STRUCTURAL STUDIES OF A FUCOGALACTOXYLOGLUCAN FROM PINUS RADIATA PRIMARY CELL WALLS

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ABSTRACT

1. The changes in carbohydrate composition of elongating Pinus radiata primary cell walls were investigated. In the hemicellulose B extracts, a large increase in the percentage of non-starch, non-cellulosic, glucose was found to occur on cessation of cell-wall elongation.

2. By fractionation of the hemicellulose B extracts, with a variety of methods involving precipitation from an aqueous solution, a xyloglucan was purified. This xyloglucan was the major hemicellulose of the Pinus radiata hypocotyl cell wall.

3. Characterisation studies on the xyloglucan involved: quantitative analysis of the monosaccharides derived by nitric acid/urea hydrolysis; identification of the partial hydrolysis products derived by trifluoroacetic acid hydrolysis; quantitation of the sugar linkages using methylation by the Hakomori method; and analysis of the anomeric configuration of component sugars using chromium trioxide oxidation.

4. From the results a tentative structure has been suggested for the xyloglucan, consisting of a backbone of β-D-glucopyranose residues linked together by 1-4 glycosidic bonds, and with sidechains of single xylose residues linked through C-6 of the glucose units. Galacto and fuco-1,2-galacto sidechains are attached to some of the xylose residues, probably through the C-2 of the xylose.

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CHAPTER 1

INTRODUCTION

The cell wall of plant cells is an intriguing biochemical system that continues to defy definitive characterization. Peter Albersheim and his co-workers have recently completed the most comprehensive characterization of the composition of the wall of tissue cultured cells (Talmadge et al., 1973; Bauer et al., 1973; Keegstra et al., 1973). Nevertheless, the model for the structural arrangements of these polymers in the wall is still tentative and the exact roles of the various fractions not well understood (Albersheim, 1976). Moreover, information on the site and pathways of biosynthesis of wall polymers is very incomplete (Christpeels, 1976).

1.1 Ontogeny Of The Cell Wall

The origin of the cell wall can be traced to the appearance of the cell plate. This arises through fusion of vesicles (derived from dictyosomes and containing polysaccharides) deposited in the equatorial plane of the phragmoplast (Newcomb, 1969). These polysaccharides, shown to be rich in pectic substances (Northcote and Pickett-Heaps, 1966), become the building materials of the cell plate, which will contribute significantly to the middle lamella of the wall deposited by each of the daughter cells. Deposition of additional wall material follows on either side of the original plate as well as over the old mother cell wall.

The wall present during elongation growth is defined as the primary cell wall. The primary wall polymers will ultimately provide resistance in the horizontal direction to turgor pressure (the in vivo driving force for cell expansion) and thus contribute to the vertical elongation of the stem. Secondary wall polymers are deposited onto the inner primary wall. Rigidity is conferred upon the wall by the deposition of partially crystalline cellulose microfibrils at all stages of their development. The

fibrils are embedded in matrix materials imparting cohesion, strength and rigidity to an otherwise potentially fluid structure.

When plant cells grow in volume they are extraordinarily dynamic, and their wall components cannot be regarded as inert or subject only to passive re-orientation under pressure. Plant-cell expansion growth is irreversible and involves a net deposition of most wall materials, including cellulose, during growth. It is evident that both biosynthesis and turnover of wall components are crucial for shaping plant development.

To begin to understand cell wall metabolism during elongation, the structure of the wall itself must be known.

1.2 Cell Wall Constituents

The basic constituents of the cell walls of dicotyledons, monocotyledons and gymnosperms studied to date show remarkable conservation within groups and considerable overlap between groups (Burke et al., 1974). Figure 1 shows some of the more common sugars which occur as constituents of the polysaccharides. Major cell wall fractions have been described (Preston, 1974) and are listed below:

(1) Pectic substances (extracted by boiling in water for 12 hours or by hot ammonium oxalate)
(2) Hemicellulose (extracted usually by 4N KOH at room temperature)
(3) Cellulose (the residue of the above and often extracted with 72% sulphuric acid)
(4) Protein
(5) Lignin

Pectic substances, hemicelluloses and protein make up the major structural components of the primary cell wall matrix. Secondary walls have a greatly increased percentage of cellulose together with hemicellulose and lignin. Lignin is a characteristic component of secondarily thickened walls

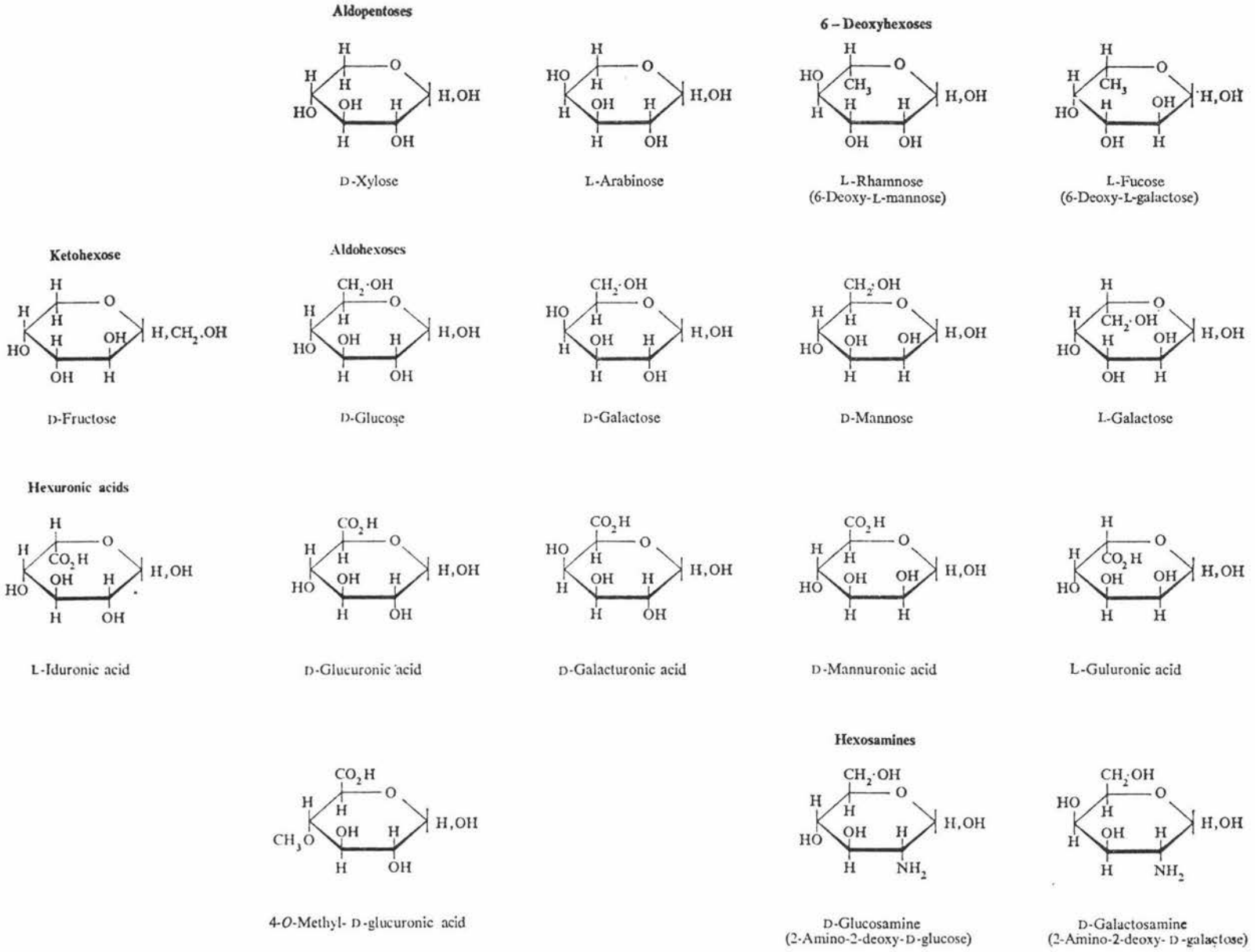


FIG. 1. Common sugar constituents of polysaccharides.

(Albersheim, 1965) and will only be described briefly.

Wilkie (1979) comments that the division implied by the variously defined terms hemicellulose, pectic substances, pentosans, linear and branched xylans, and others have limited scientific justification. They are useful laboratory terms insofar as they indicate starting-materials, procedures, preconceptions, and objectives in relation to studies of plantstuffs and their polysaccharides. Wilkie considers polysaccharides are best described by using chemical terms relating to their main structural features. However, much information is conveyed by the use of such terms as 'hemicellulose' to describe isolated fractions as it enables comparisons to be made between polysaccharides from various sources. These terms will be used in this work.

1.2.1 Cellulose

This is the major component of cell walls. Cellulose usually increases from about 20% of primary cell wall to 40% in mature walls. It is a simple linear polymer of β-1,4-linked glucopyranose residues. The glucose residues are present in the C1-chair conformation with the hydroxyl and hydroxymethyl groups all being in the more stable equatorial position. The degree of polymerisation of secondary-wall cellulose always remains constant and monodisperse in the cell walls of higher plants with a degree of polymerisation in wood between 13,000 - 16,000. The cellulose from primary cell walls however is of a much lower degree of polymerisation (2,000 - 6,000) and is more heterogeneous (Côté, 1977).

1.2.2 Lignin

Lignin is a three-dimensional polymer containing phenylpropane units linked together by C-O-C and C-C linkages. In softwoods, most phenylpropane units have one methoxyl group plus a phenolic oxygen. Lignin, a component

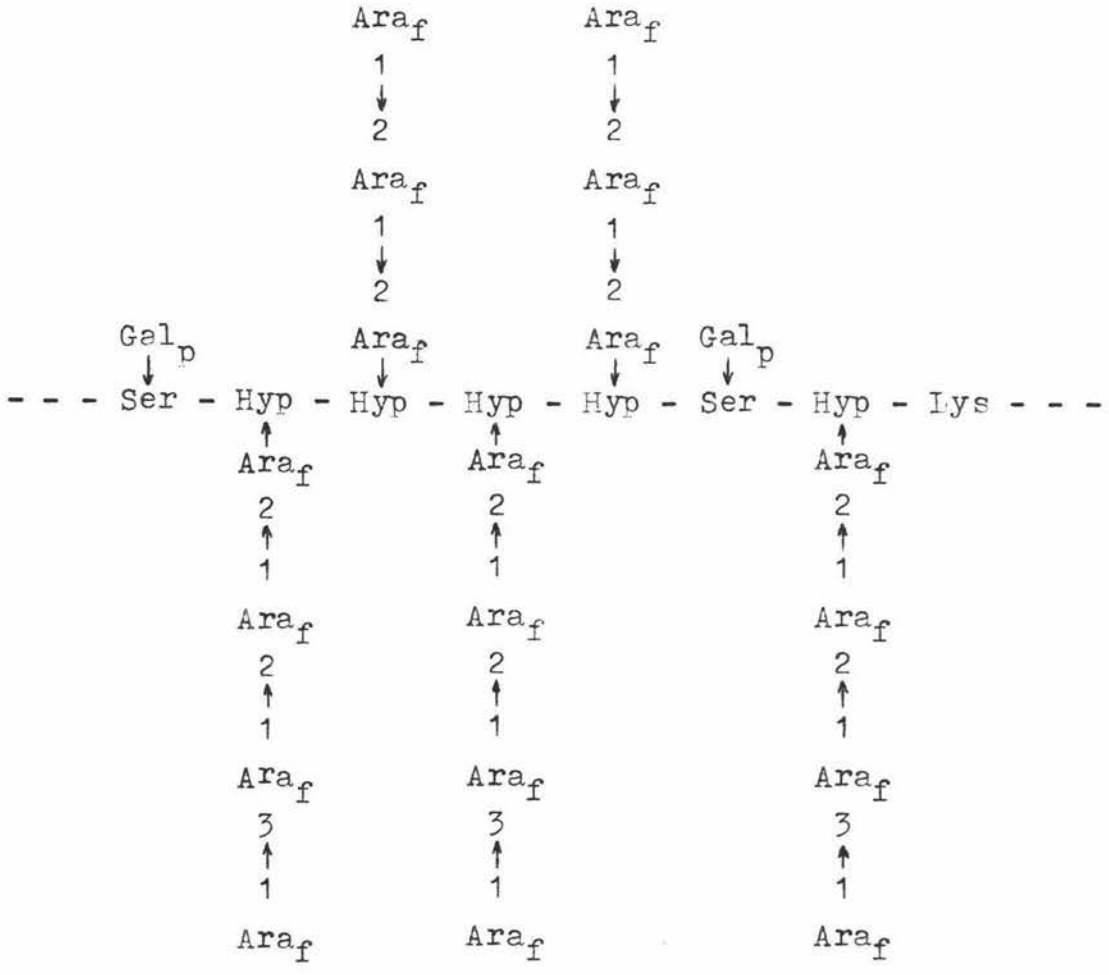
deposited with secondary wall thickening, increased from zero content in cambium cell walls (primary) of all plants, to about the same content as cellulose in mature softwood cell walls while in hardwood it reaches only half this amount (Côté, 1977). Lau et al. (1977) found lignin formation commences in developing Pinus elliotii hypocotyls only after cessation of cell wall elongation as would be expected. Evidence from several sources, such as the extraction of lignin-carbohydrate complexes, suggests that lignin is associated with or is combined with the matrix substances but not with the framework substance (Côté, 1977).

1.2.3 Protein

Protein is a quantitatively important component of the cell wall matrix (Lamport, 1965) although its role in cell wall growth is not firmly established. The cell wall protein is unusual in that about 30% of its residues are hydroxyproline (Lamport, 1970), an amino acid confined almost entirely to the cell wall in plants. Hydroxyproline is also a major constituent of animal connective tissue proteins, but is not glycosylated in these situations. In plant cell walls, L-arabinose oligosaccharides are attached O-glycosidically to most of the hydroxyproline (Lamport, 1967) and galactose by the same type of linkage to much of the serine (Lamport et al., 1973; Keegstra et al., 1973). A possible structure is given in Fig. 2.

The available evidence is in favour of the cell wall polysaccharides being connected to the hydroxyproline-containing protein through the serine residues of this protein. Due to the bonding of carbohydrate to fragments of the protein Lamport (1965) has gone as far as naming it extensin. This postulate, for control of cell wall extensibility, is supported indirectly by the finding that a decrease in growth rate is often associated with an increase in the level of cell wall hydroxyproline (Cleland and Karlsnes, 1967; Ridge and Osborne, 1970; Sadava et al., 1973).

Figure 2: Possible Structure Of Cell Wall Glycoprotein Segment (From Clarke et al., 1979).



1.2.4 Pectic Substances

The pectic polysaccharides of higher plants are a distinctive component of primary, but not secondary, wall material and are present also in the cell plate and middle lamella. Since cell-wall extension is restricted to primary growth, early theories on its mechanism laid special emphasis on a possible interrelation of wall plasticity and pectin metabolism (Stoddart and Northcote, 1967). However, cell-wall plasticity cannot be explained solely in terms of pectin metabolism, which has a specific role in new wall development.

Pectic substances are dissolved from the cell wall by aqueous solvents containing calcium chelating agents such as ethylenediaminetetraacetic acid (EDTA) or ammonium oxalate. They have been divided up by Aspinall (1973) into three polymer types.

(1) Neutral Arabinans

These are basically highly branched polysaccharides with predominantly α-L-arabinofuranose residues present. They contain α(1-5) and α(1-3) linkages (Fig. 3). They are usually in small amounts in most higher plants and their role in seeds and plant cell walls is not known. The arabinans have been isolated with arabinogalactan from lemon peel showing that the highly branched arabinans must be distinct species which cannot arise from partial degradation of the more complex neutral polysaccharide (Aspinall, 1973).

(2) Neutral Galactans and/or Arabinogalactans I (or II)

The galactans or arabinogalactans which are found in association with pectins are most commonly those based on linear chains of (1-4)-linked β-D-galactopyranose residues (Fig. 4). It is now apparent, however, that composition alone does not define structure since arabinogalactans of type II, similar in composition but of quite different structure to those of type I, have been found in association with pectins. Arabinogalactans of type II are most commonly found in coniferous woods and are particularly abundant in

Figure 3: General Structure Of An Arabinan.

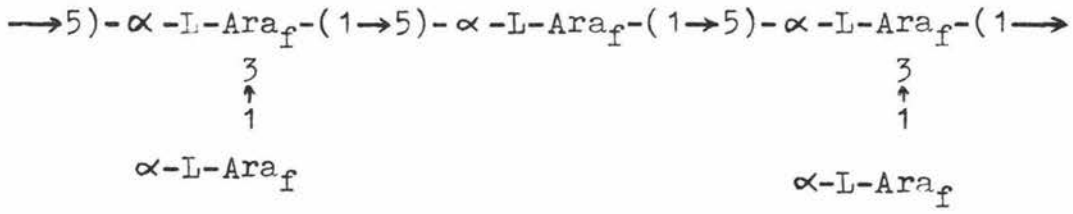
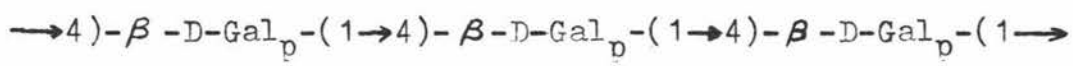


Figure 4: Structure Of Galactan.

