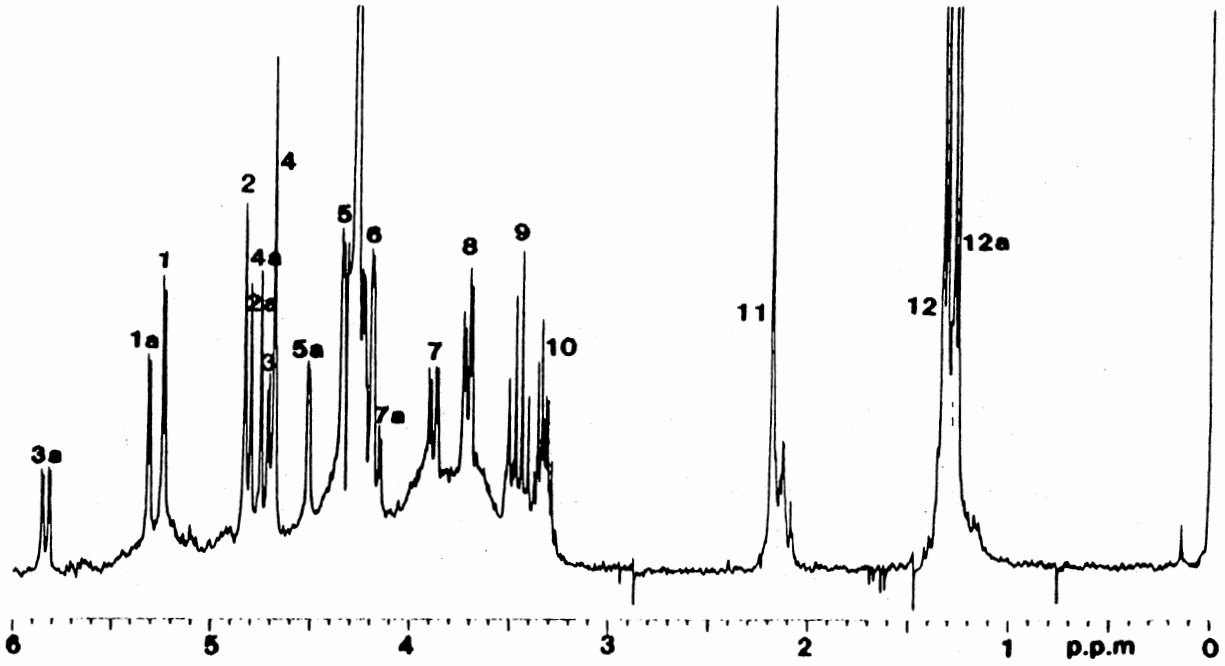

Figure 12. ^{1}H n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose (GalA-Rha) Isolated from the HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Plain numbers refer to signals in the non-acetylated disaccharide and the numbers with an "a" refer to signals in the acetylated disaccharide. Peaks are assigned as follows: 1) H-1 of GalA; 2) H-1 of Rha; 3) H-3 of GalA; 4) H-5 of GalA; 5) H-4 of GalA; 6) H-2 of Rha; 7) H-2 of GalA; 8) H-3 of Rha; 9) H-4 of Rha; 10) H-5 of Rha; 11) CH_3 of acetate; 12) CH_3 of Rha.

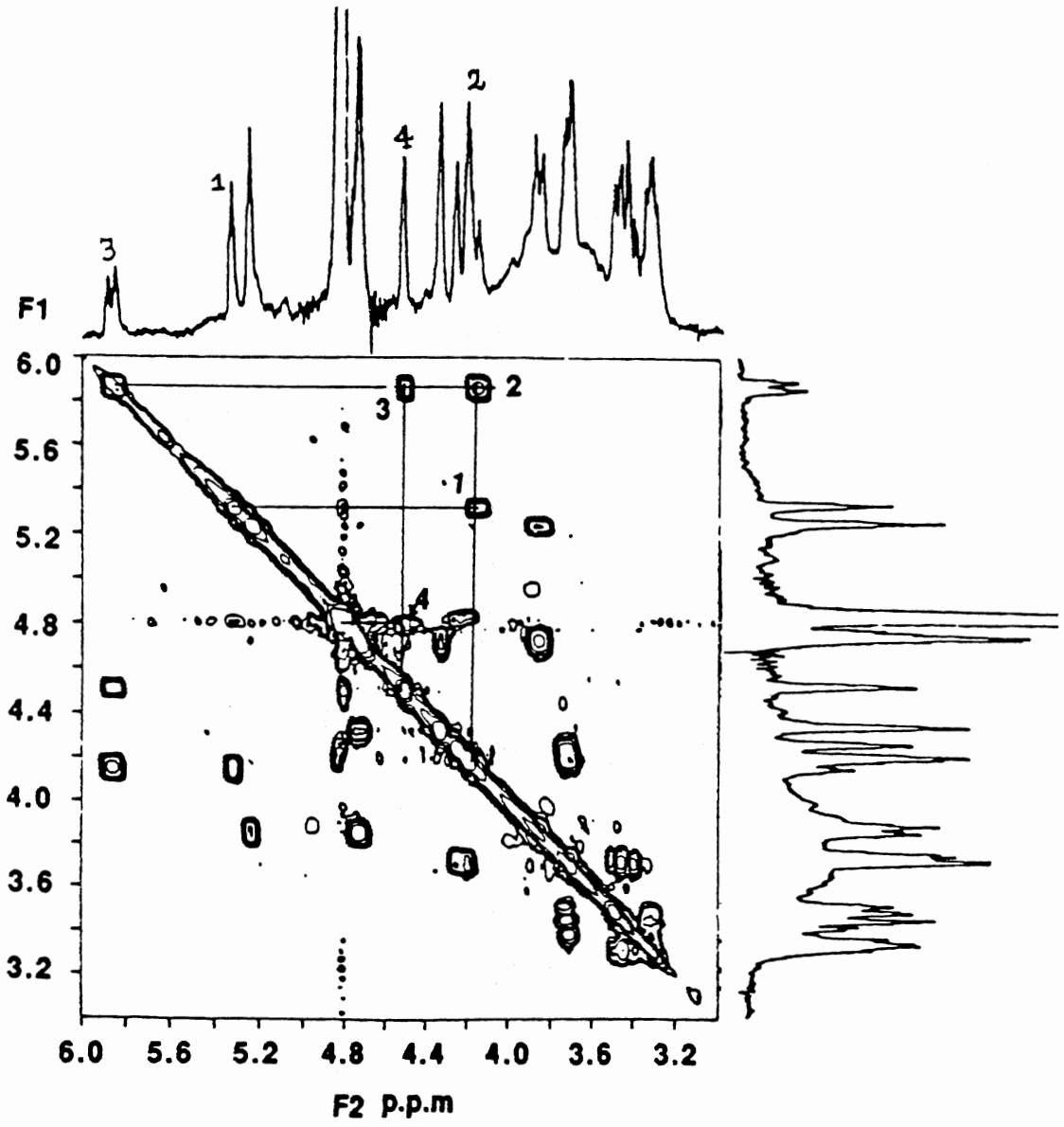

Figure 13. ^{1}H-^{1}H 2 D Homonuclear Correlated (cosy) n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose Isolated from the HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C, Together with 1 D Proton Spectrum. The spectrum was recorded using 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Off diagonal peaks show spin spin coupling between adjacent protons. Connectivities between the different positions of galacturonic acid in the acetylated disaccharide only are shown. Chemical shift of a proton at the position of acetylaton is shifted approximately 1 p.p.m. down field (refer to Table XI) and the position is identified as O-3 of galacturonic acid. Peaks are identified as follows: 1) H-1 of GalA; 2) H-2 of GalA; 3) H-3 of GalA; 4) H-4 of GalA.

This spectrum (Figure 12) shows a singlet at 2.18 p.p.m. which is from the methyl protons of an acetate ester. This signal was very small in the other spectrum (Figure 11). Thus we conclude that the disaccharides isolated from the water extract had traces of acetate ester, while the disaccharides isolated from the HF/ether fraction were rich in acetate ester. The spectrum of disaccharides from HF/ether soluble sugars (Figure 12) showed two sets of signals. One set was from the disaccharide with the acetate ester (acetylated disaccharide) and the other was from the disaccharide without an acetate ester (non-acetylated disaccharide). Integration of the ^{1}H n.m.r. spectrum showed that the disaccharides were about 40% acetylated in the HF/ether soluble sugars. The spectrum of disaccharides from the HF/ether soluble sugars showed a signal at 5.83 p.p.m. By using a 2D homocorrelation spectrum (Figure 13) this signal was identified as H-3 of galacturonic acid. From the large downfield shift of H-3 of galacturonic acid (1 p.p.m. downfield with respect to the H-3 in the non-acetylated disaccharide) it was concluded that O-3 of galacturonic acid was the position of acetylation. The presence of an acetate ester moves the signal approximately 1 p.p.m. downfield, in this case from 4.70 p.p.m. to 5.83 p.p.m. This confirms that the O-3 of galacturonic acid is the position of acetylation. The chemical shift of H-3 of galacturonic acid in was found to be anomolously high, being 4.70 p.p.m. compared to 3.91 p.p.m. The high chemical shift showed that H-3 of galacturonic acid was more shielded being adjacent to some electronegative groups. Another peculiarity was that rhamnose occurred in only one anomeric form, whereas an equilibrium mixture of anomers is expected if rhamnose had a reducing end. Both the ^{1}H and ^{13}C spectra indicated that the rhamnose was in β configuration. The H-1 of rhamnose resonated at 4.83 p.p.m. which indicated β rhamnose (Debruyn *et al.*, 1976). In the ^{13}C spectrum the signal for C-5 of rhamnose was at almost 75 p.p.m. as for all β-rhamnosyl residues not around 71 p.p.m. as it would be for α-rhamnosyl residues (Kasai *et al.*, 1979). The ^{13}C n.m.r. spectra of the disaccharides from the water extract and from the HF/ether soluble fraction are shown in Figures 14 and 15. The signals were identified by

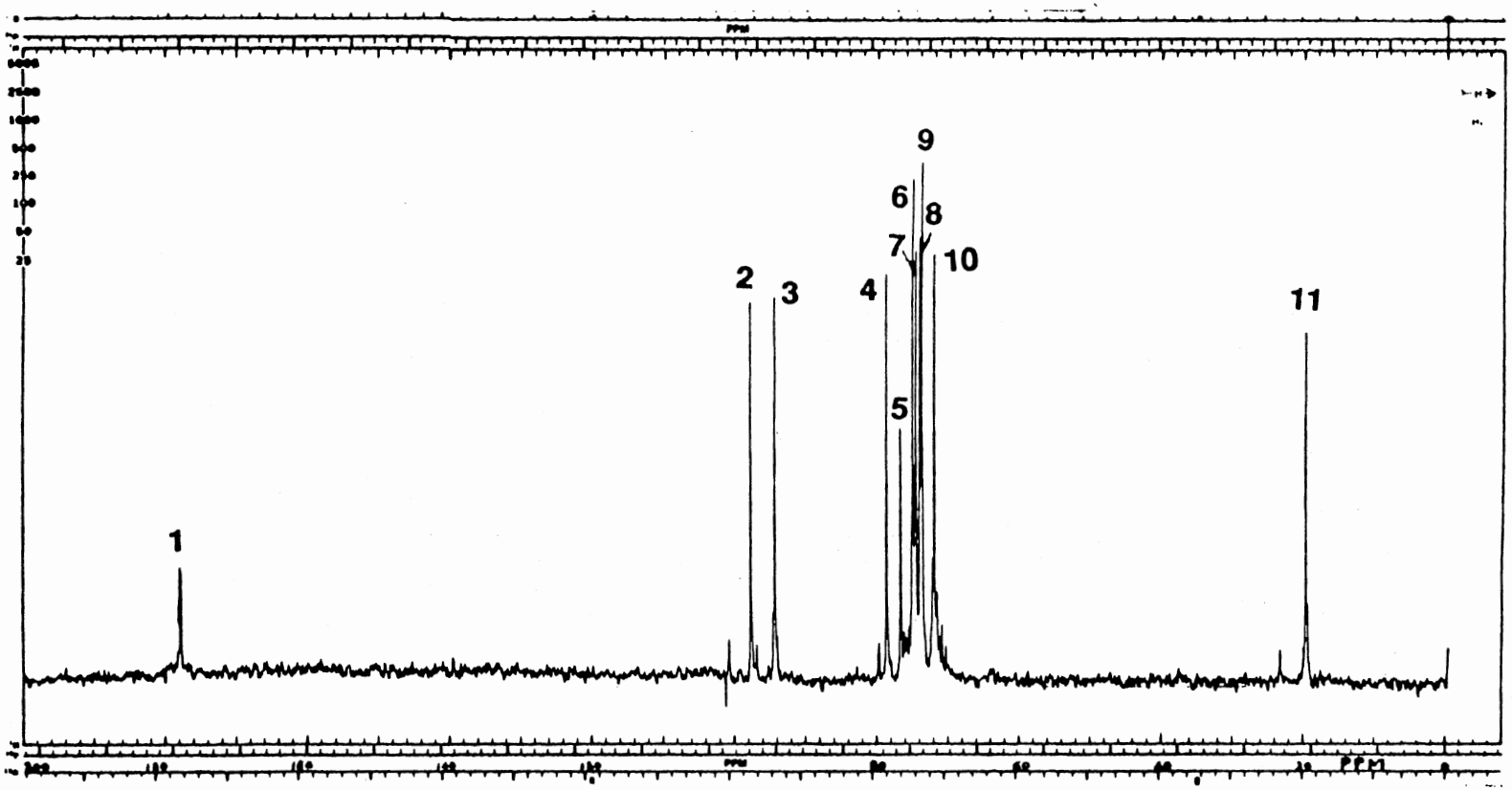

Figure 14. ^{13}C n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose (GalA-Rha) Isolated from the Water Extract of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) C-6 of GalA; 2) C-1 of GalA; 3) C-1 of Rha; 4) C-2 of Rha; 5) C-5 of GalA; 6) C-5 of Rha; 7) C-4 of Rha; 8) C-3 of Rha; 9) C-2 of GalA; 10) C-3 of GalA; 11) C-6 of Rha.

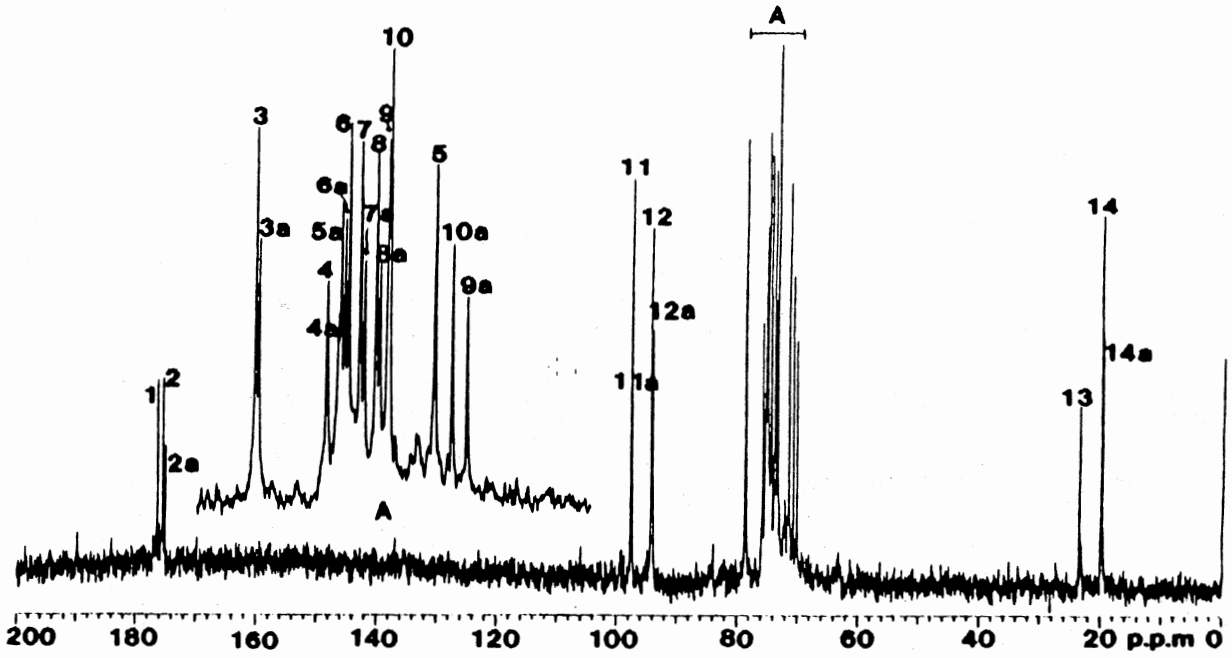

Figure 15. ^{13}C n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose (GalA-Rha) Isolated from the HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Plain numbers refer to signals in the non-acetylated disaccharide and the numbers with an "a" refer to signals in the acetylated disaccharide. A = Enlarged spectrum of the region about 60-80 p.p.m shown in the inset. Peaks are assigned as follows: 1) >C=O of acetate; 2) C-6 of GalA; 3) C-2 of Rha; 4) C-5 of GalA; 5) C-3 of GalA; 6) C-5 of Rha; 7) C-4 of Rha; 8) C-3 of Rha; 9) C-2 of GalA; 10) C-4 of GalA; 11) C-1 of GalA; 12) C-1 of Rha; 13) CH_3 of Acetate; 14) C-6 of Rha.

the 2D ^{1}H-^{13}C heterocorrelated n.m.r. spectrum of the disaccharide (Figure 16). The chemical shifts of the signals from the acetylated and non-acetylated disaccharides are shown in Table XI. Most of the signals had chemical shifts as expected from galacturonic acid and rhamnose residues. The signal at 23.46 p.p.m. represents the methyl carbon resonance of the acetate group and the signal at 176.37 p.p.m. represents the 'c=o' resonance of the acetate group. This is consistent with the conclusion of the presence of an acetate group in the disaccharide isolated from the HF/ether fraction. C-3 of the acetylated galacturonic acid was shifted 3.81 p.p.m. downfield with respect to the non-acetylated form, confirming the presence of an acetate group on C-3 of galacturonic acid. C-2 and C-4 were shifted upfield, 3.23 and 2.54 p.p.m, respectively. The downfield shift of C-2 of galacturonic acid with respect to C-2 of free α galA in the non-acetylated disaccharide suggested that it was glycosylated. This suggested that the rhamnose 1 position was reacted with the C-2 of galacturonic acid forming a β 1→2 linkage. This resulted in the formation of a cyclic disaccharide in which galacturonic acid was linked α 1→2 to rhamnose, with rhamnose linked β 1→2 back to galacturonic acid. In this structure H-3 of galacturonic acid was held in very close proximity to O-2 and O-5 of rhamnose, which explained its downfield shift in the ^{1}H n.m.r. spectrum. Using space filling models it was possible to build a cyclic disaccharide in which galacturonic acid was linked α 1→2 to rhamnose, with rhamnose linked β 1→2 back to galacturonic acid. During the solvolysis of polysaccharides in HF, the initial products are glycosyl fluorides. These are somewhat reactive, especially in liquid HF. If high concentrations of sugar fluorides are generated in HF they tend to polymerize (Mort and Parker, 1982; Kraska and Micheel, 1976). The polymerization can usually be avoided by working at low concentrations. In some cases oligomerization can occur even at low concentrations (Kuo *et al.*, 1986). In the case of galacturonic acid --rhamnosyl fluoride, the C-1 of the rhamnose is adjacent to the O-2 of galacturonic acid and this leads to the easy formation of the β 1-2 cyclic glycoside.

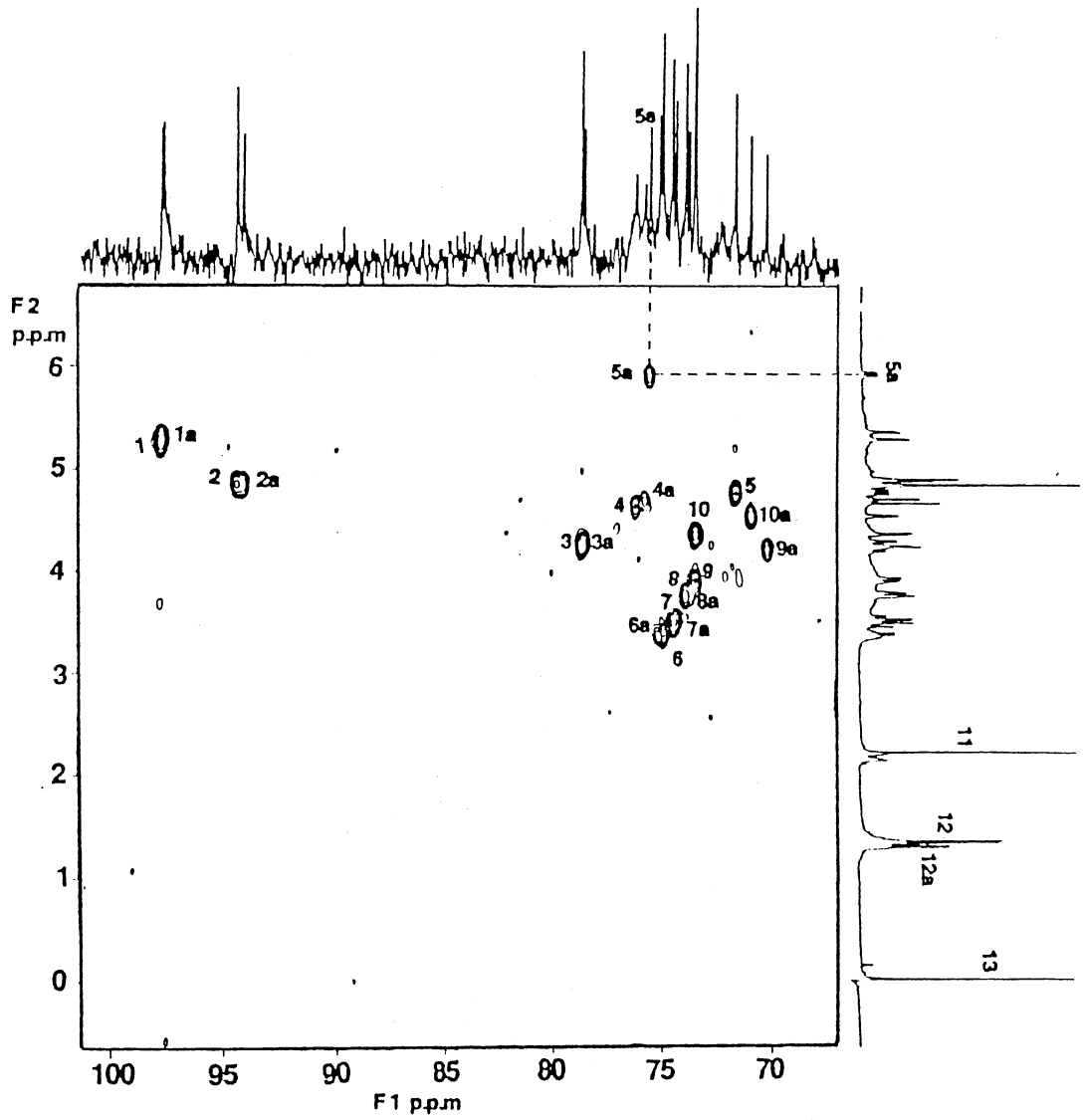

Figure 16. ^{13}C-^{1}H Heteronuclear Correlated n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose (GalA-Rha) Isolated from the HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C, Together with Proton Decoupled 1 D ^{13}C Spectrum (above) and the ^{1}H Spectrum (right). The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Plain numbers refer to signals in the non-acetylated disaccharide and the numbers with an "a" refer to signals in the acetylated disaccharide. The dashed lines illustrate how the signals correspond to both ^{13}C and ^{1}H signals. Peaks are assigned as follows: 1) C-1, H-1, GalA; 2) C-1, H-1 Rha; 3) C-2, H-2, Rha; 4) C-5, H-5 of GalA; 5) C-3, H-3 of GalA; 6) C-5, H-5 of Rha; 7) C-4, H-4 of Rha; 8) C-3, H-3 of Rha; 9) C-2,H-2 of GalA; 10) C-4,H-4 of GalA; 11) CH_3 of Acetate; 12) CH_3 of Rha; 13) CH_3 of TSP.

TABLE XI

ASSIGNMENTS OF CARBON AND HYDROGEN ATOMS TO THE SIGNALS OBSERVED IN THE ^{1}H AND ^{13}C NMR SPECTRA OF THE DISACCHARIDE (NONACETYLATED AND ACETYLATED) AND THE REFERENCE COMPOUNDS, α-GALACTURONIC ACID AND β-RHAMNOSE

	α-Galacturonic		Galacturonic acid-acetylated disaccharide		Galacturonic acid in acetylated disaccharide		β-Rhamnose		β-Rhamnose in nonacetylated disaccharide		β-Rhamnose in acetylated disaccharide	
	^{1}H[†]	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H[ƒ]	^{13}C[§]	^{1}H	^{13}C	^{1}H	^{13}C
C-1, H-1	5.30	95.26	5.24	97.60	5.31	97.49	4.86	96.35	4.83	94.29	4.80	94.02
C-2, H-2	3.81	70.73	3.88	73.37	4.16	70.14	3.94	74.17	4.20	78.71	4.22	78.57
C-3, H-3	3.91	71.58	4.70	71.45	5.83	75.26	3.60	75.59	3.71	73.85	3.71	73.68
C-4, H-4	4.26	72.58	4.33	73.29	4.51	70.75	3.36	74.66	3.47	74.49	3.44	74.31
C-5, H-5	4.41	75.24	4.68	75.82	4.75	75.36	3.40	74.88	3.35	74.95	3.35	75.11
C-6, H-6	—	174.99	—	175.51	—	175.09	1.29	19.59	1.31	19.92	1.29	19.76

CH_3 of Acetate – ^{1}H – 2.18, ^{13}C – 23.46, >C = 0 of Acetate ^{13}C – 176.37

[†]Ref. Tjan et al., (1974)
[ƒ]Ref. Debruyn et al., (1976)
[§]Ref. Kasai et al., (1979)

^{1}H and ^{13}C nmr spectra were taken in D_2O solution using as TSP internal standard.

Time Course Study of Hydrolysis of the Disaccharide with DCl Followed by N.M.R. Analysis

This experiment was done to obtain additional evidence that the disaccharides, as isolated, were cyclic in nature. The course of hydrolysis was followed by ^1H n.m.r. spectroscopy (the spectra at various time intervals are not shown). The spectrum after one hour of hydrolysis showed that the acetate ester was completely hydrolysed. This was indicated by the loss of the signal from H-3 of galacturonic acid at 5.83 p.p.m. The spectrum looked very similar to that of the non-acetylated disaccharide except for the presence of a resonance for acetic acid at 2.09 p.p.m. The signal for H-5 of galacturonic acid was shifted downfield due to the strong acidic conditions. New signals appeared in the spectrum after 4 h of hydrolysis. One signal at 5.22 p.p.m. could be attributed to H-1 of a galacturonic acid in a slightly different environment from the original sample. Two other signals at 5.1 and 4.84 p.p.m. could be attributed to H-1 of α and β forms of reducing rhamnose showing the hydrolysis of the disaccharide. Thus the disaccharide could be partly hydrolysed and yet remain a disaccharide with the original chemical shifts. The spectrum in the region of 4.5 to 3.5 p.p.m. also became complex. Up to 9 hours of hydrolysis the changes mentioned above became pronounced. But at later times new signals at the chemical shifts characteristic of H-1 of free α and β galacturonic acid started to appear. After 26 hours a large proportion of the disaccharide had been hydrolysed to monosaccharides. After reduction of the sample with sodium borohydride about 25% of the original (deacetylated) disaccharide remained and there was still some non-reducible galacturonic acid in a linkage slightly different from the original disaccharide. In the region from 1.5 to 1 p.p.m. considerable changes were also observed during the hydrolysis. After three hours three peaks representing methyl group of α and β rhamnose became significant slightly upfield of the original doublet. This set of three peaks increased during the hydrolysis and became more complex. After the 26 hours of

hydrolysis followed by reduction, the complex signals moved upfield 1.32 to 1.17 p.p.m. as one might expect for conversion of rhamnose to rhamnitol. However, the doublet from the remaining unchanged disaccharide was unaffected by the reduction. This showed that the cyclized disaccharide was very stable, that both glycosidic linkages were stable and that hydrolysis of one without the other was difficult.

^{13}C N.M.R. With Proton Coupling

^{13}C n.m.r. spectrum with proton coupling of the disaccharide isolated from the ether soluble sugars is shown in Figure 17. Most ^{13}C spectra are acquired in the ^{1}H-decoupled mode (Figure 14), which involves noise-modulated irradiation of all of the protons simultaneously. This reduces the spectrum to a group of singlets and results in a marked improvement in S/N (signal to noise) because the intensity of each signal is increased in proportion to the H splitting eliminated, as well as by nuclear Overhauser enhancement. To obtain ^{13}C-^{1}H coupling information without too large a sacrifice in S/N, irradiation of the protons is gated, so that most of the nuclear Overhauser enhancement contribution is preserved. ^{1}H -coupled spectra are complex but help to identify the anomeric configuration and gives additional structural information. The H-1, C-1 couplings of galacturonic acid were 169.7 and 170.9 Hz for the acetylated and nonacetylated forms respectively, which indicated that the galacturonic acid residue was an α-anomer (Figure 17). However, the H-1,C-1 couplings of the rhamnose residue were 166 Hz for the nonacetylated form and 167 Hz for the acetylated form. These couplings are suggestive of the α-configuration or a form intermediate between α and β (Kasai *et al.*, 1979).

Reducing End Detection of the Disaccharide

Treatment of the disaccharide with potassium borohydride and subsequent analysis of the sample by gas chromatography showed no reduction of rhamnose to rhamnitol confirming that the disaccharide did not have a reducing end.

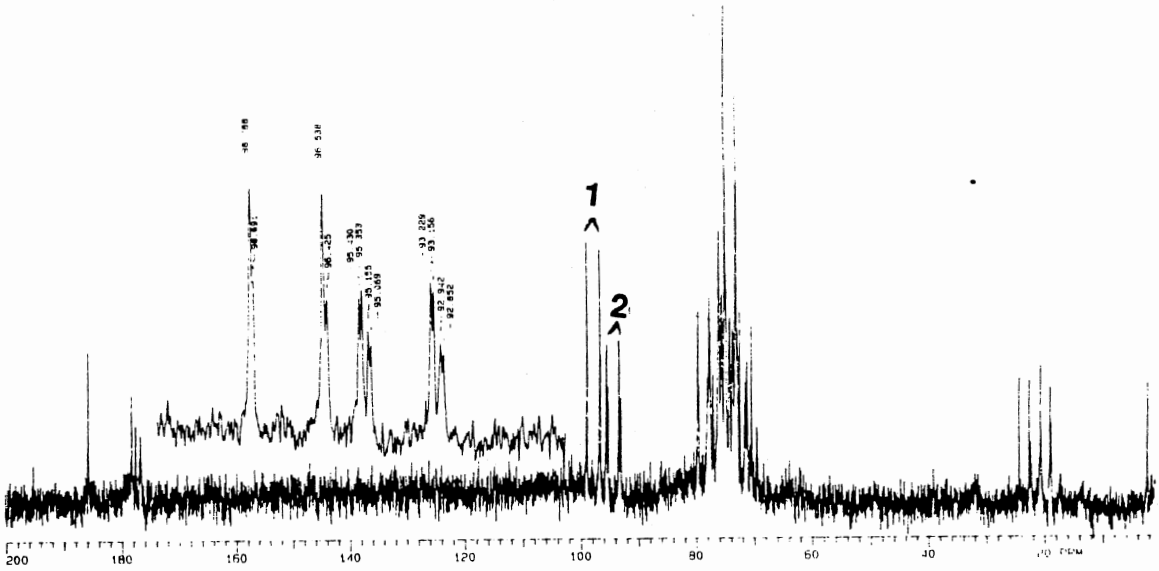

Figure 17. ^{13}C n.m.r. Spectrum with Proton Coupling of the Disaccharide Galacturonosyl Rhamnose (GalA-Rha) Isolated from the HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Anomeric carbon signals are only assigned. 1) C-1 of GalA; 2) C-1 of Rha. Enlarged spectrum of 90-100 p.p.m. is shown in the inset. The H-1, C-1 coupling of GalA is 169.7 Hz and 170.9 Hz for the acetylated and the non-acetylated disaccharide and represents α. H-1, C-1 coupling of Rha is 166 Hz and 167 Hz for the non-acetylated and the acetylated disaccharide representing α or a form intermediate between α and β.

Mass Spectroscopic and Methylation Analysis of the Disaccharide

The mass spectrum of the disaccharide is shown in Figure 18. The peaks at 321 and 363 corresponded to the molecular weights of the non-acetylated and acetylated disaccharides, respectively. In both cases the molecular weight was found to be less than the expected value by 18 mass units. This showed that a molecule of water was lost during the cyclization of the disaccharide.

The chromatographic traces of the partially methylated alditol acetates of the disaccharide galacturonosyl rhamnose without and with reduction are shown in Figure 19. Methylation was performed by the method of Hakomori which removes esters from sugar. (One half of the methylated samples containing uronic acids are reduced before hydrolysing to monosaccharides and the other half was hydrolysed without reduction. Samples are reduced after methylation and prior to hydrolysis to convert the uronic acids to alcohols for identification. In this case galacturonic acid is reduced to galactose and the amount of galactose present after reduction of the sample compared to the unreduced sample gives the amount of galacturonic acid present in the original sample). The peaks 2 (27.37 min) and 3 (51.77 min) were identified using mass spectra to be 2 linked rhamnose and 2,6 linked galactose (Figures 21 and 22). Minor amount of t-xyl was also present (signal at 20.4 min, mass spectrum shown in Figure 20). Production of 2,6 linked galactose after reduction instead of 6 linked galactose showed that galacturonic acid 2 position must be linked to rhamnose to form the cyclized disaccharide. If the disaccharide was not cyclized 2-linked rhamnose and 6 linked galactose would have been the products. Disaccharides methylated using the method of Prehm (1980) where the acetate groups are retained on the sugar during methylation showed 2 linked rhamnose and 2,3,6 linked galactose (spectrum not shown), which again confirmed the fact that acetate group was on the 3 position and 2 position of galacturonic acid was involved in the cyclization.

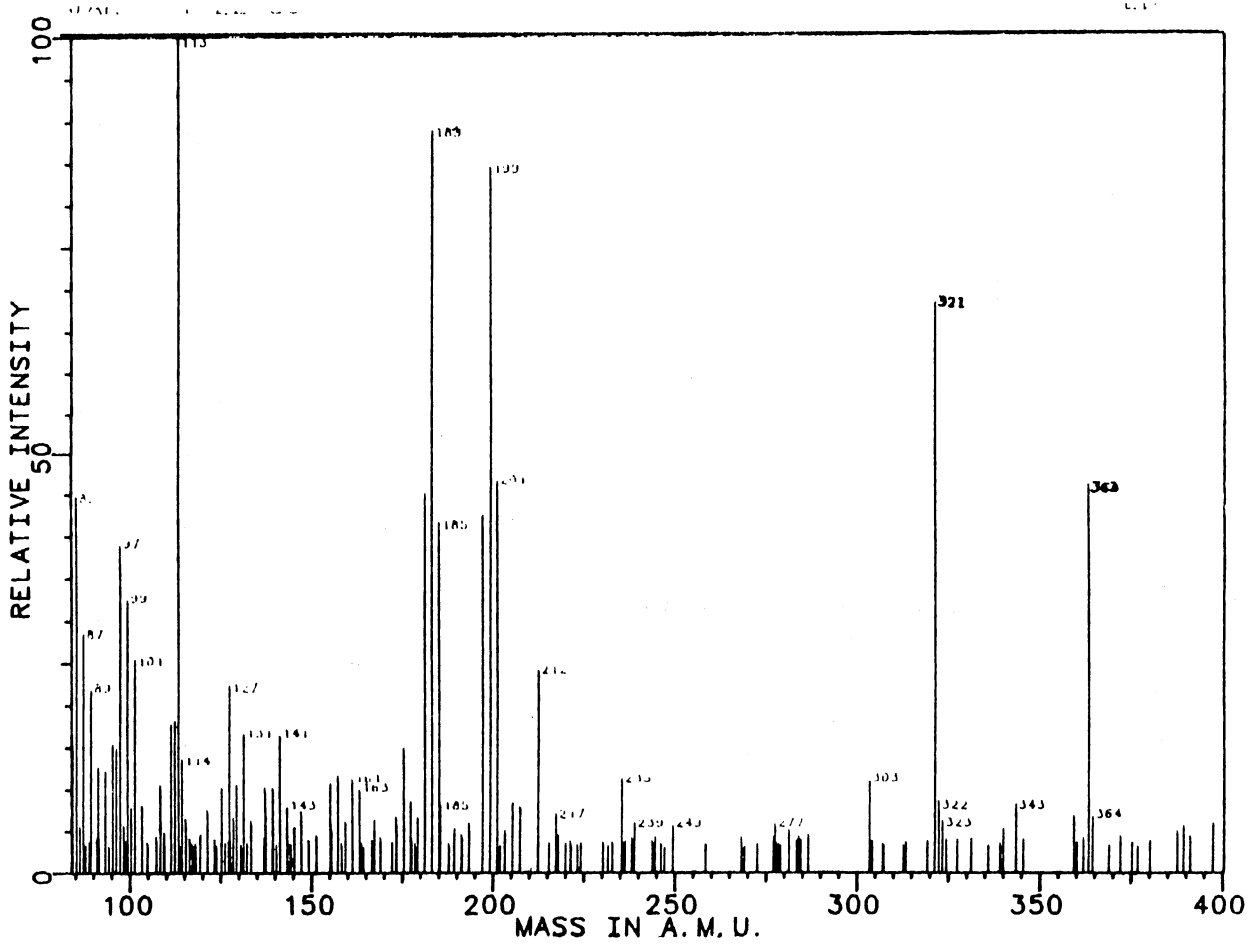

Figure 18. Mass Spectrum of the Disaccharide GalA-Rha Isolated from HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a FTMS -2000 (laser desorption mass) spectrometer. Peak with mass 321 corresponds to the molecular weight of the non-acetylated disaccharide and the peak with mass 363 corresponds to the molecular weight of the acetylated disaccharide. Both the peaks show 18 mass units less than the expected molecular weight suggesting the loss of water during cyclization of the disaccharide.