Arabinogalactans of type I are also characterized by essentially linear chains of (1—4) linked chains of β-D-galactopyranose residues.



larches where the polysaccharide is present in the lumen of tracheids and ray cells rather than as a wall component. Arabinogalactans of this type contain highly branched structures in which β-D-galactopyranose residues are mutually joined by (1-3) and (1-6) linkages, the former predominantly in interior and the latter mainly in exterior chains. L-arabinofuranose, and to a smaller extent L-arabinopyranosyl residues, terminate some of the outer chains. The distribution of (1-6) linkages is largely as indicated in Fig. 5 but not necessarily in a completely non-ramified comb-like structure. Some arabinogalactans of this type also contain terminal units of D-glucuronic acid (or its 4-methyl ether).

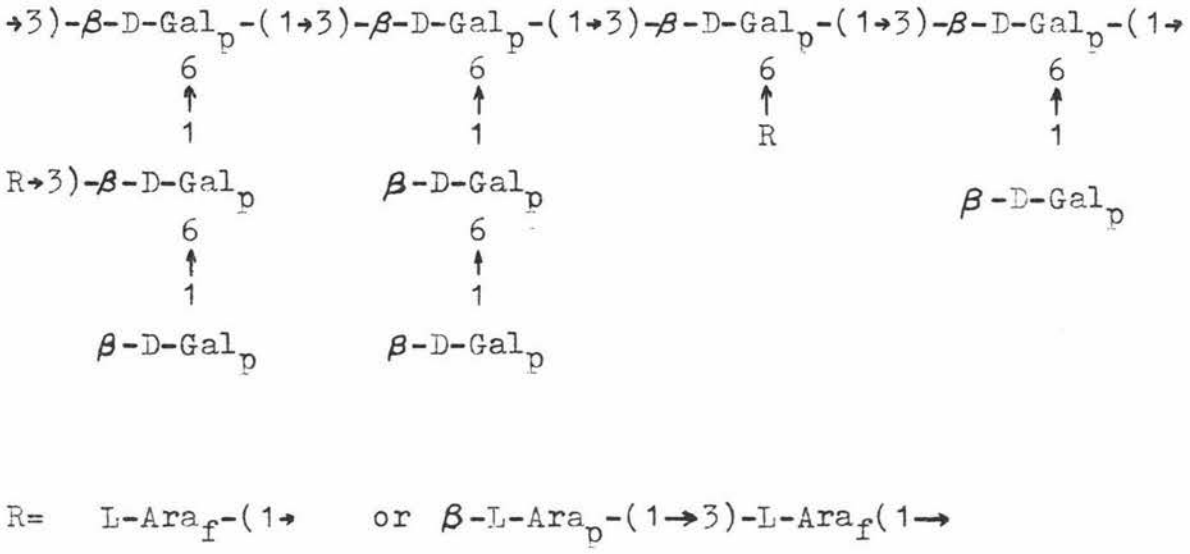
(3) Acidic Galacturonans or Galacturonorhamnans

Galacturonans are predominantly composed of linear chains of 4-linked α-D-galacturonic acid in the pyranose form (Fig. 6). In nature a large proportion of the galacturonic acid residues are methyl esterified. It is now apparent that pure galacturonans are of infrequent occurrence and that the majority of polysaccharides rich in galacturonic acid contain significant amounts of neutral sugar substituents. The 2-linked L-rhamnose occurs only in the interior chains, whereas residues of all other neutral sugars are encountered in the exterior chains only. Sidechains containing β-D-galactopyranose and L-arabinofuranose residues are the most characteristic (Fig. 6). Barrett and Northcote (1965) have shown that these sidechains in apple galacturonorhamnan are distributed irregularly in widely spaced blocks along the macromolecular chain.

Stoddart and Northcote (1967) have demonstrated a temporal linkage between the various pectic fractions of sycamore suspension-culture cell walls. Knee (1978) has produced evidence for a spacial arrangement in apple fruit cortical tissue, with a simple polymethylgalacturonate localised in the middle lamella, while a branched polymethylgalacturonate, with sidechains of arabinose and galactose residues, was concentrated in the primary wall.

Sidechains in acidic galacturonorhamnans of

Figure 5: Representative Structure For Arabinogalactans Of Type II.



R= L-Ara$_f$-(1→ or β-L-Ara$_p$-(1→3)-L-Ara$_f$(1→

Figure 6: Structure Of Galacturonan.

$\rightarrow$4)-α-D-Gal$_p$A-(1$\rightarrow$4)-α-D-Gal$_p$A-(1$\rightarrow$4)-α-D-Gal$_p$A-(1$\rightarrow$

General Structure Of A Galacturonorhamnan.

$\rightarrow$4)-α-D-Gal$_p$A-(1$\rightarrow$4)-α-D-Gal$_p$A-(1$\rightarrow$2)-L-Rha$_p$-(1$\rightarrow$

Ara$_f$, Gal$_p$, Xyl$_p$, Fuc$_p$.

D-xylopyranose residues alone or with appended D-galactopyranose or L-fucopyranose units have been found as important structural units in pectins from tissues with potential for rapid enlargement and/or rapid differentiation, such as pollen (Bouveng, 1965), cotyledons from soybeans (Aspinall et al., 1967) and white mustard seeds (Rees and Wight, 1969). In elongating cell walls, where a fluid matrix is required, it seems that pectic polysaccharides are high in rhamnose and side-chains and that these decrease in the pectic substances as the cell ages (Rees and Wight, 1969). Model building computations for galacturonans indicate that the insertion of the 2-linked L-rhamnose units interrupts the tendency to form ordered chain conformations (Rees and Wight, 1971). The rhamnose causes 'kinking' of otherwise regular chains forming a zig-zagged structure. This along with side-chains and de-esterification would prevent the pectic substances forming gels. This gelling ability depends on the formation of junction zones (Rees, 1969) where the polyuronide chains become aligned and form tightly ordered microcrystallites. It is highly possible that these physical attributes of galacturonorhamnans arising from its structure, play some role in the requirement for a fluid matrix in cell wall elongation.

1.2.5 Hemicelluloses

The term hemicellulose describes a rather indefinite group that has been variously defined. It usually designates polysaccharides of low molecular weight which normally occur in plant tissues together with cellulose, and which can be isolated from the original or delignified material by extraction with aqueous alkali. Most of the land plant hemicelluloses are heteroglycans which have a linear main backbone chain to which are attached short appendages of different types of sugars.

Most hemicelluloses have been isolated from mature cell walls. We can divide these hemicelluloses into two main types (Timell, 1964 and 1965):

(i) arabino-(4-O-methylglucurono) xylans(Fig. 7)
(ii) gluco- and galactoglucomannans(Fig. 8)

(i) The xylans make up 10 - 15% of mature softwood and 15 - 20% of hardwood cell-wall polymers. They are generally quite large molecules in gymnosperm with a degree of polymerisation of 100 - 120. 4-linked β-D-xylopyranose residues form the xylan backbone. Some of the β-D-xylose residues are substituted in the C-2 and/or C-3 positions by single unit side chains of 4-O-methyl-α-D-glucuronic acid and/or α-L-arabinofuranose residues respectively. The distribution of the acid side chains on the xylose residues is random (Rosell and Svensson, 1975). In gymnosperms the distribution of the α-L-arabinose residues along the xylan backbone is probably also random. Xylans in hardwoods generally lack arabinose, but, instead commonly bear O-acetyl groups at C-3 of the xylose residues (Timell, 1964).

(ii) Gluco- and galactoglucomannans constitute the bulk of the gymnosperm wood hemicellulose but are a minor constituent in angiosperms.

The water soluble galactoglucomannans are isolated in fairly low yields from softwoods. They are a homogeneous polymer with ratios of galactose : glucose : mannose of 1 : 1 : 3. β-D-glucose and β-D-mannose are linked (1-4) to form the backbone, while α-D-galactopyranose is linked to the C-6 position of both mannose and glucose residues. The structure is the same as the alkali-soluble glucomannans, the main difference being the increase in terminal side chain galactose residues, which may account for the water-soluble properties of the polymer.

The alkali-soluble glucomannans are the main polysaccharides present in the cell wall of mature softwoods, accounting for approximately half the hemicellulose fraction of coniferous woods. These polysaccharides are structurally similar to cellulose and seem to be closely associated with the cellulose molecules in the cell wall. The mannose to

Figure 7: General Formula For Xylans.

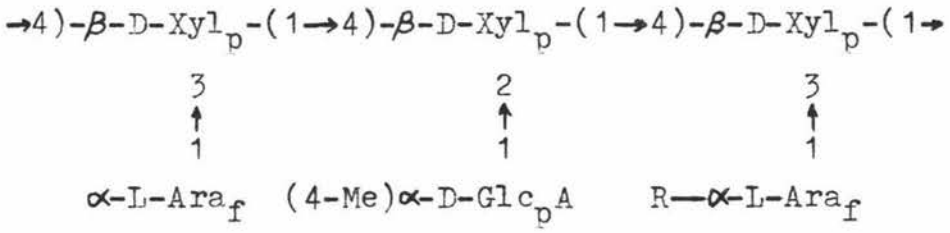
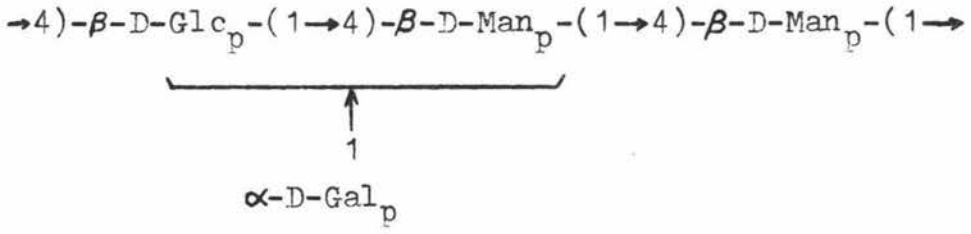


Figure 8: General Formula For Glucomannans.



glucose ratio does vary but in most softwood glucomannans it is about 3 : 1 compared with 2 : 1 in hardwoods. Also present is terminal D-galactose in quantities up to 5%. Softwood glucomannans are partially acetylated on either the C-2 or C-3 position of some of the mannose residues. These ester groups are lost under the alkaline conditions normally used for extraction of hemicelluloses.

Both the xylans and the glucomannans in mature cell walls are arranged in paracrystalline array between and in the same direction as the cellulose microfibrils and are strongly adsorbed (by hydrogen bonding) onto the microfibril surface. The linear structures of these polymers allow associations (as in the formation of microcrystallites) which have a uniting effect on wall polymers. They may therefore be important in interactions between the microfibrils and the cell wall matrix.

In some angiosperm seeds, the endosperm cell wall is greatly thickened by the deposition of galactomannans, glucomannans or mannans as a reserve polysaccharide. The galactomannans and mannans resemble the galactoglucomannans and glucomannans of wood, but lack the glucose residues.

1.2.6 Primary Cell Wall Hemicelluloses

Studies on primary cell walls have been hampered by having to deal with more than one type of primary cell wall and by the presence of secondary wall material. The use of young growing plants which contain predominantly a single cell type with little secondary thickening, together with the use of suspension-cultured cells, have however, enabled rapid progress in this area. Studies have shown the presence of a mixed-link β-1,3-β-1,4-glucan in monocotyledons and in at least one dicotyledon. Arabinoxylans have been demonstrated in monocotyledons with some results suggesting their presence also in dicotyledons. Xyloglucans have been found in dicotyledons.

Two of these polysaccharides, the β-glucan and the xyloglucan have been shown to turn over in elongating cell walls. This observation has led to their intensive investigation recently and is the primary reason for instigating this work.

1.2.6.1 Mixed-Link Glucan

The non-cellulosic mixed-linked β-D-glucan of monocots has been identified as a cell wall component of Zea, Hordeum, Sorghum, Triticum, Panicum, Arundinaria, Secale, Lolium and Avena (see Nevins et al. (1978) and refs therein cited). These polysaccharides are dissociated from the cell wall matrix of non-endospermic tissue by alkali. They have been characterised as linear homoglycans with both β-(1-3) and β-(1-4) glycosidic linkages. The mean ratio of (1-3) to (1-4) linkages was estimated at 3 : 7 (Stone, 1976). Nevins et al., (1978) using the very specific Bacillus subtilis and Rhizopus glucanases found that the cell wall β-D-glucans of five different grass species had 30.4 - 30.9% (1-3) β-D-glucosyl linkages in the molecules. These enzymes release both 3-O-β-cellobiosyl-D-glucose and 3-O-β-cellotriosyl-D-glucose with no significant disaccharides present, indicating that regions of repeating (1-3) glucosidic linkages represent at most only a small proportion of the total glucan complement.

There was at one time some argument as to whether the mixed-linked glucan was a cell wall constituent (Burke et al., 1974). Even though the glucan was found to be a component in vegetative tissues of various monocots (Buchala and Wilkie, 1973; Fraser and Wilkie, 1971) it has been postulated that this material is a storage product, not a structural wall component (Burke et al., 1974; Albersheim, 1976). In view of this criticism many workers have now given evidence for the β-D-glucan being a wall component and not a consequence of some molecular association induced during isolation (Nevins et al., 1977; Wada and Ray, 1978). It is possible that the analysis of six suspension-cultured monocots by Burke et al

(1974), which failed to reveal the presence of the glucan in all but one species, might be attributed to the use of a B. subtilis amylase preparation. This preparation is comtaminated by a β-D-glucanase capable of degrading only mixed-link glucans (Nevins et al., 1977). A similar mixed-link β-D-glucan has been reported from Phaseolus aureus - a dicot (see section 1.2.6.5).

1.2.6.2 Arabinoxylan

The other major hemicellulose present in monocot seedling primary cell walls is an acidic arabinoxylan (Darvill et al., 1978; Buchala, 1974; Wada and Ray, 1978). These arabinoxylans, like those of mature tissues already discussed, always contain a linear 1-4 linked xylan backbone. Frequent sidechains attached mainly at the 3-position (but also at the 2-position) comprise either: (a) single arabinofuranosyl residues; or (b) single 4-O-methyl glucuronosyl residues, (both (a) and (b) are common); or (c) arabinose, xylose and/or galactose oligosaccharides; or (d) glucuronic and galacturonic acid oligosaccharides. Similar arabinoxylans are found in several species of cultured monocot cells (Burke et al., 1974) and barley aleurone cells (McNeil et al., 1975). Some of these polysaccharides may also be acidic arabinoxylans although that from barley aleurone was reported to contain no uronosyl residues (McNeil et al., 1975).

The cereal arabinoxylans of both wheat endosperm (Mares and Stone, 1973) and barley aleurone cell wall (McNeil et al., 1975) can be separated into fractions with wide ranging arabinose to xylose ratios, indicating a highly irregular arrangement of the arabinosyl side groups. However, the Avena coleoptile glucuronoarabinoxylan yielded subfractions of similar arabinose : xylose ratio (Wada and Ray, 1978).

1.2.6.3 Xyloglucan

The major hemicellulose reported in dicotyledons is a xyloglucan. Kato and Matsuda (1976) have isolated xyloglucan from the hypocotyls of three leguminosae plants; Phaseolus aureus (mungbean), Glycine max and Vigna sesquipedalis. The P. aureus xyloglucan gave glucose, xylose, galactose and fucose in the approximate molar ratio of 10 : 7 : 2.5 : 1. A xyloglucan shown to turnover in Pisum sativum stem sections has been isolated and also found to have similar ratios.

The most thorough structural investigations have been done on the xyloglucans isolated from suspension-cultured cells. Suspension-cultured cells have been grown as an homogeneous tissue possessing primary, but no secondary, walls. These cells also secrete into their culture medium polysaccharides that appear to be structurally identical to the noncellulosic polysaccharides of the cell wall (Albersheim, 1976 and refs cited therein). The major isolated hemicellulose of Acer pseudoplatanus (Bauer et al., 1973), Phaseolus vulgaris (Wilder and Albersheim, 1973) and Rosa glauca (Barnoud et al., 1977) cell-suspension cultures is a fucogalactoxyloglucan of similar structure to that from Phaseolus aureus hypocotyl.

The constitutions of a wide range of dicotyledon cell walls have been examined by methylation analysis. Cell walls from tomato (Lycopersicon esculentum), soybean (Glycine max), Red Kidney bean (P. vulgaris) and sycamore cell-suspension cultures, as well as from 8-day-old Red Kidney bean hypocotyls, all gave gas chromatograms featuring as major peaks the components of a derivatised fucogalactoxyloglucan. Further, D. H. Northcote and coworkers have shown isolated sycamore cambial cell walls to have nearly identical composition to that of suspension-cultured cells and T. E. Timell and B. W. Simson found similar results for aspen cambial cell walls (quoted by Albersheim, 1976 p263). This information has convinced Albersheim and coworkers that such diverse dicot

plants as beans, tomatoes and sycamore trees, have architecturally very similar primary cell walls, containing a fucogalactoxyloglucan as a major hemicellulose.

Xyloglucan polymers have also been found in the seeds of many dicot plants. These have been designated 'amyloids' due to their blue staining property with iodine. Two subgroups are distinguished: (a) Fucogalactoxyloglucans (fucoamyloids) are found in mustard seed (Gould et al., 1971), rapeseed (Brassica) hull (Aspinall et al., 1977) and rapeseed meal (Siddiqui and Wood, 1977; Theander and Åman, 1978); (b) Galactoxyloglucans have been isolated from the seeds of Tamarindus indica (Kooiman, 1961; Srivastava and Singh, 1967), Tropaeolum majus (Hsu and Reeves, 1967; Aspinall et al., 1977), Annona muricata L. (Kooiman, 1967) and Sinapis alba (Gould et al., 1971).

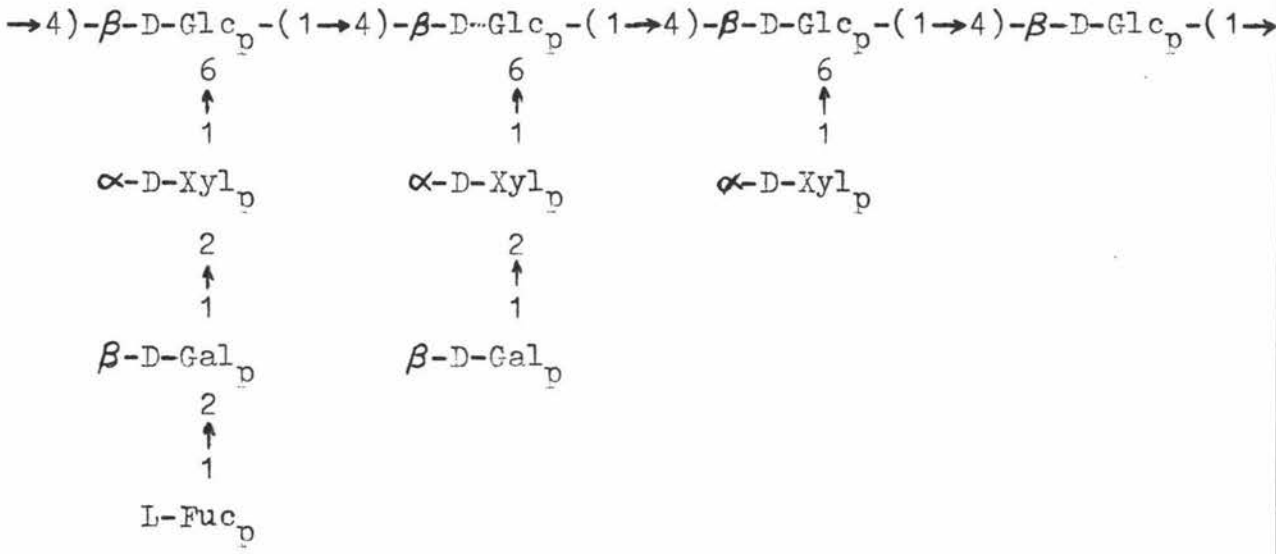
All the xyloglucan polymers isolated have a structure based on a repeating heptasaccharide unit which consists of four residues of β-(1-4)-linked glucose and three residues of terminal xylose linked to the 6-position of three of the glucosyl residues. Fucosyl-(1-2)-xylose and galactosyl-(1-2)-xylose side chains also are present in varying proportions in different species. A generalized structure for this polymer is shown in Fig. 9.

1.2.6.4 Callose

Another polysaccharide which seems to play a particularly dynamic role in cell development is callose, a linear β-1,3-glucan. Although not generally regarded as a hemicellulose, it is pertinent to consider it at this point. Callose is widely distributed, not only in the specialised plasmodesmata of sieve tube pores (Mc Nairn and Currier, 1968) but also in plasmodesmata generally. It has also been established as a polymer of the pollen cell wall eg. in Pinus mugo (Bouveng, 1963).

Most frequently, callose is classed as a wound-response material but experimental evidence also suggests it may play a role in regulating the intercellular movement of

Figure 9: General Structure Of A Fucogalactoxyloglucan.



substances in plant tissues. (McNairn and Currier, 1968). Its dynamism is documented by the cytological studies of Fulcher et al. (1975). Temporary depositions of this β-1,3-glucan are observed in cell plates of cells undergoing cytokinesis and in recently formed transverse cell walls of newly divided cells from coleoptile, primary leaf and emerging root tissues. There is little or no evidence of callose in ungerminated embryos. This suggests that a callose-like substance may be associated with cell wall formation and (or) expansion but the role and fate of the polysaccharide are unknown.

1.2.6.5 Angiosperm Primary Cell Wall

The overall impression emerging is that the matrix material of monocot and dicot primary walls is rather different. However, the generalisation that the co-occurrence of the mixed-link glucan and arabinoxylan is a distinctive characteristic of monocot primary cell walls while the xyloglucan is a component of dicot walls, must be qualified by certain facts and observations.

A water-soluble mixed-link β-D-glucan has been reported to occur transiently in the hypocotyls of Phaseolus aureus, a dicot (Buchala and Franz, 1974). This glucan has (1-3) and (1-4) linked D-glucopyranosyl residues in the molar ratio of 1.0 : 1.7.

An arabinoxyloglucan has been isolated from rice (Oryza sativa) endosperm cell walls (Shibuya and Misaki, 1978). This monocot tissue also contains the expected mixed-link β-glucan and arabinoxylan. Labavitch and Ray (1978) reported the fractionation of a xyloglucan from the coleoptile of Avena, a monocot. Enzymatic degradation gave a pentasaccharide and a trisaccharide that contain xylose and glucose in the linkages typical of xyloglucans.

Although no arabinoxylans have been isolated from dicot primary cell walls there are results suggesting a minor presence. An extracellular acidic 1,4-linked xylan was

isolated from sycamore cell-suspension culture (Keegstra et al., 1973) while fractions containing a high percentage of xylose were extracted from lupin hypocotyls (Monro et al., 1976).

It is possible that with detailed analysis of primary cell walls, the three hemicellulose types will be found present in a wide range of species but with a considerable variation in ratios between species. For example, the cell walls of the starchy endosperm of barley consist of an amorphous ground substance approximately 75% mixed-link β-glucan and 25% arabinoxylan (Fincher, 1975). In contrast, the principal classes of polymers present in the Avena coleoptile wall matrix, glucuronoarabinoxylan and mixed-link glucan, comprise approximately 55% and 30% of the hemicellulose fraction respectively, with xyloglucan a minor component (Labavitch and Ray, 1978).

1.3 Cell Wall Models

The nature of the bonds between the different polysaccharide fractions of cell walls is not clearly understood and the relationship between fractions is therefore not clear. Preston (1974) believes that the majority of bonds are of three types: (a) hydrogen bonds; (b) salt bridges; and (c) Van der Waals forces, with few if any covalent bonds, while Keegstra et al. (1973) postulated that covalent bonds were most common, and they put little emphasis on other linkages except for hydrogen bonding between xyloglucan and cellulose. From the foregoing it is obvious that the fine structure of the wall is controversial. A protein-glycan network, in general, is now supported by the studies of Albersheim and colleagues (Bauer et al., 1973; Keegstra et al., 1973; Talmadge et al., 1973). A potentially more specific dissection of the plant cell wall has been initiated by these workers, who have used enzymes in combination with chemical treatment to digest wall components and from this to deduce the nature of the interaction between the various subfractions.

In the emerging picture of the dicotyledonous cell wall, the cellulose microfibrils are coated with a single layer of xyloglucan by hydrogen bonding. The xyloglucan of one fibril may be linked to the xyloglucan of another fibril by covalent cross-linking through pectic polysaccharides Fig. 10. It is however doubtful that covalent linkages are the only linkage type between matrix polymers (Monro et al., 1976). A similar arrangement has been proposed for the walls of monocotyledonous plants, except that xyloglucan backbone is replaced by an arabinoxylan backbone (Burke et al., 1974). The picture for monocot structure is complicated by the presence of a mixed-linked β-D-glucan which undergoes rapid turnover during cell wall elongation (Huber and Nevins, 1979).

It should be emphasized that the above are simple models of the cell wall based upon analyses of very few cell types, and it seems likely that further analysis will complicate the picture.

1.4 Cell Wall Elongation

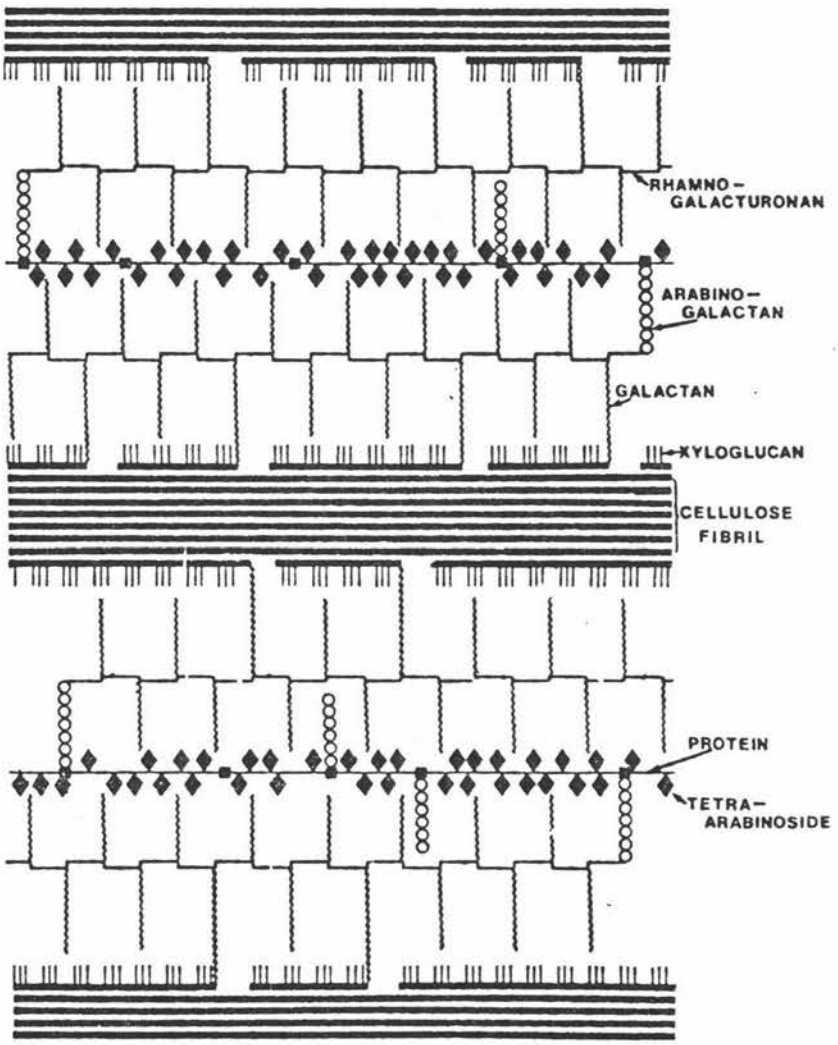
The biochemically dynamic nature of the primary cell wall has been recognised for some time (Lamport, 1970), both synthesis and breakdown of polysaccharides occurring together and leading to wall turnover. These processes are known to be strikingly increased by indole acetic acid (Lamport, 1970). In general terms, the mode of cell wall elongation in response to auxin has been studied from three major points of view: physical changes in the wall; enzymatic or non-enzymatic wall loosening; and turnover of wall components with synthesis of new wall material.

1.4.1 Physical Changes

Cleland (1971) has reviewed the considerable progress made in the study of cell wall physical changes where cell extensibility is divided into components such as plasticity, elasticity and 'creep'. He has pointed out the limitations of the physical measurements and the uncertainty which

FIGURE 10: Model of Sycamore Primary Cell Wall

(From Albersheim, 1976)



surrounds the nature of the properties being measured. Despite this, extension which is promoted by auxin and low pH can be correlated at least in part, with the measured changes in the physical characteristics of the wall.

1.4.2 Enzymatic Or Non-enzymatic Wall Loosening

A biphasic growth response has been demonstrated to occur following auxin application (Kohler, 1956) and this has since been confirmed in many tissues. The rapid effect of auxin on cell elongation is considered to be mediated by wall loosening, new wall synthesis and increased turgor pressure (Cleland, 1971). There is considerable evidence that hydrogen ions also catalyze the relaxation of the wall in a manner similar to that catalyzed by hormones (Adams et al., 1973; Evans, 1967; Rayle, 1973). There is also evidence that the hormones activate ion pumps within the cell membrane and that these ion pumps lower the pH of the wall (Cleland, 1971, 1973; Fisher and Albersheim, 1974). Auxin addition produces such a rapid response that de novo protein synthesis and de novo polysaccharide synthesis cannot participate in this initiation. An hypothesis based on these considerations would suggest that the direct action of the hormones is on the cell membrane, and that the reactions within the cell wall, which permit elongation growth, take place more efficiently at pH 5 than at pH 7. No bonds within the cell wall which would be non-enzymically degraded at pH 5 but which are stable at pH 7 have yet been found. However, Rayle and Cleland (1972) have shown that the cell walls of freeze-thawed coleoptile sections are weaker when buffered at pH 5 than at pH 7 only if the coleoptile sections have not been subjected to treatments that would denature enzymes. This supports the idea that the wall-loosening process is mediated by enzymes that remain active after freezing. A critical catalytic function for wall enzymes in plants is supported by evidence that wall enzymes play such a role in growth of bacterial cells (Fiedler and Glaser, 1973).

Serious limitations have, however, been found in the interpretations of most studies on enzyme involvement in wall extension. For example, exogenous application of enzyme mixtures containing β-1,3-glucanase and cellulase which was shown to rapidly stimulate elongation of segments of Avena coleoptile (Masuda and Wada, 1967) or Pisum epicotyl (Wada et al., 1968) could not be repeated by other workers (Cleland, 1968; Ruesink, 1969; Nevins, 1970). Also, although Datko and Maclachlan (1968) correlated auxin-induced swelling with the promotion of cellulase activity in pea epicotyls, Ridge and Osborne (1969) failed to observe a similar promotion under the same conditions. Thus results give conflicting evidence. As well, such observations are difficult to interpret unless the precise location and substrate specificity of the enzyme is known.

1.4.3 Turnover Of Wall Components And Synthesis Of New Wall Material

Turnover of various wall components is a possible mechanism whereby bond breakage and therefore wall loosening might be presumed to occur. Ray (1969) reviewed the then existing data and concluded that the early turnover studies were not interpretable for technical reasons related to the presence of starch, differential solubility of various fractions and the ill-defined nature of the wall components.

In a recent study of the effect of auxin on turnover of wall polysaccharides in pea stem segments which had been prelabelled with ^{14}C-glucose, Labavitch and Ray (1974a) noted that there was no effect of the hormone on turnover of the majority of the wall carbohydrates, but they did note changes in a pectinase-degradable xyloglucan. They found that auxin induced the conversion of an insoluble wall xyloglucan into a water-soluble form. The release of the xyloglucan was measurable within 15 min of hormone treatment, and was dependent upon cellular metabolism but independent of elongation (Labavitch and Ray, 1974b).

Subsequently Jacobs and Ray (1975 and 1976) showed that acidification could mimic the auxin effect and cause the release of a water-soluble xyloglucan from cell walls of Pisum. Labavitch has since demonstrated that the xyloglucan turned over in peas is in fact like that reported in beans and sycamore (quoted Albersheim, 1976 p270). Gilkes and Hall (1977), using gravmetric and pulse chase studies, demonstrated a turnover of polysaccharides in pea epicotyl cell walls. Their studies also suggest that auxin promotes the turnover most notably of a xyloglucan.

With monocots, auxin-induced growth of seedling sections is accompanied by a significant decrease in a non-cellulosic glucan component of the cell wall. This has led to suggestions that the glucan is involved in extension growth. Evidence consistent with the idea that glucan metabolism serves some role in this process is shown by the fact that mannitol restricts extension but not glucan metabolism (Loescher and Nevins, 1973). The results of Masuda and Satomur (1970) likewise indicate that glucan metabolism is involved in auxin-induced extension, since certain Sclerotinia glucanase preparations appear to mimic, in part, the action of auxin.

An examination of the β-glucans from Avena sativa, Triticum vulgare and Hordeum vulgare showed that with increasing plant maturity extended turnover occurs. There was a fall in the value of the ratio of β(1-3) to β(1-4) linkages, a decrease in the average molecular weight and, on a weight basis, a decrease in the amount of β-glucan present in the plant tissues (Buchala and Wilkie, 1973 and refs therein cited).

In order to avoid the removal of hydrolases that are only loosely associated with walls, glycerol has been used as a nonaqueous medium for cell wall isolation. Indeed, when incubating cell wall fragments of corn coleoptiles isolated in glycerol the original weight is considerably reduced by autolysis; the analysis of the solubilized polysaccharide has shown that at least 90% of this activity was attributed to the release of a mixed-link β-D-glucan

which constitutes as much as 11% of the cell wall (Kivilaan et al., 1971; Huber and Nevins, 1979). This autolysis reaction mimics processes which accompany auxin-induced elongation.

These findings implicate the turnover of a β-1,3-β-1,4-glucan in monocots. As well, Franz (1972) demonstrated hypocotyls of Phaseolus aureus underwent dramatic decreases in non-cellulosic glucose contents. This decline was attributed to the loss of a water-soluble mixed-link glucan (Buchala and Franz, 1974) which was absent from the water-soluble fraction of older hypocotyls. However, this decrease could also be due to the loss of a xyloglucan shown to be present in the alkali extract of P. aureus hypocotyls (Kato and Matsuda, 1976).