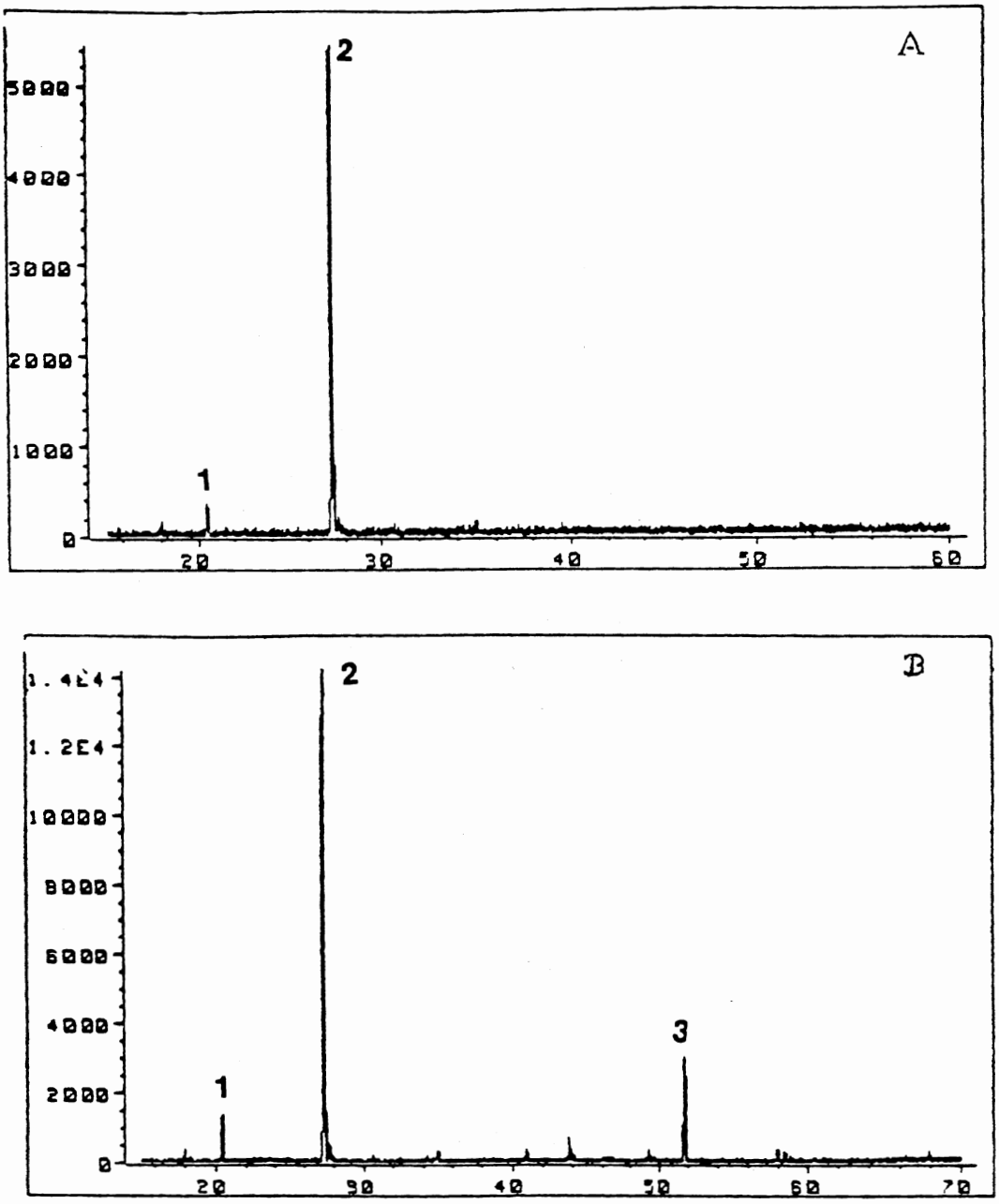

Figure 19. The Gas Chromatographic Trace of the Partially Methylated Alditol Acetates of the Disaccharide GalA-Rha Isolated from the HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. A= without reduction of the galacturonic acid before hydrolysis and B= with reduction of the galacturonic acid before hydrolysis. Peaks are identified from the mass spectrum of the corresponding peaks (see Figures 20, 21, 22). 1) t-xyl; 2) 2-linked Rha; 3) 2,6-linked Gal (Galacturonic acid is reduced to galactose).

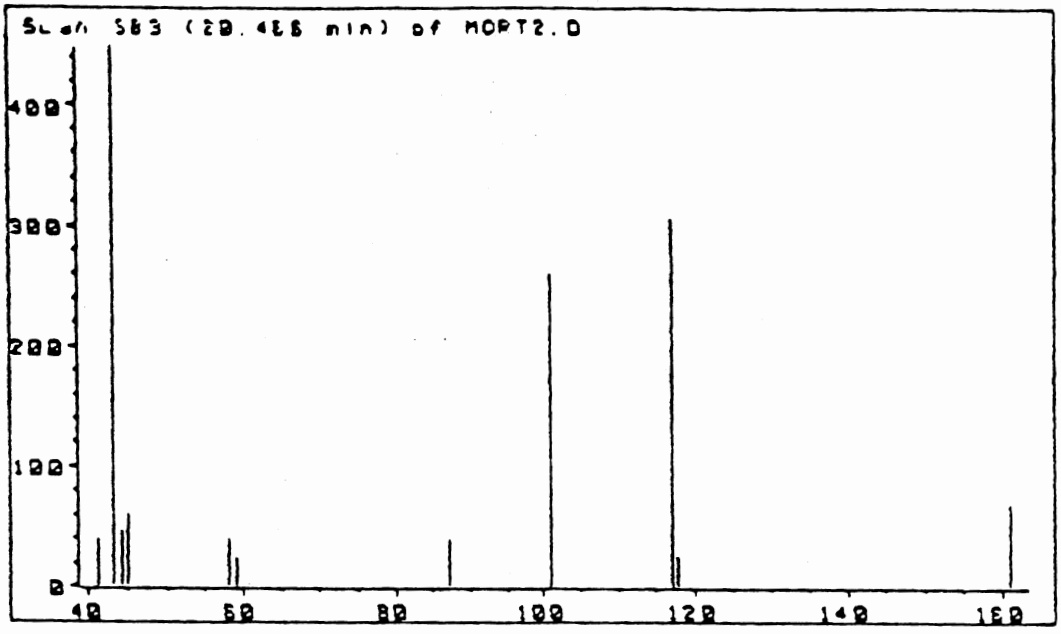

Figure 20. Mass Spectrum of the Peak Number 1 (20.46 min) of the Gas Chromatogram (Figure 19) of the Partially Methylated Alditol Acetates of the Disaccharide Gal-Rha. The fragments formed with M/Z are 43.05, 101.1, 117 and 161.05. This corresponds to the t-linked xyl fragmentation pattern and the peak 1 is thus identified as that of t-linked xyl.

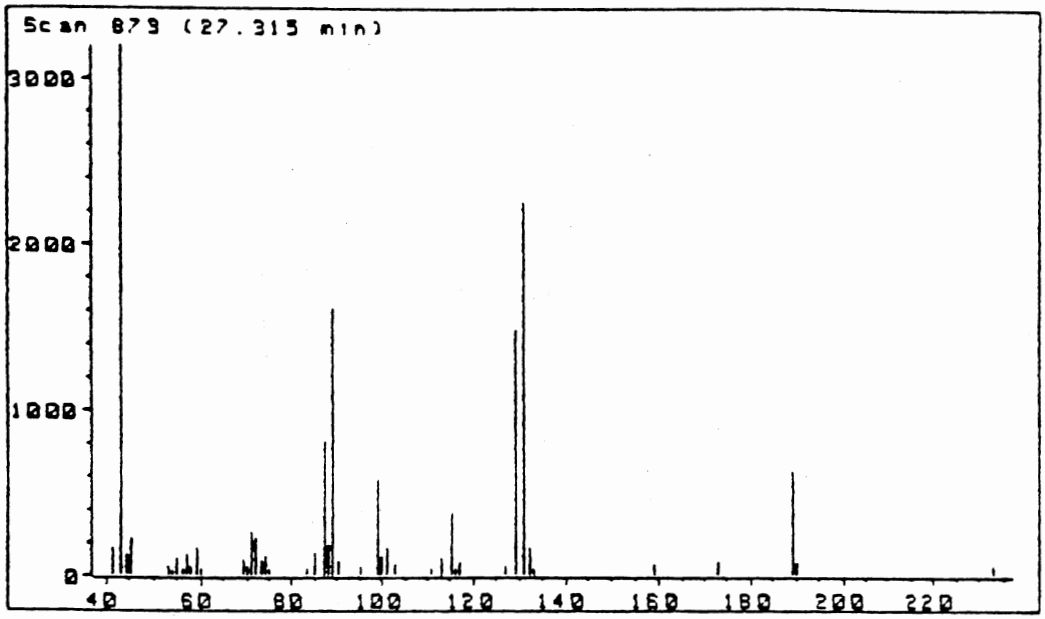

Figure 21. Mass Spectrum of the Peak Number 2 (27.315 min) of the Gas Chromatogram (Figure 19) of the Partially Methylated Alditol Acetates of the Disaccharide GalA-Rha. The fragments formed are with M/Z 43.05; 86.1; 129.1; 131.15; and 189.10. This corresponds to the fragmentation pattern of 2-linked Rha (Bjornal *et al.*, 1970) and the peak number 2 is identified as that of 2-linked rhamnose.

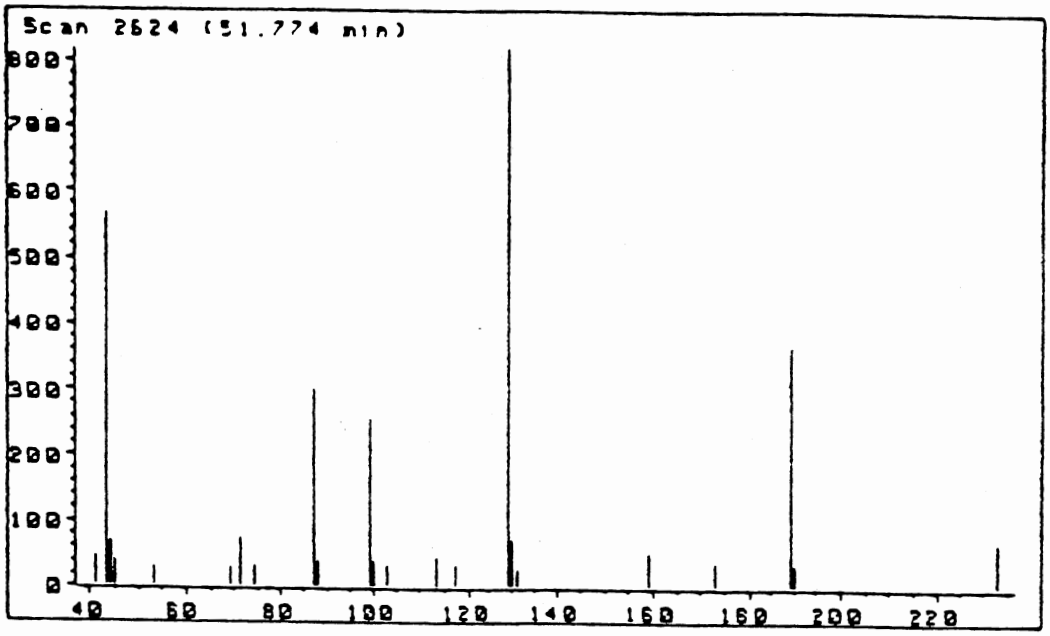

Figure 22. Mass Spectrum of the Peak Number 3 (51.774 min) of the Gas Chromatogram (Figure 19) of the Partially Methylated Alditol Acetates of the Disaccharide GalA-Rha. The fragments formed are with M/Z 43.15; 87.99; 129 and 189.1. This corresponds to the fragmentation pattern of 2,6-linked Gal and the peak 3 was identified as that of 2,6 linked Gal.

Isolation of the Disaccharide Galacturonosyl Rhamnose from IM 216 Cotton Suspension Cell Walls, Acala 44 Leaf Walls, Tomato Suspension Walls, Tobacco Leaf Walls and Carrot Root Walls

About 1 gm of the above mentioned cell walls was treated with HF at -23°C in batches of 500 mg each as described earlier for Acala 44 cotton suspension walls. The disaccharide galacturonosyl rhamnose was isolated from both the HF/ether soluble sugars and from the water extract as for the Acala 44 suspension walls (refer to Figure 5). The ^1H n.m.r. spectra of the disaccharides isolated from the ether/HF soluble sugars are shown in Figures 23 to 27. All spectra were identical to the spectrum of the disaccharide isolated from the HF/ether soluble sugars of Acala 44 cotton suspension walls except for a few signals. All spectra show signals at about 2.17 p.p.m., which is the signal for acetate ester along with the signal at 5.83 p.p.m. which represents H-3 of galacturonic acid with the acetate ester. This shows the presence of acetate on -3 of galacturonic acid residue in all these samples. The samples 23, 25 and 27 showed two signals at 2.17 p.p.m. instead of one and an addition signal at 5.6 p.p.m. which represents H-4 of galacturonic acid with an acetate ester. Samples 23, 25 and 27 were produced using $CaC0_3$ to neutralize the HF/ether mixture instead of evaporation. The ether HF soluble sugars were neutralized by adding solid calcium carbonate (about 30 gm for 10 ml HF) and the sugars were extracted with water from the calcium carbonate mixture. This procedure caused migration of acetate groups, and that was the reason for the presence of two signals at about 2.17 p.p.m. instead of one signal. Some of the acetate groups have migrated to the 4 position of galacturonic acid (signal at 5.6 p.p.m.). The 4 position of galacturonic acid was not free in the polymer. So acetate must have migrated to this position during isolation. The signal at 5.83 p.p.m. indicates the acetate group on 3 position of galacturonic acid as in the earlier cases. Changing the procedure to evaporation of the ether HF mixture instead

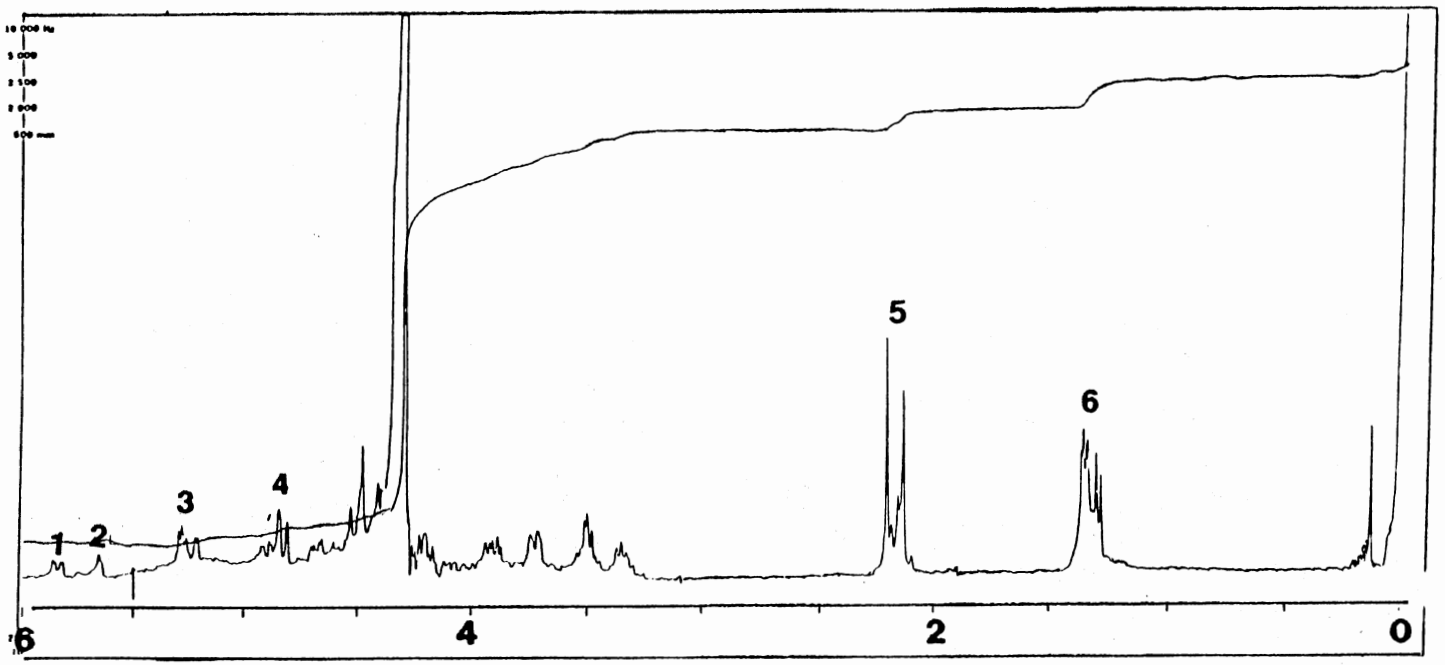

Figure 23. ^{1}H n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose Isolated from the HF/ether Soluble Fraction of Im 216 Cotton Auspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-3 of GalA, with acetate group; 2) H-4 of GalA with acetate (acetate has migrated from the 3 position during isolation) 3) H-1 of GalA; 4) H-1 of Rha; 5) CH_3 of acetate; 6) CH_3 of Rha. Two signals are present for acetate indicating two positions of acetylation, this is due to migration of acetate group during isolation in the alkaline conditions in the neutralizing step of HF/ether soluble fraction with calcium carbonate instead of evaporation.

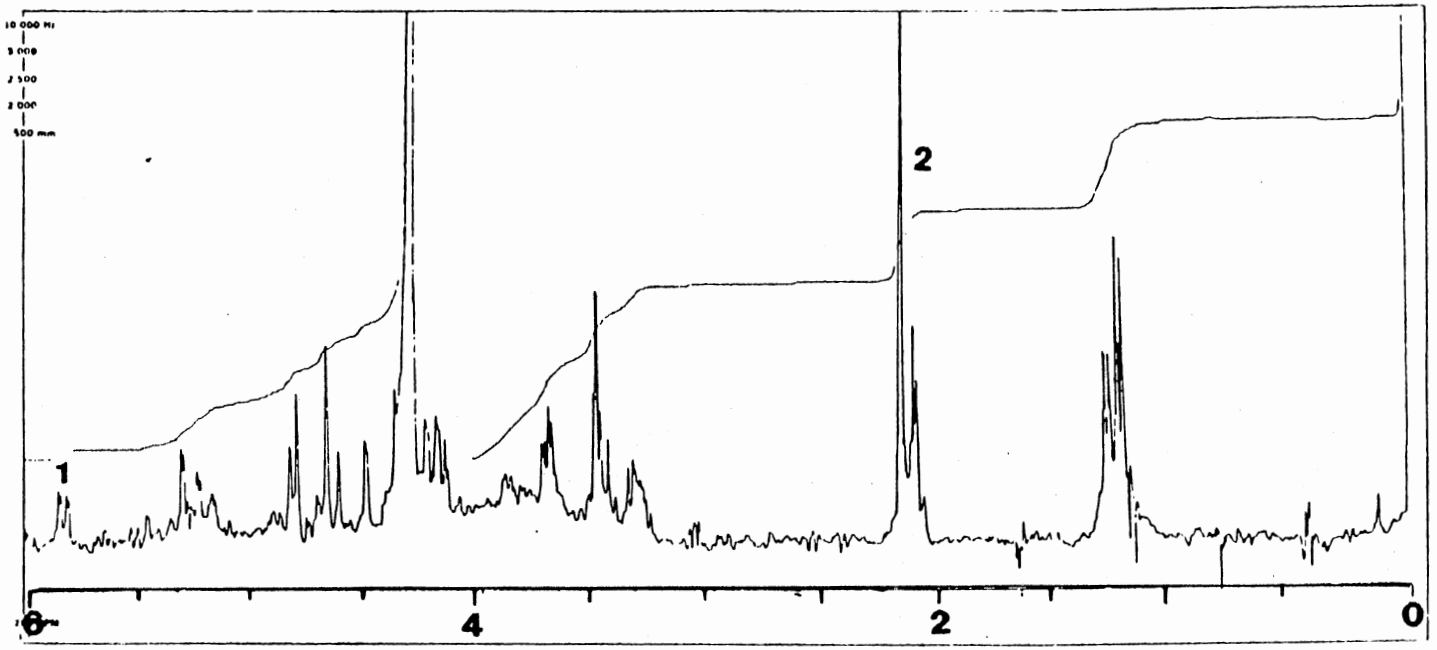

Figure 24. ^{1}H n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose Isolated from the HF/ether Soluble Fraction of Acala 44 Cotton Leaf Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-3 of GalA, with acetate group; 2) CH_3 of acetate.

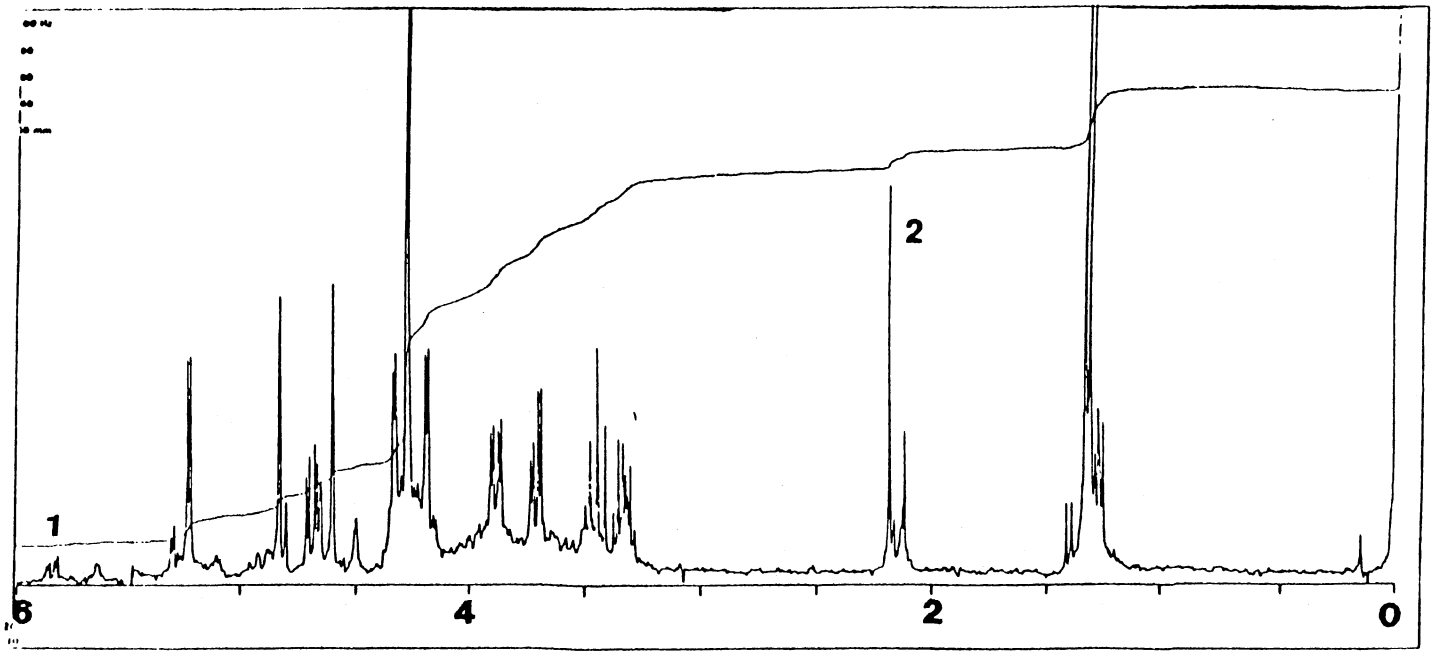

Figure 25. ^{1}H n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose Isolated from the HF/ether Soluble Fraction of Tomato Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-3 of GalA, with acetate group; 2) CH_3 of acetate.

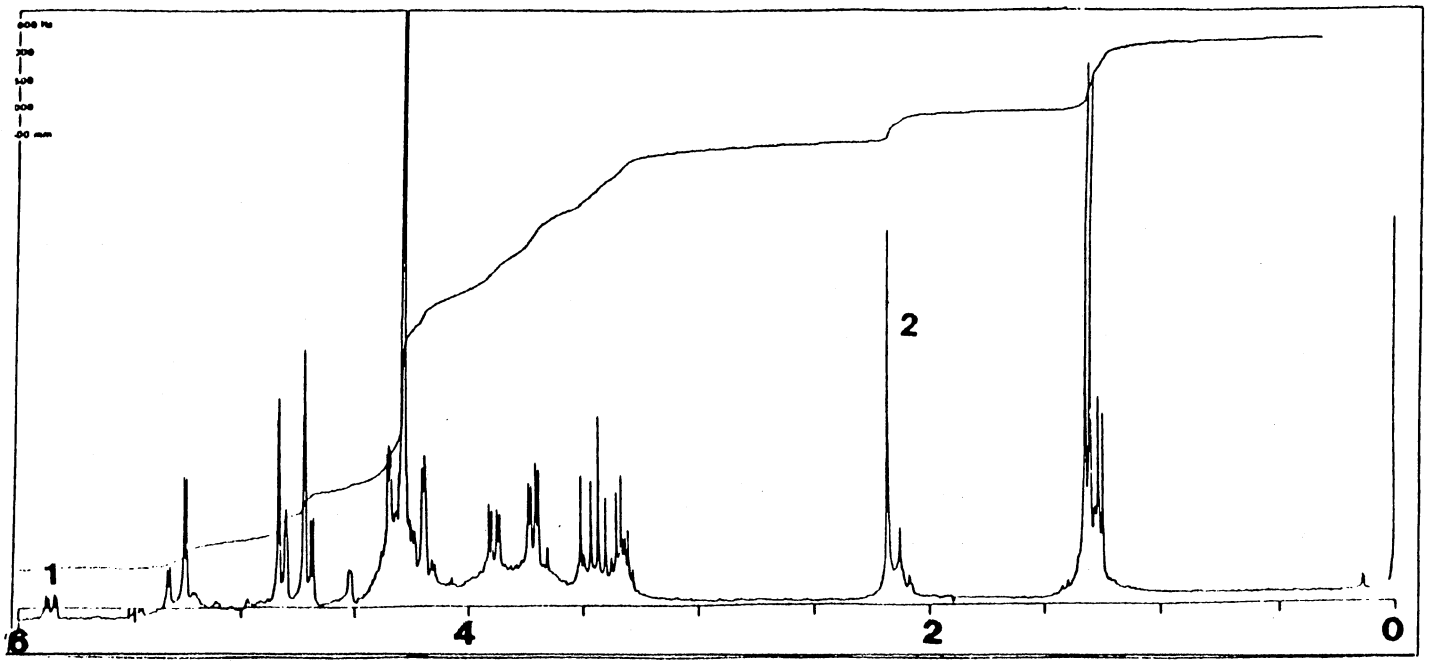

Figure 26. ^{1}H n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose Isolated from the HF/ether Soluble Fraction of Tobacco Leaf Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-3 of GalA, with acetate group; 2) CH_3 of acetate.

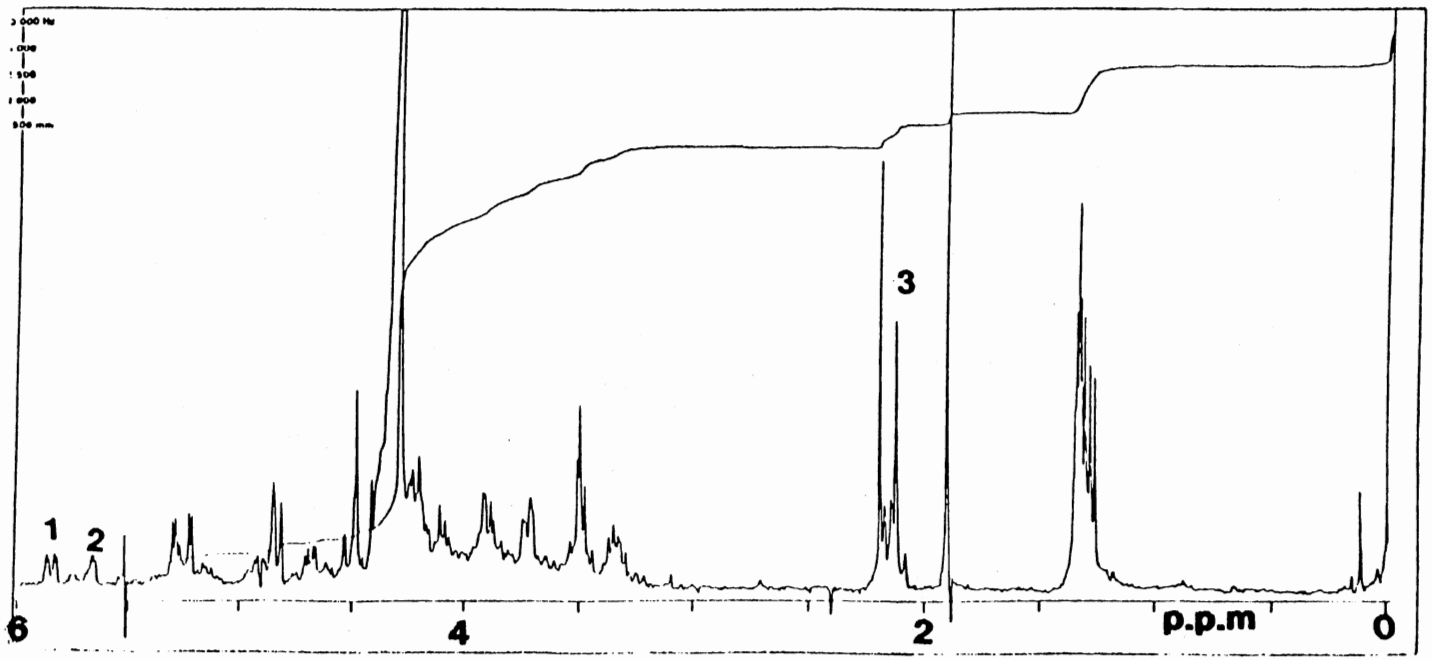

Figure 27. ^{1}H n.m.r. Spectrum of the Disaccharide Galacturonosyl Rhamnose Isolated from the HF/ether Soluble Fraction of Carrot Root Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-3 of GalA, with acetate group; 2) H-4 of GalA; 3) CH_3 of acetate. Two signals are present for acetate indicating two positions of acetylation, this is due to migration of acetate group during isolation in the alkaline conditions in the neutralizing step of HF/ether soluble fraction with calcium carbonate instead of evaporation. The signal at 1.9 p.p.m. is from the residual acetic acid from the buffer used for fractionation of disaccharides on the ion exchange column.

of $CaC0_3$ addition eliminated the migration of acetate groups from 3 position to 4 position. The disaccharide isolated from the water extract in all these cases did not have any acetylation as in the case of Acala 44 cotton suspension walls (spectra not shown).

In all the 5 cases studied a significant amount of the disaccharide galacturonosyl rhamnose was isolated. The amount of disaccharides obtained from each sample of walls varied and this indicated that the amount of RG 1 found in these samples vary. In all the cases studied the disaccharide galacturonosyl rhamnose isolated from the ether soluble sugars was acetylated at the 3 position of galacturonic acid. This shows that acetylation of RG 1 backbone is a basic structural phenomenon seen in most plants.

Analysis of the Water Extract from Acala 44 Cotton Suspension Cell Walls After HF Solvolysis at -23°C

The HPLC trace of the water extract on TSK SW 4000 and SW 2000 gel filtration columns in series is shown in Figure 28. It showed that the water extract was a heterogeneous mixture with a wide range of molecular weight, according to the pullulan (polymaltotriose polysaccharide) molecular weight standards. The water soluble sugars were fractionated as shown in scheme 1 (Figure 5). The Bio-gel P-2 fractionation of the water extract is shown in Figure 9. The mole percent of sugars in the five different fractions made is shown in Table XII.

Analysis of Bio-gel P-2 Fraction 1 (P-2 Void Volume)

The sugar composition of the P-2 void volume showed that this fraction was made up of oligomers of galacturonic acid (Table XII) and may represent the homogalacturonan region of pectins. This fraction had ~35 mole% of neutral sugars. The HPLC gel filtration trace on tandem TSK SW 4000 and SW 2000 columns in 0.3M sodium acetate buffer, of the P-2 void volume is shown in Figure 29. This fraction was found to be a mixture of oligomers with a wide range of molecular weight according to pullulan

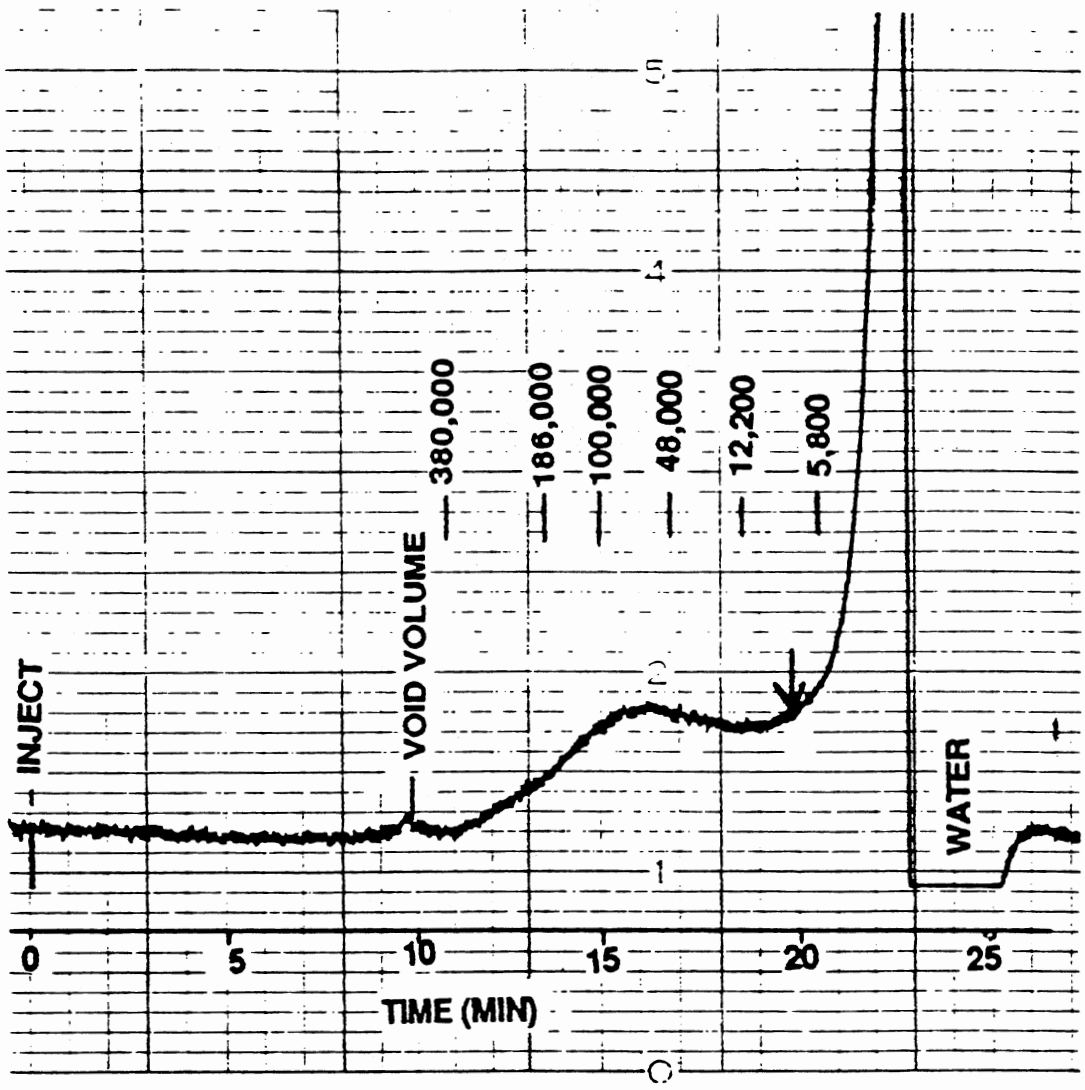

Figure 28. The HPLC Chromatogram of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. The sample was dissolved in water and injected. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. Galacturonic acid oligomers consistently elute earier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

TABLE XII

MOLE PERCENT OF SUGARS IN DIFFERENT P-2 FRACTIONS OF THE WATER EXTRACT OF THE HF -23°C REACTION

Sugar	Fr 1 P-2 void vol	Fr 2 Oligomers	Fr 3 Oligomers	Fr 4 Disaccharides	Fr 5 Monosaccharides
Ara	3.5	6.2	8.9	13.7	26.5
Rha	3.5	26.1	22.8	18.8	7.2
Fuc	0.4	-	1.1	2.1	-
Xyl	4.4	6.2	6.6	8.3	16.5
GlcA	-	-	3.9	6.2	-
GalA	63.8	30.2	23.7	19.1	8.9
Man	0.6	2.5	2.9	1.4	1.3
Gal	4.1	12.6	14.8	14.8	14.2
Glc	19.5	16.2	15.3	15.6	25.4

molecular weight standards. Galacturonic acid oligomers consistently elute earlier than expected based on pullulan standards of similar molecular weight , because they behave as rods in solution as opposed to the random coil structures of pullunans (Fishman *et al.*, 1984). This fraction was found to have an average degree of polymerization greater than 20 as determined by the elution volume of a purified 20-residue long oligogalacturonide. Gel filtration of galacturonic acid oligomers at low ionic strength was impractical because of aggregation of even small oligomers. The ^{1}H n.m.r. spectrum of the P-2 void volume showed substantial amounts of methyl esterification (Figure 30). The proton n.m.r. spectrum after demethyl esterification of the sample is shown in Figure 31. The P-2 void volume fraction was purified by a QAE Sephadex ion exchange column (Figure 32). The mole percent of sugars in different fractions is shown in Table 13. The molar ratio of rhamnose to galacturonic acid in fraction 2 was 1:10, in fraction 3 was 1:20, and in fraction 4 was 1:32. This showed that these fragments derived from the homogalacturonan region of pectins. The ^{1}H n.m.r. spectrum of the fraction 1 of QAE column showed it contained mainly β linked glucose (Figure 33) and the HPLC trace of this fraction showed it contained oligomers of small molecular weight (Figure 34). The QAE fraction 2 was highly methyl esterified (Figure 35). Fraction 3 also showed significant amount of methyl esterification (Figure 36) where as fraction 4 showed only small amounts of methyl esters (Figure 38). Oligomers of both fraction 3 and 4 had a degree of polymerization of more than 20 (Figures 37 and 39). Further analysis is needed to completely characterize each of these fragments and to determine the exact degree of methyl esterification of homogalacturonan region of pectins in cotton cell walls.