It thus appears, that specific wall components such as this mixed-link β-D-glucan, may impart structural rigidity to the primary cell wall. The lysis of these polysaccharides which takes place during cell elongation may be responsible for the necessary wall plasticity. In pea epicotyl segments, wall synthesizing enzymes undergo an increase in activity with auxin addition eg. β-glucan synthetase undergoes a two-fold increase. To reconcile these results with the release of soluble xyloglucan fractions from auxin-stimulated pea epicotyls (Labavitch and Ray, 1974) one can propose that wall elongation proceeds by a "break and repair" mechanism. Breaks might be induced by enzymatic or chemical means with a concomitant release of chopped out sections. Turgor would cause separation between breaks and these could be repaired enzymatically.

It is also possible that cellulose deposition may undergo a similar process. Maclachlan (1977) points to the fact that electron micrographs show fibril terminals readily visible in walls of algae and bacteria but absent in plants. Their absence raises the possibility that microfibrils in growing multicellular tissues extend via an insertion mechanism, wherby precursors are incorporated in such a way that breaks never appear. Several observations agree with such a "cellulose synthetase complex" including the presence of

potent cellulase that have been identified bound to the inner wall in growing regions of many higher plants.

1.5 Outline Of Present Investigation

The foregoing discussion on primary cell wall structure and elongation growth relates almost exclusively to angiosperms. Although the structures of gymnosperm wood polysaccharides have been fairly extensively studied, the studies of primary cell walls of gymnosperms have been relatively meagre. Thornber and Northcote (1961a,b) studied the monosaccharide composition of polysaccharide fractions from Pinus ponderosa cambium and Burke et al. (1974) briefly examined the linkage patterns of the sugar residues in cell walls of Pseudotsuga menziesii suspension-cultured cells. There appear to be no detailed structural studies of primary cell wall polysaccharides in any gymnosperm.

The growth and vegetative propagation of Pinus radiata is being investigated in several laboratories (see eg. N.Z. Forest Research Institute Annual Report 1977), but in order to understsnd the biochemical basis of growth in Pinus radiata it is necessary to understand something of the structure and turnover of the primary cell wall. With this in mind, an investigation was initiated on the structure and turnover of polysaccharides in the growing hypocotyl of Pinus radiata seedlings.

Since work with angiosperms has implicated a role, in the cell elongation process, for turnover of either a mixed-link β-glucan or a xyloglucan, a search for a similar polymer in the elongating hypocotyl of Pinus radiata was undertaken. The presence of a possible xyloglucan had been reported in the bark of Picea engelmanni (Ramalingham and Timell, 1964) and red spruce compression wood (Schreuder et al., 1966). Typical xyloglucan oligosaccharides were obtained on enzymic hydrolysis of a glucomannan from Pinus banksiana wood (Perila and Bishop, 1961). In addition, an acidic β-glucan, laricinan, with 1-3 and 1-4 linkages in a linear backbone, was reported in cell walls of compression

wood of Larix laricina and in Pinus resinosa xylem ray parenchyma cell walls. Laricinan was reported to have a ratio of 1-3 to 1-4 linkages of 13 : 1 (Hoffmann and Timell, 1972) which differed from the mixed-link β-glucans of monocots and Phaseolus aureus. Other non-cellulosic glucans reported from Pinus tissues include callose (Pinus sylvestris phloem, Fu et al., 1972; Pinus mugo pollen, Bouveng, 1963) and starch. However, no evidence is available in the literature on the occurrence of xyloglucans or mixed-link glucans or any other rapidly metabolising polysaccharides in elongating gymnosperm cell walls.

Consequently the project fell into two natural categories. The initial phase was to identify a glucan-type polymer showing changes in absolute levels during elongation growth of Pinus radiata hypocotyls. It was therefore necessary to monitor the proportions of glucose and other sugar residues in polysaccharide fractions from hypocotyls at different stages of growth, and to distinguish the glucose-containing component from starch and cellulose.

The second phase of the work was a structural characterisation of the glucan found in the first phase. For this, methylation analysis, partial hydrolysis and chromium-trioxide oxidation were all employed in determining sugar linkages and anomeric configurations.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Pinus radiata certified seeds were obtained from the Forest Research Institute, Rotoura. The seeds were sown in trays of peat/pumice (1 : 1, v/v) potting mixture obtained from the Plant Physiology Division, D.S.I.R., Palmerston North.

Reagents used in the present study were obtained from the following sources: 2-deoxy-D-glucose Grade II, maltose, D-galactose, glucose oxidase (E.C. 1.1.3.4.), peroxidase Type II (Horseradish) and amyloglucosidase (E.C. 3.2.1.3, *Rhizopus*) from Sigma Chemical Company, U.S.A.

Cetyl trimethyl ammonium bromide (cetavlon), chromium trioxide (CrO_3), O-dianisidine, L-arabinose, galacturonic acid and barium hydroxide ($Ba(OH)_2 8H_2O$) from B.D.H. Chemicals Ltd., Poole, England.

Procion blue (M3G) from I.C.I. (N.Z.) Ltd, dimethyldichlorosilane (Repelcote) from Hopkins and Williams Ltd and meta-hydroxydiphenyl from Eastman, Kodak Co., U.S.A.

All other reagents were obtained from May and Baker Ltd., Dagenham, England or Koch-Light Laboratories Ltd., England.

P.G.O. Reagent was made up as follows:
9.8ml 0.2M sodium phosphate buffer, pH 6.0.
0.1ml 1% o-dianisidine (in 95% ethanol).
0.1ml 0.1% peroxidase (in sodium phosphate buffer).
2.0mg glucose oxidase.

2.2 General Methods

2.2.1 Solvent Exchange Drying

The method used was based on that of Green (1963). Polysaccharide material to be dried was suspended successivily in ethanol, methanol and n-hexane. The material was stirred in two aliquots of each solvent for at least five minutes each and recovered by centrifugation. Residual n-hexane was removed in a vacuum dessicator over potassium hydroxide.

2.2.2 Gas Liquid Chromatography

The following columns were used for gas liquid chromatography (g.l.c.).

Column A: A stainless steel column, 2m x 2mm I.D., containing 3% OV-225 coated on Varaport 30, mesh 100-120.

Column B: A glass column, 2.5m x 3mm I.D., containing 3% SP-2340 coated on 100-120 supelcoport.

Column C: A glass column, 3m x 3mm I.D., containing 3% PCNSS-M (silicone-polyester copolymer) coated on Gas Chromosorb W (AW-HMDS).

Column D: A glass fibre SCOT (support coated open tubular) column, of length 60-80m with 0.5mm I.D., containing a non-polar liquid phase SF 30. Column is Grade D with Neff 40,000+. (Neff = effective number of theoretical plates).

The g.l.c. columns were fitted in the following:

Column A was used in the Varian 1400 aerograph fitted with a flame ionisation detector (FID).

Columns B and C were fitted into the Pye 104 gas chromatograph (FID).

Both machines used nitrogen as a carrier gas with a flow rate of 30ml/min through the column and operated isothermally for the separation of alditol acetates. For the separation of partially methylated alditol acetates

both temperature programming and isothermal operation were used.

Column D was used in the Varian 2700 aerograph (FID) which used a carrier gas (hydrogen) flow rate of 7 ml/min and operated isothermally (210°C). Only trimethylsilylated derivatives were separated on this column.

2.2.3 Gas Liquid Chromatography - Mass Spectrometry

For gas liquid chromatography - mass spectrometry (GC-MS), column A was used in a Varian 1740 gas chromatograph (FID) coupled with a V.G. Micromass 12F mass spectrometer.

2.3 Analytical Methods

2.3.1 Determination Of Total Carbohydrate

Total carbohydrate in solutions was measured by the method of Dubois et al. (1956) as modified by Immers (1964).

Aqueous phenol (0.5ml of 5% solution) was added to 0.5ml of sample, containing up to 80ug glucose or equivalent, in a test-tube. Uniform test-tubes of 1.7cm internal diameter were used in all tests. Concentrated sulphuric acid (3ml) was added rapidly from a 'Zipette' dispenser (2 x 1.5ml) and the tubes immediately stirred in a vortex mixer for five seconds. Tubes were then left at room temperature to cool for twenty minutes and the absorbance measured at 490nm in a Hitachi 101 spectrophotometer.

Rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and galacturonic acid standards (10-100ug) all gave linear standard curves (Fig. 11).

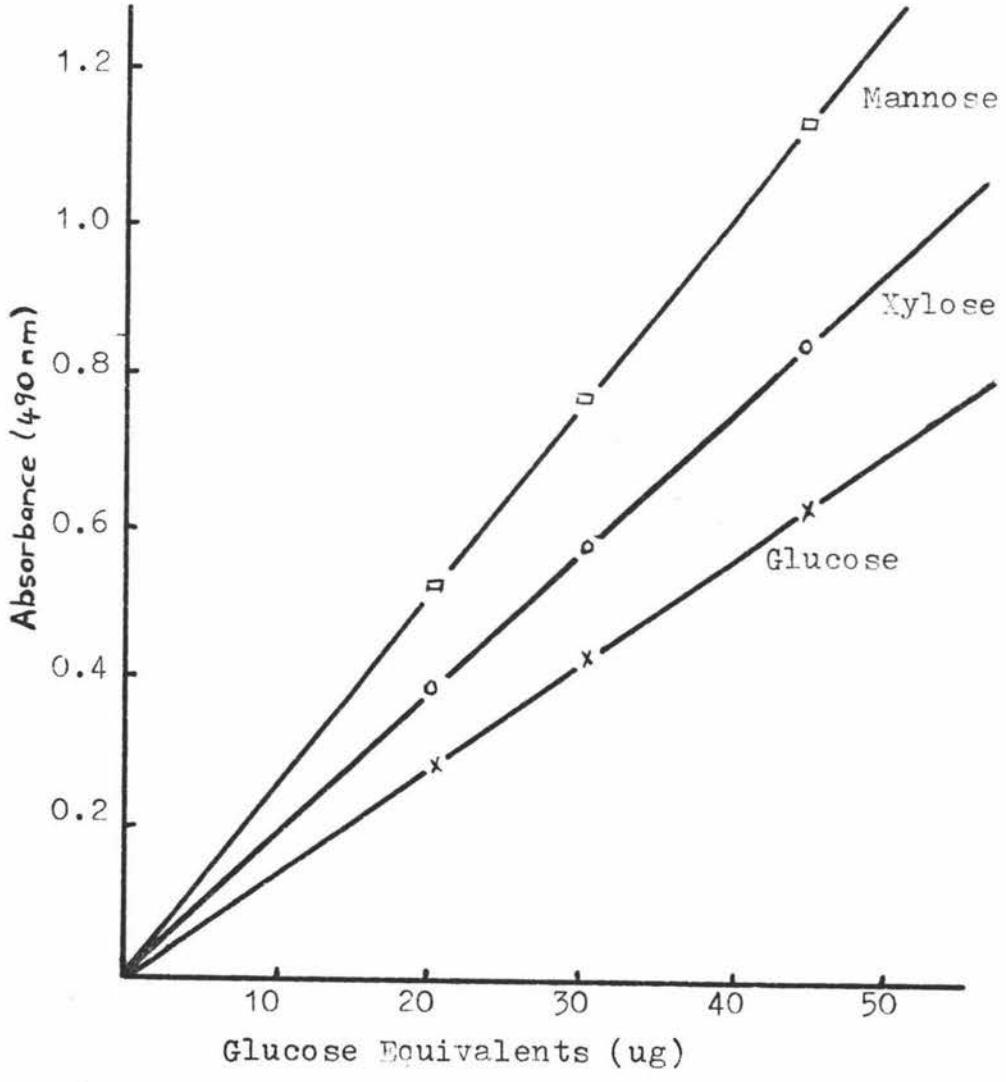
As there is a variation in molar absorbance between monosaccharides, the method by itself can only be used to give an approximation to total carbohydrate when a mixture of sugars is present, as in the fractions isolated in this project. To overcome this problem, first the individual

Table 1: Values Of Standard Monosaccharides

Standard (50ug)	Rha.	Fuc.	Ara.	Xyl.	Man.	Gal.	Glc.	GalA*
Absorbance (490nm)	0.48	0.86	0.61	0.92	1.26	0.79	0.70	0.30

* All uronic acid found by spectrophotometric analysis was assumed galacturonic acid (see text).

Figure 11: Phenol-Sulphuric Standard Curves



neutral monosaccharide percentages found by g.l.c. were combined with the uronic acid percentage found by spectrophotometric analysis and summed to 100%. The absorbance contribution of each monosaccharide was then calculated (multiply the absorbance found for 50ug of the standard sugar (Table 1) by the percentage weight of its composition in the sample) and summed. The observed absorbance was then used in conjunction with the total absorbance calculated for 50ug of polysaccharide and the percentage of carbohydrate worked out. This procedure gave a more accurate indication of total carbohydrate in the assayed fractions, resulting in good agreement with the predicted percentage based on g.l.c. plus uronic analysis.

2.3.2 Determination Of Uronic Acid

Uronic acids were determined by the method of Blumenkrantz and Asboe-Hansen (1973).

To 0.5ml of sample containing 0.5-30ug uronic acids in a test-tube, was added 3.0ml of sulphuric acid-tetraborate (0.0125M solution of tetraborate in concentrated sulphuric acid). The tubes were refrigerated in crushed ice for five minutes and the mixture shaken in a vortex mixer. The tubes were heated in a water bath at 100°C for five minutes, cooled in ice-water for two minutes and 50ul of the m-hydroxydiphenyl reagent added (0.15% solution of meta-hydroxydiphenyl in 0.5% sodium hydroxide. The reagent solution can be kept in the refrigerator covered with aluminium foil for at least a month without effect.). Tubes were shaken and read at 520nm after 15 minutes in a Hitachi 101 spectrophotometer. A vivid purple colour developed and remained fairly stable for an hour.

As carbohydrates produced a pinkish chromogen with sulphuric acid-tetraborate at 100°C, a blank sample was run in which the m-hydroxydiphenyl reagent was replaced by 50ul of 0.5% sodium hydroxide. The absorbance of the blank sample was subtracted from the total absorbance.

A standard curve was calibrated with galacturonic acid for each batch of samples assayed.

2.3.3 Determination Of Starch

The method adapted for determination of starch was based on that of Messer and Dahlquist (1966).

The polysaccharide material (1-5mg) in 2ml of water was placed in a boiling water bath for 60 minutes to gelatinise the starch and allowed to cool. Samples and standards (soluble starch) were then incubated with 2ml 0.2M sodium acetate buffer (pH 4.5) and 1ml of amyloglucosidase preparation in a shaking water bath, 60 minutes at 55°C, and finally centrifuged (2,000 r.p.m. for 3 minutes). Solutions were transferred to test-tubes containing charcoal (0.1g), stirred for 10 minutes on a vortex mixer and centrifuged (3,000 r.p.m. for 20 minutes). The supernatant was filtered to remove traces of charcoal. From the filtrate, 0.1-1ml aliquots were taken for colorimetric analysis and made up to 1ml with water, P.G.O. reagent (1ml) was added and the mixtures incubated 37°C for 30 minutes. (Blank of 1ml water plus 1ml P.G.O. reagent). Addition of 1ml of 50% sulphuric acid produced a magenta colour which was stable for several hours and absorbances were read at 530nm.

2.3.4 Zone Electrophoresis

2.3.4.1 Unmodified Polysaccharides

The method used was that of Jarvis et al. (1977). Preparation of electrophoresis paper strips: Glass-fibre paper (Whatman G.F. A) was cut into strips (usually 15x4cm) and heated at 400°C for 2 hours to burn out organic material, then immersed for 18 hours in a glass dish of carbon tetrachloride containing 2% dimethyldichlorosilane, rinsed in toluene and dried. The trimethylsilylated glass fibre strips were wetted by floating for 18 hours in trays of the appropiate buffer to which Tween-20 (0.2% by volume) had been added.

Analytical electrophoresis: Using a 0.05M sodium tetraborate buffer, pH 9.0, strips were blotted only lightly and placed in the electrophoresis tank powered by a Vokam power pack. Jarvis et al. (1977) found it often advantageous to preequilibrate the strips before sample application for 10-20 minutes at the running voltage. The sample, usually 1-5ug in ca 2ul of buffer, was applied at or near the midpoint of the strip as a narrow band 1-2cm long. Separation conditions involved a potential gradient of 3-10 Vcm^{-1}, a current 1-2.5 $mAcm^{-1}$ strip width and a running time of 10 minutes to 6 hours. To limit evaporation, power input was not allowed to exceed $8mWcm^{-2}$ unless the running time was very short.

Location of polysaccharides: Strips were immersed in ethanol (to immobilise the polymers) and dried in hot air. If necessary, repeated soakings in ethanol removed the buffer. The spray reagents used in locating the polysaccharides were:
(1) α-naphthol spray (Barrett and Northcote, 1965) (200mg α-naphthol and 1ml sulphuric acid in 50ml methanol). After heating the sprayed electropherogram 10 minutes at 110°C, polysaccharides appear as purple bands which turned brown with time.
(2) orcinol spray (Moczar, 1973) (0.1% orcinol in 1N sulphuric acid). Polysaccharides again appear as purple spots after heating 10 minutes, 100°C. This spray was the more sensitive with a limit of detection 0.1-0.2ug.

2.3.4.2 Dyed Polysaccharides

This followed the method of Dudman et al. (1968).
Preparation of dyed polysaccharides: To a solution of the polysaccharide (10mg) in water (1ml) was added 1ml of a freshly prepared 1% solution of the dye, Procion Blue M3G. After 5 minutes, sodium chloride was added, either as a solid or as a small volume of a concentrated solution, to give a final concentration of 2%. Thirty minutes later sodium carbonate was added to give a final concentration of

0.5% (Anderson et al., 1971). The coupling of the dye to the polysaccharides is thought to be complete within an hour, however, the mixtures were set aside for 18 hours (overnight) to allow most of the unreacted dye to be hydrolysed. The reaction mixtures were diluted to reduce viscosity and added directly to a column (21x1.1cm) of Sephadex G-25. Elution of the columns with water produced two widely separated zones. The dyed polysaccharides, excluded from the gels, were eluted in the void volume; salts and unreacted dye were retarded. To avoid contamination by inorganic salts, which were eluted more rapidly than the unreacted dye, care was taken to collect only the strongly coloured portion of the polysaccharide eluate. The dyed polysaccharides were recovered by freeze-drying.

Analytical electrophoresis: The cellulose acetate, trimethylsilylated glass fibre or Whatman Number I paper strips were soaked in the buffer solution (0.1M sodium tetraborate-sodium chloride; pH 9.3) for 5 minutes. The strip was then pressed between two sheets of Whatman 3MM filter paper to remove excess buffer and the starting line was marked with a pencil. Samples (1% solutions) were applied as thin lines by using fine glass capillaries. The strip was then positioned in a Shandon Southern electrophoresis chamber so that the starting line was midway between the terminals which were 10cm apart. Voltage was applied, 250-350V, 7mA, and migration of the bands followed visibly. When the electrophoresis was finished, the strips were removed and dried immediately in a stream of hot air. If this was not done, the bands diffused and became distorted.

2.3.5 Monosaccharide Analysis Of Carbohydrate Fractions

2.3.5.1 Acid Hydrolysis Of Polysaccharides

This followed the method of Jermyn and Isherwood (1956). Polysaccharides (1-5mg) were hydrolysed in 4ml of 0.5M nitric acid containing 0.5% (w/v) urea at 100°C for

3.5 hours in stoppered glass tubes. Urea was added to minimise the destruction of sugars during hydrolysis. After 3.5 hours, the tubes were cooled, opened and the hydrolysates neutralised with 2.5M potassium hydroxide to the bromothymol blue endpoint (pH range 6.0-7.6).

2.3.5.2 Preparation Of Alditol Acetates

The monosaccharides released by acid hydrolysis of the polysaccharides were analysed by g.l.c. as their volatile alditol acetate derivatives. The method used was based on that of Albersheim et al. (1967).

A sample of the hydrolysate containing 1-5mg of sugars was reduced for 2 hours at room temperature with 1ml of 1M aqueous ammonia (NH_4OH) containing 20mg sodium borohydride ($NaBH_4$). 2-deoxy-D-glucose (0.4mg) was added as an internal standard. The excess borohydride was converted to borate by dropwise addition of glacial acetic acid until evolution of hydrogen ceased. (The acetate added acts as a catalyst during subsequent acetylation). Samples were evaporated to dryness under an air stream (60°C) and the borate removed as the volatile methyl borate by five successive additions of approximately 4ml of methanol, followed by evaporation to dryness each time. (It is necessary to remove the borate completely otherwise the borate-alditol complex prevents complete acetylation (Albersheim et al., 1967))

Samples were acetylated either using;

(1) 1ml of acetic anhydride, heated 8 hours at 120°C, or
(2) 2ml of 1:1 acetic anhydride-pyridine, 8 hours at room temperature.

2ml water was added, the mixture stirred and allowed to stand till homogeneous. The alditol acetates were extracted with methylene chloride (2x2ml), mixing thoroughly each time and the bottom layers of methylene chloride (withdrawn with a pasteur pipette) were combined, evaporated to dryness and taken up in methylene chloride (0.2ml) prior to injection (ca 1ul aliquots) in the g.l.c. column.

2.3.5.3 Gas Liquid Chromatography Of Alditol Acetates

G.l.c. was routinely performed on column A (OV-225). Operating conditions (isothermal) were: column temperature, 206°C; detector temperature, 270°C; injection port temperature, 240°C. Carrier gas flow (nitrogen) 30ml/minute; hydrogen gas flow 30ml/minute and air flow 300ml/minute. A good separation was achieved, under these conditions, of all the monosaccharides encountered except rhamnose and fucose (Fig. 12).

Column B (SP-2340), which was not initially available, afforded good separation of rhamnose and fucose as well as all the other monosaccharides (see Fig. 13). This column was used in the Pye 104 gas chromatograph (FID) operated isothermally with an oven temperature 230°C, and a carrier gas flow (nitrogen) 30ml/minute.

Authentic alditol acetates of rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose were prepared and used to identify the peaks of the alditol acetates of the carbohydrate samples in the gas liquid chromatograph. The method used was based on a comparison of the retention time of the unknown component with that obtained from a known compound analysed under identical conditions (Table 2).

2.3.5.4 Quantitation Of Alditol Acetates

As the peaks that eluted first (rhamnose/fucose, arabinose, and xylose) were very narrow, it was difficult to obtain accurate peak areas. The relative peak areas for each sugar were thus obtained from the ratio of the peak height of any component to the peak height of an internal standard, 2-deoxy-D-glucitol penta-acetate, multiplied by the ratio of the retention time of the component to the retention time of the internal standard. This ratio gave a relative peak area that was linearly proportional to the amount of monosaccharide present (Fig. 14).

To enable direct comparison between the individual

Figure 12: The Separation By Gas-Liquid Chromatography Of Monosaccharides As Alditol Acetates.

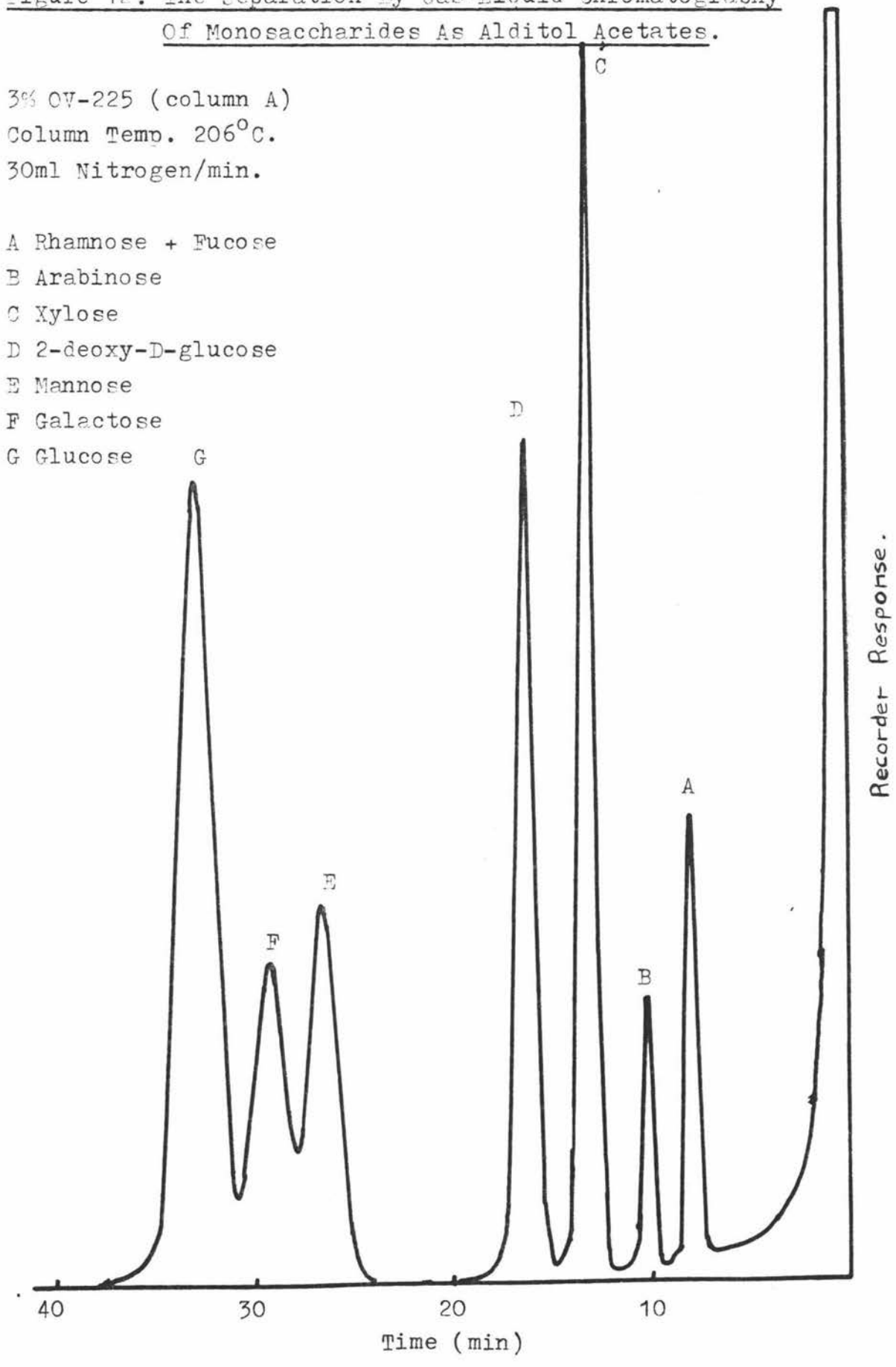


Figure 13: The Separation Of Rhamnose And Fucose By Gas-Liquid Chromatography.

3% SP 2340 (column)
Column Temp. 230°C.
30ml Nitrogen/min.

Peaks correspond to:
A1 Rhamnose
A2 Fucose
B Arabinose
C Xylose
D 2-deoxy-D-glucose
E Mannose
F Galactose
G Glucose

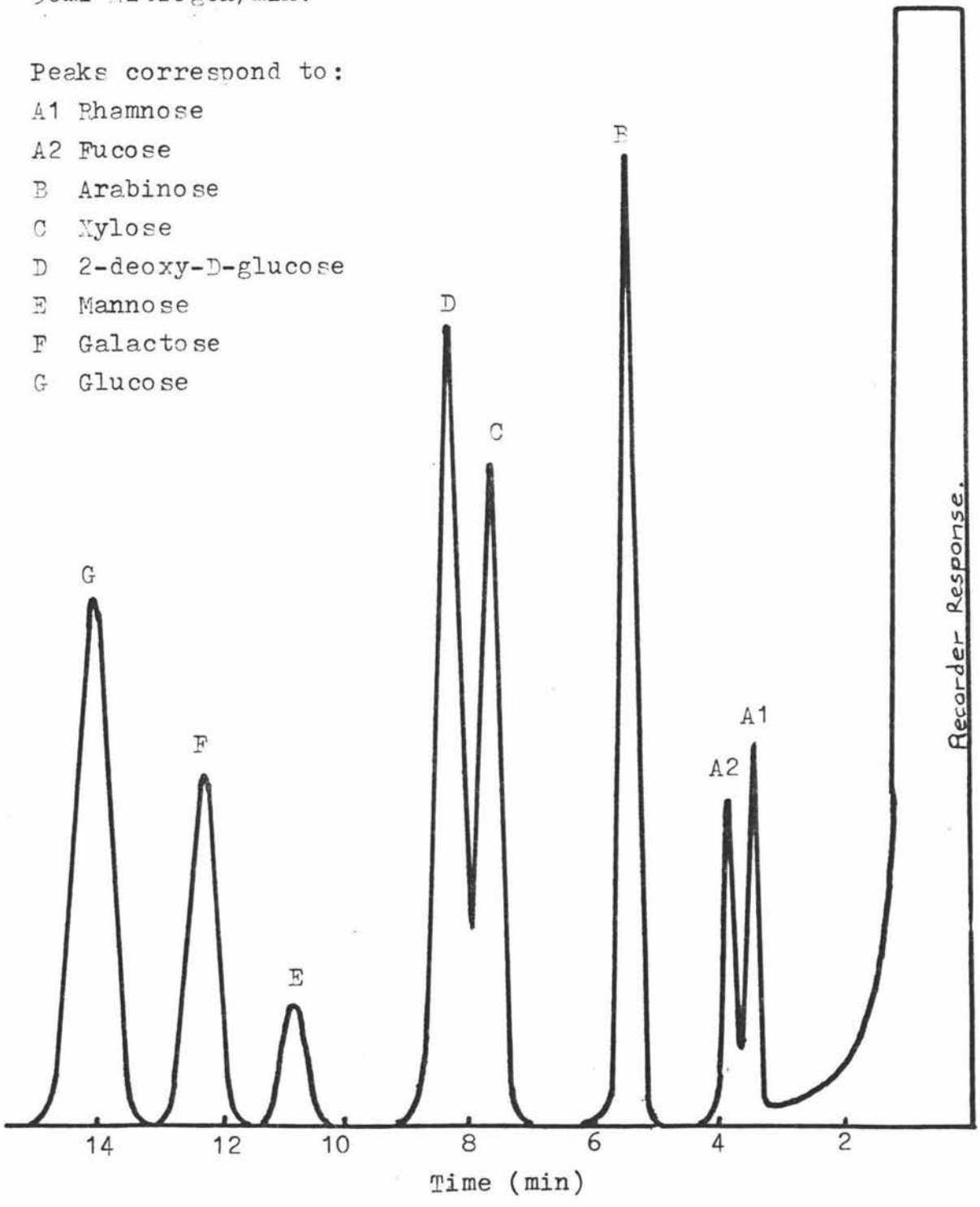
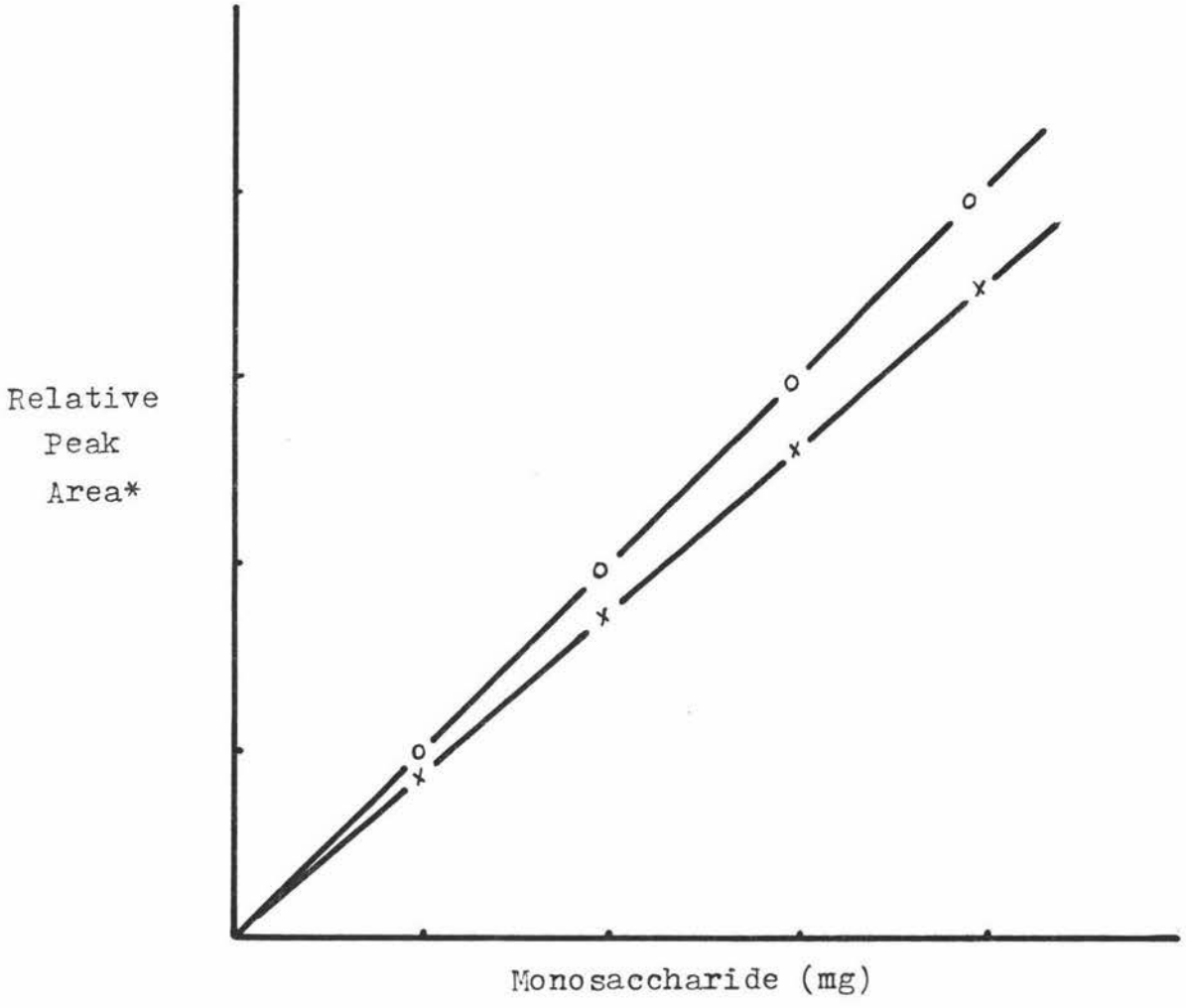


Table 2: Retention Time Of Fully Acetylated Glycitols Relative To 2-deoxy-D-glucoypranoside Penta Acetate

Sample	Acetylated Product	Relative Retention Time OV-225	SP2340
L-Rhamnose$_p$	Rhamnitol penta acetate	0.49	0.44
L-Fucose$_p$	Fucitol penta acetate	0.49	0.48
L-Arabinose$_f$	Arabinotol penta acetate	0.62	0.68
D-Xylose$_p$	Xylitol penta acetate	0.80	0.92
2-deoxy-D-glucose$_p$	2-deoxy-D-glucitol penta acetate	1.00	1.00
D-mannose$_p$	Mannitol hexa acetate	1.62	1.36
D-galactose$_p$	Galacitol hexa acetate	1.78	1.53
D-glucose$_p$	Glucitol hexa acetate	2.00	1.75
myo-inositol$_p$	myo-inositol hexa acetate	2.18	2.00

Figure 14: Relative Peak Area Of Standard Monosaccharides.



o-o Glucose.

x-x Rhamnose.