Analysis of P-2 Fractions 2 and 3 (Oligomers)
of the Water Extract

The sugar composition of the two fractions from Bio-gel P-2 (Figure 9) is shown in Table XII. Both the fractions were found to have rhamnose and galacturonic acid in 1:1

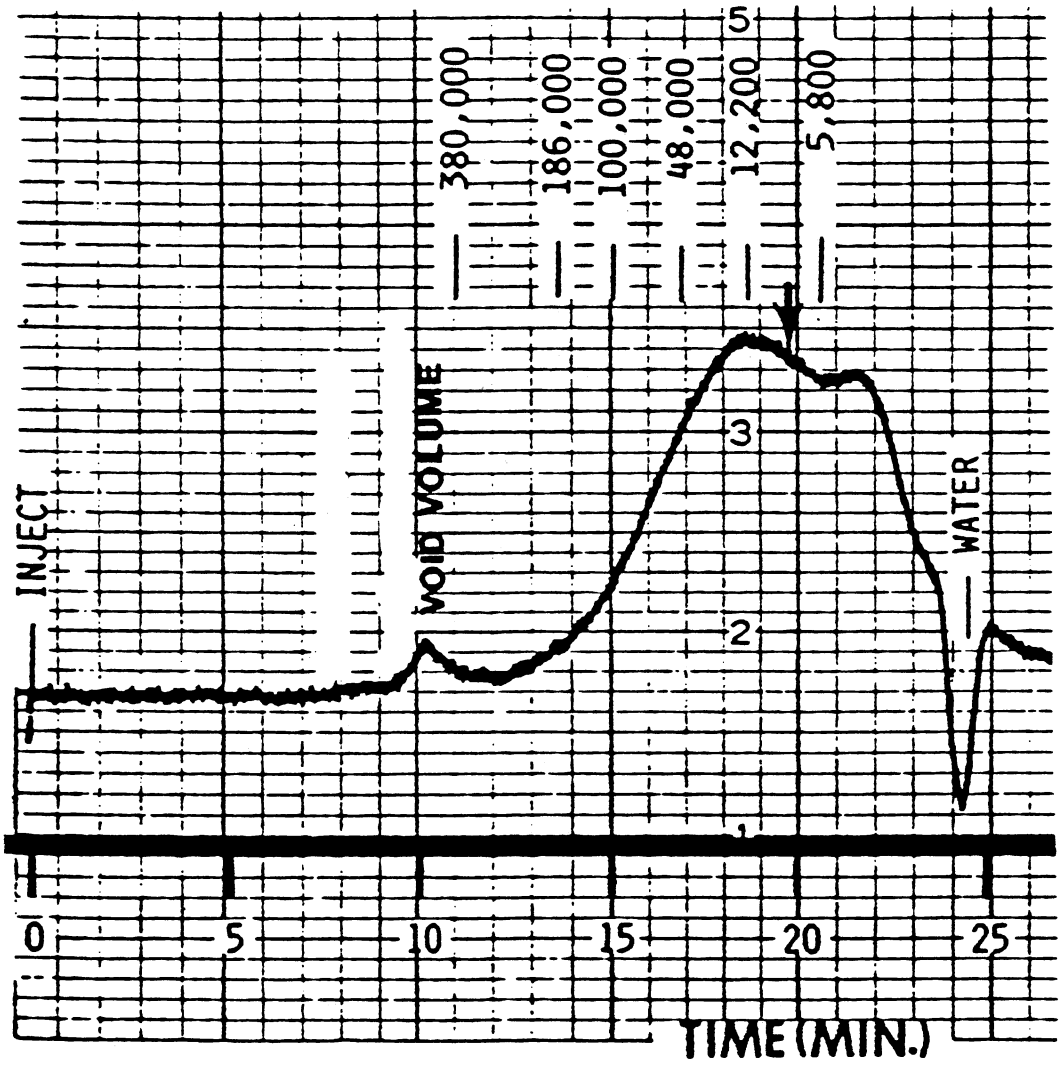

Figure 29. The HPLC Chromatogram of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. Galacturonic acid oligomers consistently elute earier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

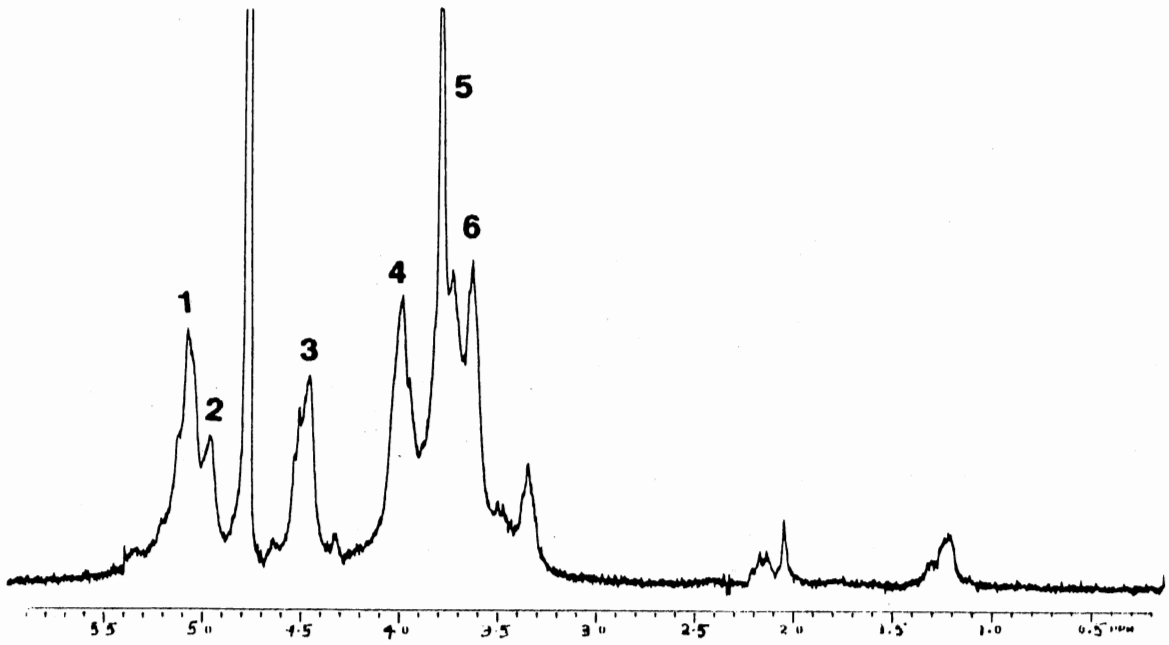

Figure 30. ^{1}H n.m.r. Spectrum of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-1 α GalA; 2) H-5 GalA; 3) H-4 GalA; 4) H-3 GalA; 5) CH_3 of methyl ester; 6) H-2 GalA.

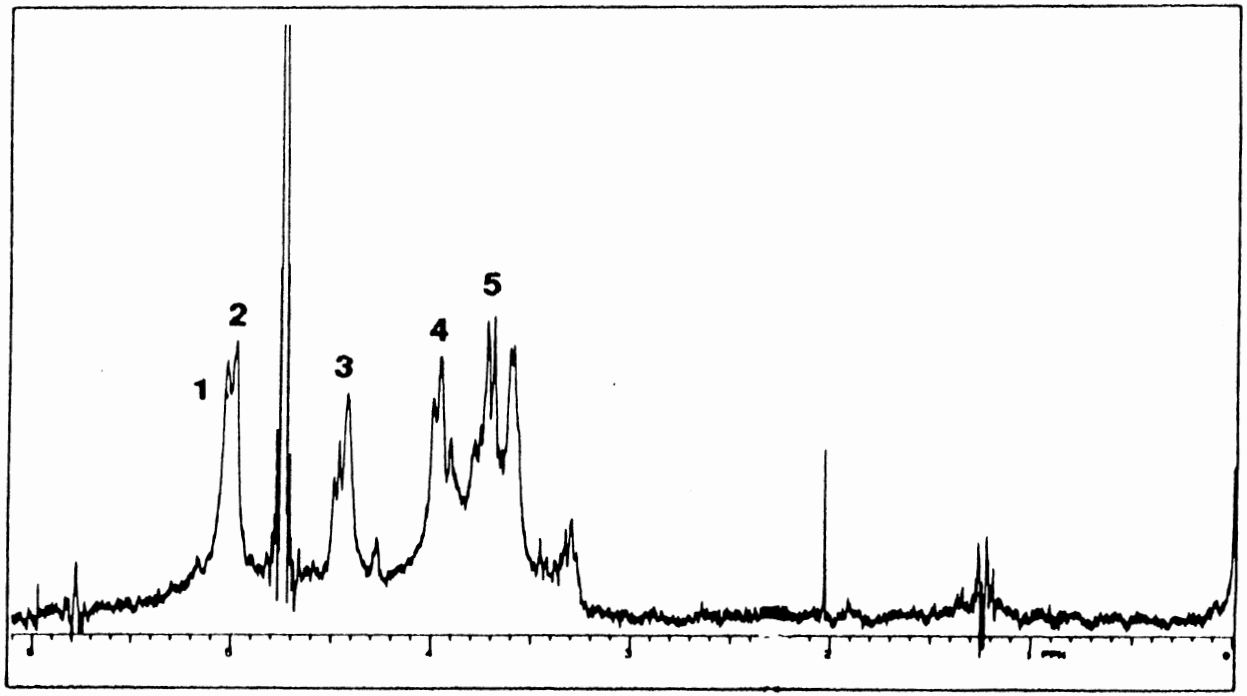

Figure 31. ^{1}H n.m.r. Spectrum of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C, after demethyl esterification of the sample. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-1 α GalA; 2) H-5 GalA; 3) H-4 GalA; 4) H-3 GalA; 5) H-2 GalA.

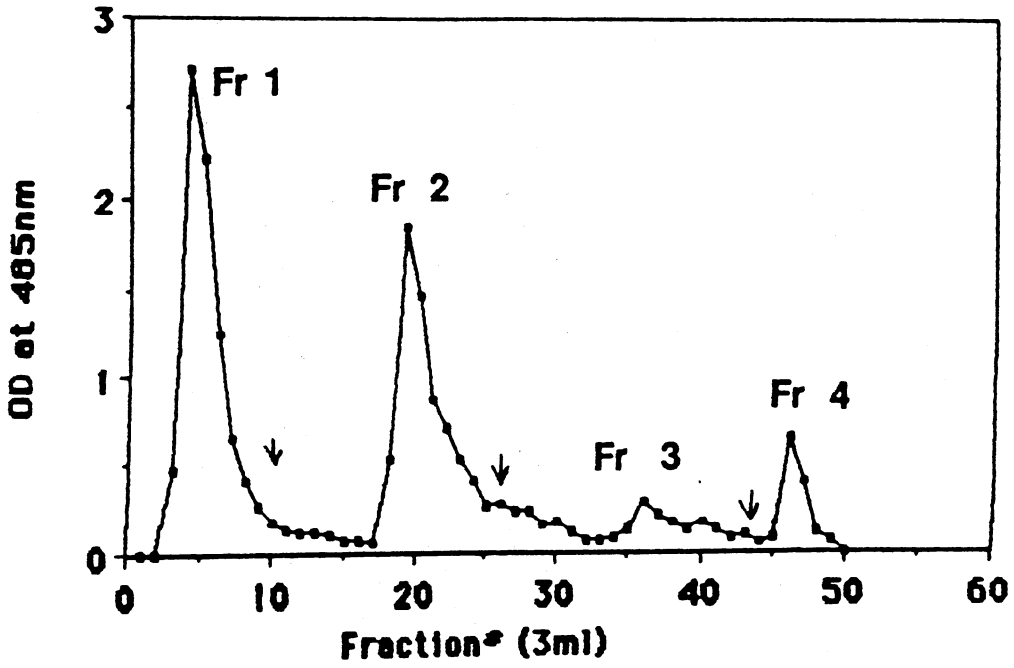

Figure 32. Chromatography on a QAE Sephadex Anion Exchange Column (5 x 1 cm) of Bio-gel P-2 Void Volume of the Water Extract from Acala 44 cotton Suspension Walls After HF Solvolysis at -23°C. Sample was applied on to the column in 0.05 M sodium acetate buffer pH 5.2 and eluted with 0.05 M, 0.3 M, 0.5 M and 1 M sodium acetate buffer pH 5.2 forming fractions 1, 2, 3 and 4, respectively. Fractions were tested for sugar by phenol sulfuric acid test.

TABLE XIII

MOLE PERCENT OF SUGARS IN THE QAE FRACTIONS OF THE P-2 VOID VOLUME OF THE WATER EXTRACT

Sugar	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Ara	10.6	3.6	2.2	1.2
Rha	3.8	6.8	4.1	2.7
Fuc	1.4	-	1.3	-
Xyl	12.2	4.6	2.8	2.1
GalA	6.5	67.7	81.8	87.8
Man	0.6	-	-	-
Gal	9.8	4.4	1.7	0.9
Glc	55.1	12.9	6.1	5.3

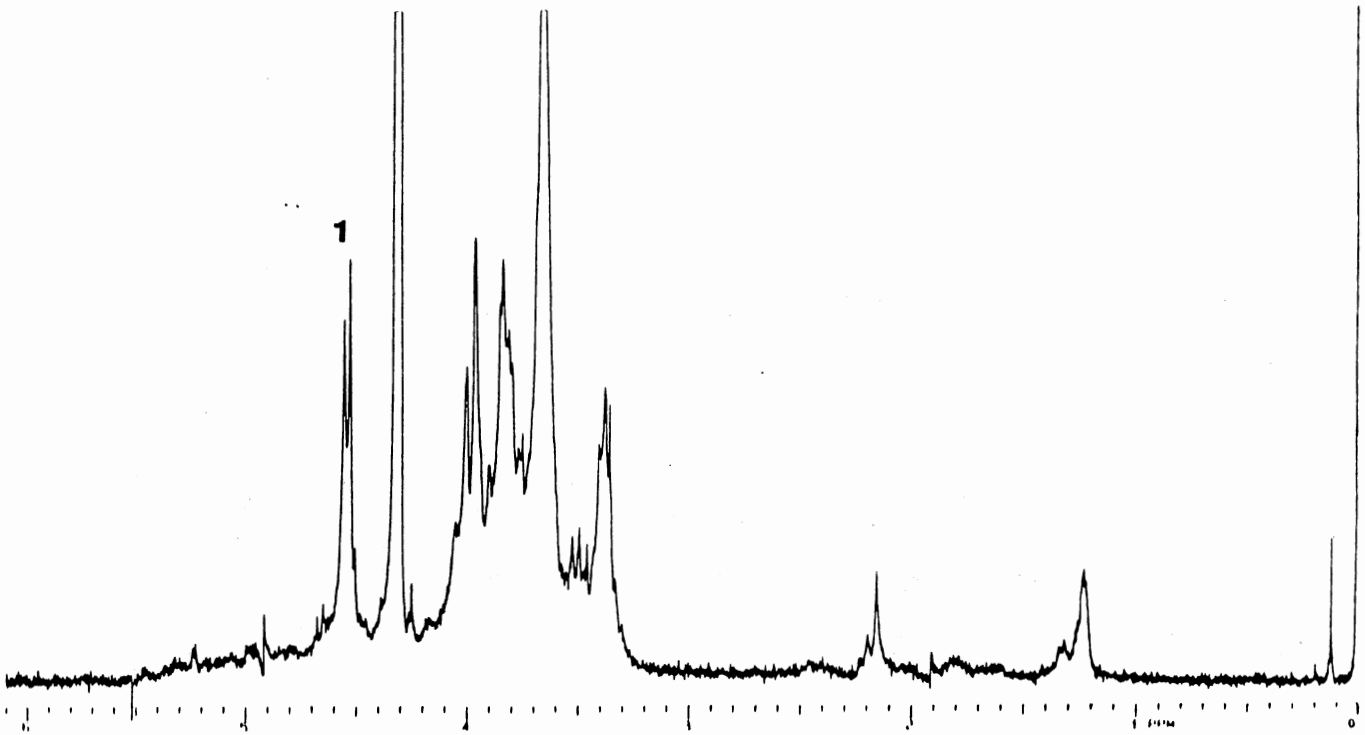

Figure 33. ^{1}H n.m.r. Spectrum of the QAE Sephadex Fraction 1 of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C, After Demethyl Esterification of the Sample. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Signal (1) at 4.6 p.p.m. represents H-1 of β Glucose. There are traces of acetate (signal at 2.18 p.p.m.).

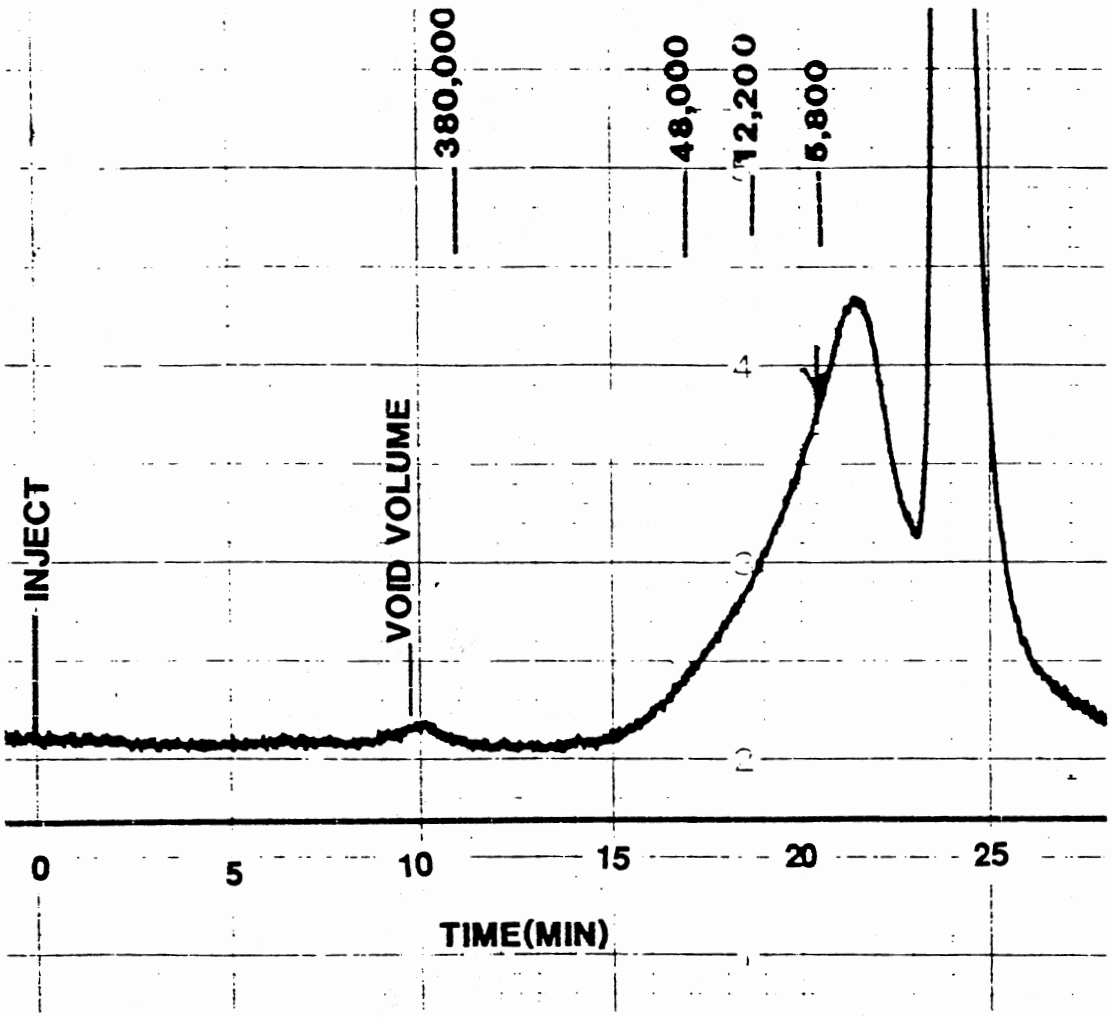

Figure 34. The HPLC Chromatogram of the Fraction 1 of the QAE Sephadex Column of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight.

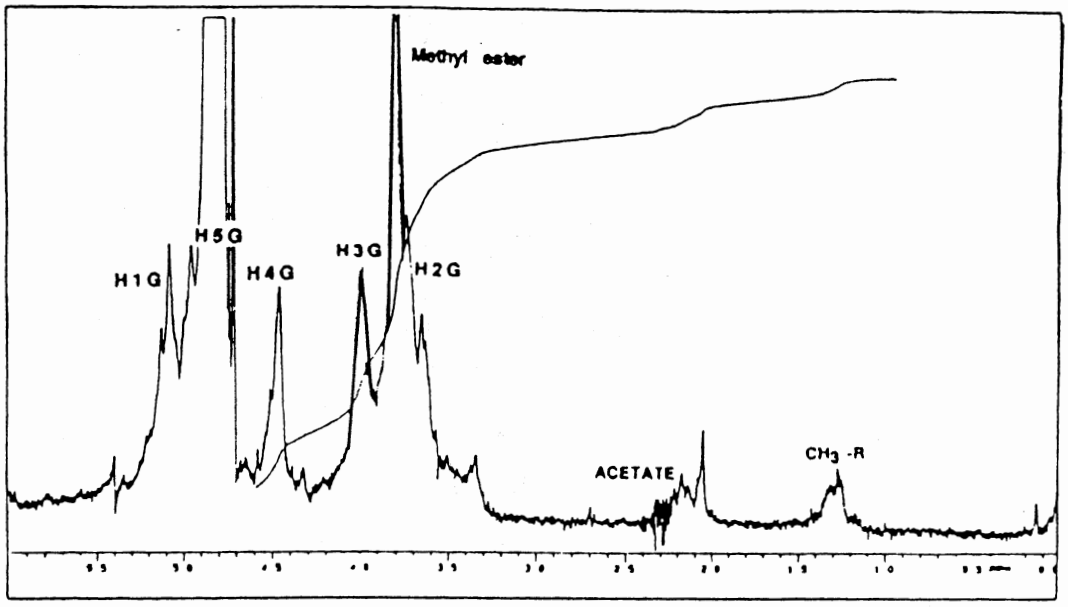

Figure 35. ^{1}H n.m.r. Spectrum of the QAE Sephadex Fraction 2 of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. G = galacturonic acid.

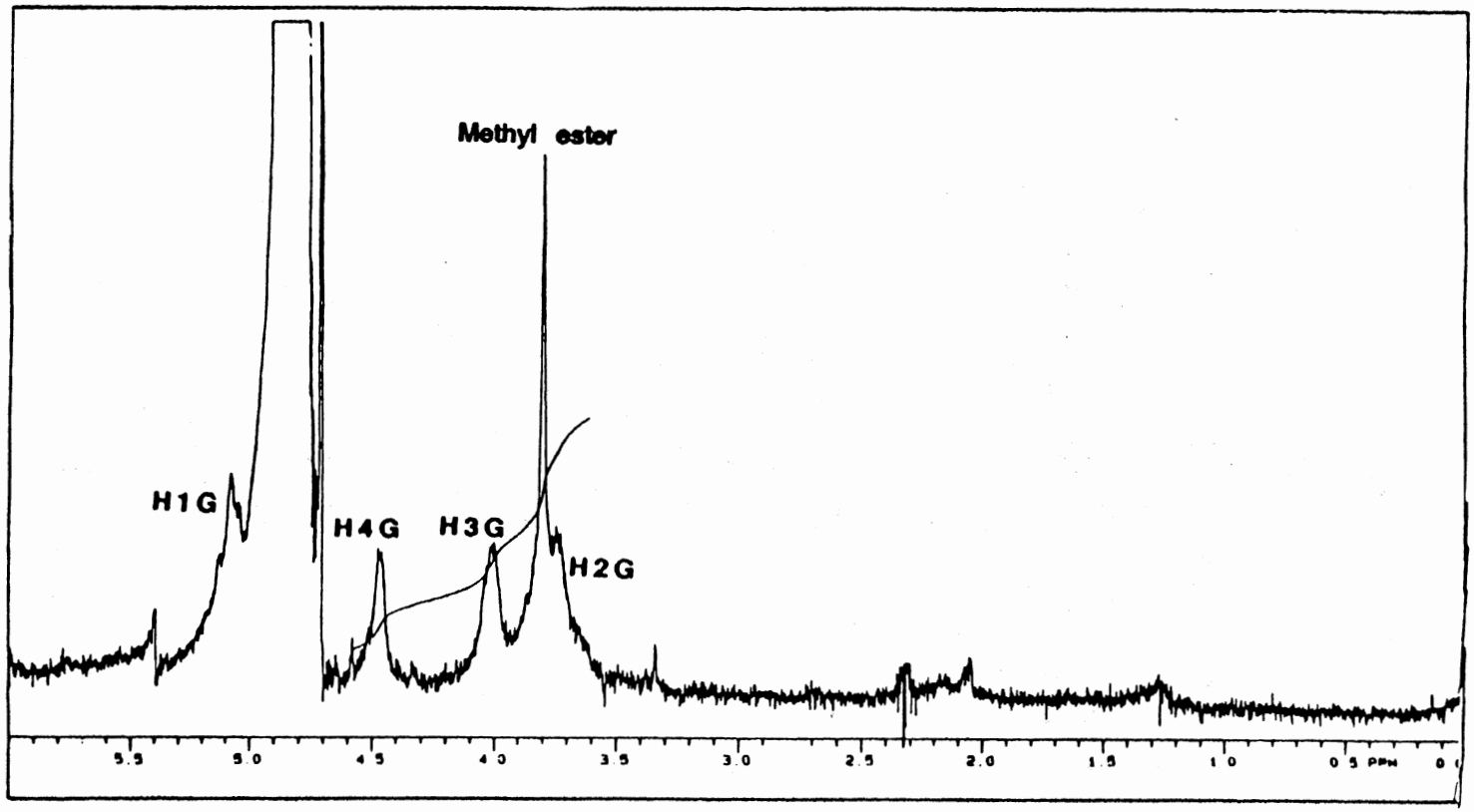

Figure 36. ^{1}H n.m.r. Spectrum of the QAE Sephadex Fraction 3 of theBio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. G = galacturonic acid.

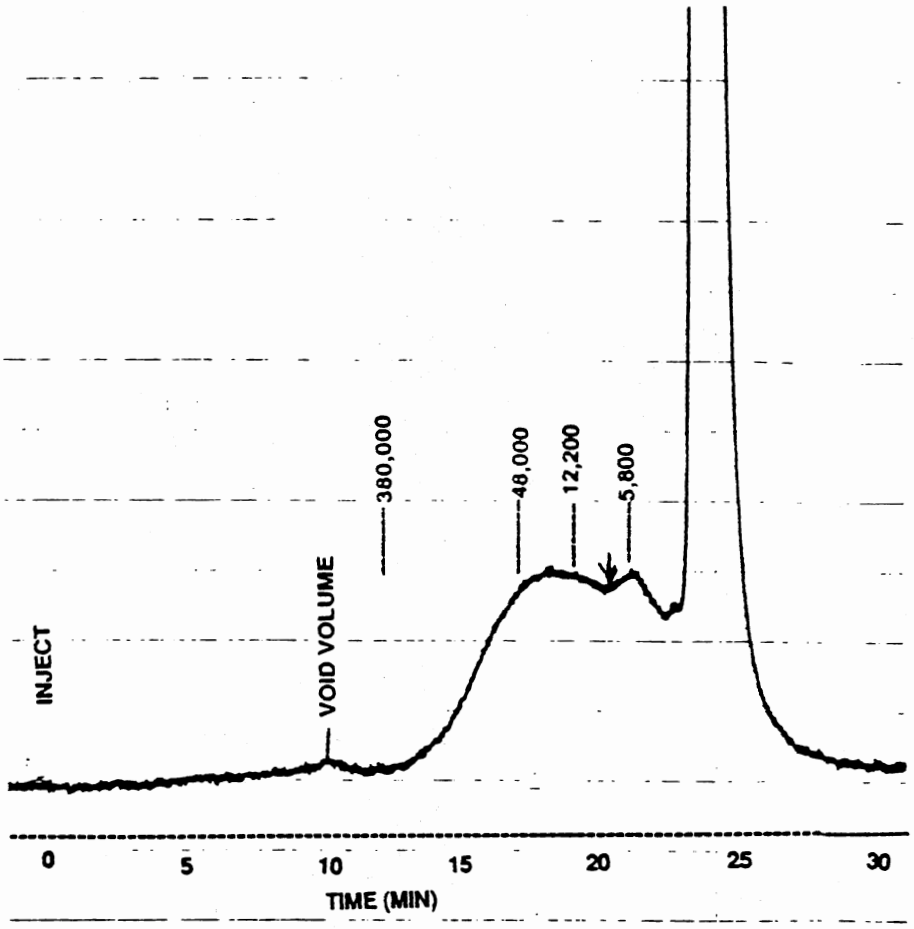

Figure 37. The HPLC Chromatogram of the Fraction 3 of the QAE Sephadex Column of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. GalA oligomers consistently elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

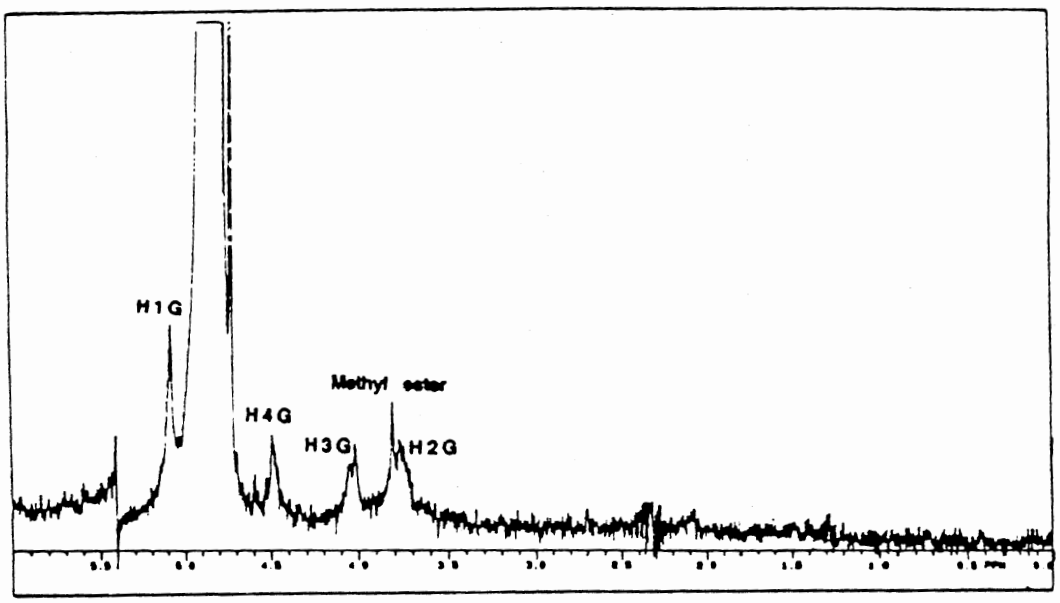

Figure 38. ^{1}H n.m.r. Spectrum of the QAE Sephadex Fraction 4 of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. G = galacturonic acid.

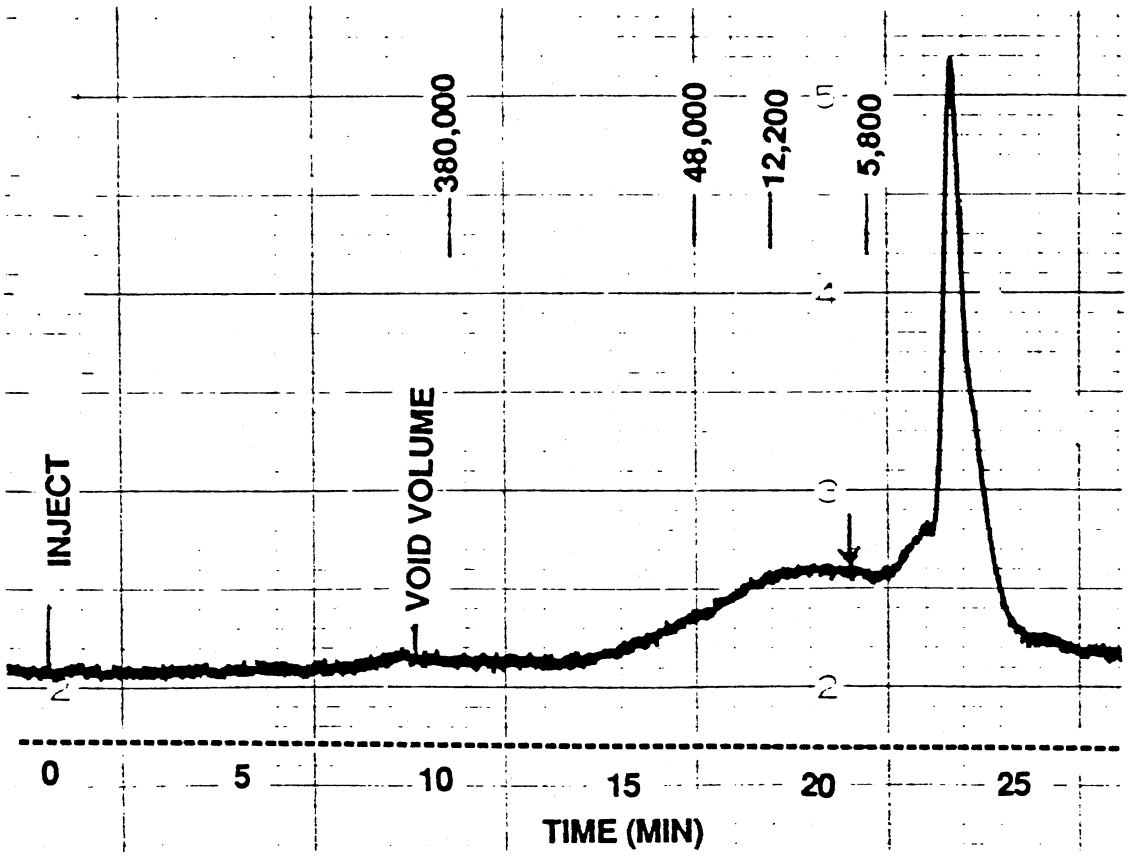

Figure 39. The HPLC Chromatogram of the Fraction 4 of the QAE Sephadex Column of the Bio-gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in series. The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. GalA oligomers consistently elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

ratio, therefore should have derived from the rhamnogalacturonan I portion of pectins. Since these fragments also have considerable amounts of arabinose and galactose, these fractions may contain substituted disaccharides of galacturonosyl rhamnose. These disaccharides have some of the side chains of RG I attached to them. The ^{1}H n.m.r. spectra of the two fractions are shown in Figures 40 and 41. Both the fractions were acetylated and no methyl esters were found. Methylation analysis of these fractions had shown 2 and 4 linked rhamnose showing that the side chains are present on the 4 position of rhamnose. The acetate groups were on the 3 position of galacturonic acid as determined from the signal at about 5.88 p.p.m. in the proton n.m.r. spectra (Figures 40 and 41). It is likely that some acetate groups are also present on the side chains. Further characterization of these fractions will shed more light on the length of side chains present and the different sugars found in the side chains of RG I region of pectins.

Analysis of the Water Insoluble Residue

Isolation of Homogalacturonan (HG) Region of Pectin

The water insoluble residue (refer to scheme 2, Figure 6) was found to contain mainly galacturonic acid and glucose. The total weight percent of sugar in this residue was 32%. Most of the pectin that was left behind in the residue was extracted with 0.5 M imidazole buffer pH 7. This fraction referred to as imidazole extract was found to be ~15% of the original weight of the walls. The amount of sugar accounted for sugar in this fraction was 48.5%. The composition of this fraction is shown in Table IX. From the composition it showed that this fraction was a polymer of galacturonic acid, the homogalacturonan region of pectins. The ^{1}H n.m.r. spectrum of the imidazole extract is shown in Figure 42. The chemical shifts for the signals were the same as expected for polygalacturonic acid. The chemical shifts were found to be H-1 (5.09 p.p.m.), H-2 (3.78 p.p.m.), H-3 (3.97 p.p.m.), H-4 (4.42 p.p.m.), and H-5 (4.73 p.p.m.) and were

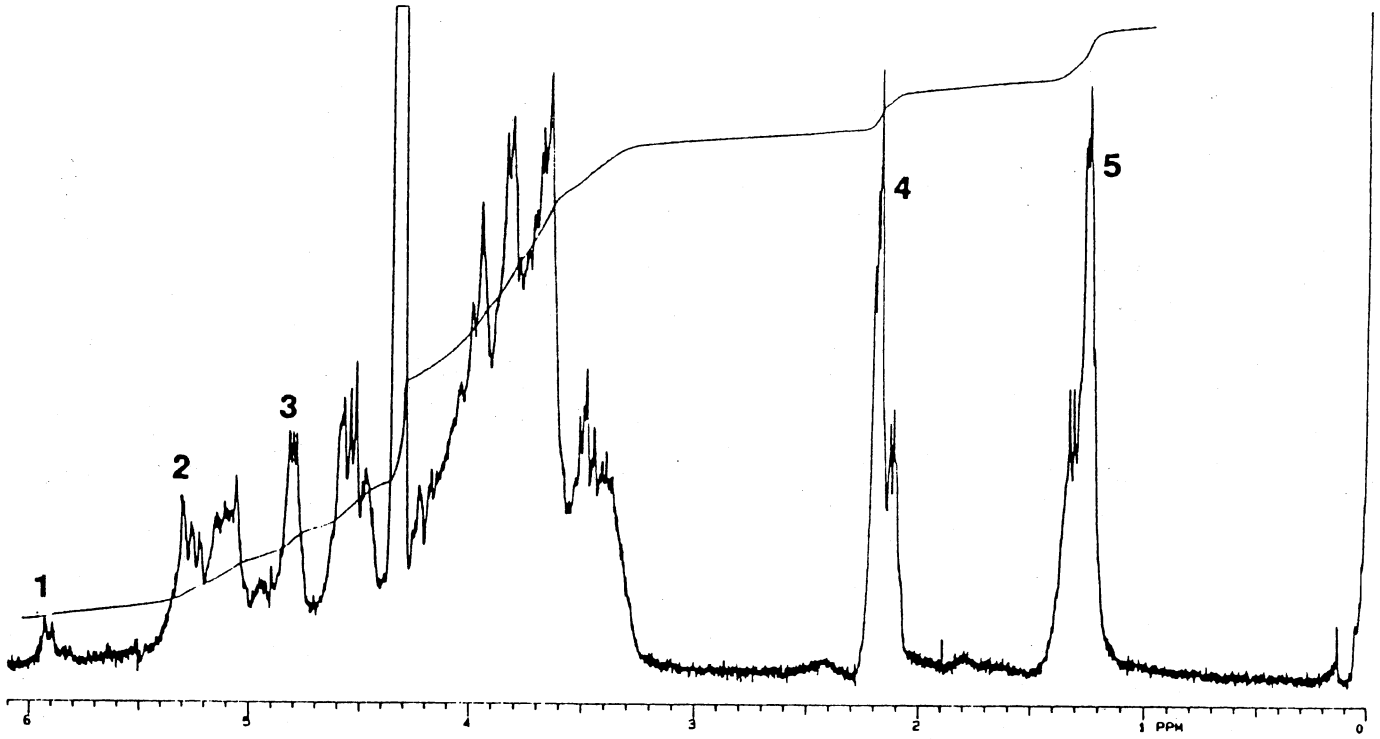

Figure 40. ^{1}H n.m.r. Spectrum of the Bio-gel P-2 Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C, After Demethyl Esterification of the Sample. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 70°C in D_2O Using TSP as Internal Standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-3, GalA (position of acetylation); 2) H-1 of α GalA; 3) H-1 of β Rha; 4) CH_3 of acetate; 5) CH_3 of Rha.

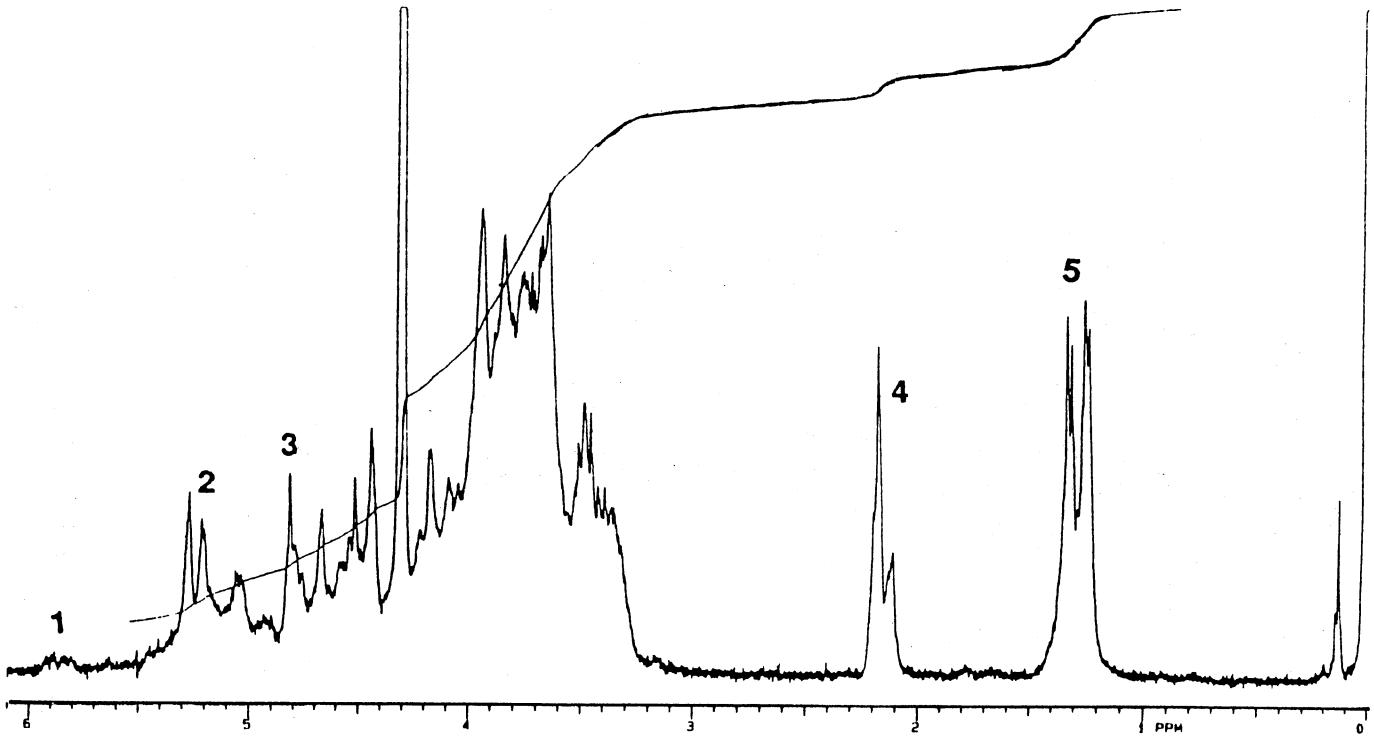

Figure 41. ^{1}H n.m.r. Spectrum of the Bio-gel P-2 Fraction 3 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C, after de70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) H-3, GalA (position of acetylation); 2) H-1 of α GalA; 3) H-1 of β Rha; 4) CH_3 of acetate; 5) CH_3 of Rha.

comparable to the values in the literature (Tjan *et al.*, 1974). The degree of methyl esterification of this fraction was found to be very low and could not be detected by n.m.r. spectroscopy as the methyl protons resonate along with the ring protons at 3.8 p.p.m. This fraction did not have any acetate esters. The ^{13}C n.m.r. spectrum was quite consistent with the results of the proton spectrum. The chemical shift values in p.p.m. were C-1 (101.83), C-2 (70.94), C-3 (71.68), C-4 (80.69), C-5 (74.19), and the C=O of acid group at 178.25 p.p.m.

The relative size of this fraction was determined by HPLC on tandem gel filtration columns as described above for the analysis of homogalacturonan region isolated from the water extract (Figure 43). Average degree of polymerization of the polymers was more than 20. The apparent size of these fragments was found to be larger than that of the water soluble homogalacturonan fragments isolated earlier from the water extract. Molecular weight range seems to overlap between the two extracts.

The residue after imidazole extraction still contained more galacturonic acid and was extracted with 100 mM EDTA. The composition of the EDTA extract is given in Table IX. The EDTA extract was found to be about 7% of the original weight of the Acala 44 cotton suspension walls, however this extract did have residual EDTA making the total weight percent of sugars very low (15.5%). Chelators like EDTA and CDTA are useful in extracting pectins, but a large amounts of these chelators binds to pectin and is very difficult to remove from the polygalacturonic acid by dialysis after extraction. Moreover these chelators show signals in the n.m.r. spectrum where the CH_3 of the methyl ester protons resonate, making it very difficult to identify methyl esterification of polygalacturonic acid. On the contrary, imidazole can be removed from the extracted pectins by dialysis and thus is a better reagent for extracting pectins than EDTA and CDTA. It does not interfere with the interpretation of the n.m.r. spectrum as imidazole shows signals beyond 6 p.p.m. in the proton n.m.r. spectrum. The ^{1}H n.m.r. spectrum of the EDTA extract showed very little methyl esterification (Figure 44) and no acetylation

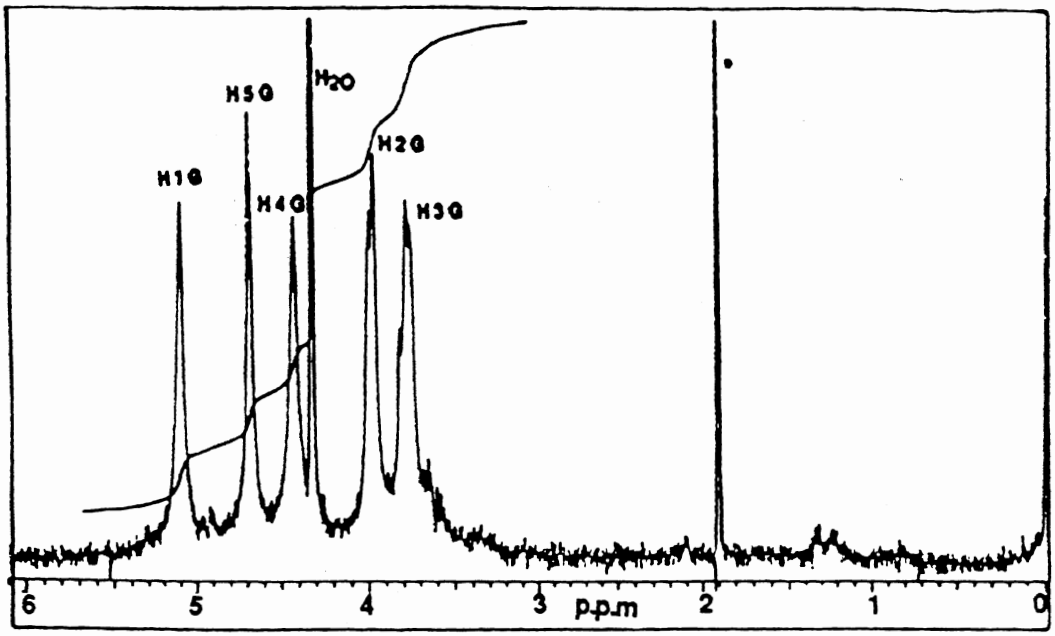

Figure 42. ^{1}H n.m.r. Spectrum of the Imidazole Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. G = galacturonic acid. *Free acetate from the imidazole buffer used to solubilize the sample.

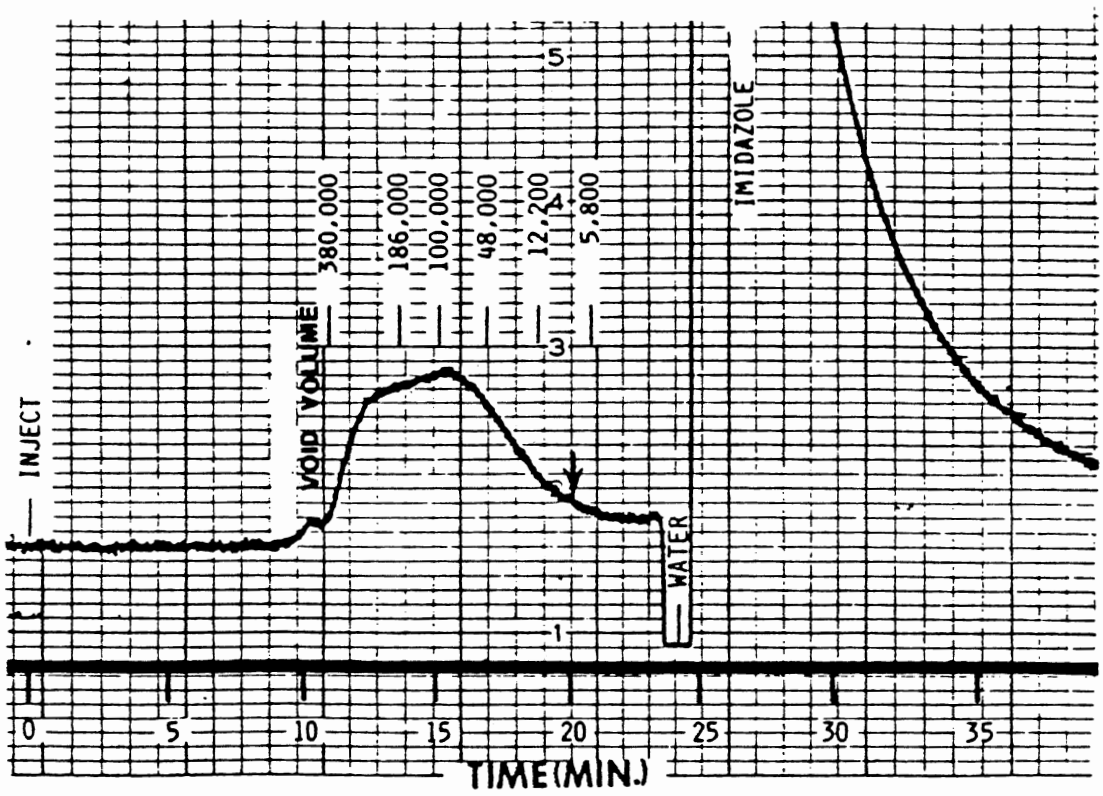

Figure 43. The HPLC Chromatogram of the Imidazole Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The sample was dissolved in 0.5 M imidazole buffer pH 7.0 and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. GalA oligomers consistenly elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

in these fragments. The sizes of these fragments determined by gel filtration were similar to those of the imidazole extract (Figure 45).

Imidazole buffer extraction and the subsequent EDTA extraction of the residue removes most of the pectins (the homogalacturonan region) from the HF treated walls. These two extracts together constitute ~18% in Acala 44 cotton suspension walls. The homogalacturonan extracted with water constituted about 25% of the total homogalacturonan region of pectins in cotton walls. This shows that in Acala 44 cotton suspension walls homogalacturonan represents ~18% of the walls of which ~15-20% of the polygalacturonic acid show methyl esterification.

Analysis of the Final Residue

After the water, imidazole and EDTA extractions of the HF treated wall the residue left behind contained about 5% of the original galacturonic acid and glucose (Table IX). Sugar accounted for ~20% of the residue. This residue mainly contained cellulose and cell wall protein. When this residue was subjected to HF solvolysis at 0°C for 30 mins cellulose was completely broken down to glucose (~8% of the original walls) and the residue left behind was protein(~2% of the original walls) with minor amounts of galacturonic acid and glucose (14% of the dry weight was accounted for sugar). The composition of these fractions is shown in Table IX. It is not known how much of the original cellulose and protein are left behind in the residue after the sequential extractions. Cellulose is not broken down significantly by HF solvolysis at -23°C, but undergoes structural changes and becomes more susceptible to methanolysis. When pure cellulose fibers were treated with HF at -23°C for 30 mins, about 2-4% was made soluble in the HF/ether mixture. Another 5-10% became sparingly water soluble and by HPLC gel filtration on 2000 PW column of the soluble part showed this fraction to be bigger than dextran T 10. The sugars recovered from the HF/ether fraction were mainly xylose and mannose and some glucose from the contaminating xyloglucan and mannans found in

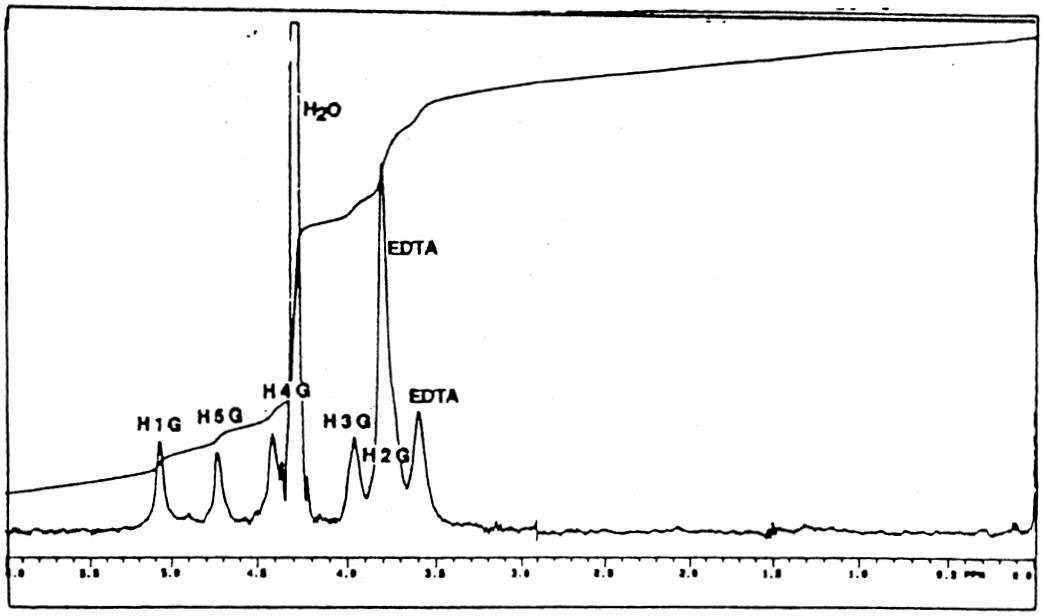

Figure 44. ^{1}H n.m.r. Spectrum of the EDTA Extract from Acala 44 Cotton Suspension Walls After [illegible] Solvolysis at -23°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. G = galacturonic acid. Residual EDTA present in the sample.

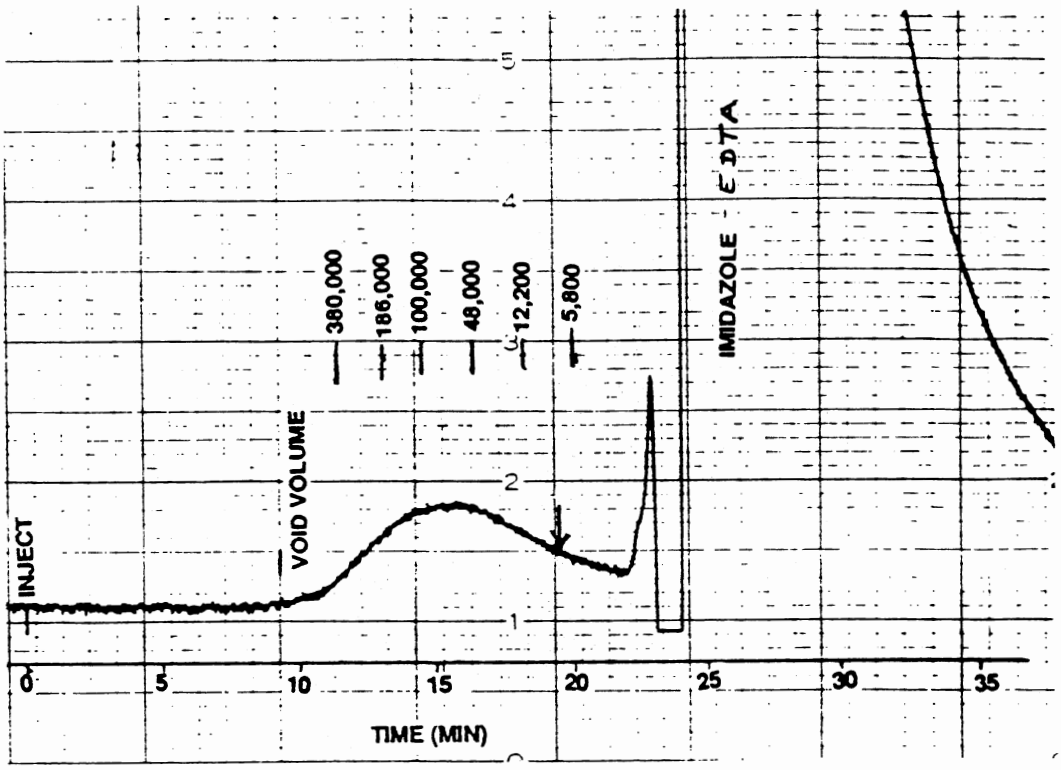

Figure 45. The HPLC Chromatogram of the EDTA Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -23°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The sample was dissolved in 0.5 M imidazole buffer pH 7.0 and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. GalA oligomers consistently elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

commercial cellulose. About 17% of the pure cellulose fibers undergo methanolysis while after HF treatment at -23°C about 70% of the cellulose becomes methanolysed. This suggests that even though cellulose is not broken down during HF solvolysis at -23°C, it undergoes structural changes and becomes more susceptible to methanolysis.

Solvolysis of Acala 44 Cotton Suspension Cell Walls at -40°C and Structural Analysis of the Extracted Polymers

HF solvolysis at -23°C isolated the backbone of RG I as disaccharides and substituted disaccharides. As lowering the temperature of the reaction results in a smaller number of cleavages, larger oligomers should be produced if solvolysis of cell walls is carried out at a temperature lower than -23°C. In order to see whether this was true, about 500 mg Acala 44 cotton suspension cell walls were treated with HF at -40°C for 30 min and fractionated as outlined in the scheme III (Figure 46). The sugar composition of different fractions of cell walls after HF solvolysis at -40°C is shown in Table XIV. The total weight percent of sugars in the water extract was 69%, residue after water extraction was 40%, imidazole extract was 60%, residue after imidazole extraction was 33%, EDTA extract was 5% and the residue after EDTA extraction was 9%.

About 20% of the weight of the walls was soluble in the HF/ether mixture when ether was added to quench the reaction. This fraction was rich in arabinose and xylose (Table XIV). Significant amounts of gal, glc, rha and fuc were also present. These were mainly monosaccharides and were not investigated any further. About 18% of the original weight of the walls became water soluble when the residue was extracted with water. The mole percent of sugars in the water extract is given in Table XIV. The sugar composition shows that this fraction contain fragments of RG I and xyloglucan. From the sugar composition (assuming that all of the rhamnose present was in the disaccharide unit) it was calculated that the disaccharide repeat represents ~6% of the original walls. To determine the average size of the oligomers the water extract was fractionated on HPLC

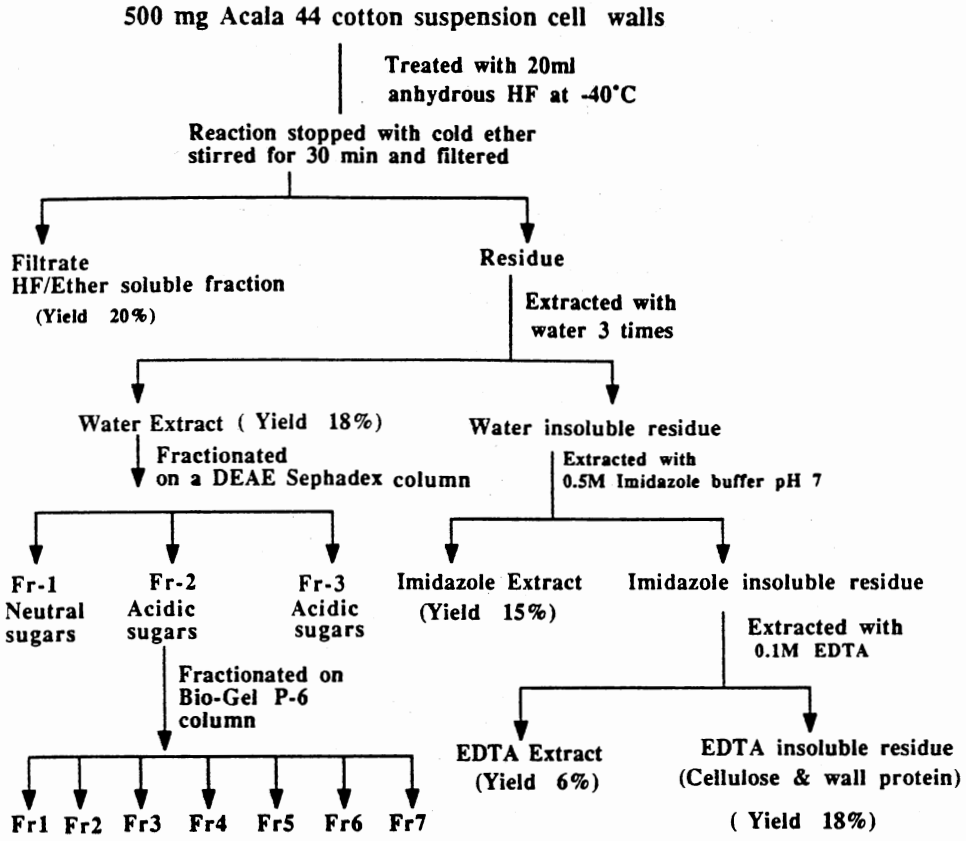

Figure 46. Scheme III, for HF Solvolysis and Fractionation of Cell Walls at -40°C.

TABLE XIV

MOLE PERCENT OF SUGARS IN DIFFERENT FRACTIONS OF ACALA 44 COTTON SUSPENSOION CELL WALLS AFTER HF TREATMENT AT -40°C

Materials from Acala44 cotton cell walls after HF treatment at -40 C	Percentage of starting weight recovered	Ara	Rha	Fuc	Xyl	GlcA	GalA	Man	Gal	Glc
Ether soluble sugars	20	38.7	4.8	5.1	31.7	1.3	2.4	0.2	11.9	3.9
Water extract	18	11.6	21.1	Trace	10.8	Trace	25.1	1.3	13.7	16.4
Residue after water extn	48	2.5	5.3	-	7.9	-	60.7	-	3.1	20.5
Imidazole extract	15	1.6	3.9	-	6.7	-	82.8	-	1.5	3.5
Residue after imidazole extraction	30	3.5	5.9	-	11.9	-	46.1	-	3.4	29.2
EDTA extract	6	Trace	2.8	-	6.7	-	82.6	-	2.9	4.8
Residue after EDTA extn	18	3.9	6.7	-	14.2	-	35.7	-	0.6	38.9

gel-filtration column 2000 SW (Figure 47), and was found to be a very heterogeneous mixture with a wide range of molecular weight. Treatment of the water insoluble residue with HF at -40°C again did not remove any more RG I from the walls suggesting that all of the RG I can be removed by one HF treatment at -40°C.

The neutral sugars associated with the water extract was separated from the acidic sugars by fractionation on a DEAE Sephadex column (Figure 48) and the sugar composition of the three fractions made is shown in Table XV. Composition analysis of the fraction 1 showed that it contained neutral sugars and was rich in xyl, glc, ara and gal. HPLC profile of the fraction 1 (Figure 49) showed that this fraction contained oligomers of small molecular weight. The ^{1}H n.m.r. spectrum (Figure 50) and ^{13}C n.m.r. spectrum (Figure 51) suggests this may be mainly fragments of xyloglucan along with oligomers of ara and gal, may be originated from arabinogalactan or from the side chains of pectic polymers.

Analysis of the DEAE Sephadex Fraction 2 of the Water Extract After HF Solvolysis at -40°C

Molar sugar composition of the DEAE fraction 2 (Table XV) showed that it contained rhamnose and galacturonic acid in about 1:1 ratio, indicating its origin was the RG I region of pectin. This fraction was found to be a heterogeneous mixture with a wide range of molecular weight determined by HPLC gel filtration (Figure 52). A major portion of the oligomers were in the molecular weight range of ~6000 according to pullulan standard used to standardize the column. Some of the oligomers were in the range of 10,000 to 100,000 but it should be remembered that pectin always elutes earlier than the pullulan standard which is made up of neutral sugars. The ^{1}H n.m.r. and the ^{13}C n.m.r. spectra of the DEAE fraction 2 (Figures 53 and 54) showed the presence of acetylation and methyl esterification (signal at 2.18 p.p.m. in the proton spectrum and the signal at 56 p.p.m. in the carbon spectrum respectively). By 2D ^{1}H homonuclear

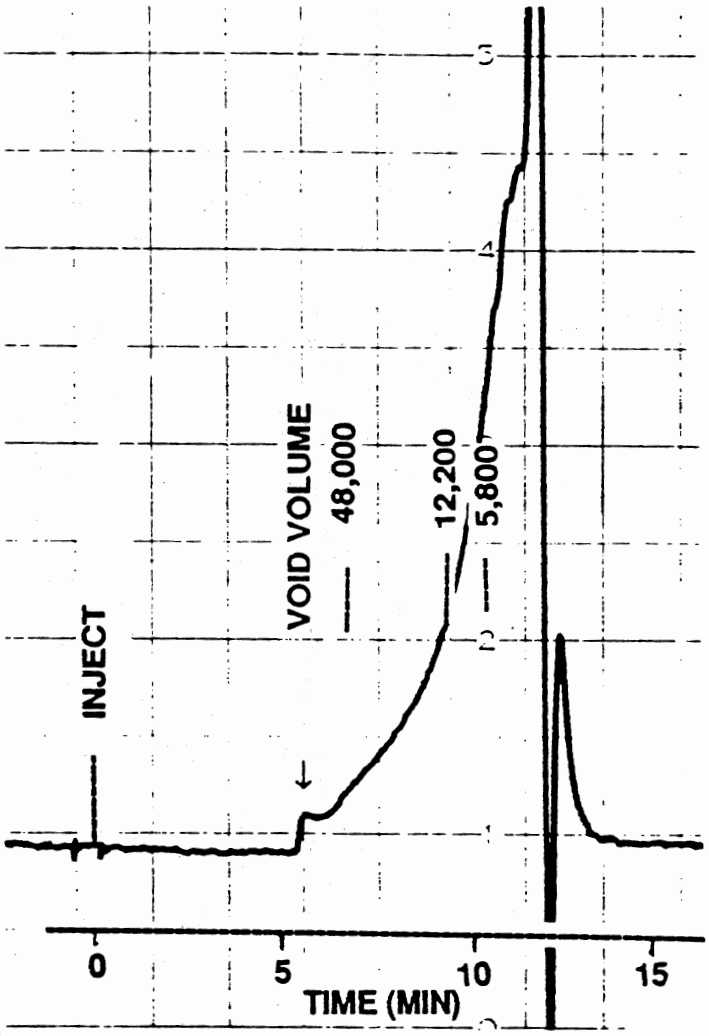

Figure 47. The HPLC Chromatogram of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C on TSK SW 2000 Gel Filtration Column (7.5 mm x 30 cm). The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. GalA oligomers consistenly elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

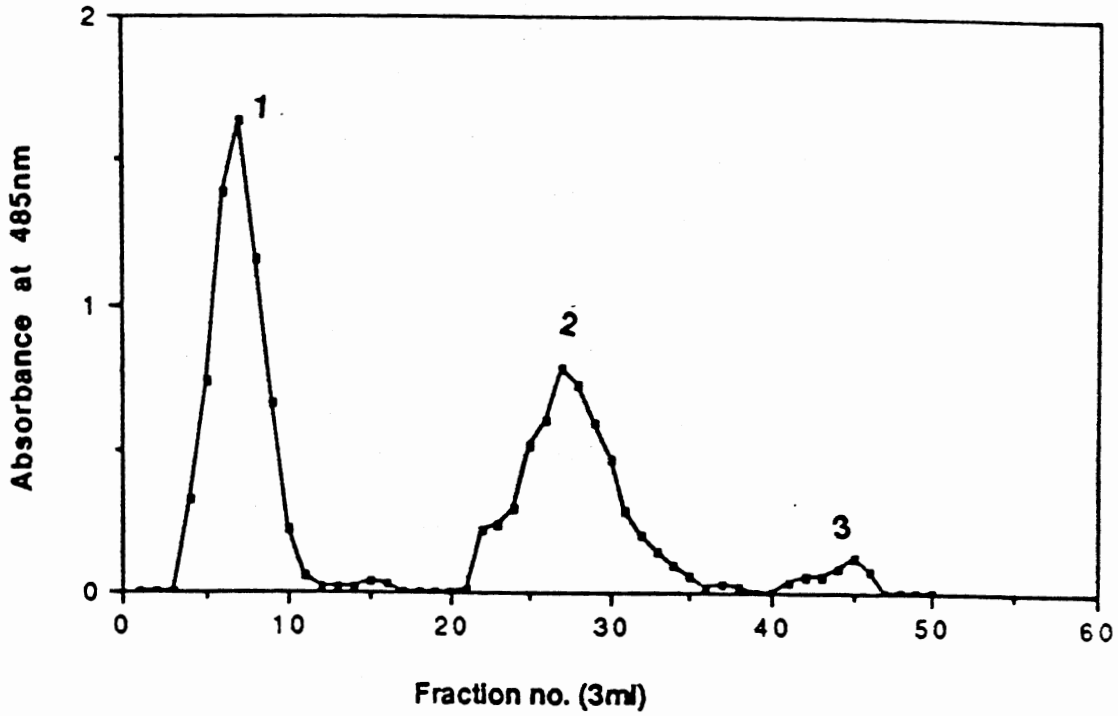

Figure 48. Chromatography on a DEAE Sephadex Anion Exchange Column (20 x 2 cm) of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. Sample was applied on to the column in 0.05 M sodium acetate pH 5.2 and eluted with 0.05 M, 0.5 M and 1 M sodium acetate buffer pH 5.2 forming fractions 1, 2 and 3, respectively. Fractions were assayed for sugar by phenol sulfuric test.

TABLE XV

MOLE PERCENT OF SUGARS IN THE DEAE FRACTIONS OF THE WATER EXTRACT FROM COTTON WALLS AFTER HF SOLVOLYSIS AT -40°C

Sugar	Fraction 1	Fraction 2	Fraction 3
Ara	20.1	2.7	2.1
Rha	1.3	34.4	39.2
Fuc	1.9	-	-
Xyl	19.2	3.7	2.4
GlcA	-	Trace	-
GalA	-	42.6	46.7
Man	2.8	0.6	-
Gal	17.1	11.3	5.9
Glc	37.6	4.7	3.3

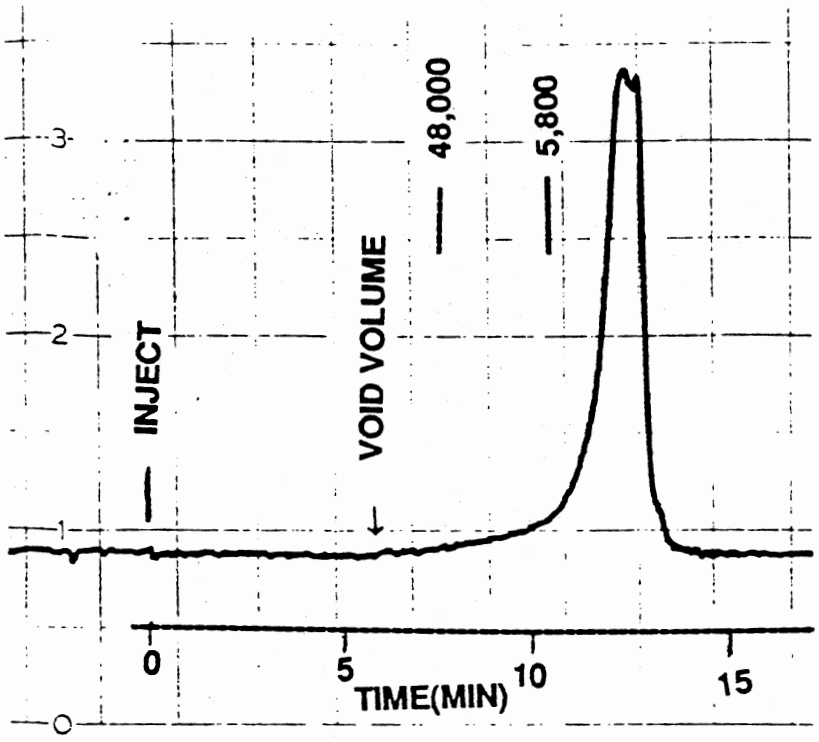

Figure 49. The HPLC Chromatogram of the DEAE Fraction 1 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C on TSK SW 2000 Gel Filtration Column (7.5 mm x 30 cm). The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards.

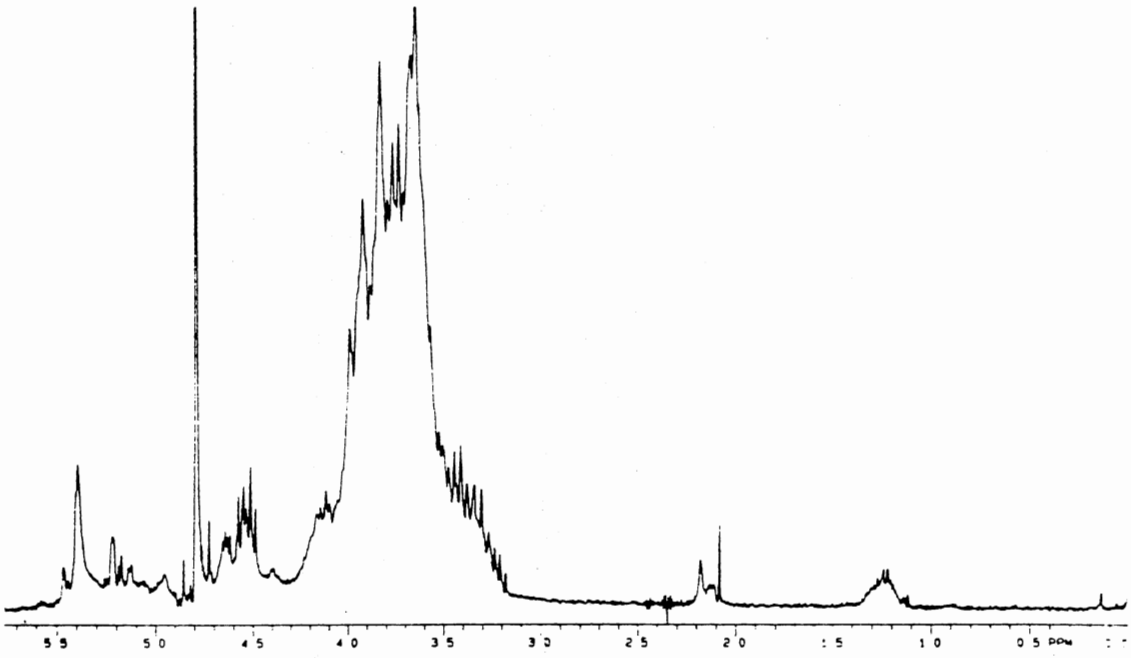

Figure 50. ^{1}H n.m.r. Spectrum of the DEAE Fraction 1 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m.

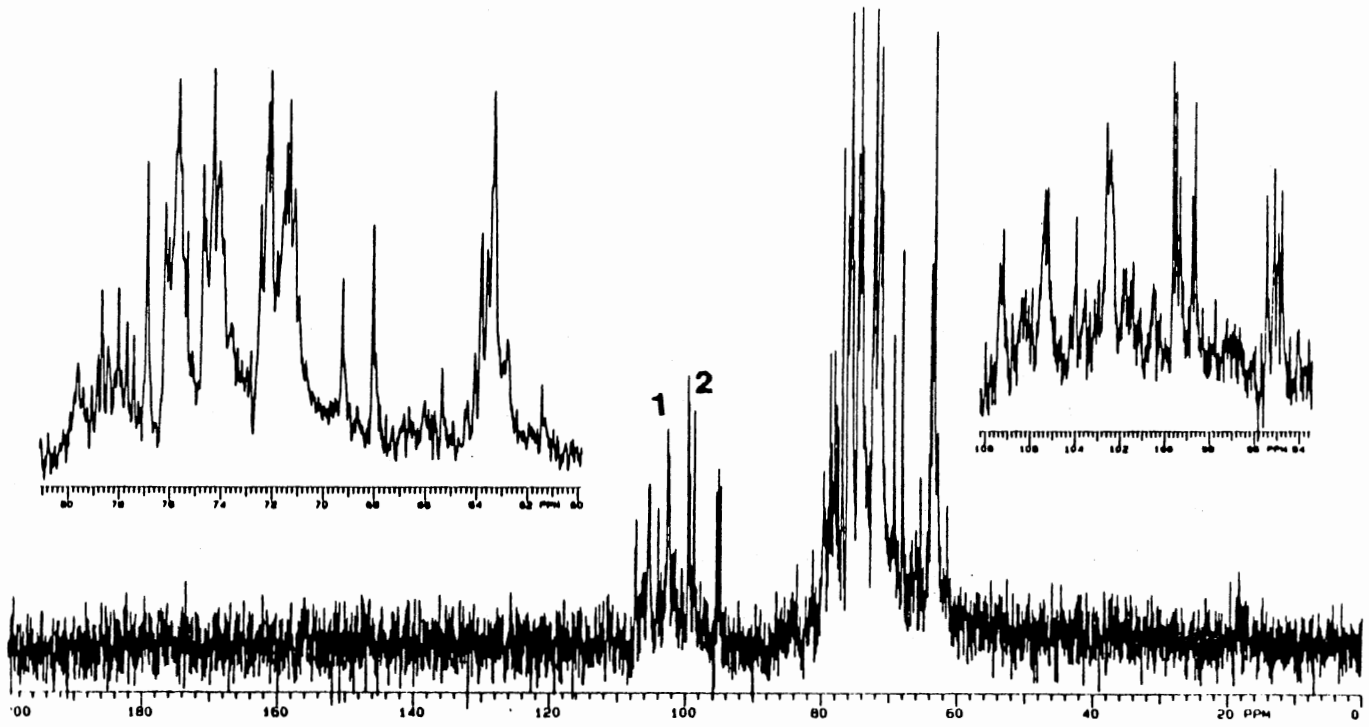

Figure 51. ^{13}C n.m.r. Spectrum of the DEAE Fraction 1 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. 1) C-1 β Glc; 2) C-1 xyl.

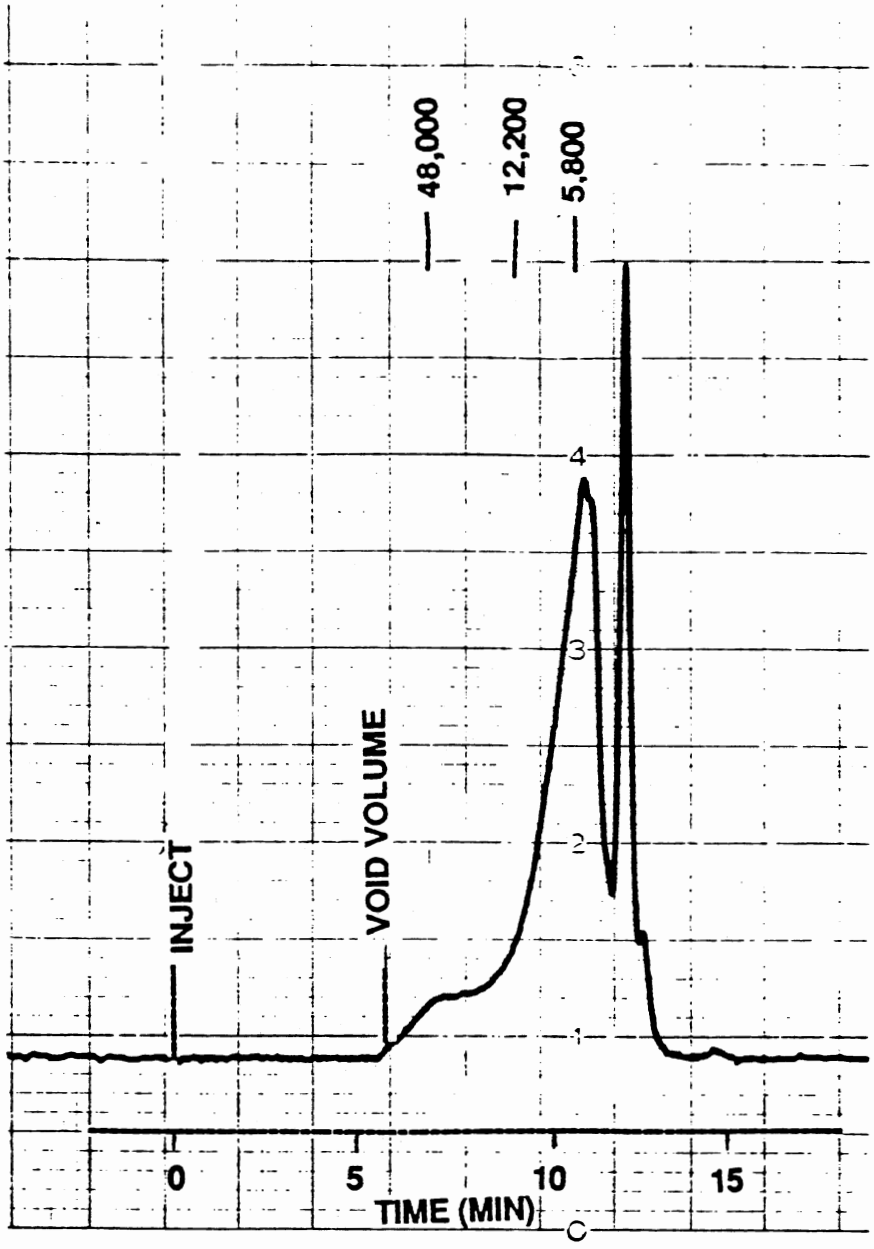

Figure 52. The HPLC Chromatogram of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C on TSK SW 2000 Gel Filtration Column (7.5 mm x 30 cm). The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. GalA oligomers consistently elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

correlation spectroscopy (Figures 55 and 56), and 2D ^{13}C-^{1}H heteronuclear correlation spectroscopy (Figure 57) it was possible to identify most of the proton and carbon resonances. The results suggested that the oligomers were at least 6 sugars in length with a side chain attached in many cases as indicated by the presence of a branched rhamnose. Acetylation was found to be exclusively on the 3 position of galA residue. The backbone of the polymer consisted of α 1→2 linked galA-rha, with the L-rha linked α 1→4 to the next galA residue. Integration of the 1D proton spectrum showed about 50% of the oligomers to be acetylated. Methylation analysis followed by mass spectroscopy showed the presence of 2-linked rha, 2,4-linked rha, t-gal, t-glc, t-xyl, 4-gal without reduction and after reduction showed in addition to 6-gal and 4,6-gal (Figures 58 to 67). This gives further evidence for the presence of side chains on the rha. The length of side chains/remnants of side chains is to be determined. The distribution of acetylation with respect to side chains also has to be determined. This may be possible by analysis of deacetylated sample by n.m.r., and also by obtaining oligomers of different size by varying the time of the reaction.