* Peak area relative to peak area of internal standard, 2-deoxy-D-glucose.

monosaccharides, weight response factors were calculated. Prepared standard monosaccharide alditol acetates were injected onto column A. The necessary weight response correction factor for each standard monosaccharide was calculated to give the same relative peak area for any particular weight (Table 3).

As polysaccharides were hydrolysed in nitric acid for 3.5 hours, the released monosaccharides always underwent some degradation. To get a correction factor for this monosaccharide degradation, preparations of standard monosaccharides were subjected to nitric acid hydrolysis conditions. After conversion to their alditol acetate derivatives and injection onto column A, relative peak areas were calculated incorporating the relevant weight response correction factors. Maximum degradation correction factors (D.C.F.) were worked out so as to give the same relative peak areas for each standard monosaccharide for any weight. As polysaccharides release their monosaccharides at a decreasing rate with time, the minimum polysaccharide D.C.F. for this work was assumed as 75% of the maximum monosaccharide D.C.F. (Table 3).

Both correction factors were combined and used in all g.l.c. calculations for polysaccharide compositions.

2.3.6 Partial Hydrolysis Of Carbohydrate Fractions

2.3.6.1 Acid Hydrolysis

The method used was based on the method of Albersheim et al. (1967).

Polysaccharides (1-5mg) were partially hydrolysed in 1ml of 2M trifluoroacetic acid (T.F.A.) at 100°C for 1 hour. The tubes were then evaporated to dryness under a stream of air (40-45°C) ready for separation by paper chromatography.

Table 3: Correction Factors

Sample	Weight Response	Degradation	Combined
Rhamnose Fucose	1.173	1.101	1.292
Arabinose	1.045	1.094	1.143
Xylose	1.020	1.112	1.134
Mannose	1.063	1.034	1.099
Galactose	1.006	1.057	1.063
Glucose	1.013	1.011	1.023

2.3.6.2 Descending Paper Chromatography

Descending paper chromatography was performed on Whatman Number I chromatography paper which had previously been washed in deionised water. Separation of the oligosaccharides was by the following solvent systems;
Solvent 1: n-propanol : ethylacetate: water (7 : 1 : 2)
Solvent 2: n-propanol : ethylacetate: water (32 : 57 : 13)

After 48 hours oligosaccharides were cut out and eluted with deionised water (Laidlaw and Reid, 1950). These solutions were freeze-dried and trimethylsilylated for g.l.c. injection. Standards of the 1,3 series (laminaribiose, laminaritriose, etc.) were obtained by partial hydrolysis of laminarin with 11M hydrochloric acid, (1h at 22°C), (Aronson et al., 1967).

Reducing sugars were detected with alkaline silver nitrate according to the method of Trevelyan et al. (1950).

2.3.6.3 Preparation And G.L.C. Of Trimethylsilyl (T.M.S.) Derivatives

As based on the method of Sweeley et al., (1963).

A solution of the disaccharide eluted from Whatman Number I chromatography paper was freeze-dried and dissolved in 0.1ml T.M.S. reagent (pyridine-trimethylchlorosilane-hexamethyldisilazane, 5 : 1 : 1) under nitrogen. The mixture was shaken and heated for 0.5 hours at 60°C then left at room temperature 2 hours. The T.M.S. derivatives were evaporated to dryness under nitrogen and dissolved in dry n-hexane for injection. Samples could be centrifuged to get rid of the insoluble ammonium chloride.

Standard samples of trimethylsilylated sucrose, cellobiose and laminaribiose were used for co-injection with the sample. A small volume (0.5ul) of the sample or standard was injected into column C (SE 30 SCOT column) fitted into a Varian 2700 gas chromatograph (FID) operating isothermally with column temperature 210°C; detector temperature 295°C, and injection port temperature 285°C with a carrier gas (hydrogen) flow 7ml/min and nitrogen gas flow 100ml/min.

2.3.7 Determination Of The Anomeric Configurations Of The Glycosidic Bonds

This was performed according to the method of Lindberg et al. (1977).

2.3.7.1 Polysaccharide Acetylation

The polysaccharide (10mg) was dissolved with ultrasonication in formamide (6ml). Acetic anhydride-pyridine (1 : 1, 6ml) was added, the solution was left at room temperature overnight then poured into water and dialyzed exhaustively against deionised water. The material was recovered by freeze-drying.

2.3.7.2 Chromium Trioxide Oxidation

The freeze-dried acetylated polysaccharide was dissolved in glacial acetic acid (0.6ml) with myo-inositol hexa-acetate (2mg) as internal standard. After withdrawal of a zero-time sample (0.2ml), finely powdered chromium trioxide (A.R. 40mg) was added to the remainder (0.4ml) of the solution and agitated ultrasonically at 50°C in a water bath. After 1 hour and again after 3-4 hours, further 0.2ml aliquots were withdrawn. The 0 hour, 1 hour and 3-4 hour aliquots were each pipetted into water (10ml) and extracted with methylene chloride (3x3ml). The combined organic phases were evaporated to dryness.

The residue then remaining was hydrolysed with 0.25M aqueous sulphuric acid (2ml) at 100°C overnight. Solutions were neutralised with barium carbonate ($BaCO_3$) and filtered through a double layer of washed Whatman Number 541 filter paper. The filtrate was evaporated *in vacuo*, then 2ml of 1% sodium borohydride in 1M ammonia added and left overnight. Acetic acid (4N) was added dropwise until effervescence ceased, the sample evaporated to dryness, co-evaporated with methanol (5x5ml) and acetylated for 20 minutes at 100°C with 2ml pyridine-acetic anhydride (1 : 1). Water was added, the alditol acetates extracted with methylene

chloride and injected into column A and column B.

A comparison of the three analyses using relative peak areas for each monosaccharide shows which sugar residues have been oxidized.

2.3.8 Methylation Analysis Of Polysaccharides

2.3.8.1 Preparation Of Methylsulphinyl Anion

The method used was that of Conrad, (1972).

NaH (1.5g) placed in a 250ml three-necked round-bottomed flask containing a magnetic bar and fitted with a condenser and thermometer. Throughout the preparation, the flask was flushed with nitrogen. The mineral oil was removed by washing with aliquots of n-hexane (3x20ml). Residual n-hexane was removed by evaporation under nitrogen. Using a hypodermic syringe 10ml dimethyl sulphoxide was added and the mixture stirred at approximately 50°C until hydrogen evolution ceased (about 1 hour) when the solution had become a dark sea-green colour. After a further 20 minutes, the solution was transferred to glass stoppered tubes, flushed with nitrogen and stored frozen in this state.

NaH was dissolved in ethanol prior to disposal (NaH when freed of the mineral oil is an extremely powerful base which will react explosively with water).

1ml of the methysulphinyl anion solution was mixed with 15ml water and titrated against 0.1N HCl to the phenolphthalein endpoint. 23.0ml of HCl used meant an anion concentration of 2.3N.

2.3.8.2 Methylation

The method followed was that of Hakomori (1964) as modified by Bjorndal *et al*. (1970).

Freeze-dried polysaccharides (2-20mg) were dried *in vacuo* over phosphorous pentoxide for at least 48 hours. The dried samples were flushed with nitrogen, then dissolved in 5ml dimethyl sulphoxide, heating the mixture to 60°C with ultrasonication to aid dissolution (Sandford and Conrad, 1966). In some cases polysaccharides were acetlyated prior

to methylation in order to aid dissolution (Haslemore, 1974). The methyl sulphinyl anion (1ml) was added slowly to the sample and a gel formed which gradually dispersed. Stirring was maintained for 2 hours and then 200ul methyl-iodide (B.D.H.) was added slowly, taking care to maintain the temperature at 20-25°C, over a period of 30 minutes. Stirring was continued for an additional 30 minutes. The variously coloured solutions became clear and the viscosity declined indicating that methylation of free hydroxyl groups was occurring (Sandford and Conrad, 1966). Chloroform/methanol (1 : 1, v : v, 5ml) was then added. The solutions were exhaustively dialysed to remove dimethyl sulphoxide and other reagents, and then freeze-dried. In some cases methylated polysaccharides were extracted into the chloroform phase of a water/chloroform mix (Hakomori, 1964).

Where g.l.c. revealed that methylation was incomplete a second methylation was performed. All methylations were found to be incomplete with a single Hakomori methylation, so multiple methylations were performed without isolation of the intermediate methylated products using the method of Sweet et al., (1975).

2.3.8.3 Hydrolysis

The method was modified after Lindberg et al. (1972).

A 2-5mg sample of methylated polysaccharide was treated with 2ml 90% (w : w) formic acid for 1 hour at 100°C. Formic acid was removed under a stream of nitrogen and the residue dissolved in approximately 1ml 0.25M sulphuric acid and held at 100°C for 16 hours. The hydrolysate was neutralised with barium carbonate and the insoluble barium sulphate removed by filtration.

Reduction: The method of Albersheim et al. (1967) was used. The filtrates from the neutralisation were dried in vacuo and reduced for 4 hours at room temperature in 2ml of a 1% (w : v) solution sodium borohydride in 1M ammonia. The excess sodium borohydride was destroyed by adding 4N acetic

acid until hydrogen evolution ceased. The samples were then evaporated in vacuo at 35°C (rotary evaporation) and the borate removed by 5 washes with 10ml of methanol followed by evaporation in vacuo at 35°C of the volatile methyl borate.

Acetylation: The samples were acetylated in 1ml acetic anhydride for 1 hour at 100°C. The addition of pyridine as a catalyst was found unnecessary for complete acetylation.

2.3.8.4 G.L.C. Of Partially Methylated Alditol Acetates

The acetic anhydride was hydrolysed with 2ml water (removal of acetic anhydride by evaporation under nitrogen can also remove volatile methylated components). The partially methylated alditol acetates were extracted into methylene chloride for g.l.c. injection. Columns A, B and C were used under the following conditions:

Column A fitted into a Varian 1400 (FID) was operated isothermally with a column temperature 180°C, injection port temperature 230°C, detector temperature 260°C, a carrier gas (nitrogen) flow rate of 30ml/minute and a hydrogen gas and air flow rate of 300ml/minute.

Column B fitted into a Pye 104 gas chromatograph (FID) operated with an initial temperature 180°C, temperature programmed 2°C per minute rise to 200°C, held 10 minutes and then 2°C per minute rise to 230°C. It had a carrier gas (nitrogen) flow rate of 30ml/minute.

Column C fitted into a Pye 104 gas chromatograph (FID) operated isothermally with an oven temperature 150°C and a carrier gas (nitrogen) flow rate of 30ml/minute.

Gas Liquid Chromatography - Mass Spectrometry: Column A was fitted into a Varian 1740 gas chromatograph and temperature programmed from 130°C to 160°C (1°/min), held 70min, then 1°/min to 210°C. The V.G. Micromass 12F mass spectrometer was operated at an inlet temperature of 230°C, an ionization potential of 70eV, and an ion source temperature of 250°C.

2.3.8.5 Identification And Quantitation Of Peaks

Peaks were identified by co-chromatography with standard partially methylated alditol acetates and comparing the measured retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol using the values given by Lonngren and Pilotti (1971). Combined g.l.c. - mass spectrometry is often used for identification (Bjorndal et al., 1970; Talmadge et al., 1973) and this was used to identify standards derived by partial methylation of soluble starch as well as a sample of the methylated xyloglucan.

Quantitation of the partially methylated alditol acetates was based on measuring the relative peak areas by the triangular method (see Mefferd et al., 1968).

2.4 Growth And Harvesting Of Plants

Certified Pinus radiata seed was germinated on moist filter paper and then grown in pumice-peat mix at 22-25°C in the dark with daily watering. Growth ceased about 2-3 weeks after germination by which time the etiolated hypocotyls averaged 7cm in length.

Seedlings were harvested by cutting the hypocotyl at soil level approximately 1-2 weeks after germination (hypocotyls 4cm in length) or at 2-3 weeks (hypocotyls 7cm). The harvested plant was divided into three zones; cotyledons (incorporating about 1mm of stem) and the upper and lower hypocotyl arising from division of the hypocotyl (Fig. 15). These sections were placed in foil-wrapped, airtight containers and kept in the freezer (-14°C) until used.

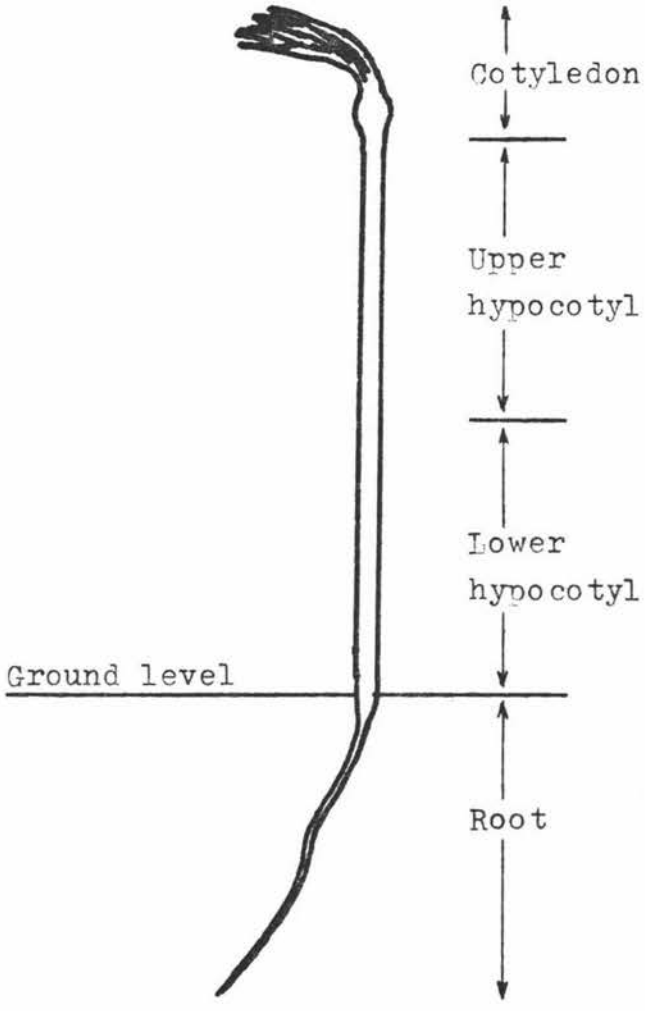
2.5 Polysaccharide Extractions

2.5.1 Extraction Of Needle Polysaccharides

The procedure was based on the method of Fu et al., (1972).

Mature Pinus radiata needles (5.1g) frozen at -14°C were ground up in acetone with a mortar and pestle to give a fine

Figure 15: Sections Of Pinus Radiata Seedings.



suspension. After 3 extractions centrifuging (4000g, 5 minutes) each time to separate the ground material, the residue was extracted once with cold water (room temperature, 30 minutes, 50ml) and then once with hot water (100°C, 60 minutes, 50ml). Both water extracts were centrifuged 4,000g for 5-10 minutes and the supernatant combined with a water washing of the residue left. After the hot water extraction the residue was further extracted in order with: 0.5% ammonium oxalate (50ml, 75°C, 60 min); 10% sodium carbonate (50ml, room temperature, 60 min); 10% potassium hydroxide (50ml, room temperature, 60 min); and 25% potassium hydroxide (50ml, room temperature, 60 min).

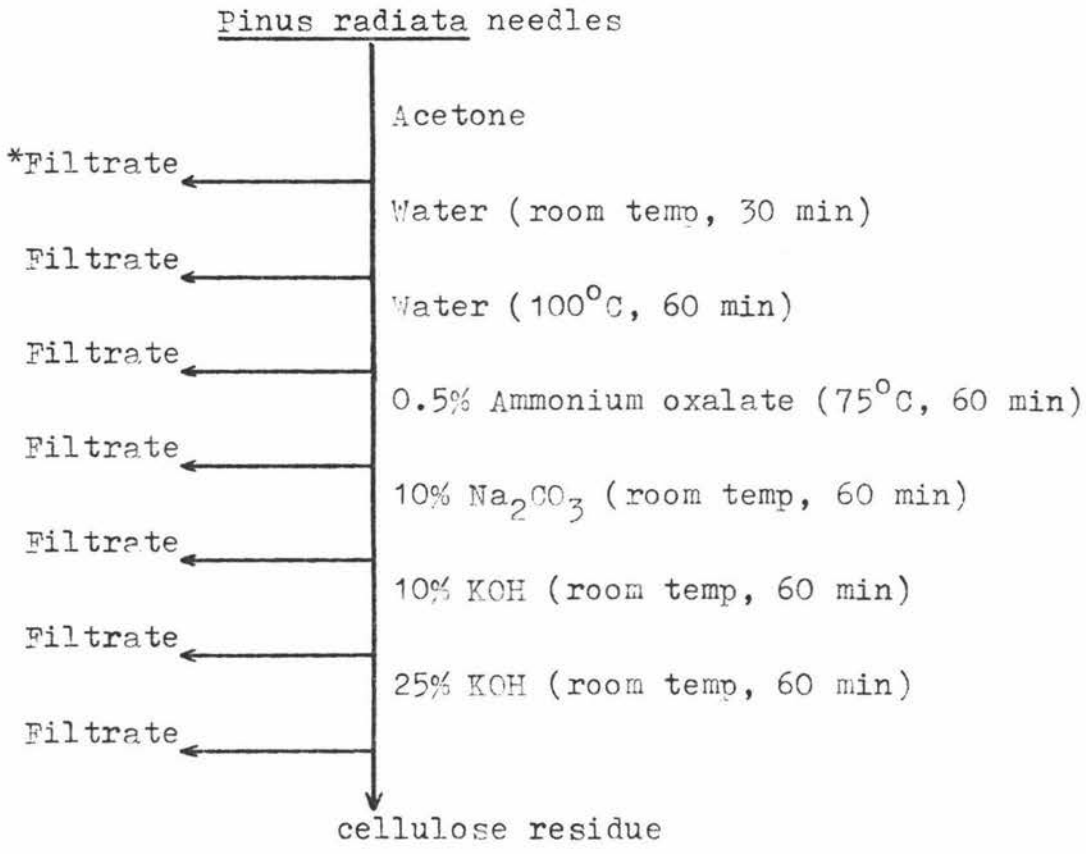
The sodium carbonate and potassium hydroxide extraction supernatants were neutralised with glacial acetic acid (added dropwise with stirring) and the precipitates centrifuged off. To each neutral supernatant (i.e. from the cold water supernatant through to the 25% potassium hydroxide supernatant) was added, dropwise and with stirring, four volumes of ethanol. The precipitates were then dried by solvent exchange (section 2.2.1) and stored in a vacuum dessicator over potassium hydroxide. A summary of the needle extraction procedure is given in Fig. 16.

2.5.2 Extraction Of Hypocotyl Polysaccharides: Procedure A

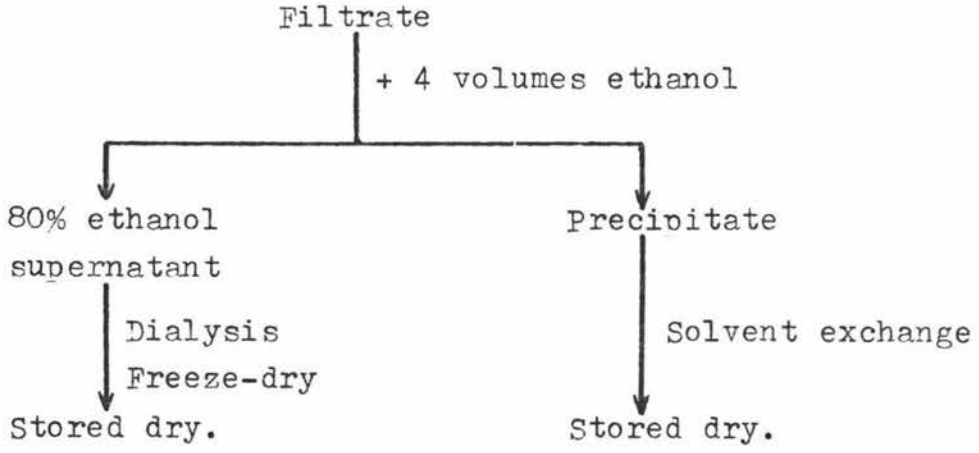
Preparation of cell-wall fraction: Frozen hypocotyl or cotyledon sections were placed in liquid nitrogen (oxygen free) and ground up. The ground tissue was extracted with acetone (0°C), three to six times, and the residue centrifuged off (4,000g, 10min).

Ammonium oxalate extraction: The extracted material was suspended in hot 0.5% ammonium oxalate (10ml/g fresh weight, 75°C, 60 min) and stirred constantly. The extraction mixture was centrifuged and the insoluble material washed with 0.5% ammonium oxalate. These washings were combined with the extraction supernatant and to this was added, dropwise and with stirring, four volumes of ethanol. The

Figure 16: Pine Needle Extraction.



All filtrates underwent the following



* Filtrates obtained after centrifugation.

precipitated material was dried by solvent exchange and stored in a vacuum dessicator over potassium hydroxide.
Potassium hydroxide extraction: The ammonium oxalate residue was extracted with 10% potassium hydroxide containing 0.5% sodium borohydride (10ml/g fresh weight), 60 minutes at room temperature, with constant stirring. The insoluble material (α-cellulose) was washed with 10% potassium hydroxide and dried by solvent exchange. The combined potassium hydroxide extract and washings were neutralised with glacial acetic acid to give a precipitate (hemicellulose A). The supernatant on addition of four volumes of ethanol, also formed a precipitate (hemicellulose B) and both precipitates were dried by solvent exchange and stored in a vacuum dessicator over potassium hydroxide. (Fig. 17 gives summary)

2.5.3 Extraction Of Hypocotyl Polysaccharides: Procedure B

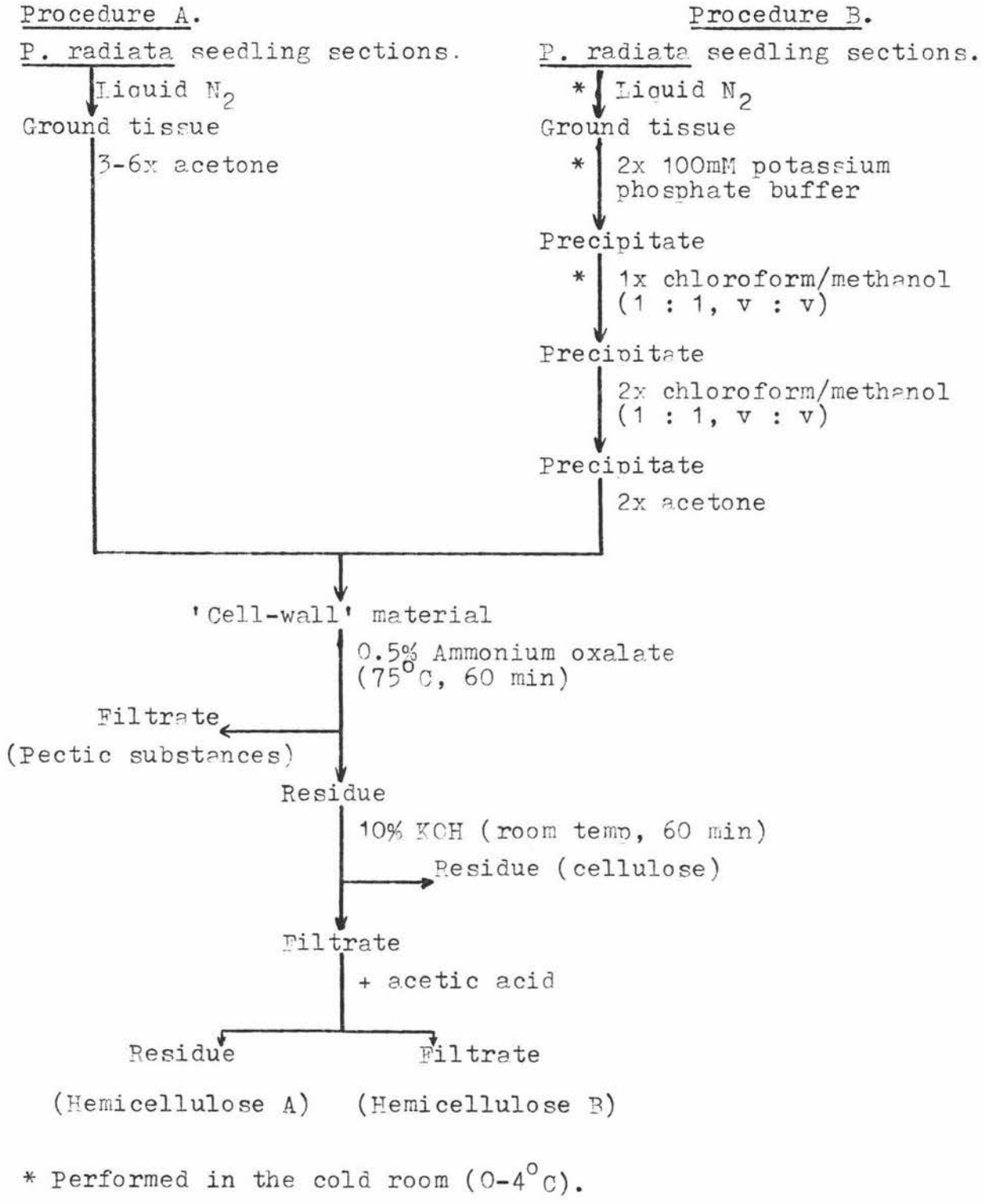
In this procedure, based on the method of English et al., (1971) a 'cell-wall' fraction was first prepared as follows. (Initial steps were performed in the cold room at 0-4°C.)

Frozen seedling sections were ground in liquid nitrogen (oxygen free) to a fine powder. The ground material was then extracted with cold 100mM potassium phosphate buffer pH 7.0 (4ml/g fresh weight) twice in a Waring Blender for five minutes. After centrifugation (2,000g, 10 min), the residue was washed (by suspending in one volume of cold distilled water for five minutes and recentrifuging) and the washed residue was extracted with three volumes of a cold 1 : 1 chloroform/methanol mixture for five to ten minutes in a Waring Blender. The insoluble material was collected by centrifugation.

(Subsequent operations were conducted at room temperature.)

The residue was twice more extracted with two volumes of chloroform/methanol by continual stirring and the supernatants discarded. The residue was now put through two acetone washes with stirring and the insoluble material ('cell-wall' fraction)

Figure 17: Hypocotyl And Cotyledon Extractions.



* Performed in the cold room (0-4°C).

air dried and placed in a vacuum dessicator over potassium hydroxide. (See Fig. 17 for summary)

Both the water and buffer extracts were concentrated by rotary evaporation, dialysed, freeze-dried and stored in the vacuum dessicator over potassium hydroxide.

Ammonium oxalate extraction: The 'cell-wall' preparation was extracted twice with 0.5% aqueous ammonium oxalate (10ml/g fresh weight) and the combined supernatants concentrated by rotary evaporation, dialysed and stored in the refrigerator in airtight containers with 0.02% sodium azide (NaN_3) as an anti-bacterial agent.

Potassium hydroxide extraction: The residue after ammonium oxalate extraction was then extracted twice with 5% potassium hydroxide containing 0.5% sodium borohydride (10ml/g fresh weight) and the insoluble material (α-cellulose), dried by solvent exchange and stored *in vacuo* over phosphorous pentoxide. The combined extracts were adjusted to pH 5.5 with glacial acetic acid and left 18 hours at $0^{o}C$. After centrifugation on the bench centrifuge, the precipitate (hemicellulose A) was dried by solvent exchange and stored *in vacuo* over phosphorous pentoxide. The supernatant (containing hemicellulose B) was concentrated by rotary evaporation, dialysed and stored in 0.02% sodium azide. Dry polysaccharide material was recovered by freeze-drying.

2.6 Fractionation Of Polysaccharides

Various extracted polysaccharide fractions were fractionated with the following procedures:

2.6.1 Barium Hydroxide

The procedure used followed the method of Meier, (1965).

To a 1% solution of the hemicellulose (15ml) was added, dropwise and with stirring, a saturated aqueous solution of barium hydroxide octahydrate. The resulting precipitate was washed with a 5% potassium hydroxide solution (containing 0.25% sodium borohydride), acidified with acetic acid, and

added to 150ml of ethanol. After stirring 10 minutes the material was centrifuged, washed on the centrifuge twice with 80% aqueous ethanol, dried by solvent exchange and stored over phosphorous pentoxide.

The barium hydroxide supernatant and potassium hydroxide washings were combined, neutralised with glacial acetic acid, precipitated with four volumes ethanol, dried by solvent exchange and stored over phosphorous pentoxide.

2.6.2 Cetyl Trimethyl Ammonium Bromide And Borate

The method used was that of Barker et al., (1957).

A 5% cetyl trimethyl ammonium bromide solution was added slowly, with stirring to an equal volume of a 1% hemicellulose solution at room temperature. Sodium sulphate (Na_2SO_4) was added to give a concentration no more than 0.02M to aid flocculation of the precipitate. The aqueous solution was left at least five minutes standing and then centrifuged on a bench centrifuge to separate the precipitated acidic polysaccharides.

A 5% boric acid solution (equal in volume to the original volume of the 1% hemicellulose solution) was then added and the solution adjusted first to pH 8.0 and then to pH 10.0 with 2N sodium hydroxide. After each addition of sodium hydroxide any precipitate formed was collected by centrifugation and the supernatant cooled to $4^{\circ}C$ (refrigerator) for further precipitation of borate complexes (collected by centrifugation). Addition of four volumes of ethanol to the final borate supernatant caused precipitation of most of the remaining polysaccharide in solution.

All precipitates were dissolved in 2N acetic acid to decompose the cetyl trimethyl ammonium complexes and the free polysaccharides reprecipitated with four volumes of ethanol. Precipitates were washed with acidified ethanol and then ethanol, dispersed in deionised water and freeze-dried.

2.6.3 Iodine-Potassium Iodide

The method of Gaillard, (1965) was used.

The polysaccharide preparation was dissolved in a solution of calcium chloride (3.7M) to give a 1% solution, stirred overnight, and clarified by brief centrifugation at 20,000g. A volume (15% of the calcium chloride volume) of an aqueous solution of iodine (3%) and potassium iodide (4%) was added. After leaving it to settle for two hours, centrifugation gave a dark blue precipitate (linear polymer) and a clear brown supernatant (branched polymer).

Branched hemicellulose B: The brown supernatant was neutralised with sodium thiosulphate and poured, with stirring, into five volumes of ethanol to precipitate the branched polymer. To remove Ca^{++}, the branched polymer was dissolved in 0.1N hydrochloric acid (5ml) and reprecipitated with ethanol. The polysaccharide was washed with ethanol, dissolved in a minimum volume of deionised water and freeze-dried.

Linear hemicellulose B: The dark blue precipitate containing the linear polymer was washed with calcium chloride solution containing 15% iodine-potassium iodide solution. The washed residue was dissolved in hot water, the iodine neutralised with sodium thiosulphate and the polymer reprecipitated by pouring into five volumes of ethanol. To remove Ca^{++}, the precipitate was dissolved in a minimum volume of 1M potassium hydroxide under nitrogen, neutralised with 1M hydrochloric and again precipitated with five volumes of ethanol. This was then dissolved in a minimum amount of deionised water and freeze-dried.

2.6.4 Aqueous Ethanol

The procedure used followed the method of Whistler and Sanella, (1965).

The hemicellulose was dissolved in deionised water to give a solution which was adjusted to pH 7.0 by the dropwise addition of sodium hydroxide solution. The insoluble substance was removed by centrifugation at 3,000 r.p.m.

Ethanol (from a burette) was added dropwise with stirring to incipient turbidity. After standing for five minutes, the dispersion was centrifuged. The precipitate was washed with ethanol several times then dissolved in deionised water and freeze-dried. The procedure was repeated on the supernatant as before until a concentration of 80% ethanol was obtained.

CHAPTER 3

PINUS RADIATA HEMICELLULOSE COMPOSITIONAL CHANGES.

3.1 Introduction

Seedling hypocotyls were selected for following elongating gymnosperm primary cell wall changes because they: (a) give cell types that are very similar as most cells are undifferentiated; and (b) have regions where the cell walls are still elongating and regions which have recently ceased elongation, all with very little secondary wall thickening. Since etiolation leads to greater gypocotyl growth, such seedlings were used for cell-wall analysis. Etiolation also gives minimal secondary thickening and lignification of the hypocotyl cell wall (I.G. Andrew, personal communication).

In the elongating hypocotyl of Pinus radiata seedlings, only the upper 2cm is actively elongating (A. Laan, personal communication). A comparison of upper and lower regions of the elongating hypocotyl therefore, would demonstrate if there were changes between cell walls undergoing elongation and those which had recently ceased elongation. The cotyledons, which were also investigated, are initially green and remain unexpanded at 1-2 weeks but within the next week have undergone a small amount of growth so that the cotyledons are up to 2cm in length. Starch is present in the upper hypocotyl and cotyledons but is rapidly depleted with growth (see results).

In the work reported here the etiolated Pinus radiata hypocotyl has been divided into two equal regions. Both these two zones and the cotyledon were analysed for cell wall carbohydrate. One group of seedlings was harvested when hypocotyls were 4cm in length and another group when 7cm in length. Thus hypocotyl sections of different ages were obtained, from the youngest upper zone of the 4cm hypocotyl to the oldest lower zone of the 7cm hypocotyl.