In order to learn more about the size distribution of these oligomers of RG I, the DEAE fraction 2 was further fractionated on a Bio-gel P-6 column (Figure 68). Part of the sample did elute in the void volume of the column whereas the rest of the sample fractionated in the entire elution range of the column. The sugar composition of the pooled fractions 1 to 7 is shown in Table XVI. The void volume (fraction 1) contained rhamnose to galacturonic acid in about a 1:2 ratio. It had significant amounts of glc, gal, xyl and ara. The n.m.r. analysis of this fraction showed a substantial amount of methyl esterification (Figure 69) and small amounts of acetylation. This suggests that this fraction may have fragments of polygalacturonic acid with methyl esterification. It has to be determined whether these oligomers with methyl esterification are attached to the oligomers from RG I or whether these are coeluted due to similar size.

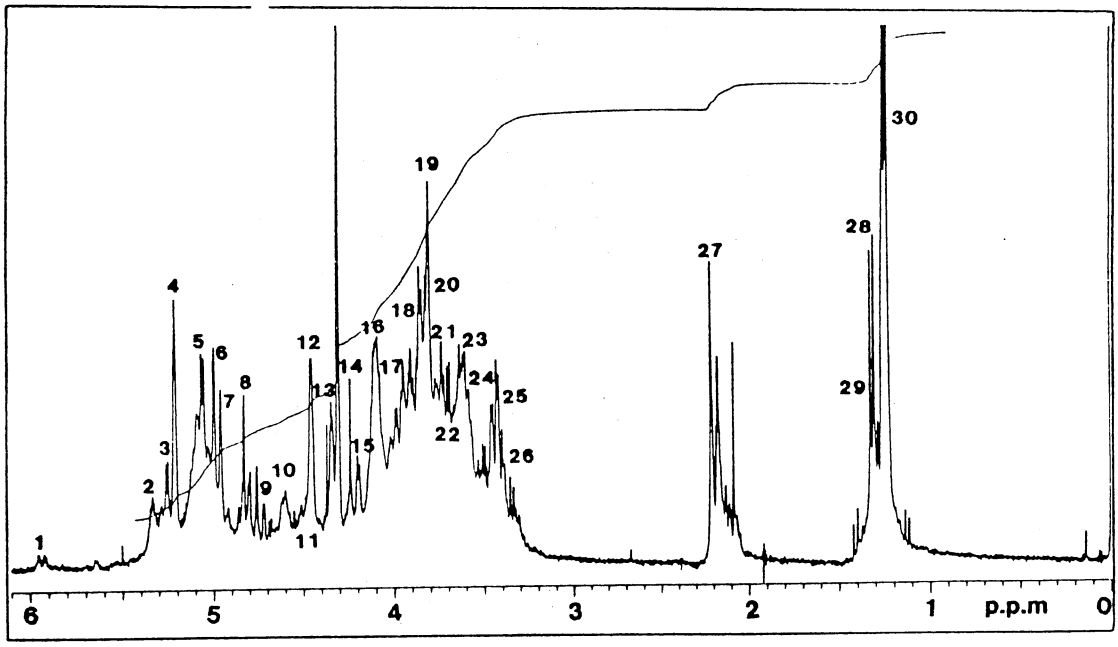

Figure 53. ^{1}H n.m.r. Spectrum of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 70°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are identified using the 2D, homo and hetero correlation spectra shown in Figures 55, 56, and 57. Integration of the spectrum shows about 50% of the fractions being acetylated. Peaks are identified as follows: G = Galacturonic acid; C = Cyclized; A = Acetylated; R = Rhamnose; L = linear; (---) not acetylated; I = Internal; N = Non reducing end. 1) H-3 GCA; 2) H-1 GCA, H-5 GCA; 3) H-1 GIA, H-1 GC---; ; 4) H-1 of α R; 5) H-1 GI---, H-5 GIA, H-5 G, H-1 GN---; 6) H-5 GC---; 7) H-3 GIA; 8) H-1 β RC; 9) H-3 GC---; 10) H-4 GCA; 11) H-1 β Gal; 12) H-1 GIA, H-4 GI---; 13) H-2 GCA, H-4 GC---, H-4 GN---; 14) H-2 β RC; 15) H-2 β RC; 16) H-2 α R, H-3 GI---; 17) H-3 GN---; 18) H-2 GIA; 19) CH_3 of methyl ester; 20) H-2 GC---, H-2 GI---; 21) H-2 GN---, H-3 α R; 22) H-3 β RC; 23) H-5 α R; 24) H-4 α R; 25) H-4 β RC; 26) H-5 β RC; 27) CH_3 of acetate; 28) CH_3 of β Rha.; 29) CH_3 of α R (with branch); 30) CH_3 of α R.

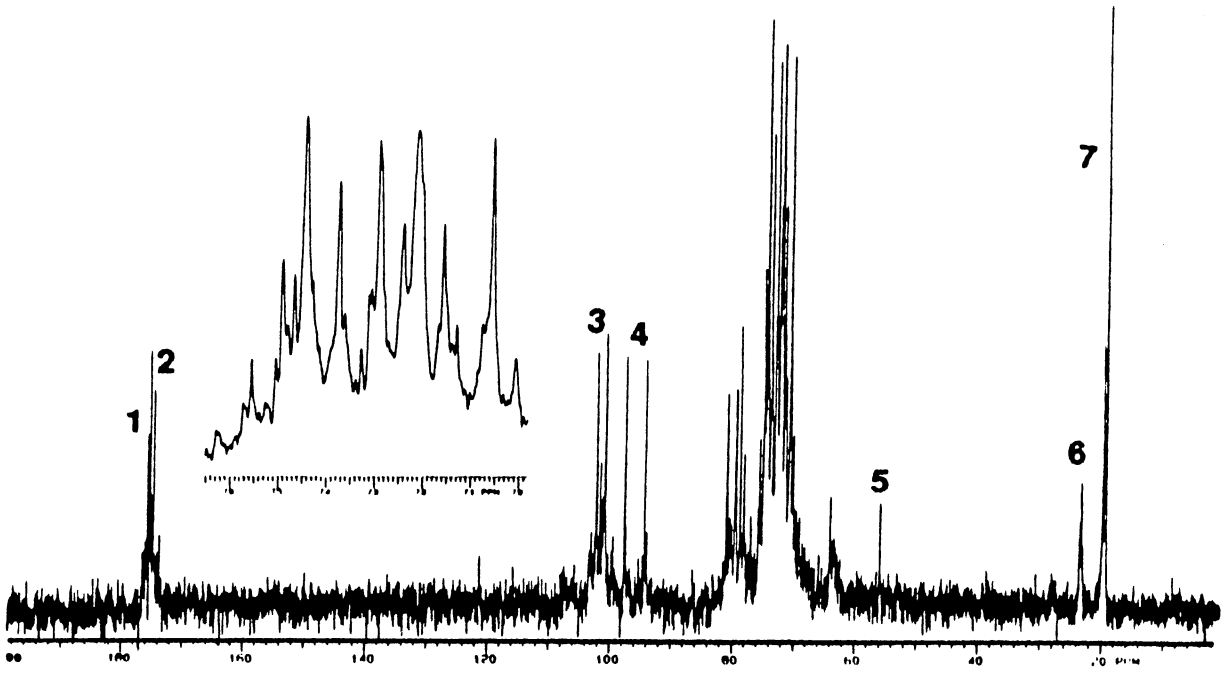

Figure 54. ^{13}C n.m.r. Spectrum of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Only some peaks are identified as follows: 1) >C=O of acetate; 2) C-6 of GalA; 3) C-1 of α Rha; 4) C-1 of α GalA (cyclized); 5) CH_3 of methyl ester acetate; 6) CH_3 of acetate ester; 7) CH_3 of Rha. Signal at 62 p.p.m is from Gal. Refer to the 2D heterocorrelation spectrum for complete assignment of peaks (Figure 57).

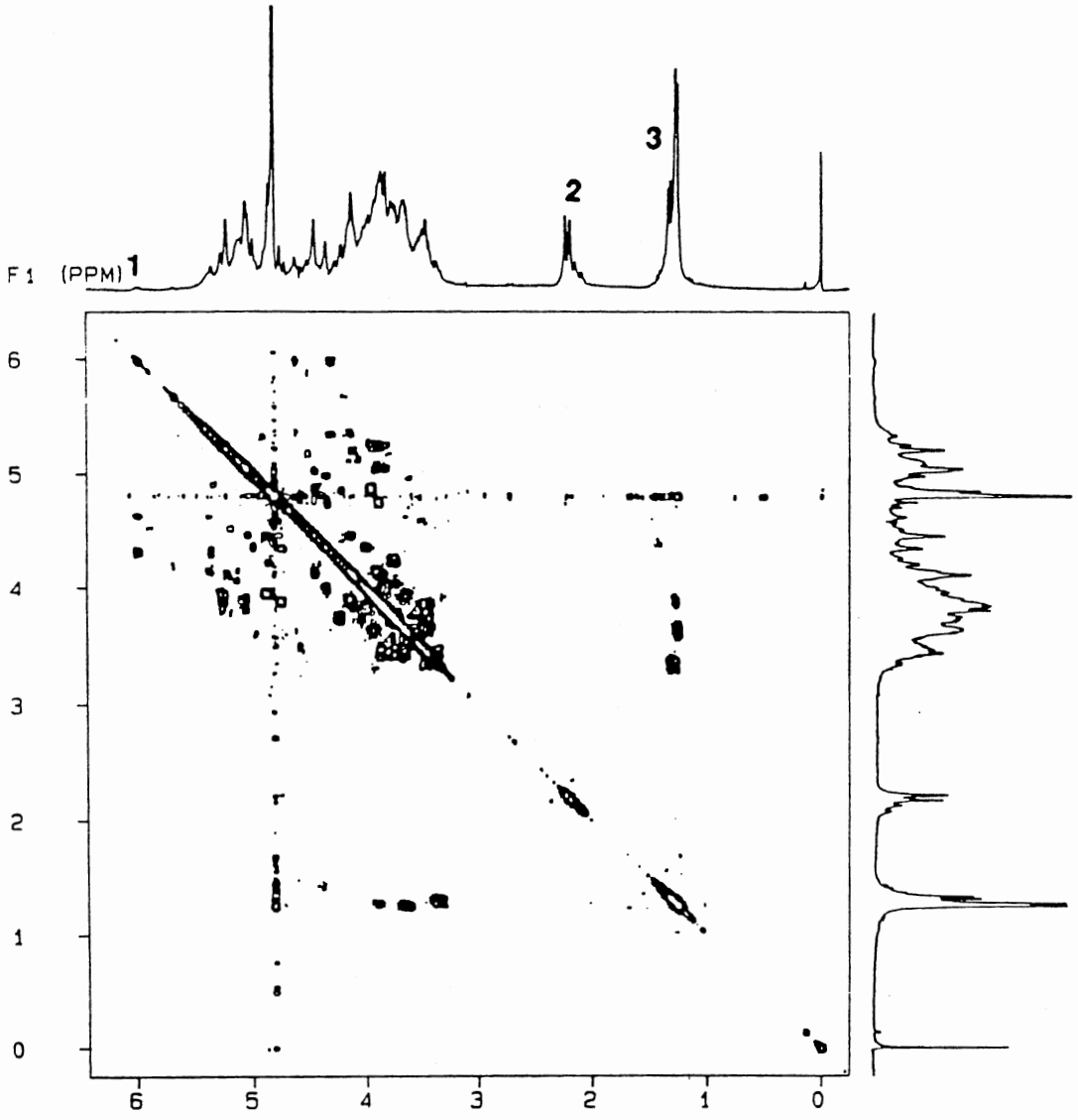

Figure 55. ^{1}H-^{1}H homonuclear (cosy) n.m.r. Spectrum of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. 1) H-3 of GalA; 2) CH_3 of acetate; 3) CH_3 of Rha. See next figure for detailed peak assignment.

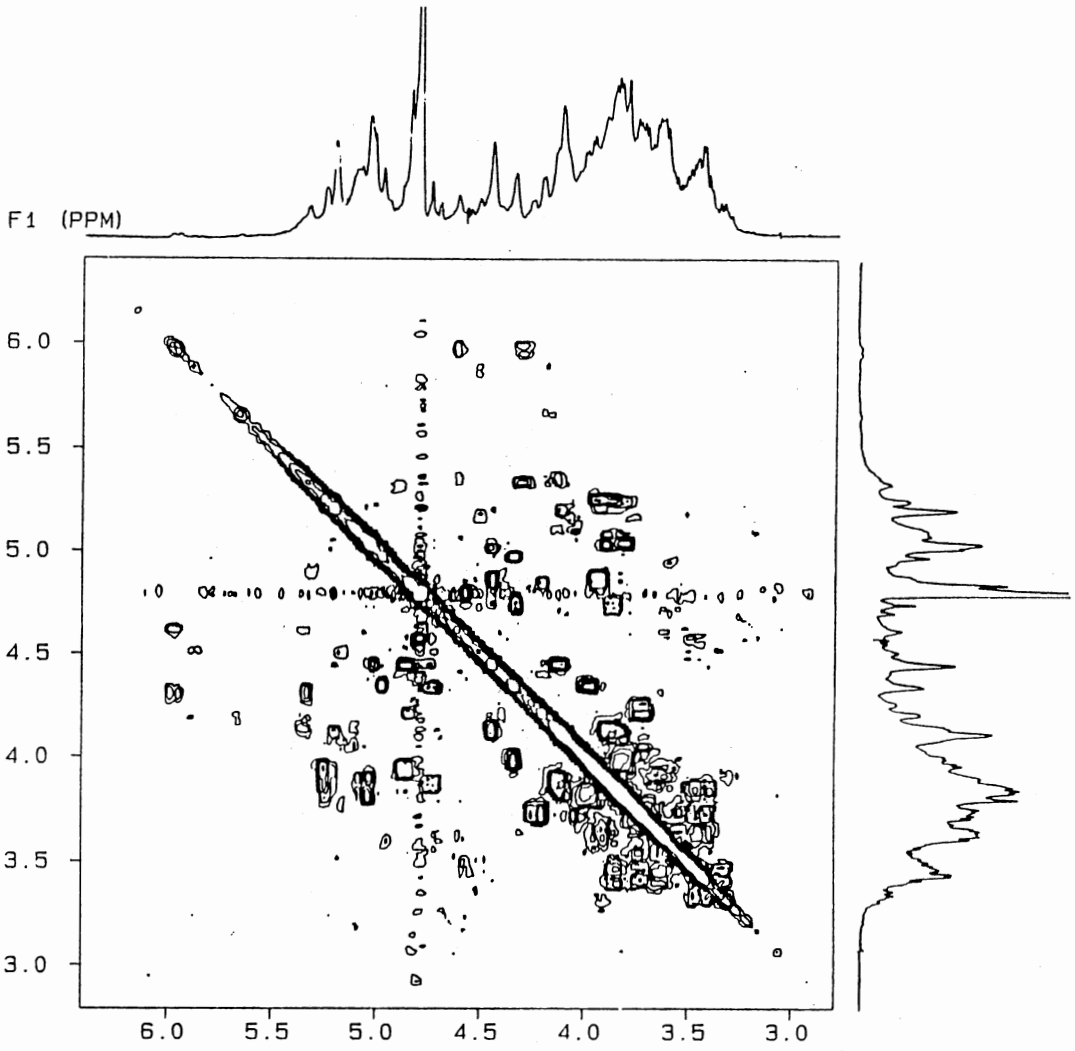

Figure 56. Enlarged Spectrum of 3-6 p.p.m. of the ^1H-^1H Homonuclear (cosy) n.m.r. Spectrum of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are identified as follows: G = Galacturonic acid; C = Cyclized; A = Acetylated; R = Rhamnose; L = linear; (---) not acetylated; I = Internal; N = Non reducing end. 1) H-3 GCA; 2) H-1 GCA, H-5 GCA; 3) H-1 GIA, H-1 GC---; ; 4) H-1 of α R; 5) H-1 GI---, H-5 GIA, H-5 G, H-1 GN---; 6) H-5 GC---; 7) H-3 GIA; 8) H-1 β RC; 9) H-3 GC---; 10) H-4 GCA; 11) H-1 β Gal; 12) H-1 GIA, H-4 GI---; 13) H-2 GCA, H-4 GC---, H-4 GN---; 14) H-2 β RC; 15) H-2 β RC; 16) H-2 α R, H-3 GI---; 17) H-3 GN---; 18) H-2 GIA; 19) CH_3 of methyl ester; 20) H-2 GC---, H-2 GI---; 21) H-2 GN---, H-3 α R; 22) H-3 β RC; 23) H-5 α R; 24) H-4 α R; 25) H-4 β RC; 26) H-5 β RC.

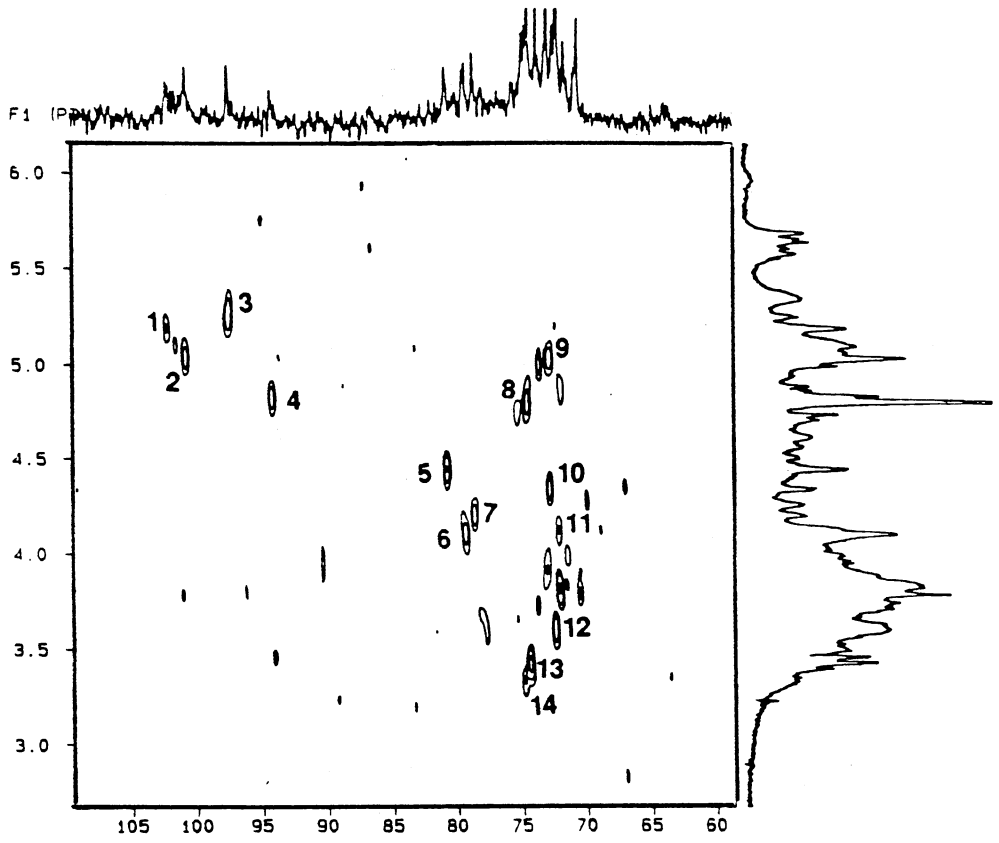

Figure 57. ^{13}C-^{1}H Heteronuclear Correlated n.m.r. Spectrum of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded on a 300 MHz n.m.r. spectrometer at 22°C in D_2O using TSP as internal standard at 0.00 p.p.m.Only some peaks are identified as follows: 1) C1,H1 α R; 2) C1,H1 α G; 3) C 1,H1 α G cyclized; 4) C1,H1 β R; 5) C4,H4 G; 6) C2,H2 α R; 7) C2,H2 β R; 8) C3, H3 G (acetylated); 9) C5,H5 G; 10) C4,H4 G; 11) C3,H3 G (non-acetylated); 12) C5,H5 α R; 13) C4,H4 α + β R; 14) C5,H5 β R.

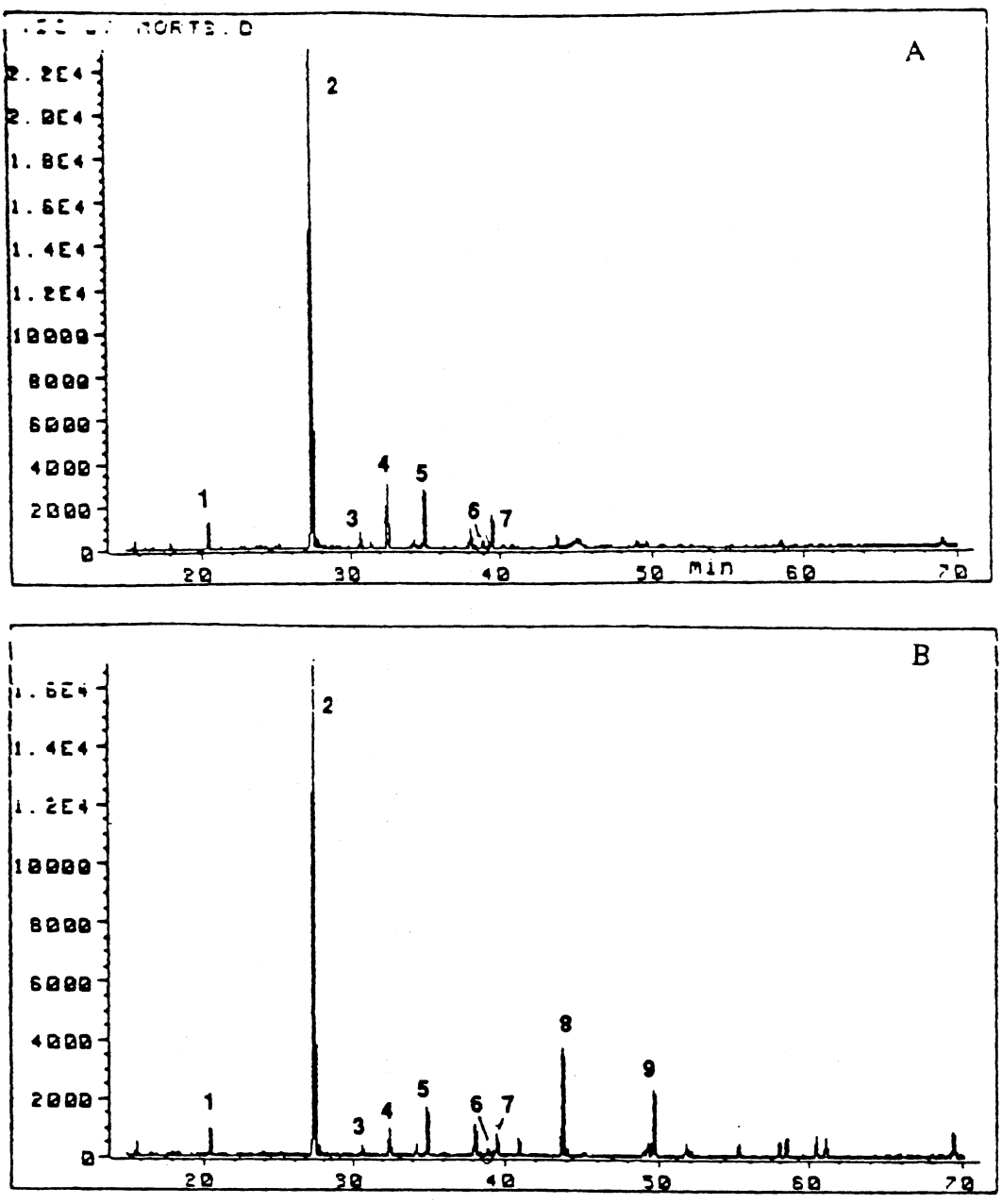

Figure 58. Gas Chromatograph of the Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls Without (A) and with (B) Reduction of Galacturonic Acid Before Hydrolysis. Peaks are identified from the mass spectra of these signals (Figures 59-67). 1) t -xyl; 2) 2-rha; 3) t -glc; 4) t -gal; 5) 2,4-rha; 6) 4-gal; 7) 4-glc; 8) 6-gal; 9) 4,6-gal.

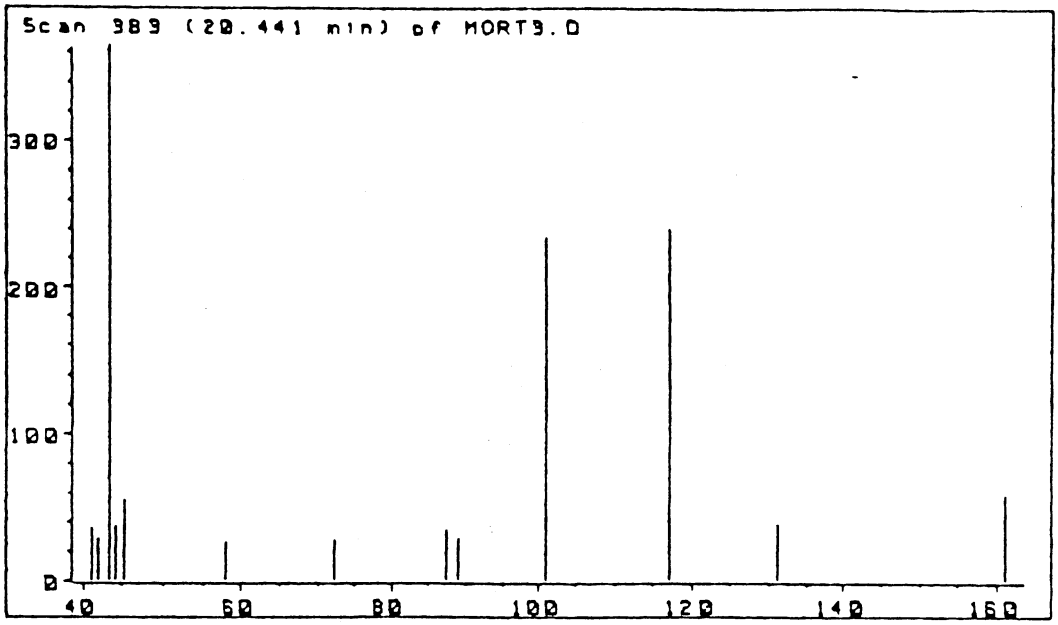

Figure 59. The Mass Spectrum of the Peak 1 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The most abundant peaks were M/Z 43.15, 101.1 and 117.1 identified as the fragmentation pattern of t-xyl.

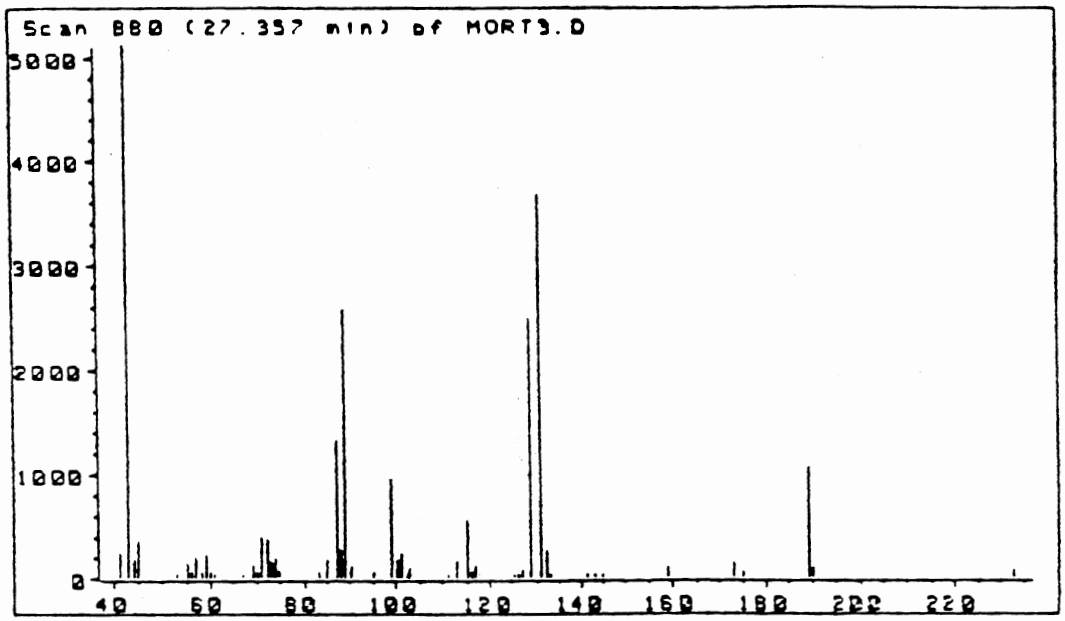

Figure 60. The Mass Spectrum of the Peak 2 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The most abundant peaks were M/Z 43.15, 129 and 131.15 and identified as that of 2-linked rhamnose.

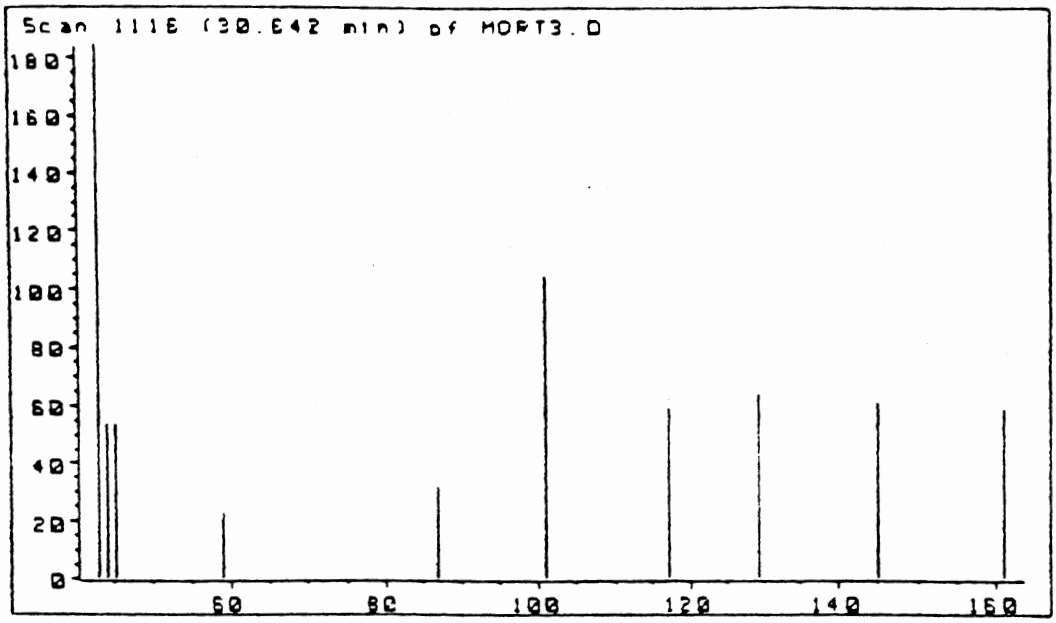

Figure 61. The Mass Spectrum of the Peak 3 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The most abundant peaks were M/Z 43.15, 101.1, 129.1, 145.05 and 161.05 and identified as that of t-linked glucose.

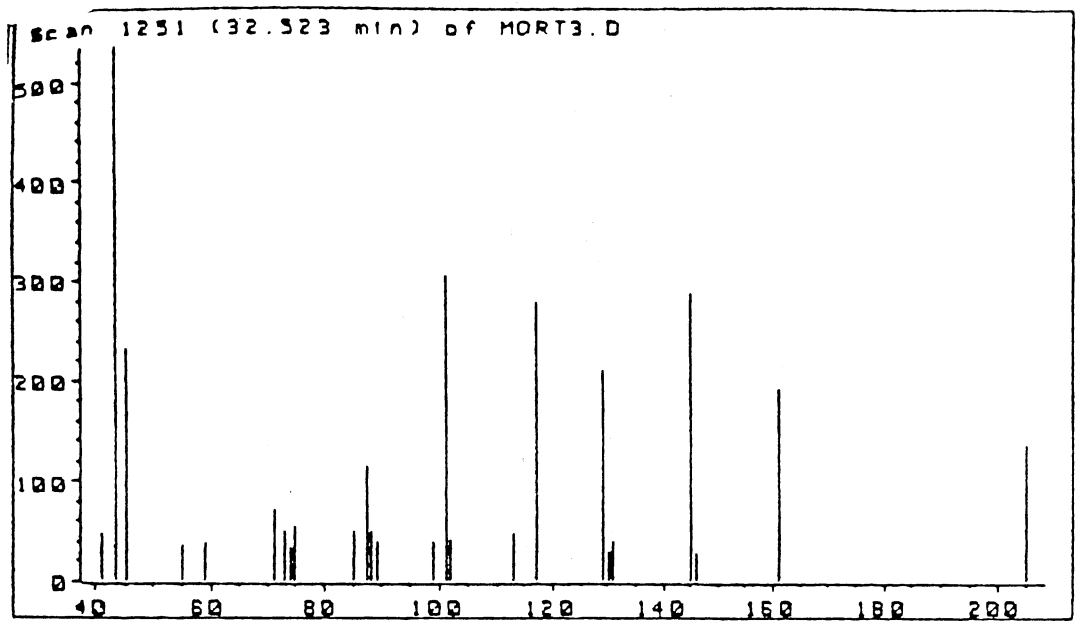

Figure 62. The Mass Spectrum of the Peak 4 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The fragmentation pattern was that of t-linked galactose.

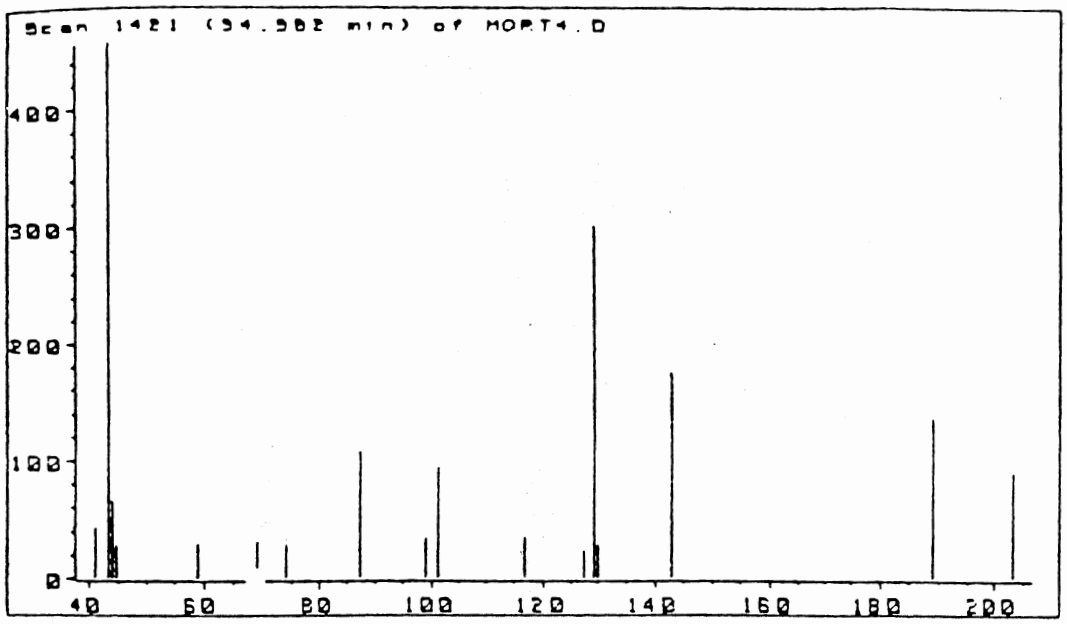

Figure 63. The Mass Spectrum of the Peak 5 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The fragmentation pattern was that of 2,4 linked rhamnose.

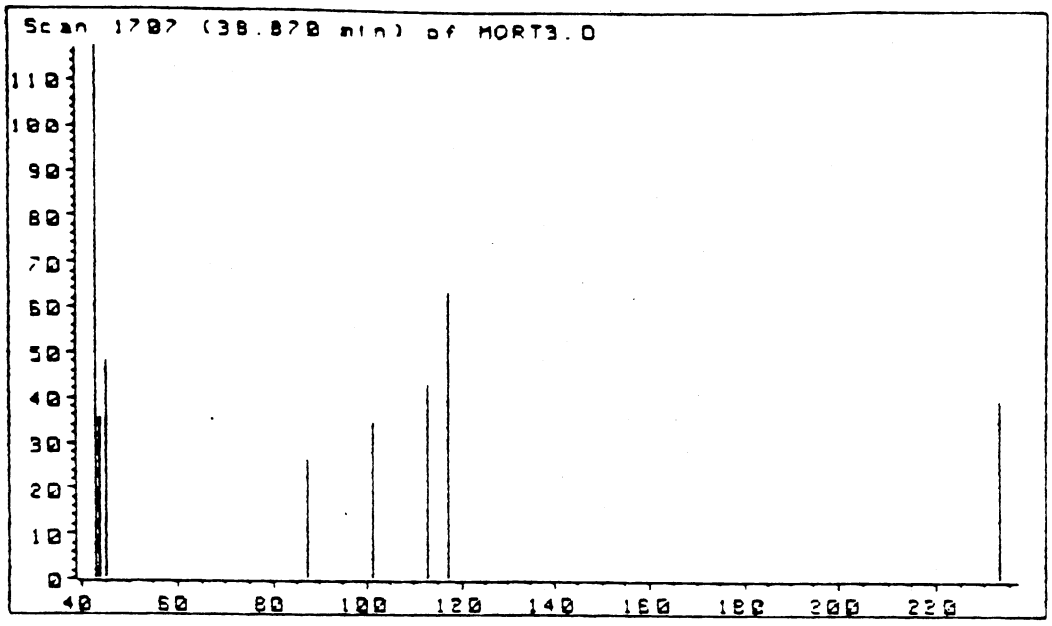

Figure 64. The Mass Spectrum of the Peak 6 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The fragmentation pattern was that of 4-linked galactose.

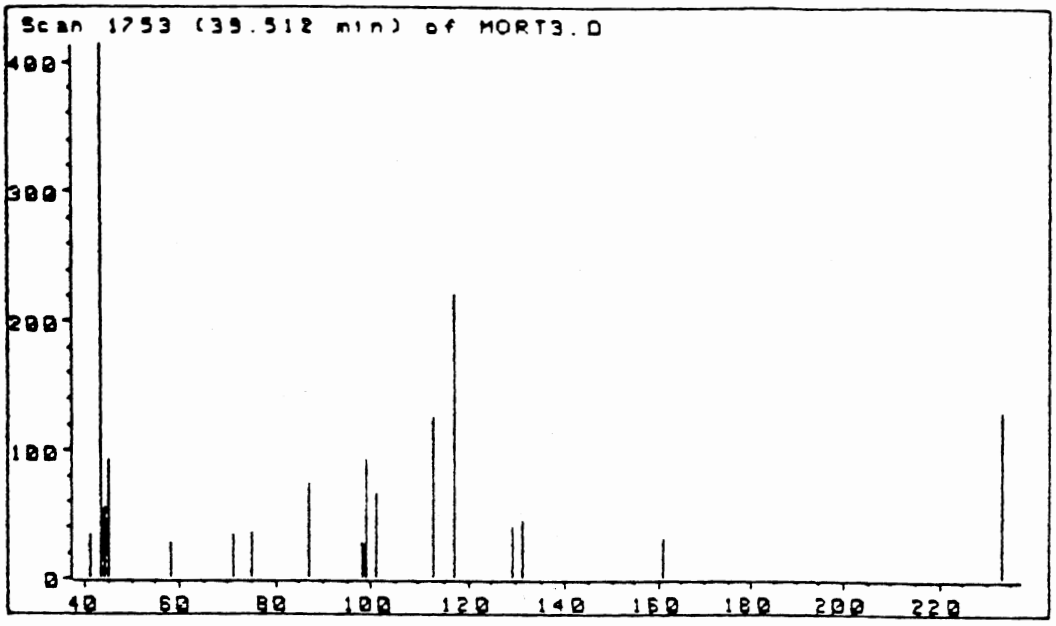

Figure 65. The Mass Spectrum of the Peak 7 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The fragmentation pattern was that of 4-linked glucose.

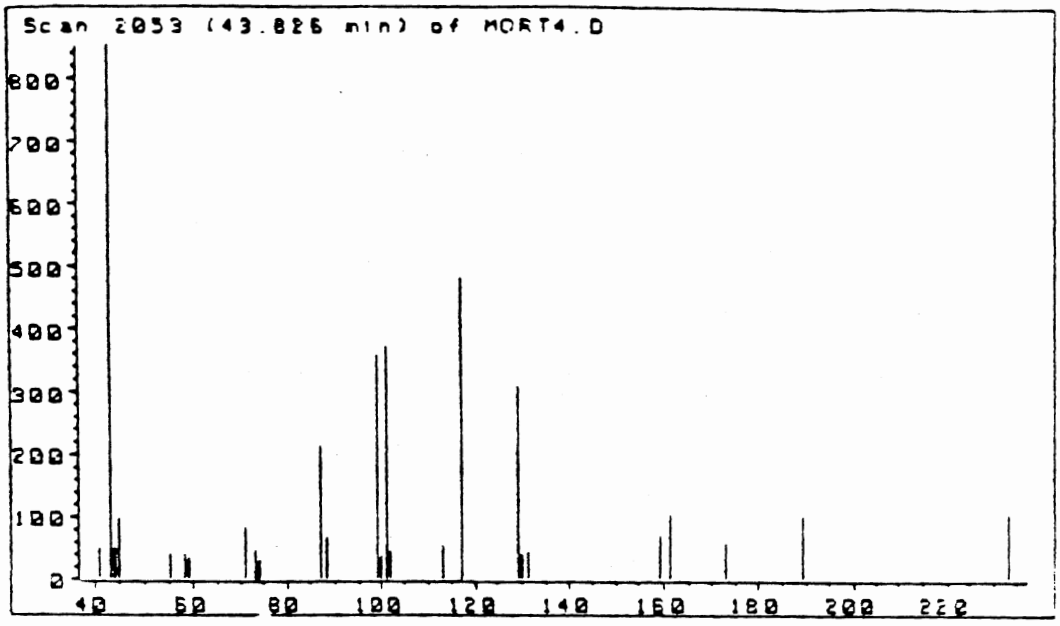

Figure 66. The Mass Spectrum of the Peak 8 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The fragmentation pattern was that of 6-linked galactose.

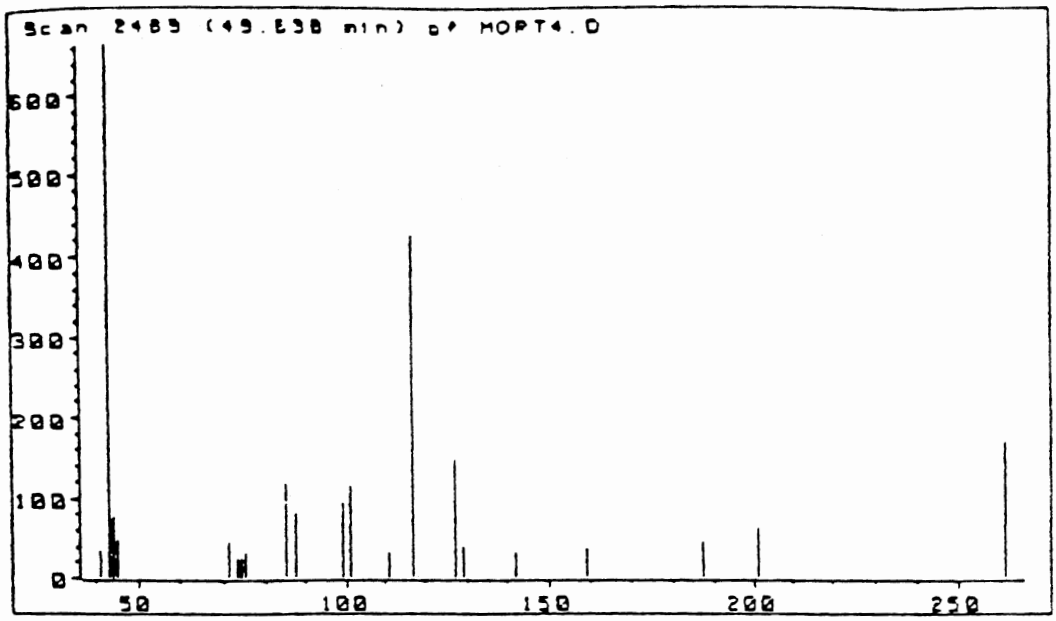

Figure 67. The Mass Spectrum of the Peak 9 of the Gas Chromatograph of Partially Methylated Alditol Acetates of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The fragmentation pattern was that of 4,6-linked galactose.

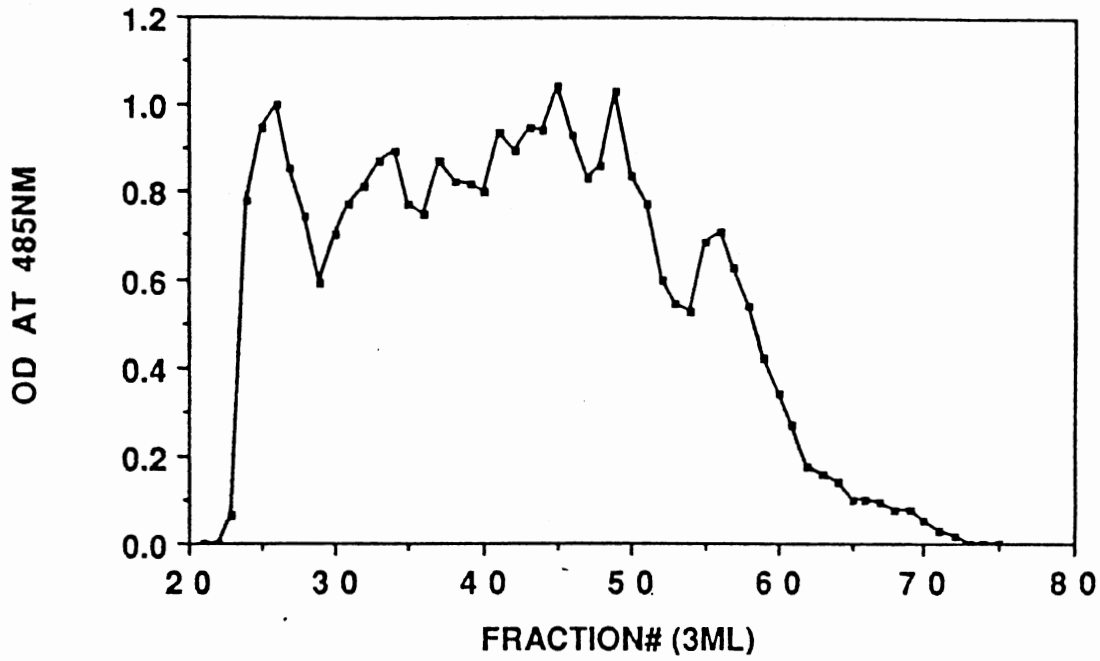

Figure 68. Chromatography on a Bio-gel P-6 (50 x 2.5 cm) Column of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C in 0.3 M Sodium Acetate Buffer pH 5.2. 3 ml fractions were collected and the fractions were tested for sugar by phenol sulfuric acid test. Tubes 21-29 were pooled into fraction 1, tubes 30-35 into fraction 2, tubes 36-40 into fraction 3, tubes 41-46 into fraction 4 , tubes 47-54 into fraction 5, tubes 55-61 into fraction 6 and tubes 62-75 were pooled into fraction 7.

TABLE XVI

MOLE PERCENT OF SUGARS IN THE BIO-GEL P-6 FRACTIONS OF THE DEAE FRACTION 2 OF THE WATER EXTRACT FROM COTTON WALLS AFTER HF SOLVOLYSIS AT-40°C

Sugar	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	Fr-6	Fr-7
Ara	2.5	2.3	3.2	2.4	2.7	8.5	-
Rha	25.2	36.8	37.2	39.9	34.6	22.5	Trace
Xyl	3.5	2.7	3.3	2.3	3.9	7.9	Trace
GalA	50.2	44.3	39.8	40.1	32.9	25.9	-
Man	0.9	0.6	0.8	0.5	0.9	0.5	-
Gal	7.5	7.7	9.6	8.1	12.5	24.6	-
Glc	10.1	5.7	6.2	6.7	12.5	9.9	Trace

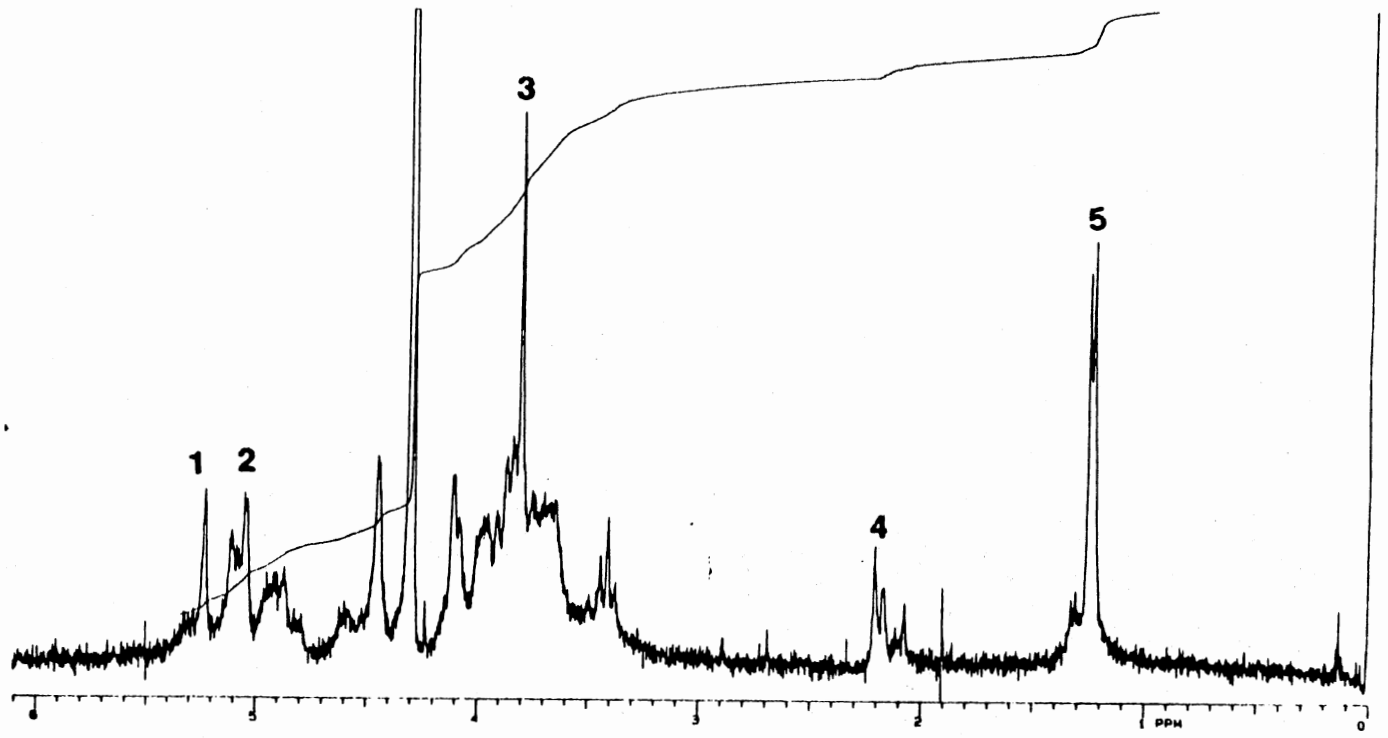

Figure 69. The ^{1}H n.m.r. Spectrum of the P-6 Fraction 1 of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard. Peaks are identified as follows; 1) H-1 αGalA; 2) H-1 GalA (internal); 3) CH_3 of methyl ester; 4) CH_3 of acetate ester; 5) CH_3 of Rha.

The molar composition of fractions 2 through 6 of the P-6 column showed the ratio of rhamnose to galacturonic acid to be about 1:1 with significant amounts of gal, glc, xyl and ara (Table XVI). ^{1}H n.m.r. analysis of fractions 2 and 3 showed acetylation and no methyl esterification (Figures 70 and 71). These represent oligomers of the RG I backbone with side chains/remnants of side chains attached to them.

Analysis of DEAE Fraction 3 of the Water Extract Obtained After HF -40°C Reaction

Fraction 3 of the DEAE column was found to contain oligomers with 1:1 ratio of rhamnose and galacturonic acid (Table XV) and the proton n.m.r. spectrum showed acetylation (Figure 72). These oligomers were bigger in size compared to the fraction 2 of the DEAE column (HPLC trace, Figure 73).

The Water Insoluble Residue

The residue after water extraction had a substantial amounts of galacturonic acid (Table XIV). Sugar accounted for 40% of the dry weight of the residue. This galacturonic acid was extracted with 0.5 M imidazole buffer pH 7.0 followed by 0.1 M EDTA. The composition the imidazole extract and the EDTA extract showed that these contained about 80% of galacturonic acid (Table XIV) and hence represents the homogalacturonan region of pectin. These two extracts were not very different in composition and in size. The imidazole extract was about 15% of the walls where as the EDTA extract was about 6% of the walls. The size distribution of oligomers in these extracts showed that these were slightly larger than those in the extracts obtained after HF solvolysis at -23°C (Figures 74 and 75). The proton n.m.r. spectrum of the imidazole extract showed very little methyl esterification (Figure 76). The residue left behind after the series of extractions described above contained mainly cellulose and cell wall protein. It also had about 6% of the original galacturonic acid in the wall and small amounts of xyl,

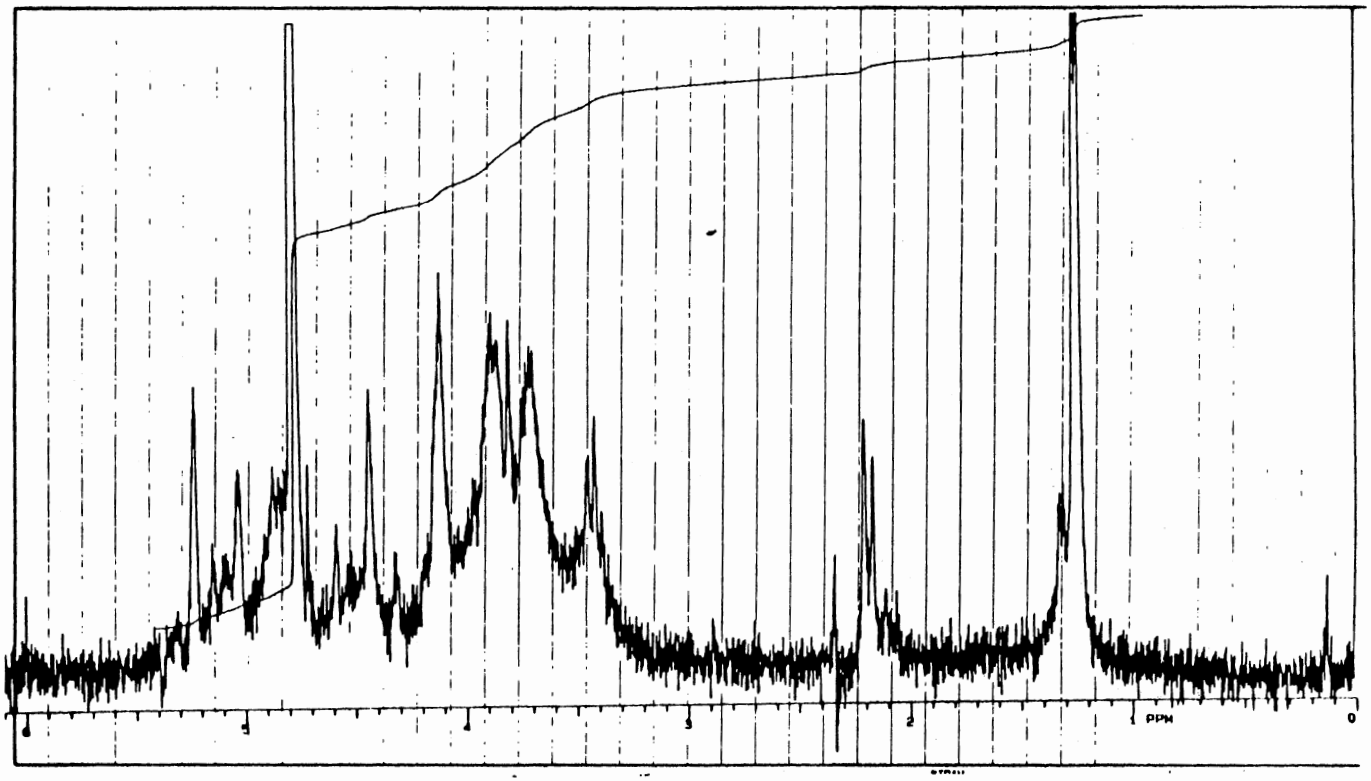

Figure 70. The ^{1}H n.m.r. Spectrum of the P-6 Fraction 2 of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 22°C in D_2O, using TSP as internal standard. Signal at 2.18 p.p.m. represents acetate ester.

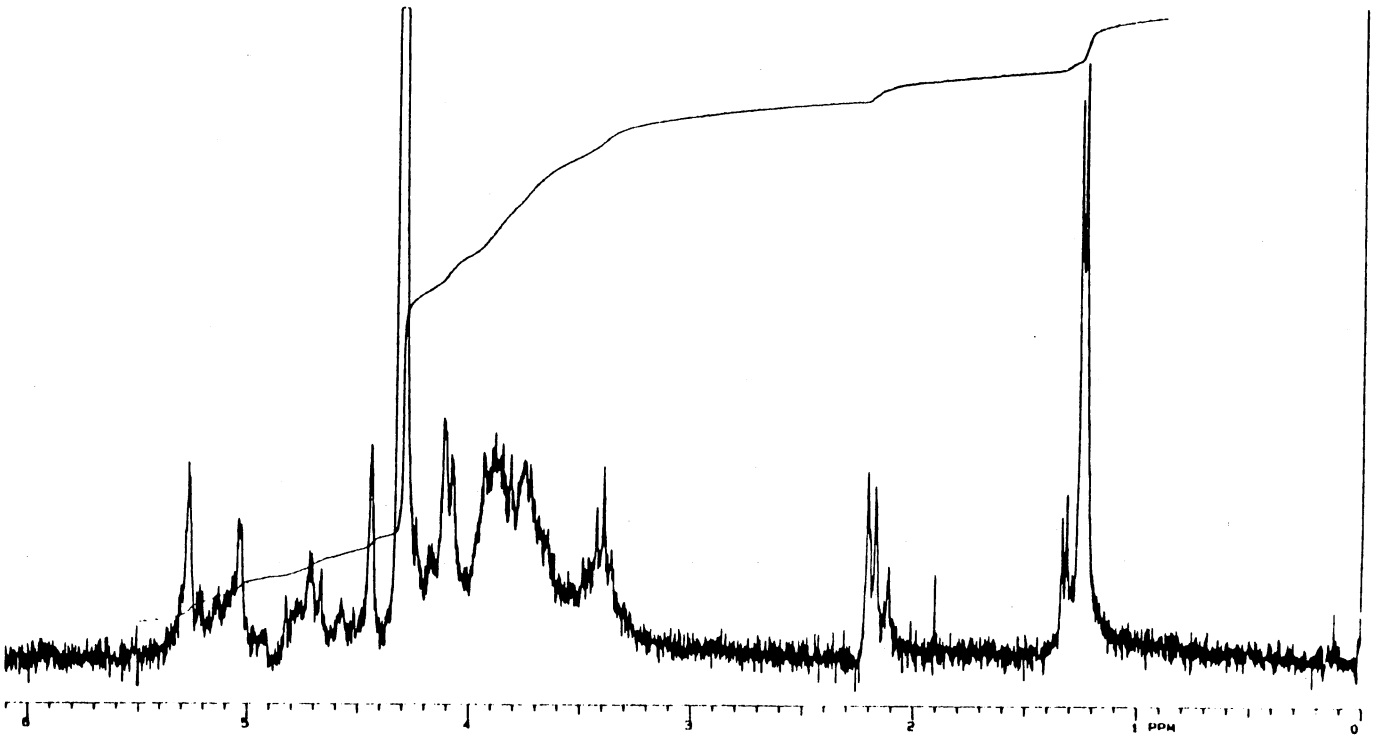

Figure 71. The ^{1}H n.m.r. Spectrum of the P-6 Fraction 3 of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard. Signal at 2.18 p.p.m. represents acetate ester.

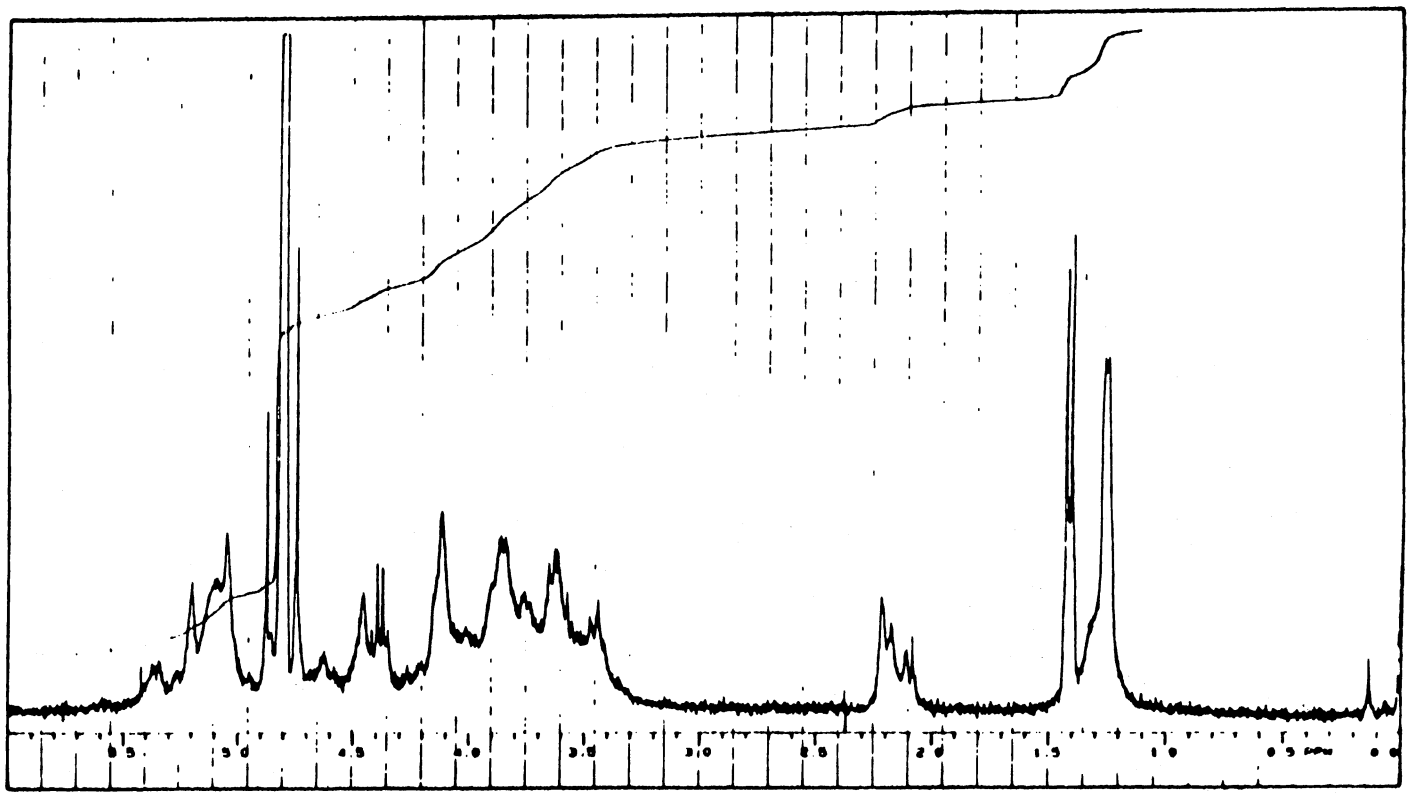

Figure 72. The ^{1}H n.m.r. Spectrum of the DEAE Fraction 3 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 22°C in D_2O, using TSP as internal standard. Signal at 2.18 p.p.m. represents acetate ester.

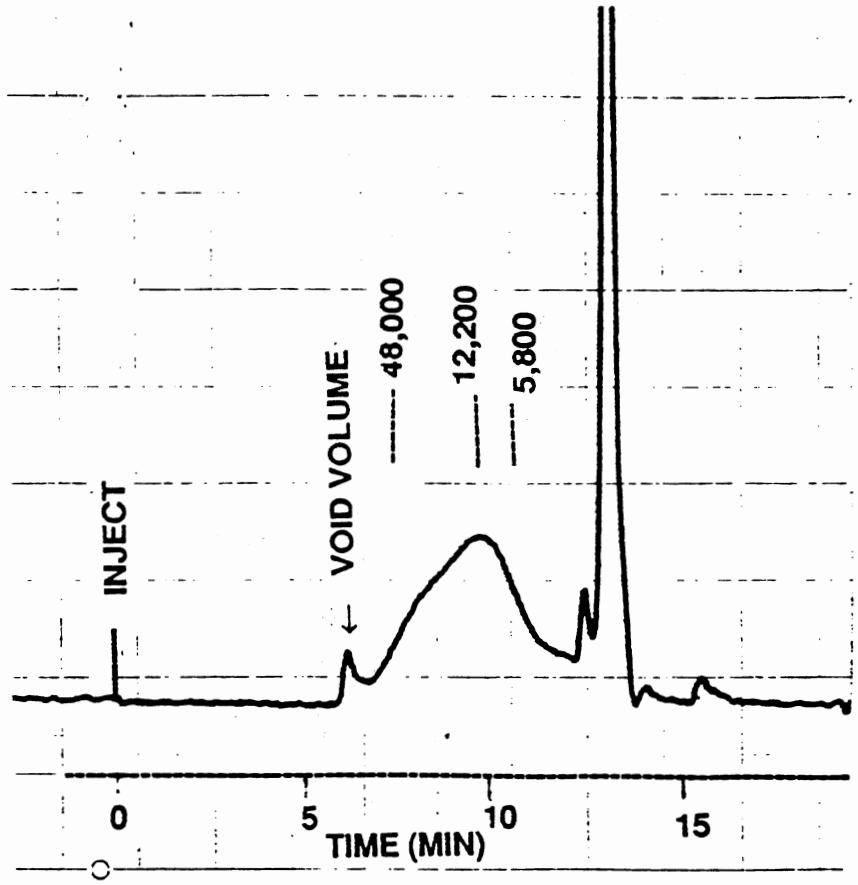

Figure 73. The HPLC Chromatogram of the DEAE Fraction 3 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C on TSK SW 2000 Gel Filtration Column (7.5 mm x 30 cm). The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. GalA oligomers consistenly elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulan.

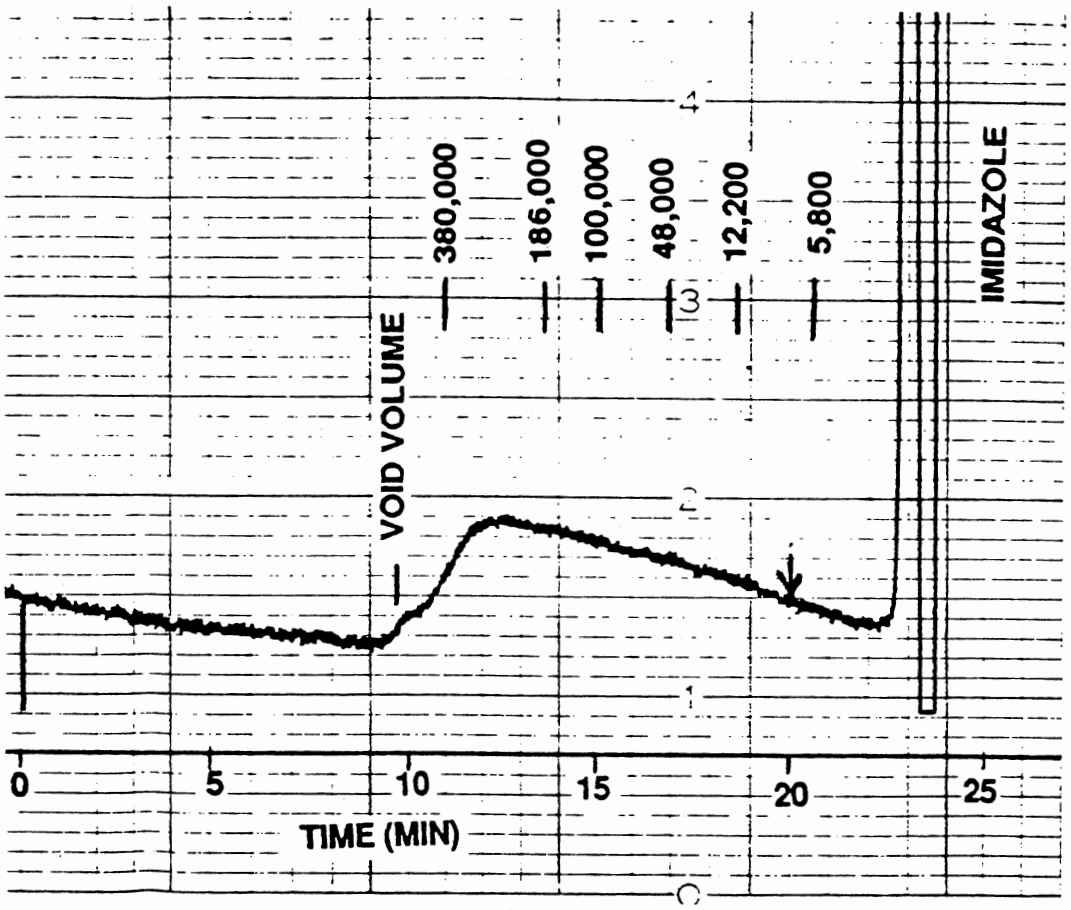

Figure 74. The HPLC Chromatogram of the Imidazole Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The sample was dissolved in 0.5 M imidazole buffer pH 7.0 and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. GalA oligomers consistenly elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

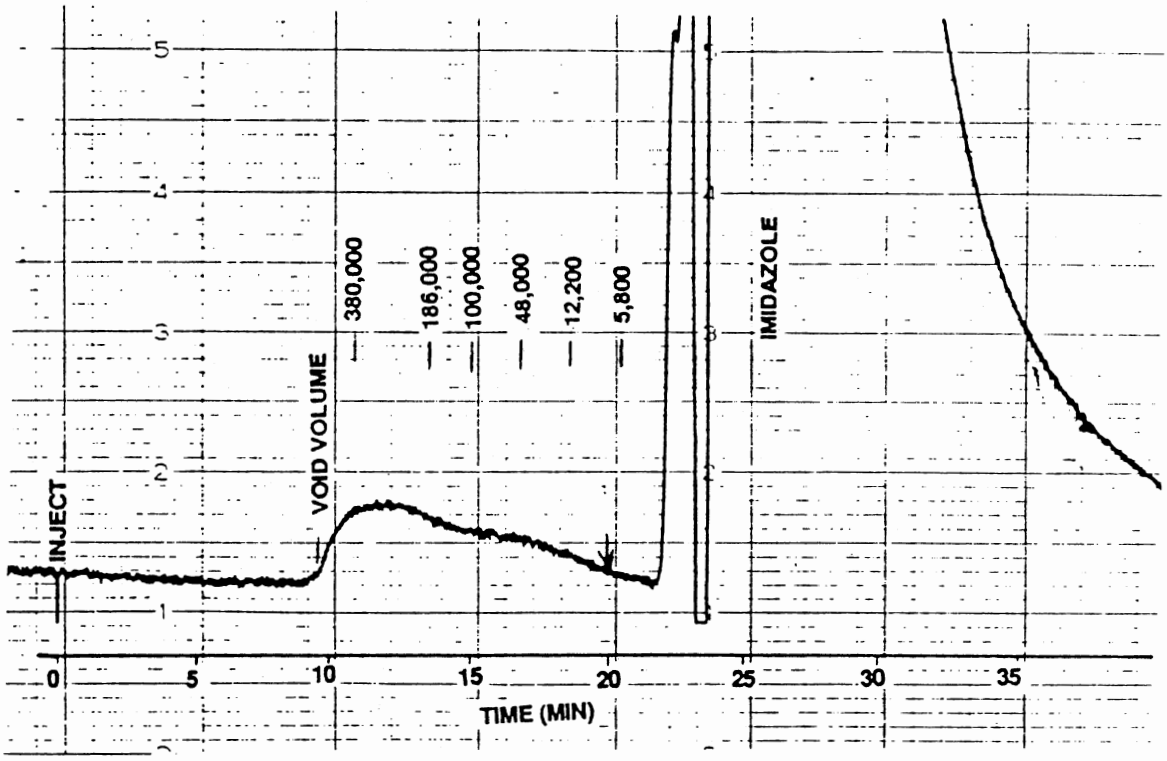

Figure 75. The HPLC Chromatogram of the EDTA Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C on TSK SW 4000 and SW 2000 Gel Filtration Columns (7.5 mm x 30 cm) in Series. The sample was dissolved in 0.5 M imidazole buffer pH 7.0 and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. The arrow indicates the elution volume of a galacturonic acid oligomer of 3,500 molecular weight. GalA oligomers consistenly elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

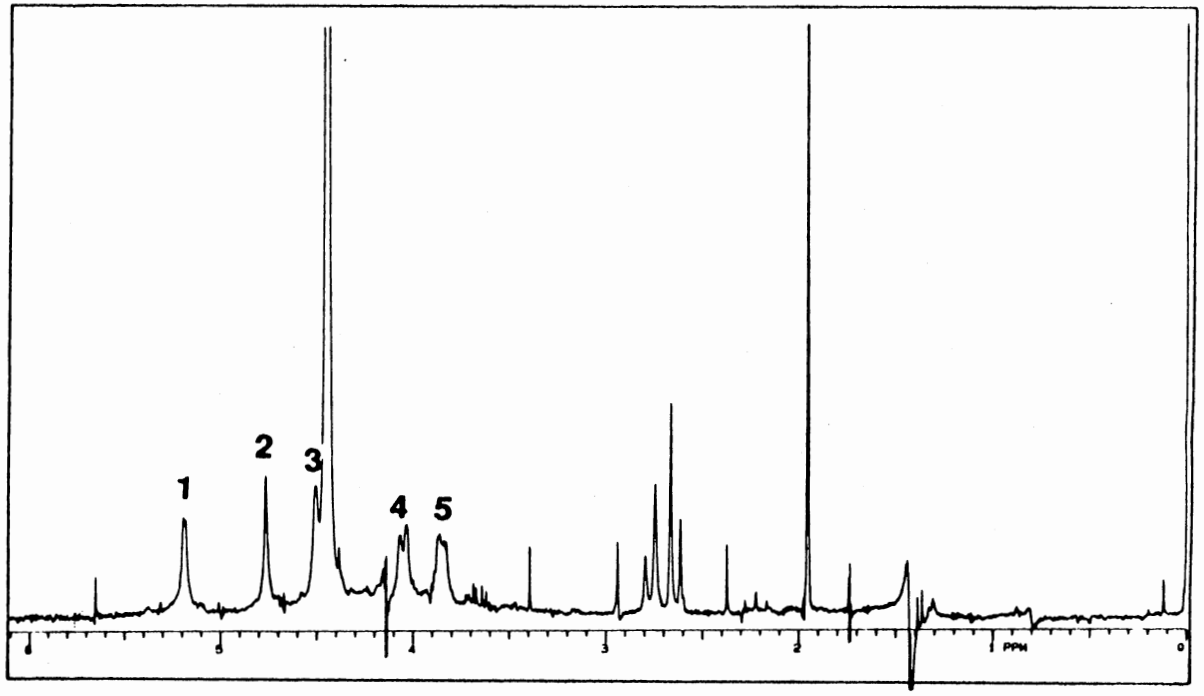

Figure 76. The ^{1}H n.m.r. Spectrum of the Imidazole Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -40°C. The spectrum was recorded using a 300 MHz nmr spectrometer, at 70°C in D_2O, using TSP as internal standard. Sample was dissolved in 0.5 M imidazole buffer pH 7.0. 1) H-1 α GalA; 2) H-5 GalA; 3) H-4 GalA; 4) H-3 GalA; 5) H-2 GalA.

ara, rha, gal and glc (Table XIV). The reason for the release of RG I from walls after HF treatment at -40°C involves breakage of covalent bonds but the specificity of this cleavage is not clear at present and needs to be investigated further.

Solvolysis of Acala 44 Suspension Cell Walls at -73°C

About 2 gm of cell walls were treated with HF at -73°C for 30 min in four batches of 500 mg each as described earlier. About 6-8% of the walls became soluble in the HF/ether mixture. Another 17% of the original weight became water soluble and the residue left behind weighed approximately 50% of the original weight. There seems to be loss of sample during filtration using scintered glass funnel which lowers recovery. This could be minimized by the use of a teflon filter unit. Analysis of the HF/ether soluble sugars showed it contained about 81% arabinose showing that the main linkage that was broken during this treatment was arabinose (Table XVII). This fraction also had small amounts of fucose and xylose. The sugar composition of the water extract is shown in Table XVII and this showed that it had hemicellulose and pectin fragments. HPLC analysis of the water extract on SW 2000 gel filtration column showed that part of the water extract eluted in the void volume (Figure 77). In order to see whether these two polymer fragments (hemicellulose and pectin) could be separated from each other the water extract was fractionated on a DEAE Sephadex column (Figure 78). The sugar compositions of the 3 DEAE fractions showed that both fractions 2 and 3 contained equimolar composition of rhamnose and galacturonic acid suggesting that these oligomers originated from RG I region of pectin (Table XVIII). Both fractions also had high amounts of xylose. The fraction 1 of the DEAE column contained mainly xylose and glucose and arabinose and may have originated from hemicelluloses and arabinogalactan. This fraction also had small amounts of acetylation (Figure 79). The ^{1}H n.m.r. spectra of the fractions 2 and 3 showed the presence of acetylation (Figures 80 and 81). Integration of the proton spectra of these two fractions showed the acetylation to be ~50% . Both

TABLE XVII

MOLE PERCENT OF SUGARS IN THE DIFFERENT FRACTIONS OF ACALA 44 COTTON SUSPENSION CELL WALLS AFTER HF SOLVOLYSIS AT -73°C

Sugar	Ether fraction	Water extract	Residue after water extn
Ara	81.5	22.4	3.7
Rha	2.4	9.5	10.1
Fuc	5.6	2.7	-
Xyl	7.6	27.5	14.5
GalA	-	8.3	48.9
Man	-	0.7	0.5
Gal	2.9	12.5	8.3
Glc	Trace	16.4	14.1

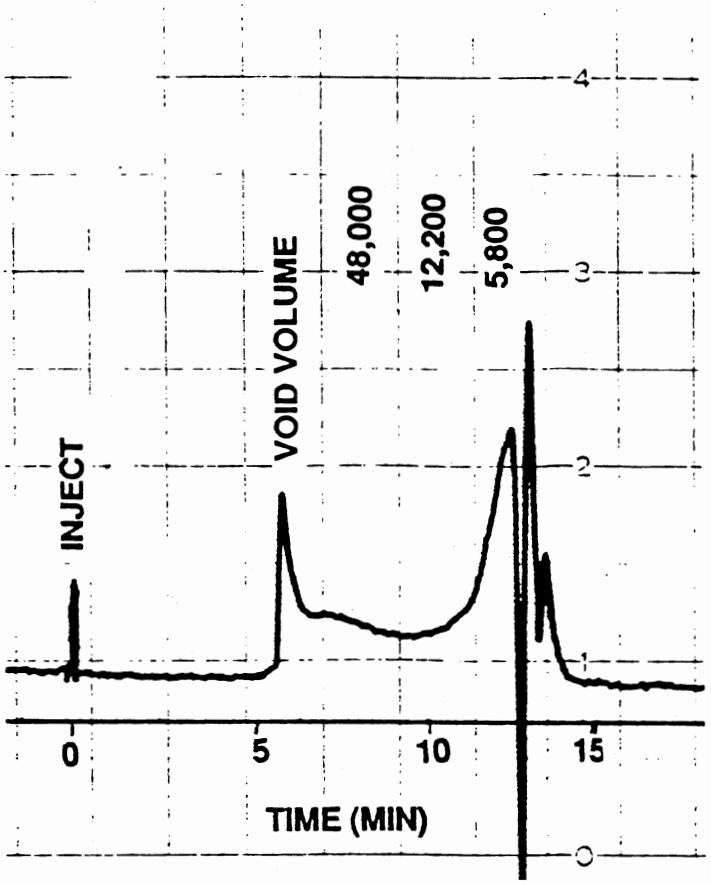

Figure 77. The HPLC Chromatogram of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -73°C on TSK SW 2000 Gel Filtration Column (7.5 mm x 30 cm). The sample was dissolved in water and injected. The elution buffer was 0.3 M sodium acetate pH 5.2 and the flow rate was 1 ml/min. Detection was by refractive index monitor. Molecular weights indicated were from pullulan standards. GalA oligomers consistenly elute earlier than expected based on pullulan standards because they behave as rods in solution as opposed to the random coil structures of pullulans.

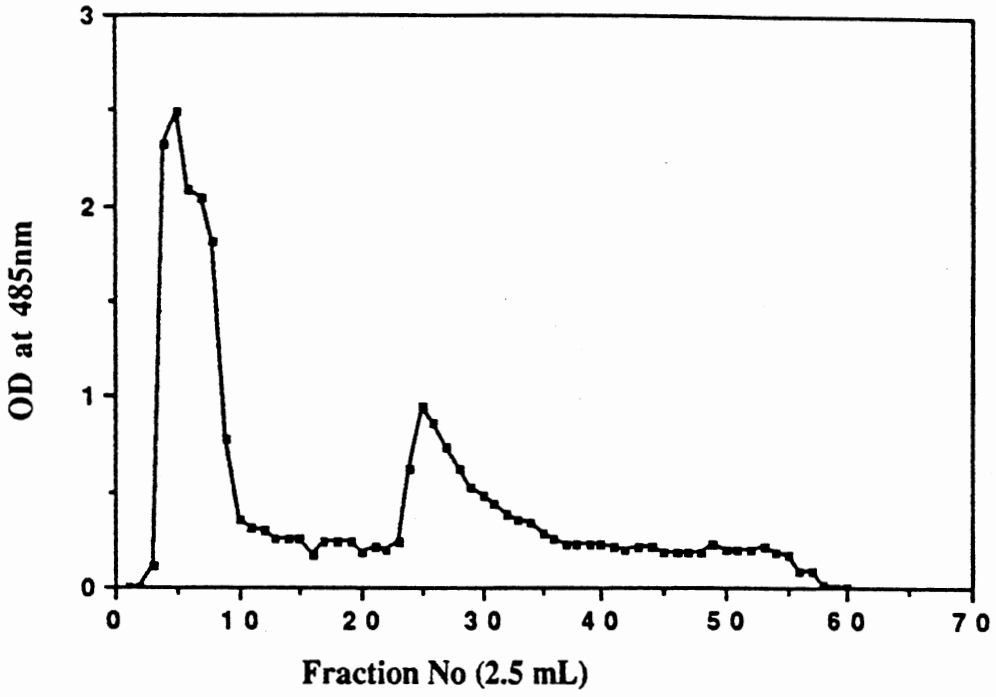

Figure 78. Chromatography on a DEAE Sephadex Anion Exchange Column (20 x 2 cm) of Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -73°C. Sample was applied in 0.05 M sodium acetate buffer pH 5.2 and eluted with 0.05 M, 0.5 M and 1 M sodium acetate buffer pH 5.2. Tubes 1-15 pooled into fraction 1, tubes 16-35 pooled into fraction 2 and tubes 36-60 pooled into fraction 3. Fractions were tested for sugar by phenol sulfuric acid test.

TABLE XVIII

MOLE PERCENT OF SUGARS IN THE DIFFERENT DEAE FRACTIONS OF THE WATER EXTRACT FROM COTTON WALLS AFTER HF SOLVOLYSIS AT -73°C

Sugar	Fraction 1	Fraction 2	Fraction 3
Ara	24.7	12.9	8.4
Rha	2.7	17.3	26.5
Fuc	2.5	Trace	-
Xyl	31.4	25.9	42.4
GlcA	-	Trace	-
GalA	-	20.3	43.1
Man	0.7	-	-
Gal	10.7	16.1	13.8
Glc	27.3	7.5	5.8

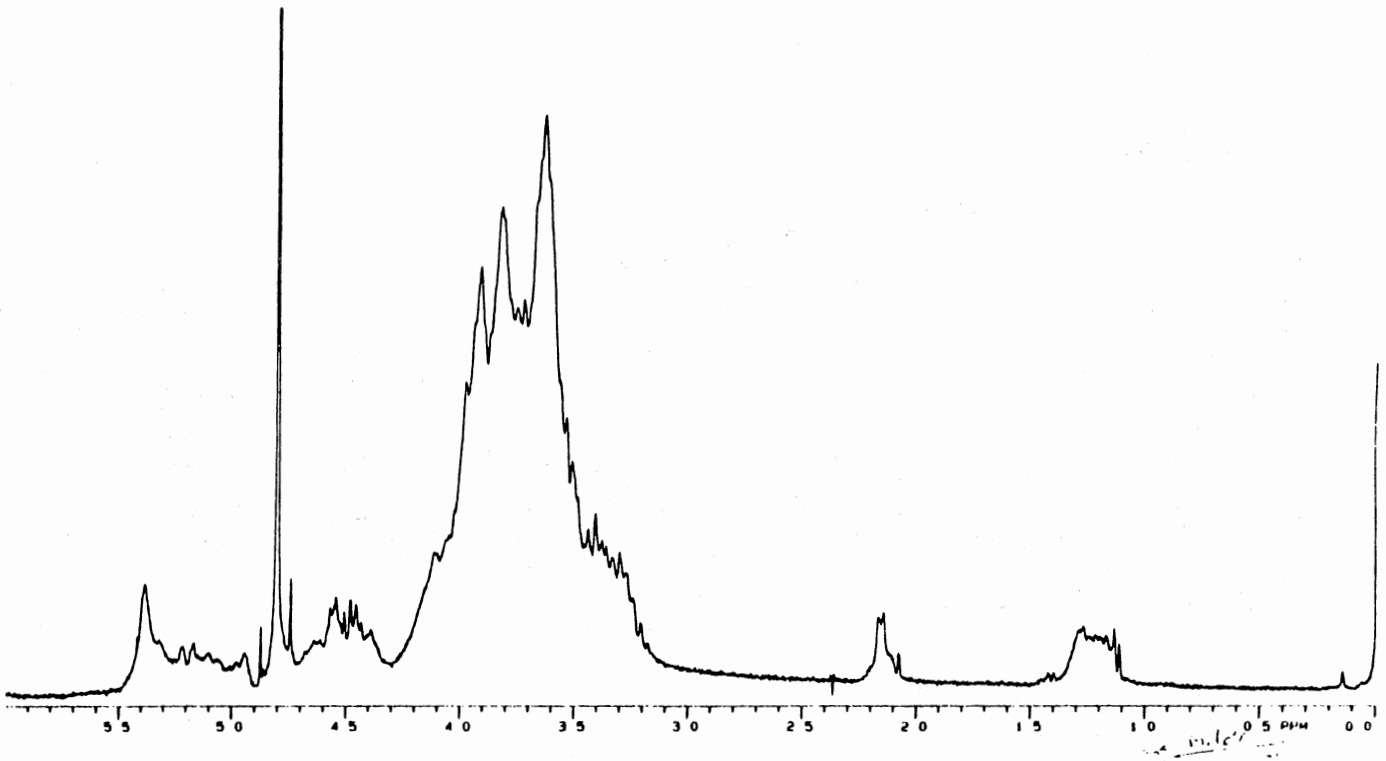

Figure 79. The ^{1}H n.m.r. Spectrum of the DEAE Fraction 1 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -73°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 22°C in D_2O, using TSP as internal standard. Signal at 4.7 p.p.m. suggests β glucose.

these fractions also had significant amount of xylose and glucose, more than the amount found in the DEAE fractions of the -40 C reaction. The DEAE fraction 2 was fractionated on a Bio-gel P-6 column (Figure 82) and the sugar composition of different fractions is shown in Table XIX. A major portion of the sample had eluted in the void volume of the column and the fractionation pattern was different from that of the DEAE fraction 2 of the -40°C HF reaction. This shows that at -73°C the oligomers formed are larger in size than those formed in the -40°C reaction. Most of the P-6 fractions had a 1:1 ratio of rhamnose and galacturonic acid. These fractions had significant amounts of xylose which made in some cases interpretation of the n.m.r. spectra rather difficult (Figure 83). Significant amounts of xylose and glucose are present along with the oligomers of RG I. The release of RG I from walls after treatment with HF at -73°C involves breakage of covalent linkages between polymers but the exact nature of this reaction has to be determined.

Solvolysis of Acala 44 Cotton Suspension Cell Walls at 0°C

Acala 44 suspension cell walls (250 mg) were treated with HF at 0°C for 30 min. 34% of the original weight of the walls was dissolved in HF/ether mixture. About 30% of the original weight of the walls became soluble in water and the residue left behind was about 5% of the walls. The compositions of different fractions are shown in Table XX. HF solvolysis at 0°C removes almost all sugars from the walls including those in cellulose. It essentially deglycosylates cell wall protein. The water insoluble residue was found to contain ~25% sugars which was mainly galacturonic acid and small amounts of glucose, arabinose and xylose. The rest of the sample was protein and some unknown water insoluble materials in the wall.

The ether-HF soluble sugars were separated on a P-2 column (Figure 84) and the proton n.m.r. spectra of the two fractions are shown in Figures 85 and 86. Fraction 1 had small amounts of acetate while fraction 2 containing mainly monosaccharides, did not have any acetate esters. The water extract was fractionated on a Bio-gel P-2 column

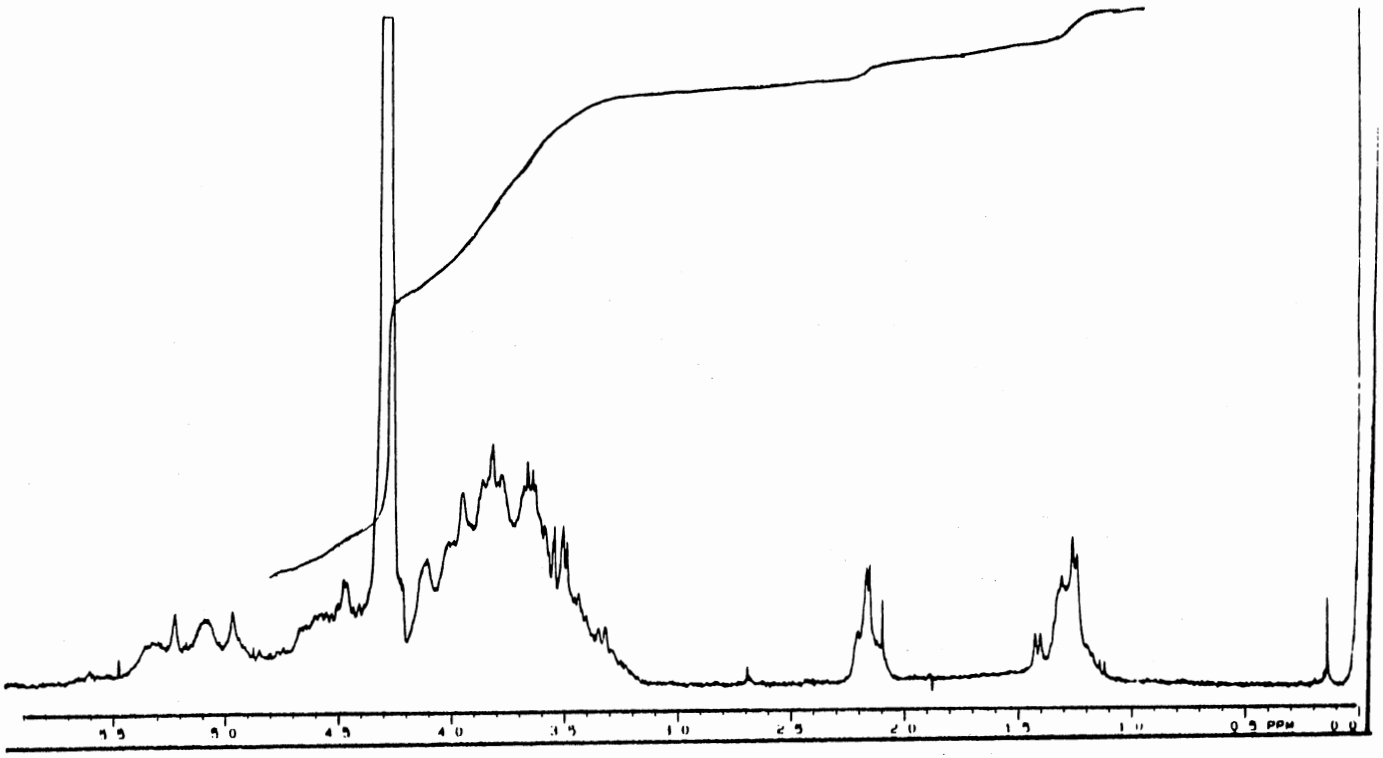

Figure 80. The ^{1}H n.m.r. Spectrum of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls after HF Solvolysis at -73°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard. Tentative assignments are 2,18 p.p.m., acetate ester, 1.3 p.p.m., CH_3 of rhamnose, 5.23 p.p.m. H-1 α rhamnose; 5.1 p.p.m. H-1 α galacturonic acid.

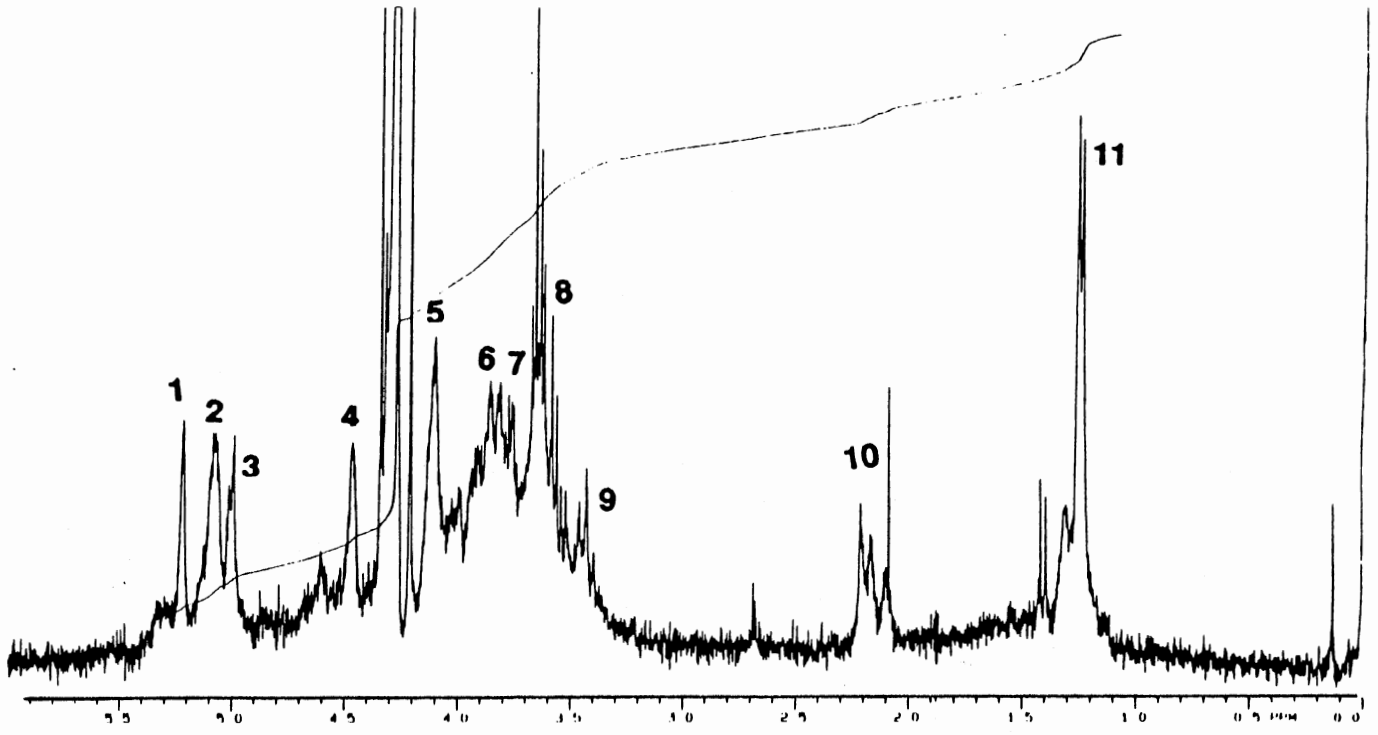

Figure 81. The ^{1}H n.m.r. Spectrum of the DEAE Fraction 3 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -73°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard. 1) H-1 α rha; 2) H-1 α galA; 3) H-5 galA; 4) H-4 GalA; 5) H-3 galA; 6) H-2 rha; 7) H-3 rha; 8) H-5 rha; (0 H-4 rha; 10) CH_3 of acetate; 11) CH_3 of rha.

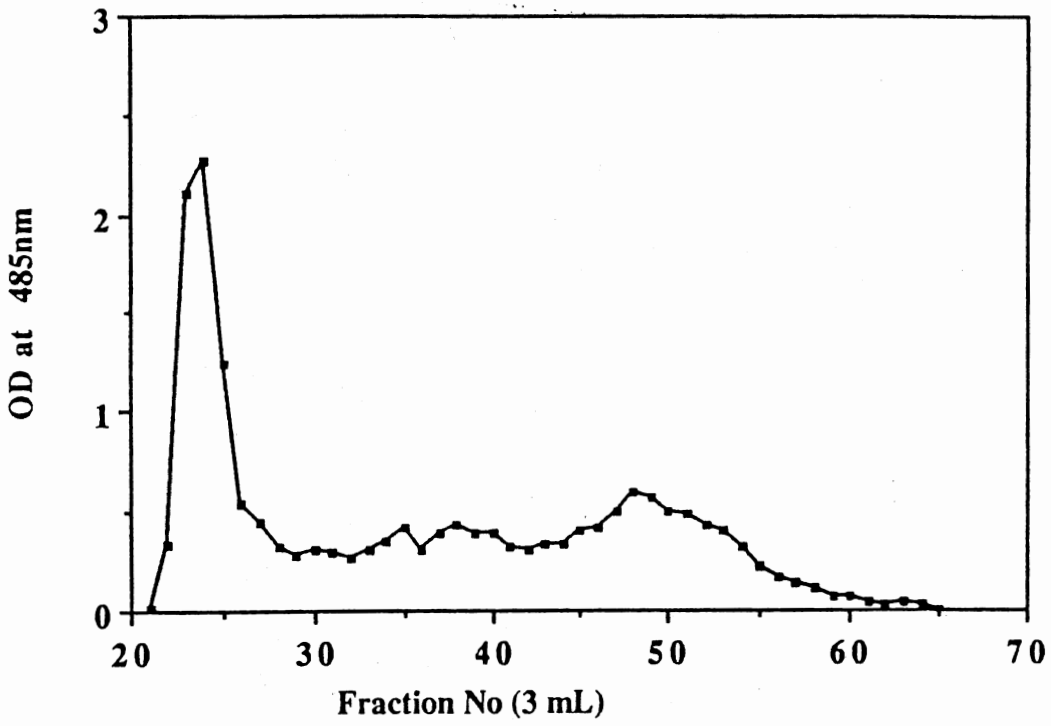

Figure 82. Chromatography on a Bio-gel P-6 (50 x 2.5 cm) Column of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -73°C in 0.3 M Sodium Acetate Buffer pH 5.2. 3 ml fractions were collected and the fractions were tested for sugar by phenol sulfuric acid test. Tubes 42-46 into fraction 4, tubes 47-55 into fraction 5, tubes 56-65 into fraction 6.

TABLE XIX

MOLE PERCENT OF SUGARS IN THE DIFFERENT P-6 FRACTIONS OF THE DEAE FRACTION 2 OF THE WATER EXTRACT OF THE HF -73°C REACTION

Sugar	Fr-1	Fr-2	Fr-3	Fr-4	Fr-5	Fr-6
Ara	8.3	11.7	12.6	14.2	14.1	26.5
Rha	22.1	12.5	10.6	14.2	8.3	6.6
Fuc	-	-	Trace	3.2	4.6	-
Xyl	13.9	30.1	45.8	25.1	23.9	29.2
GlcA	-	-	-	11.3	16.9	-
GalA	29.4	13.3	13.4	13.7	10.7	-
Man	1.2	6.3	0.9	0.3	-	-
Gal	15.4	10.4	8.6	13.4	15.4	23.1
Glc	9.7	15.7	8.1	4.6	6.1	14.6

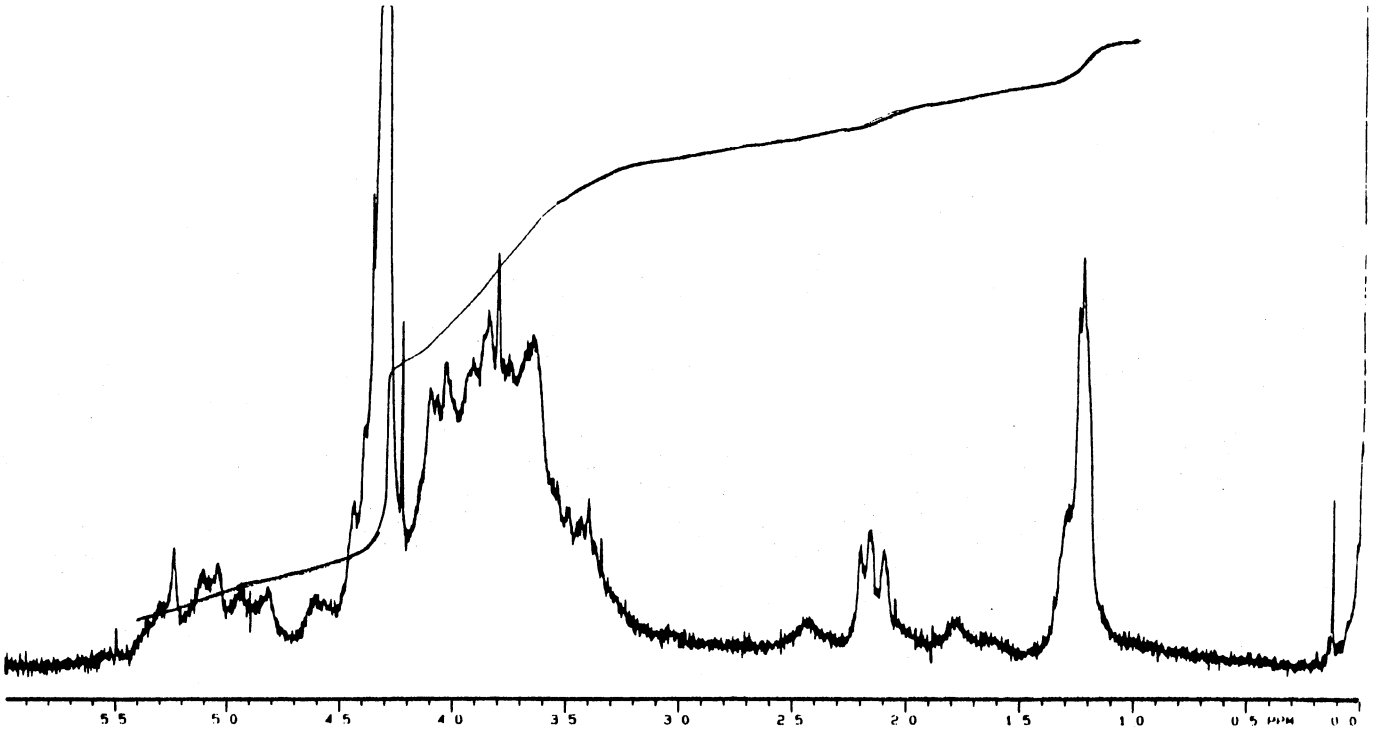

Figure 83. The ^{1}H n.m.r. Spectrum of the Bio gel P-6 Fraction 1 of the DEAE Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at -73°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard. Tentative assignments are 2,18 p.p.m., acetate ester, 1.3 p.p.m., CH_3 of rhamnose, 5.28 p.p.m. H-1 α rhamnose; 5.1 p.p.m. H-1 α galacturonic acid.

TABLE XX

MOLE PERCENT OF SUGARS IN THE DIFFERENT FRACTIONS OF ACALA 44 COTTON SUSPENSION WALLS AFTER HF SOLVOLYSIS AT 0°C

Materials	Percentage of starting weight recovered	Ara	Rha	Fuc	Xyl	GlcA	4 (o) Me GlcA	GalA	Man	Gal	Glc
Ether/HF soluble sugar	34	17.1	7.3	1.7	12.7	Present	Present	9.8	0.6	7.1	43.7
Water extract	30	8.3	3.3	Trace	6.1	-	-	52.5	0.9	7.1	21.8
Residue after water extn	5	4.1	1.5	-	4.1	-	-	81.7	-	-	8.6

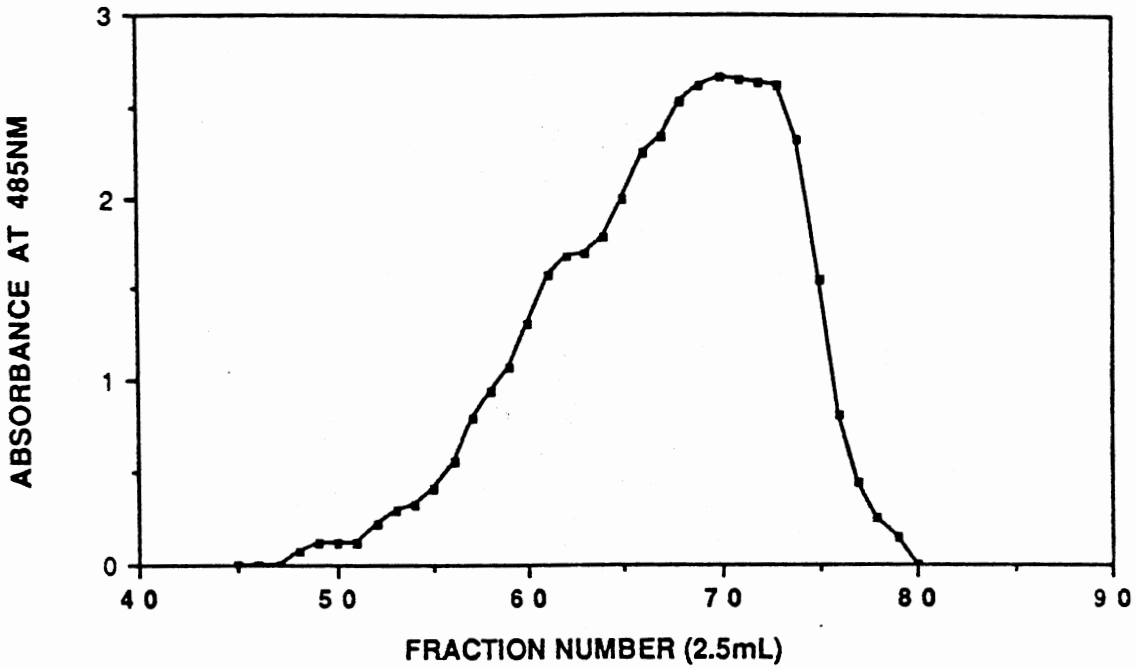

Figure 84. Chromatography of a Bio-gel P-2 Column (62 x 2.5 cm) of the HF/ether Soluble Fraction of Acala 44 Cotton Suspension Walls After HF Solvolysis at 0°C in 0.05 M Sodium Acetate Buffer pH 5.2. Fractions 45-65 were pooled into fraction 1 and tubes 66-80 were pooled into fraction 2.

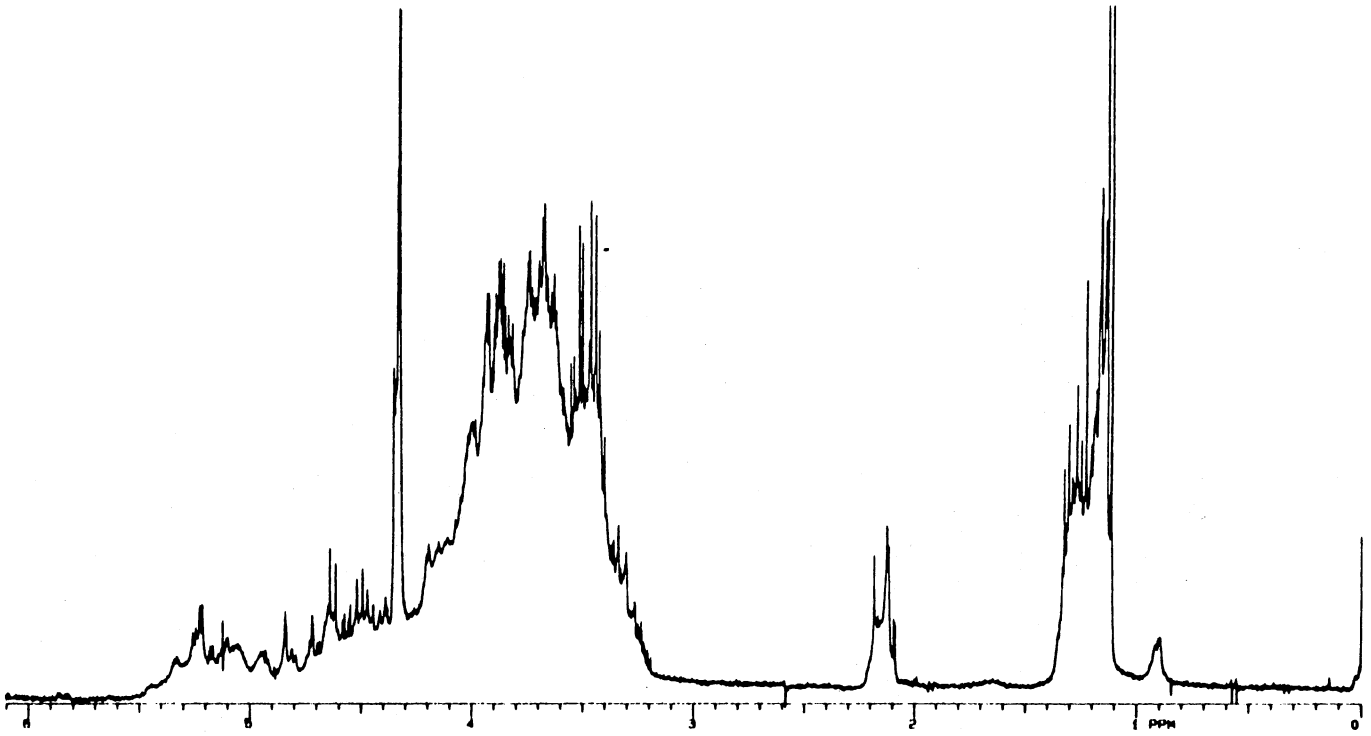

Figure 85. The ^{1}H n.m.r. Spectrum of the Bio gel P-2 Fraction 1 of the HF/ether Soluble Fraction from Acala 44 Cotton Suspension Walls After HF Solvolysis at 0°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard.

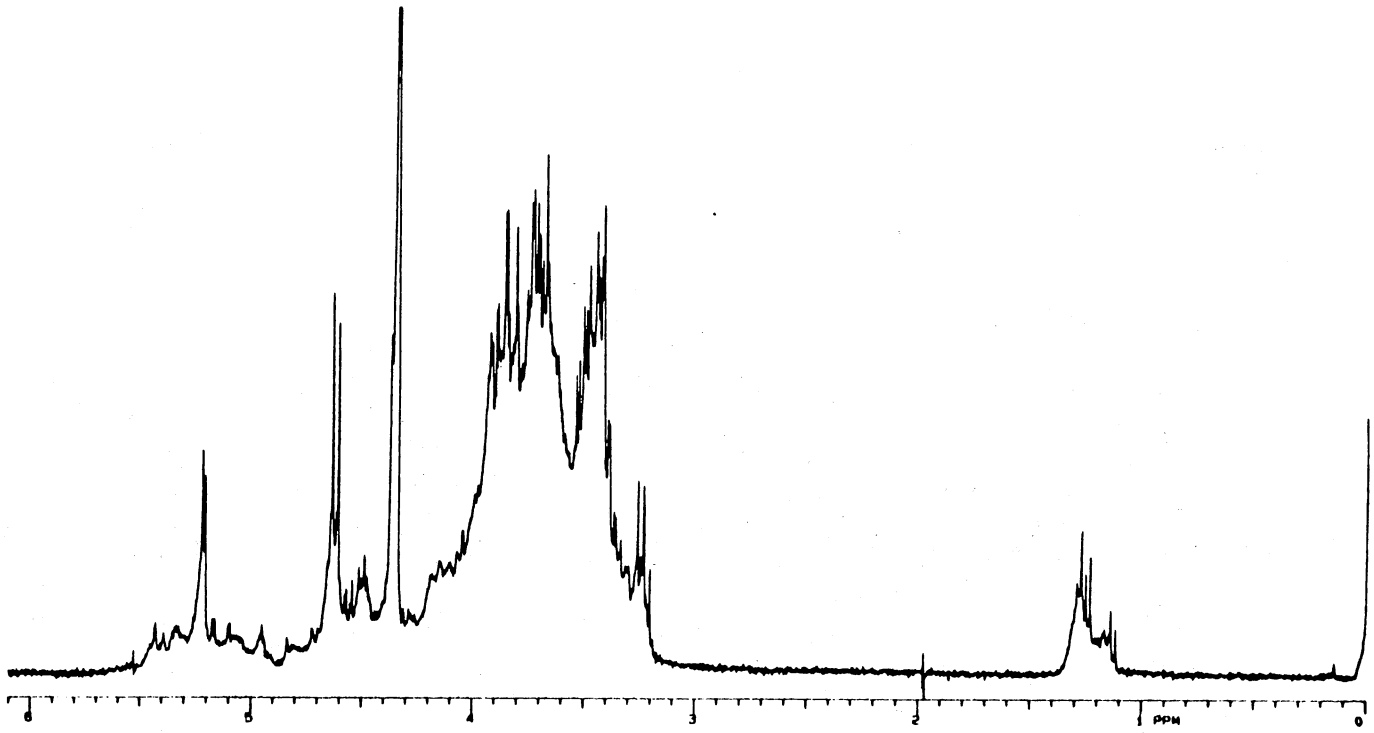

Figure 86. The ^{1}H n.m.r. Spectrum of the Bio gel P-2 Fraction 2 of the HF/ether Soluble Fraction from Acala 44 Cotton Suspension Walls After HF Solvolysis at 0°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard. H-1 of α glc at 5.24 p.p.m.; H-1 of β glc at 4.62 p.p.m.

(Figure 87) and the sugar composition of the different fractions is shown in Table XXI. The fraction 1 which is the void volume of the column was about 90% galacturonic acid. The proton n.m.r. spectrum of this fraction showed it to be a polymer of galacturonic acid with α 1→4 linkages (Figure 88). This fraction did not have any acetate esters and had only small amounts of methyl esters. It represents the homogalacturonan region of pectin. Fraction 2 had acetate esters (Figure 89) while fraction 3 did not have any acetate esters (Figure 90). In this reaction the useful fractions helpful in structure determination are the P-2 void volume of the water extract (homogalacturonan region of pectin) and the water insoluble extract which is rich in protein.

TABLE XXI

MOLE PERCENT OF SUGARS IN THE DIFFERENT P-2 FRACTIONS OF COTTON WALLS AFTER HF SOLVOLYSIS AT 0°C

Sugar	Ether fraction		Water extract			
	Fr-1	Fr-2	Fr-1	Fr-2	Fr-3	Fr-4
Ara	22.1	15.6	-	15.4	15.1	12.9
Rha	11.1	5.5	-	5.9	7.9	2.5
Fuc	2.8	Trace	-	-	1.5	-
Xyl	14.2	13.7	3.8	10.1	11.4	9.9
GlcA	Trace	Trace	-	-	-	-
4(o) methyl GlcA	-	Trace	-	-	-	-
GalA	11.4	9.1	89.4	26.2	22.8	18.1
Man	0.4	0.6	-	0.9	0.7	1.1
Gal	8.4	6.4	-	7.4	8.1	7.8
Glc	29.6	49.1	6.8	34.1	32.5	47.9

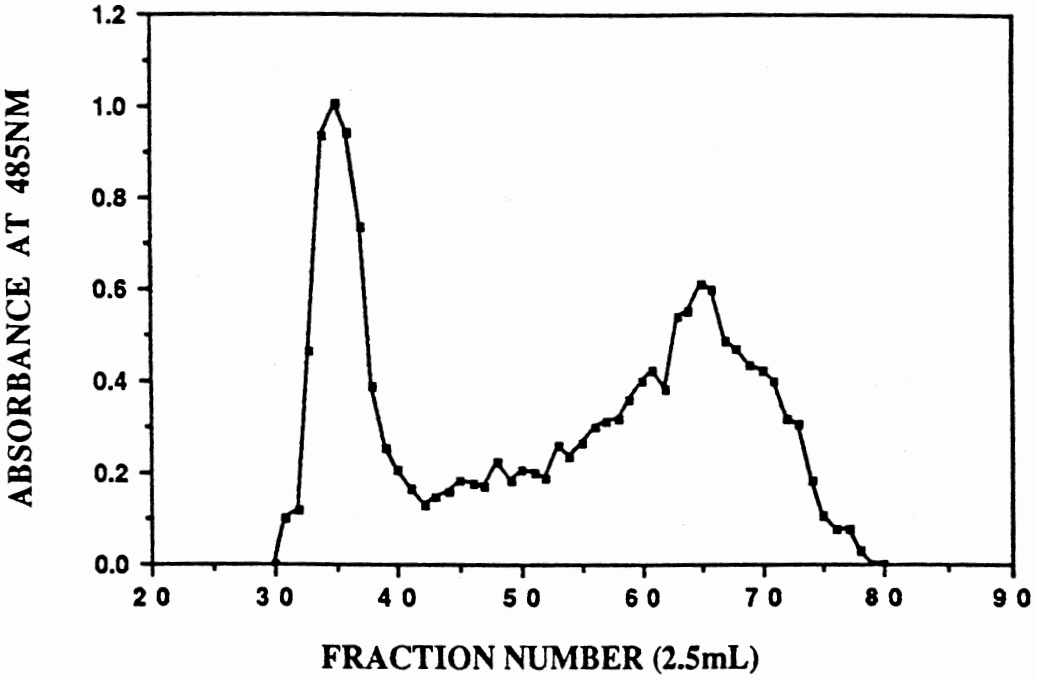

Figure 87. Chromatography of a Bio-gel P-2 Column (62 x 2.5 cm) of the Water Extract of Acala 44 Cotton Suspension Walls After HF Solvolysis at 0°C in 0.05 M Sodium Acetate Buffer pH 5.2. 2.5 ml fractions were collected. Tubes 30-40 were pooled into fraction 1, tubes 41-55 into fraction 2, 56-64 into fraction 3 and 65-80 into fraction 4.

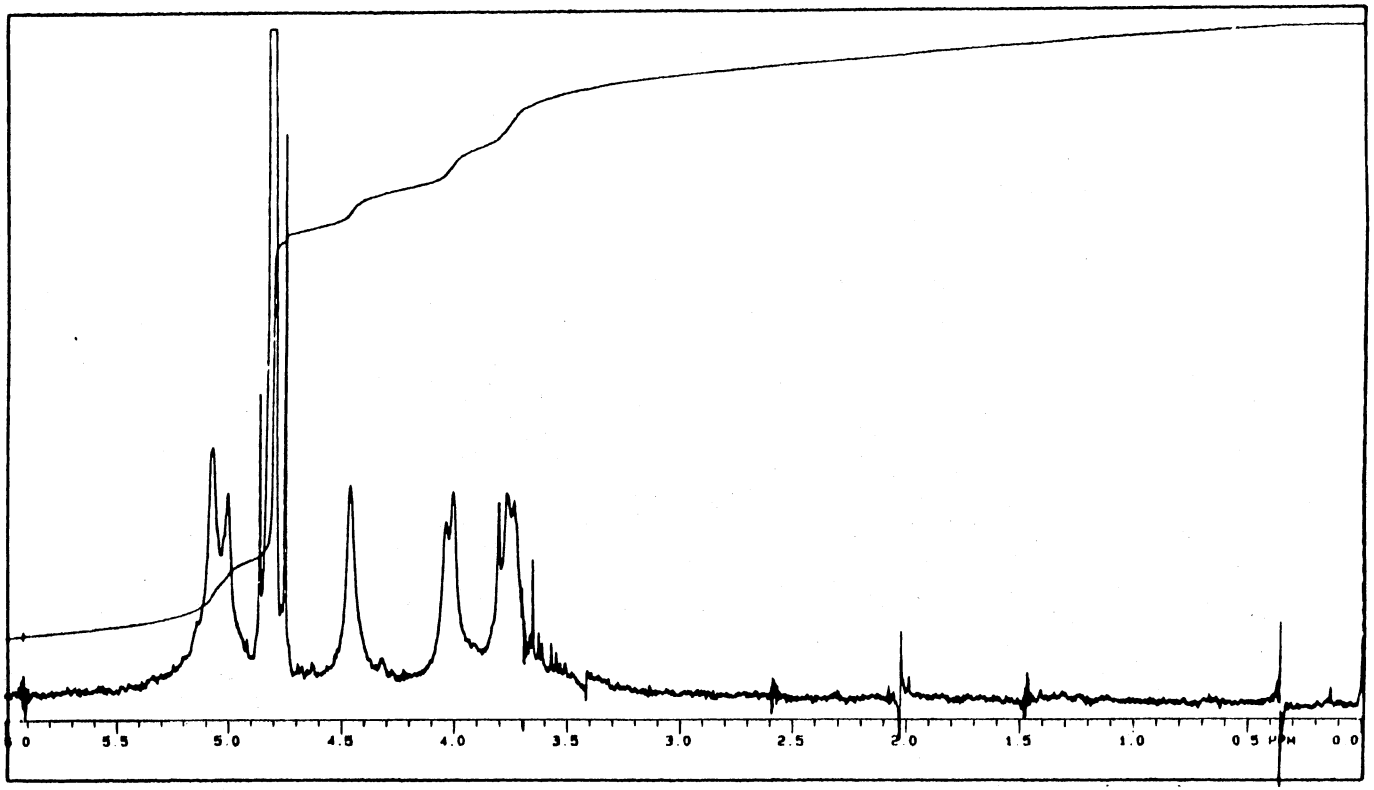

Figure 88. The ^{1}H n.m.r. Spectrum of the Bio gel P-2 Void Volume of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at 0°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 22°C in D_2O, using TSP as internal standard. 1) H-1 α galA; 2) H-5 galA; 3) H-4 galA; 4) H-3 galA; 5) H-2 galA.

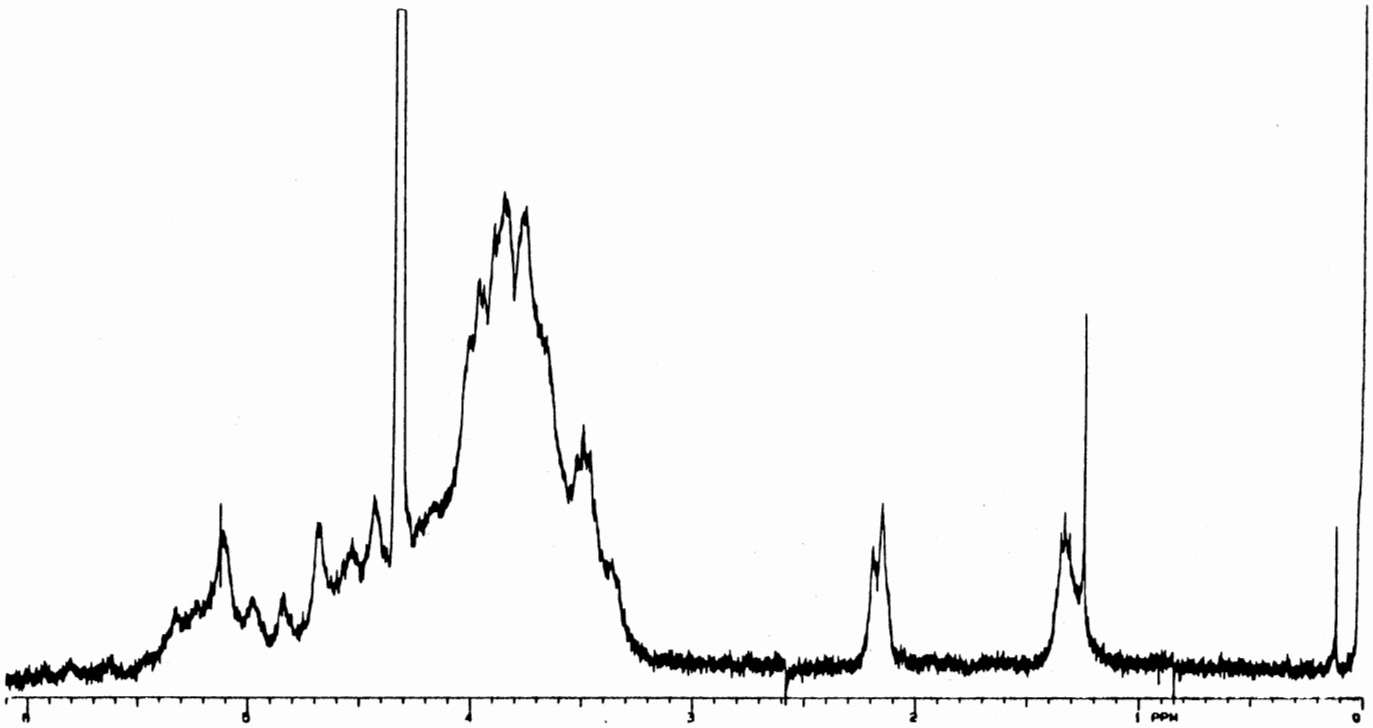

Figure 89. The ^{1}H n.m.r. Spectrum of the Bio gel P-2 Fraction 2 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at 0°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard. The signal at 2.18 p.p.m. is acetate ester.

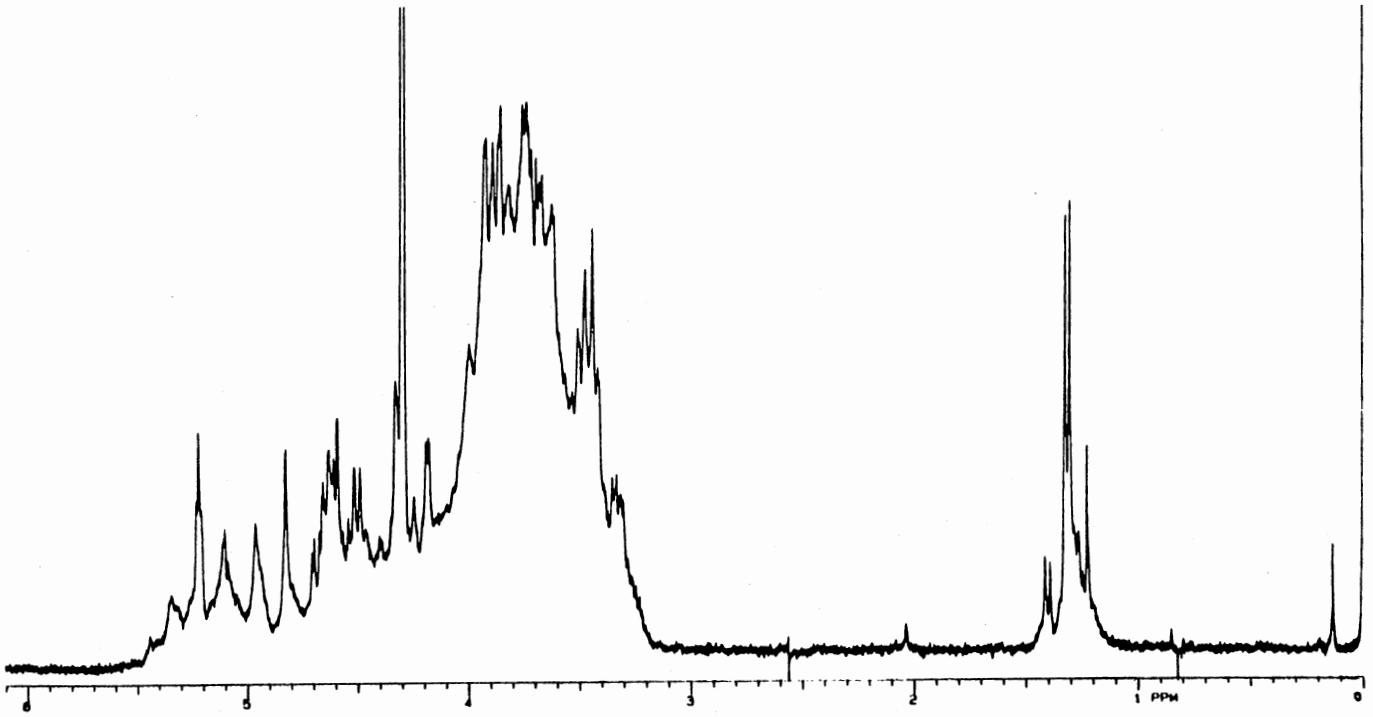

Figure 90. The ^{1}H n.m.r. Spectrum of the Bio gel P-2 Fraction 3 of the Water Extract from Acala 44 Cotton Suspension Walls After HF Solvolysis at 0°C. The spectrum was recorded using a 300 MHz n.m.r. spectrometer, at 70°C in D_2O, using TSP as internal standard.

CHAPTER V

SUMMARY

Cell walls are no longer considered to be just inactive outer envelopes that enclose cells. These are now recognized to have very specific functions essential to the continuation of the plant itself. Pectins are a major component of all dicot primary cell walls, but their complete structure is still unknown. It is necessary to know the complete structure of pectins to fully explain their functions. This project was undertaken to isolate and characterize pectins from suspension cultured cells of cotton.

Cell walls were prepared from two varieties of cotton suspension cells Acala 44 and Im 216, which are susceptible and resistant to cotton blight, and also Acala 44 leaves, tomato suspension cells, tobacco leaves and carrot root cells. Mole percents of galacturonic acid were 38% in Acala 44 suspension cell walls, 29% in Im 216 suspension cell walls, 36% in Acala 44 cotton leaf walls, 40% in tomato suspension walls, 42% in tobacco leaf walls and 45% in carrot root walls. Mole percents of rhamnose were 11% in Acala 44 suspension walls, 6% in Im 216 walls, 9% in Acala 44 leaf walls and tomato suspension walls, 8% in tobacco leaf walls and carrot root walls. Compared with Acala 44 suspension walls, Im 216 walls contained less rhamnose rich pectin and significantly more noncellulosic glucose. The composition of cotton cell walls was very different from that of sycamore suspension cell walls. Cotton cell walls were richer in galacturonic acid and rhamnose, poorer in arabinose and galactose than sycamore walls (Talmadge *et al.*, 1973), indicating that cotton contains more pectin than sycamore walls, and that since cotton cell walls had less arabinose and galactose, pectin side chains might also be shorter. The percent dry weight accounted for in Acala 44 cotton suspension walls was 74%, in

Im 216 walls it was 79% and in Acala 44 leaf walls it was 57%. The cellulosic glucose was found to be about 20 to 25% of cotton walls. The protein content of Acala 44 and Im 216 cotton suspension walls was 6-8% and Acala 44 leaf walls was 8-10%. Hydroxy proline was 0.5 to 0.6% of Acala 44 and Im 216 suspension walls and 0.1% of Acala 44 leaf walls. Cotton cell wall protein was rich in val, leu, ser and gly.

Sequential treatment of cotton cell walls with CDTA and sodium carbonate extracted pectin with about 60% galacturonic acid that accounted for about 40-50% of the dry weight. Extraction of the depectinated residue with 1 M and 4 M KOH and sodium borohydride extracted polymers rich in xylose and glucose that accounted for about 15-20% of the walls. The final residue consisted mainly of α cellulose that accounted for about 15-20% of the dry weight . However sugar analysis of the final residue showed that it still had both pectic and hemicellulosic polymers attached to the cellulose. One of the problems with this extraction procedure was that almost all sugars were present in each of the different extracts making them not suitable for characterization of individual polymers. Moreover use of harsh chemicals for extraction changes the polymer properties. In most cases it was very difficult to remove the reagents associated with the extracts and this interfered with further characterization of the polymers.

Extracellular polysaccharides (EPS) were isolated from the suspension culture mediums of both Acala 44 and Im 216 cotton cells. Sugar analysis of the EPS showed it to be rich in xylose, galactose and glucose. Small amounts of arabinose, rhamnose, fucose, mannose and galacturonic acid were also present, showing that EPS consisted mainly of hemicellulose and not pectin.

HF solvolysis of cell walls at -23°C extracts all of the sugars from the wall except those in cellulose and the homogalacturonan region of pectin. Since, at this temperature, HF cleaves rhamnosyl linkages and not galacturonosyl linkages; it was possible to extract pectins from the wall by solvolysis at -23°C. Specificity of cleavage of bonds during solvolysis depends on the temperature of the reaction making it possible to extract

oligomers of different size and type by varying the reaction temperature. HF retains acyl substituents on the sugars which helped to identify these substituents in pectin. Thus HF solvolysis at -23°C is a very good reagent for extraction of pectins from cell walls.

Cell walls isolated from cotton, tomato, tobacco and carrot were separated into 3 fractions, a HF/ether soluble fraction, a water extract and a water insoluble residue after HF solvolysis at -23°C. HF/ether soluble sugars when fractionated on a P-2 gel filtration column followed by ion exchange column, yielded an acidic disaccharide fraction (Scheme 1, Figure 5). This acidic disaccharide fraction contained an equimolar ratio of rhamnose and galacturonic acid. The structure of the disaccharide was determined by 1D, ^{1}H and ^{13}C n.m.r, 2D, ^{1}H-homonuclear correlation n.m.r, 2D, ^{1}H-^{13}C heteronuclear correlation n.m.r spectroscopy and mass spectroscopy. The 1D proton n.m.r spectrum showed this disaccharide fraction to be a mixture of acetylated and non-acetylated disaccharide. From the integration of the n.m.r spectrum, about 50% of the disaccharides were acetylated. The signal for the acetate group was identified at 2.18 p.p.m. in the proton spectrum. The signal at 5.83 p.p.m. was identified as the position of acetylation which was the O -3 of galacturonic acid. Presence of an acetate ester shifts the chemical shift of the ring hydrogen about 1 p.p.m. downfield with respect to the non-acetylated sugar and makes it easier to identify thier location. The position of acetylation on galacturonic acid (O-3) was confirmed by 2D homo and heteronuclear correlation spectroscopy.

When compared with free galacturonic acid most of the chemical shifts of the disaccharide were quite close to those expected, except that C-2 of galacturonic acid in the disacharide was shifted downfield in the ^{13}C spectrum and H-3 of galacturonic acid was shifted downfield in the proton spectrum. This suggested that C-2 of galacturonic acid was glycosylated and H-3 of galacturonic acid was in a different electronic environment. Further peculiarities were the occurrence of rhamnose in only the β anomeric form and the fact that the disaccharide did not have a reducing end. These observations suggested that the rhamnose 1 position was linked. Combining all the information, it was concluded that

the disaccharide was cyclized during the isolation, with the rhamnose 1 position linked β-1→2 back to the galacturonic acid. This explains the anomolous chemical shift of C-2 of galacturonic acid and the occurrence of rhamnose in only one anomeric form. Cyclization of the disaccharide was confirmed from the mass spectrum that showed the molecular weight of both acetylated and non-acetylated disaccharide to be 18 mass units less than the expected value. Cyclization was also confirmed from the methylation analysis results where 1,2,5,6 tetra-acetyl, 3,4 dimethyl galactitol was obtained rather than the 1,5,6 tri-acetyl 2,3,4 trimethyl galactitol, the expected derivative from the non cyclized disaccharide. Using space filling models it was possible to build a cyclic disaccharide in which galacturonic acid was linked α-(1→2) to rhamnose, with rhamnose linked β-(1→2) back to galacturonic acid. Cyclization seems to be an artifact of the HF reaction, occurring due to the high reactivity of the rhamnosyl fluorides formed initially. The structure of the isolated disaccharide was found to be

$$\alpha\text{-D-Gal}_p\text{A}\ \underset{3\ \ 2}{\text{-(1}\rightarrow\text{2)-}}\beta\text{-L-Rha}_p\ \ \underset{1}{}$$
$$\underset{(\text{OAc})}{}$$
$$\underset{0.3}{}$$

The disaccharide galacturonosyl rhamnose was also isolated from the HF/ether soluble sugars extracted from Im 216 cotton suspension cell walls, Acala 44 leaf cell walls, tomato suspension cell walls, tobacco leaf cell walls and carrot root walls. In all these cases the disaccharide was acetylated at the O-3 of galacturonic acid. This showed that all the plant species studied contained RG I like polymer with a fairly strict repeating disaccharide backbone that was acetylated at O-3 of the galacturonic acid residue. Acetylation of RG I has not been reported before, perhaps because most of the conventional methods used for isolation of pectin remove acetate esters. Apart from being able to produce characterizable oligomers from cell wall polymers, HF solvolysis retains the acyl substituents on the sugars making it possible to identify their location. Acetylation

of pectin is an important phenomenon. The physiological function of the acetylation of RG I remains to be determined. It may be involved in controlling the shape of the RG I backbone and thus the orientation of the side chains that protrude from it. It may well be involved in directing placement of sidechains during the biosynthesis of the region.

The disaccharide fraction with equimolar amounts of rhamnose and galacturonic acid was also purified from the water extract of the cell walls after treatment with HF at -23°C (Scheme 1). N.m.r. analysis showed very little acetylation on this disaccharide but it was cyclized to the structure below.

$$\underset{2}{\alpha\text{-D-Gal}_p\text{A}}\text{-}(1\rightarrow 2)\text{-}\underset{1}{\beta\text{-L-Rha}_p}$$

This disaccharide was also generated from the RG I backbone.

Another pectic fraction with about 60% galacturonic acid was purified from the water extract of cotton walls after HF treatment at -23°C. This fraction had neutral sugars like rha, gal, ara, xyl and glc and was highly methyl esterified. Fractionation on an ion exchange column produced oligomers containing rha and galA in the ratio of 1:10 , 1:20, and 1:32. The oligomers with a 1:10 ratio of rha and galA were highly methyl esterified, oligomers with a 1:20 ratio showed lesser methyl esterification and the oligomers with a 1:32 ratio showed even less methyl esterification. The last two sets of oligomers did not have any acetate esters while the first set of oligomers had traces of acetylation. These oligomers originated from the homogalacturonan region of pectin. By HPLC gel filtration analysis of elution volume, these oligomers had an average degree of polymerization greater than 20 when compared to the elution volume of a purified 20-residue long oligogalacturonide. Further analysis of this fraction will reveal the distribution of methyl esters and the exact length of these oligomers.

Homopolymeric galA was extracted from the water insoluble residue remaining after HF solvolylsis of cell walls (Scheme 2) with 0.5 M imidazole buffer followed by 0.1 M

EDTA. These extracts were rich (90%) in galA. The relative size of these oligomers was found to be larger than the polygalacturonic acid oligomers extracted with water from the HF treated walls. However there was some overlap. N.m.r. analysis showed very little methyl esterification in the imidazole and EDTA extracted homogalacturonan fragments and no acetylation was observed. Imidazole extracts more than 75% of the galacturonic acid left behind in the HF treated residue of walls after water extraction. The homogalacturonan region accounted for ~18% of the Acala 44 cotton suspension walls; about 15-20% of the galacturonic acid being methyl esterified.

The final residue left behind after the series of extractions described above contained about 5% of the original galA, cellulose and cell wall protein. Treatment of this residue with HF at 0°C removed cellulose as glucose leaving behind the cell wall protein with some water insoluble materials (unknown) along with some galA.

Treatment of Acala 44 suspension cell walls with HF at -40°C allowed extraction of RG I as larger oligomers. This helped in quantitation of acetylation and also confirmed the position of acetylation in the original polymer. 90 mg of water extract were obtained from 500 mg of walls. From the sugar composition (assuming that all of the rhamnose present was in the disaccharide repeat unit) it was calculated that the disaccharide repeat represents ~6% of the original cell walls. Integration of the n.m.r. spectrum of the purified oligomers showed 50% acetylation.The acetate group was found exclusively at O-3 of galacturonic acid. The oligomers were at least 3 to 4 repeating disaccharide units. Some of the rhamnose residues had side chains or remnants of side chains attached to the 4 position. Side chains were made up of gal, xyl, ara and glc. The backbone was made up of repeating disaccharide unit in which galA is linked
α-(1→2) to L-rha, and the rha is linked α-(1→4) to the next galA.

HF solvolysis of Acala 44 cell walls at -73°C also extracted some of RG I from the walls. Arabinose was the main sugar linkage broken during this reaction. These oligomers were larger than the oligomers isolated by HF -40°°C reaction, had more side

chains attached to rhamnose, and were about 50% acetylated with the acetate at O-3 of galacturonic acid. These RG I oligomers were associated with large amounts of xylose and glucose, which could be part of xyloglucan or xylan.

Future Considerations

The next step in this project is to characterize the RG I oligomers extracted from walls by HF solvolysis at -40°C and -73°C. The specificity of cleavage of linkages by HF at -40°C and -73°C has to be determined. By varying the reaction time it may be possible to get more uniform oligomers. Analysis of the more uniform oligomers will be less complicated. This may also provide oligomers with more side chain attached to the backbone. Structural analysis of these oligomers might explain the distribution of acetate esters in the RG I backbone. Experiments can also be directed towards isolating the side chain fragments from the oligomers isolated after HF -73°C reaction. The exact length of the side chain cannot be determined this way as some of the arabinose residues will be lost, however, it will help to identify if any acyl substituents are located on the side chains.

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APPENDIX A

HF SOLVOLYSIS OF CELL WALLS–APPARATUS AND PROCEDURE

Apparatus Necessary

There is no commercially available apparatus for performing the HF reactions. Most of the components, however, are commercially available. For careful control of reaction temperatures and time, it is necessary to be able to start the reaction with reagents pre-equilibrated to the desired temperature and to stop the reaction rapidly after the desired reaction time. To accomplish this, one must be able to transfer HF as a liquid from an equilibration vessel to the reaction vessel. A suitable apparatus was described recently (Mort, 1983). In this apparatus the HF is transferred from vessel to vessel by means of 3-mm (o.d.) Teflon tubes reaching to the bottom of the vessel, using slight N_2 pressure and vacuum as the driving force.

The apparatus is a modified version (refer to Figure 2) of the HF Reaction Apparatus Type IB obtained through Peninsula Laboratories, Inc., San Carlos, California. It contains six Teflon or Kel-F vessels and Teflon or Kel-F tubing which connects these vessels. The two fittings into which the vessels (12, 13) of the commercial unit attach are replaced by ones machined from 4 cm Teflon rod. These differ from the original in that the stem is made 2.5-cm long rather than 1.25 cm to allow for insertion of, in one case one and in the other two, lengths of 3-mm Teflon tubing through tightly fitting holes drilled through the fittings. The small stopcocks, and the fluid-flow needle valve for making connections between the vessels are obtained from Berghof/America Inc., Raymond, New Hampshire.

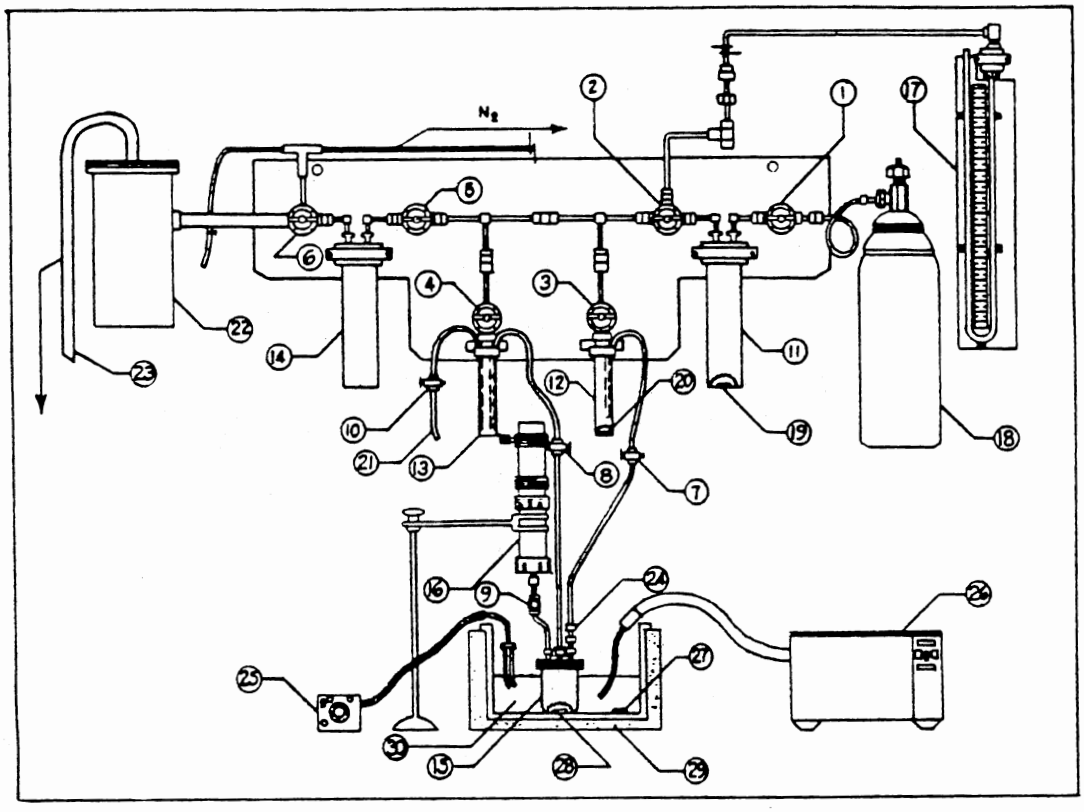


Figure 2. Schematic Representation of the Hydrogen Fluoride Solvolysis Apparatus. 1-8, 10, stopcocks; 9, teflon needle valve; 11-16, teflon and Kel-F vessels; 17, manometer; 18, hydrogen fluoride tank; 19, 20, stirrer bars; 21, exit to the sink for pressure release, if necessary; 22, calcium oxide trap; 23, connection to the vacuum pump; 24, 3 mm to 6 mm adaptor; 25, heater/regulator; 26, immersion cooler; 27, 28, stirrer bars; 29, insulated container; 30, 95% ethanol.

Vessel 15, used as a reaction vessel is a Teflon container with 500-ml capacity from Savillex Corporation, Minnetonka, Minnesota. It has a screw cap with three inlets. Through the first 6-mm inlet 3-mm Teflon tubing from vessel 12 is connected to vessel 15 using a 3-mm-to-6-mm adaptor (24). To the other 6-mm inlet is connected to vessel 16 (ether reservoir composed of two Teflon column segments (Savillex Corporation) joined together) by 6-mm Teflon tubing with needle valve 9. Vessel 13 is connected to the reaction vessel 15 via the 3-mm hole through 3-mm Teflon tubing.

Procedure

1. TRANSFER OF HF FROM HF TANK TO THE HF RESERVOIR

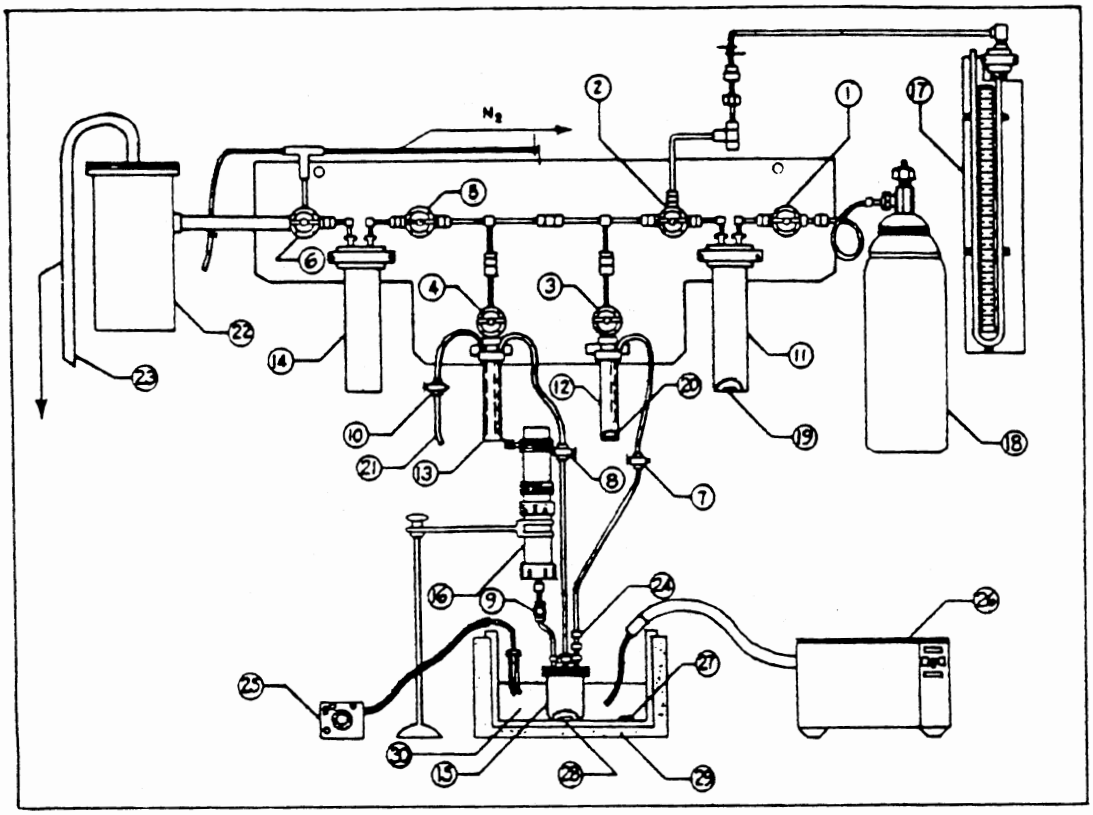
Hydrogen fluoride needed for 2 or 3 experiments was transferred from an HF tank to the HF reservoir 11 for ready use by the following procedure:

1) Stopcocks 3 and 4 were closed.
2) Stopcocks 2, 5, and 6 were opened and the vacuum pump was turned on.
3) Reservoir 11 was evacuated and any leaks in the system were detected by closing stopcock 5 and observing the manometer. There were no leaks if the manometer reading remained constant.
4) Slowly, stopcock 1 was opened and the line to the HF tank was also evacuated for five minutes. The presence of leaks in the line was checked as before.
5) Reservoir 11 was cooled by immersing it in liquid nitrogen.
6) After 20-30 minutes stopcock 2 was closed and the valve on the HF tank was opened slowly. The manometer reading did drop at this point.
7) The amount of HF transferred into reservoir 11 was checked occasionally. When enough HF was collected in reservoir 11 the HF tank was closed as was stopcock 1.
8) The HF in reservoir 11 was allowed to come to room temperature before use.

2. HF SOLVOLYSIS OF CELL WALLS

The apparatus for solvolysis of cell walls was as shown in Figure 2.

1) At least one hour before the reaction, the cooling system, a liquid immersion cooler (FTS Systems, Inc., Stone Ridge, NY) was turned on. The cooling liquid used was 95% ethanol.
2) Before attaching the different parts together, all vessels were checked to see whether they were clean and completely dry.
3) Approximately 500 mg of dry cell walls from Acala 44 cotton suspension cells were placed in reaction vessel 15 along with a 2.5-cm long stirr bar.
4) Stopcocks 1, 2, 9 and 10 were closed.
5) Stopcock 6 was opened to the vacuum pump.
6) Stopcocks 3, 4, 5, 7 and 8 were opened.
7) The whole apparatus was evacuated for 15 minutes.
8) The system was checked for leaks by closing stopcock 5 and observing the reading on the manometer. When a leak was detected its source was found by closing stopcocks to particular vessels. For example, if stopcocks 4, 7 and 8 were closed and the mercury still dropped in the manometer then the leak was in vessel 12.
9) Once the system was leak proof the next step was to transfer HF from reservoir 11 to vessel 12. For 500 mg cell walls 20 ml HF was required.
10) Stopcocks 1, 2, 4, 7, 8, 9, and 10 were closed.
11) Stopcocks 3, 5 and 6 were opened.
12) Vessels 11 and 12 were stirred using magnetic stirrers.
13) Vessel 12 was cooled by immersing it in a cooling bath prepared by adding dry ice to acetone.



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14) After about 20-30 minutes when vessel 12 was well cooled stopcock 5 was closed and stopcock 2 was opened slowly. HF was then distilled into vessel 12 from reservoir 11. The manometer reading dropped when stopcock 2 was opened. It took about 15 to 20 minutes to transfer 20 ml of HF into vessel 12.

15) When 20 ml of HF had transferred, stopcocks 2 and 3 were closed.

16) The acetone cooling bath was removed.

17) Both vessel 12 and reaction vessel 15 were immersed in the cooling bath of ethanol, the temperature of which was maintained at -23°C using the immersion cooler offset by a regulated immersion heater (FTS Systems, Inc., Stone Ridge, NY). The cooling bath was stirred to ensure uniform temperature throughout. Stirring was continued for 30 minutes to obtain temperature equilibrium.

18) During the time that temperature equilibrium was being reached, stopcock 5 was opened partially very slowly to remove HF in the line. Stopcock 3 was closed during this procedure or else HF from vessel 12 would be sucked into the calcium oxide trap (22). The bubbles which formed in the manometer, which escape from the fluorocarbon oil trapped on the sides of the manometer, were released by tapping the manometer gently so as to prevent excessive bursts of gas which would propel the mercury out of the manometer. Once all the bubbles were gone, the vacuum was released and vacuum pump was turned off.

19) Stopcock 6 was turned to the nitrogen line. The next step was to transfer HF from vessel 12 to the reaction vessel 15. This was achieved using slight nitrogen pressure.

20) The stopcocks 4, 8, 9 and 10 were closed.

21) Stopcock 7 was opened slowly and then stopcock 3 was opened.

22) Stopcock 5 was opened to let nitrogen blow HF into the reaction vessel. It took only a few seconds for the transfer.

23) Once all the HF was transferred into the reaction vessel stopcock 7 was closed.

24) The nitrogen was turned off and the reaction was timed.

25) The reaction vessel was stirred and the reaction was allowed to continue for 30 minutes.

26) Ten minutes before the end of the reaction, about 300 ml of anhydrous diethyl ether was added to vessel 16. Small pieces of dry ice were added to the vessel to cool the ether. Dry ice was added until the vessel was frosty.

27) The HF reaction was stopped by opening stopcock 9 and allowing cold ether to enter vessel 15.

28) Slight vacuum was used to enhance transfer of ether by opening stopcocks 7, 3, and 5 with the stopcock 6 opened to vacuum.

29) The cooling bath was removed. The reaction mixture was allowed to stir for 30 minutes and warm to room temperature.

30) After 30 minutes the reaction mixture was filtered using a Teflon filter (50 mm diameter, fine grade, Savillex Corporation)

3. FILTRATION OF THE REACTION MIXTURE

1) To filter the reaction mixture vessel 15 was removed and an inline Teflon filter unit (Savillex Corporation) was attached to it.

2) Nitrogen was used to push the liquid through the filter.

3) The insoluble material was rinsed with ether.

4) The HF/ether/sugar mixture (filtrate) was collected in a Teflon container.

5) The filter unit was sealed using a sealing ferrule at the outlet and the whole apparatus was evacuated for 30 to 45 minutes to dry the insoluble material and to remove the ether left behind in the whole HF line.

6) The vacuum was released and the residue on the filter unit was extracted with water three times.

7) The water extract was freeze dried to obtain the water-soluble oligomers.

8) The water-insoluble residue was also freeze dried.

4. RECOVERY OF SUGARS FROM THE HF/ETHER SOLUBLE FRACTION

The sugars, mainly monosaccharides and some disaccharides, present in the HF/ether-soluble fraction (filtrate) were recovered by evaporating the mixture.

1) The filtrate was put in the reaction vessel 15, never to more than half of its capacity.
2) A stirrer bar was placed in the vessel.
3) One 6-mm inlet and the 3-mm inlet were sealed using sealing ferrules.
4) Vessel 15 was attached, through the other 6-mm inlet directly to the main line of the HF apparatus where vessel 13 is normally attached, using an 8-mm to 6-mm adaptor.
5) Stopcocks 2 and 3 were closed, and 4 and 5 were opened.
6) Stopcock 6 was opened to the vacuum.
7) The vessel 14 was immersed in liquid nitrogen. When it was well cooled (about 15 minutes), the vacuum pump was turned on.
8) Vessel 15 was immersed in a warm water bath and stirred on the magnetic stir/heater plate. The HF/ether complex was evaporated from vessel 15 and condensed in vessel 14. Sugars were retained in vessel 15.
9) When vessel 15 was dry, the vacuum was released, and the sugars were dissolved in water and freeze dried.
10) The HF/ether complex in vessel 14 was allowed to come to room temperature and then neutralized by pouring it into excess (~ 60 g) of solid calcium carbonate.

    At this point the starting material had separated into three parts (refer to Scheme 1): the HF/ether-soluble fraction (filtrate) containing mainly monosaccharides and some disaccharides, the water extract containing mono-, di-, tri-, and

oligosaccharides, and the water-insoluble residue containing polysaccharides and other water-insoluble materials.

VITA

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Candidate for the Degree of

Doctor of Philosophy

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