Before commencing the search for a glucan in hypocotyls, it was considered desirable to establish optimum conditions for fractionation by using pine needles as these were much more readily available than the seedling hypocotyls.

The wall composition was analysed by conventional polysaccharide fractionation and quantitative analysis of the monosaccharides comprising each of the fractions taken.

3.2 Results

3.2.1 Pinus Radiata Needle Fractionation

The fractionation procedure used for the needles was based on that of Fu et al. (1972) and is described in Section 2.5.1. The results of this extraction sequence (Table 4) showed that the polysaccharides of the hot water and ammonium oxalate extracts contained a preponderance of arabinose, mannose and galactose. This suggested the presence of pectic material or arabinogalactan together with water-soluble galactoglucomannan. Such a conclusion would agree with the work of Brasch and Jones (1959) who isolated a cold water soluble arabinogalactan from Pinus radiata wood meal and Roudier and Eberhard (1965, 1967) who isolated both an arabinan and galactoglucomannan from maritime pine (Pinus pinaster var. maritime) wood meal by hot water extraction. The other two quantitatively important extracts (sodium carbonate and 10% potassium hydroxide) both contained greatly increased quantities of xylose and glucose.

Since the sodium carbonate and 10% potassium hydroxide extracts contained most of the non-starch, non-cellulosic polymeric glucose and since these two fractions had very similar composition, it should be possible to employ a simplified extraction procedure to obtain this glucose material. This would involve prior extraction with acetone, followed by hot ammonium oxalate to extract pectic material, and then 10% potassium hydroxide to extract the required 'glucan'. The hot water, sodium carbonate, and 25% potassium hydroxide extraction steps could be omitted. Such an extraction sequence might be equally applicable to hypocotyl tissue, as it was expected that needles and hypocotyl would have similar composition except for lower proportions of secondary wall components (and hence less mannose) in the elongating

Table 4: Monosaccharide % Of Total Neutral Carbohydrate In Pine Needle Extracts.

Monosaccharide	Hot Water	Ammonium Oxalate	Sodium Carbonate* Neutral	Ethanol	10% KOH
Rhamnose** Fucose**	1.5	15	3	8	3
Arabinose	27	21	14	39	16
Xylose	3	5	31	8	38
Mannose	31	23	20	10	11
Galactose	23	22	11	17	11
Glucose#	15	14	21	18	21
Starch##	-	-	6.3	4.5	8.4
Carbohydrate material (mg)	7	9	28	5	48

* The fraction has been divided into the precipitate obtained with acetic acid neutralisation and that obtained by the addition of ethanol.

** Rhamnose and fucose could not be separated on the column used.

Includes glucose from starch.

% of total neutral carbohydrate (Found by decrease in glucose after addition of pig pancreatic α-amylase and subsequent dialysis of hydrolysed sample.).

- Iodine negative test indicated no starch.

hypocotyls. This method (procedure A) was therefore adopted in the earlier work with hypocotyl (section 3.2.2).

The 80% ethanol supernatants (Fig. 16) were dialysed and the material recovered by freeze-drying to ensure no major polysaccharide fraction was unprecipitated. Gas-liquid chromatographic analysis revealed only trace quantities of polysaccharide of which a large percentage was arabinose. Many arabinans, for example that from Pinus pinaster var. maritima, are found to be soluble in relatively concentrated aqueous ethanol (Roudier and Eberhard, 1965).

3.2.2 Analysis Of Hypocotyl Of Different Ages

The sugar composition of carbohydrate fractions isolated with procedure A (section 2.5.2) are given in Tables 5, 6 and 7. The ammonium oxalate fraction consists primarily of arabinose- and galactose-containing polymers (Table 5B). These polymers are typical of those found in pectic fractions isolated from the cell walls of higher plants. Looking at the distribution of neutral sugars between the oxalate and potassium hydroxide fractions of two-week old plants (Table 5A), the data shows that oxalate extracted most of the starch plus much of the pectic polysaccharide. Xylose, mannose and glucose are almost exclusively found in the alkali extract.

As the etiolated plant sections age, the starch content decreases (Table 6). This starch decrease is matched by a corresponding increase in hemicellulose content. The composition of the hemicellulose fractions isolated from hypocotyl and cotyledon samples of different age are given in Table 7. Summarized below are the main points arising from the results presented in these tables.

(1) Starch is rapidly depleted in line with it being a storage polymer and not a structural polysaccharide of the cell wall. As the plant is growing in the dark no replenishment of the depleted starch reserves would be expected.

Table 5: Hypocotyl Fractionation

Carbohydrate Component	A* Average % extracted by Oxalate	KOH	B* Average % composition (by weight) of oxalate fraction (excluding starch)
Rhamnose Fucose	35	65	7
Arabinose	57	43	36
Xylose	7	93	6
Mannose	10	90	2
Galactose	49	51	42
Glucose	6	94	7
Total non-starch	25	75	100
Starch	92	8	

A Distribution of carbohydrates between oxalate and potassium hydroxide fractions.

B Monosaccharide composition of oxalate fraction.

* Both A and B show average values for several two-week and older hypocotyl and cotyledon samples

Table 6: Starch, Pectin And Hemicellulose Contents*

Sample	Hypocotyl				Cotyledon	
	1 week		2 weeks		1 week	2 weeks
	Upper	Lower	Upper	Lower		
Starch	7.5	0.3	0.8	0.1	8.9	1.3
Pectin**	1.4	1.7	1.8	1.7	2.3	2.7
Hemicellulose-B***	3.4	4.4	4.2	6.8	3.2	6.1
Hemicellulose B/Pectin	2.4	2.6	2.3	4.0	1.4	2.3

* Values are expressed as % by weight of total oxalate-plus alkali-extracted polymeric material.

** Carbohydrate extracted with ammonium oxalate.

*** Carbohydrate extracted with alkali not precipitated on neutralisation.

Table 7: Monosaccharide Composition Of Hemicellulose Preparations From Plants Of Different Age*

Monosaccharide	Hypocotyls 1 week Upper (107)	Hypocotyls 1 week Lower (107)	Hypocotyls 2 weeks Upper (127)	Hypocotyls 2 weeks Lower (127)	Cotyledons 1 week	Cotyledons 2 weeks (129)
Rhamnose Fucose	7	5	4	4	5	5
Arabinose	15	7	8	6	26	10
Xylose	42	40	36	35	20	30
Mannose	6	7	11	16	3	12
Galactose	15	11	11	10	26	15
Glucose**	16	30	29	30	21	28
Starch***	35	2	tr	tr	37	2
	→ Increasing age.					

* Expressed as weight % of total neutral monosaccharide.

** Excluding starch.

*** Starch glucose expressed as weight % of total neutral monosaccharides.

(2) There is an apparent decrease in the pectic material as a percentage of wall polysaccharide with an increase in the hemicellulose fraction. This is only an apparent change because cellulose was not quantitated. The percentage of cellulose in plant cell walls typically increases with maturity, particularly with the deposition of secondary wall. In etiolated tissue, the amount of secondary wall thickening is much reduced therefore the changes in cellulose levels cannot be predicted.

(3) There is an increase, with age, of the hemicellulose/pectin ratio. It almost doubles between young tissue and that tissue from the oldest region where secondary wall thickening would have progressed the furthest.

(4) The differences between the upper and lower regions in 4cm hypocotyls are quite marked. The two regions in the 7cm hypocotyls are very similar to each other and the lower 4cm sections, except in their mannose content. As the upper 2cm of hypocotyl is the actively elongating region only the upper sections of 4cm hypocotyls would consist of rapidly elongating cell walls. All other hypocotyl sections would have non-elongating cell walls undergoing limited secondary wall thickening. As a consequence an especially large difference is seen between the carbohydrate composition of hemicellulose from the youngest hypocotyl cell walls and those from other hypocotyl sections.

(5) Rhamnose/fucose, arabinose and galactose all decrease as a percentage of wall polysaccharide in line with the decreasing percentage content of pectic material. It is likely that a high proportion of these monosaccharides in the hemicellulose fractions are components of pectic polysaccharides not extracted with ammonium oxalate due to covalent linkage with hemicellulosic polysaccharides (Talmadge et al., 1973).

(6) Xylose content stays fairly constant while both the mannose and non-starch, non-cellulosic glucose increase rapidly with tissue age. The glucose undergoes almost all its increase as the cell wall ceases elongation while the mannose percentage continues increasing constantly with age.

(7) Cotyledon analysis shows marked differences to the hypocotyl data. Cotyledons show much more (a) oxalate-extractable material i.e. pectic substances and (b) arabinose and galactose in their alkali extracts. This results in a lower hemicellulose B/pectin ratio. With increasing tissue age cotyledons undergo increases in typical hemicellulosic monomers (xylose, glucose and mannose) and large decreases in 'pectic monomers' (arabinose and galactose). This is expected because cotyledon cell walls are much younger than the hypocotyl tissue - undergoing elongation only after the first week of hypocotyl growth.

(8) The percentage of carbohydrate recoverable from the isolated material was not very high. The carbohydrate content only reached a maximum of around 40% by weight of the total material extracted in the oldest hypocotyl sections. Most of the non-carbohydrate material may have been protein, although there could have been some lignin and non-extracted lipid material. The hydroxyproline containing cell wall protein found in many higher plants (Lamport, 1970) contains both arabinose and galactose as sidechains. This protein is sometimes extracted in the alkali fraction and could explain partly why these results show both a low carbohydrate yield and the presence of arabinose and galactose. It is possible that the extraneous material might interfere with hydrolysis and quantitation of the derivatives. To improve on the quantity of recoverable carbohydrate in the extracts an alternative extraction procedure (procedure B) was therefore instigated.

3.2.3 Procedure B Extraction Of Hypocotyls

Only fully extended hypocotyls were extracted by procedure B (section 2.5.3) as the small differences between the upper and lower hypocotyl of 7cm seedlings (Table 7) suggested a longitudinal homogeneity in the hypocotyl at this stage of development. It also gave increased material for the isolation of the 'glucan' apparent in the hemicellulose fractions. Results in Table 8 show that the recovery of carbohydrate was increased only slightly by procedure B. However, procedure B did cause the mannose content present in the hemicellulose fraction to decrease when compared with procedure A fractions. This decrease could be accounted for to some extent by a relatively high yield of mannose in the initial buffer plus oxalate extracts (see Table 8 and compare Table 5B). As this mannose component was very difficult to separate from the 'glucan' with the fractionation methods used here (see Chapter 4), the alternative procedure provided an attractive route to a more homogeneous final 'glucan' fraction.

We may note the composition of the hemicellulose fraction of hypocotyl extracted by procedure B is quantitatively similar to that for upper and lower segments of 7cm hypocotyl sections (see Table 7 and 8). As the fractions were obtained using non-specific alkali extraction, this similarity in results demonstrates in particular the defined nature of the hemicellulose cell wall polysaccharides at this stage of cell wall development.

3.3 Discussion

The major aim of these extractions from various aged tissues was to find a non-cellulosic 'glucan' fraction which showed 'turnover', in elongating gymnosperm primary cell walls. A non-starch, non-cellulosic glucan fraction does seem to have been demonstrated by the results in Table 7. These results also indicate that the 'glucan' increases with age (notably between the upper elongating zone and the older lower non-elongating zone of 4cm hypocotyls). It is arguable whether

Table 8: Carbohydrate Composition Of Fractions Isolated From Cell Walls Prepared By Procedure B.

Monosaccharide	Hypocotyl			Cotyledon		
	Buffer	Oxalate	Alkali*	Buffer	Oxalate	Alkali
Rhamnose Fucose	11**	14	5	10	15	9
Arabinose	23	25	11	21	35	18
Xylose	4	5	36	4	4	30
Mannose	4	9	4	6	9	5
Galactose	42	30	11	30	22	16
Glucose	15	16	33	30	15	24
Starch***	1	7	tr	2	5	tr
% carbohydrate in isolated material	31	9	40	8	8	20
% distribution carbohydrate	40	9	51	37	17	47

* Fraction C.

** Values are weight %.

*** Starch glucose is weight % of total neutrals.

we can strictly speak of 'turnover' of the cell wall polysaccharides here when a degradation of these polysaccharides has not been demonstrated although it is known that both synthesis and breakdown of polysaccharides occur together and lead to wall turnover during elongation, (Lamport, 1970). The change in monosaccharide composition indicates only a change in net carbohydrate and does not tell which polymers are altering in level. However, the large percentage increase found in the glucose content between elongating cell walls and those cell walls which have ceased elongating, would suggest a major glucose-containing polysaccharide intimately involved in the elongating cell wall. Whether this 'glucan' undergoes turnover as shown by the xyloglucan from pea epicotyl (Labavitch and Ray, 1974) it is impossible to say.

The increase in the glucan fraction here is in marked contrast to the rapid decrease observed, for example, in elongating Phaseolus aureus hypocotyl (Nevins et al., 1968; Franz, 1972), Avena coleoptile (Loescher and Nevins, 1972), Hordeum (barley) coleoptile (Sakurai and Masuda, 1978) and Zea mays (corn) first leaf lamina (Nevins et al., 1968). It is however noteworthy that glucose polymers undergoing rapid gross content changes, seem to be an attribute of elongating primary cell walls in angiosperm and a representative gymnosperm - Pinus radiata.

Even though mannose, a known secondary wall component, continually increases with age, the glucose component no longer undergoes any gross changes in cell walls which are not undergoing elongation. Since Harwood (1972 and 1973) has shown that mature Pinus radiata wood contains mainly glucomannan and arabino-(4-O-methyl glucurono)xylan with no major non-cellulosic glucan present it is quite clear that the primary gymnosperm cell-wall contains a major polysaccharide not found in secondarily-thickened wall. This 'glucan', present in the early stages of growth could either be degraded in the cell wall by the action of glucanases known to be present in cell walls (Clarke and Stone, 1962; Buchala and Meier, 1973) or it could be diluted by the

formation of other hemicelluloses during plant maturation.

Further investigation was carried out to purify and analyse the 'glucan' structure in line with the aim of the thesis. Although the experiments were not designed to study the whole picture of carbohydrate 'turnover' some pertinent points can be raised.

Nevins et al. (1968) found large changes in the proportion of various monosaccharides accompanying hypocotyl cell wall elongation. After cessation of elongation however, there was little change in the relative proportions of sugars.

Consistent with the results found for Pinus radiata hypocotyl cell wall changes, previous workers have found a decrease in arabinose with wall maturation (Nevins et al., 1968; Reid and Wilkie, 1969) and established clearly that mannose and xylose become predominant in the secondary wall (Northcote, 1969). This has been essentially what has been found with Pinus radiata hypocotyl and confirms that the changes occurring in etiolated tissue mimic those in normal growing tissue.

Mannose is usually an indicator of secondary wall growth (see, for example, Thornber and Northcote, 1961). With regard to seedling hypocotyls, Albersheim (1976) using methylation analysis, compared the cell walls of 8-day-old hypocotyls of Red Kidney bean with those of Red Kidney bean cell-suspension cultures. The major difference between the two resulting chromatograms is a large 4-linked mannose peak present in the hypocotyl tissue. As cultured cells can be grown as a homogeneous tissue possessing primary, but no secondary, walls (Albersheim, 1976) the 4-linked mannose would originate from secondary wall thickening. The Pinus radiata hypocotyl mannose probably originates from an alkali-soluble glucomannan similar to that present in mature wood (Harwood, 1973) and possibly more significantly, a water-soluble galactoglucomannan (compare with that isolated from the wood of Pinus pinaster var. maritima by Roudier and Eberhard, 1967), as extraction procedure B removed a large proportion of the mannose in the buffer and oxalate extracts.

In Pinus radiata hypocotyl the glucomannan (or galactoglucomannam) and xylan are only a minor proportion of the

total hemicellulose. The 'glucan' is the major polymer present with greater than 60% of the hemicellulose content (see Chapter 4). However, in Pinus radiata wood only the glucomannan and arabino-(4-O-methyl glucurono)-xylan seem present in large quantities - the 'glucan' has either been degraded or 'diluted out' by the formation of other hemicelluloses. These two results combined give a simplified analysis of the hemicellulose changes from the Pinus radiata hypocotyl primary cell wall through to the highly secondarily-thickened cell wall of wood. Thornber and Northcote (1961a and b) working on Pinus ponderosa analysed the hemicellulose changes during development of a cambial cell into a mature secondary-thickened heartwood cell. As found with Pinus radiata hypocotyl, glucose and xylose were dominant in cambium wood hemicellulose fractions. From cambium to heartwood, glucose decreased greatly, together with arabinose and galactose, while xylose, uronic acid and especially mannose increased. Thus, in concordance with the proposed 'disappearance' of the Pinus radiata primary cell wall glucan, Pinus ponderosa also undergoes a similar hemicellulose change. Other changes are also in line with those expected from the development of Pinus radiata primary cell wall to secondarily-thickened cell wall.

The pectic polymers of all higher plants contain predominantly rhamnose, galactose and arabinose as neutral sugar components. It is likely these monomers present in the oxalate and alkali fractions, mainly arise from pectic polymers present in the Pinus radiata hypocotyl as very little secondary wall growth has occurred. In this work, rhamnose (and/or fucose), arabinose and galactose all decrease as a percentage of extracted carbohydrate with increasing tissue age. Similarly, Thornber and Northcote (1961) found pectic substances are 'lost' during Pinus ponderosa cell-wall secondary thickening.

3.4 Appendix: Discussion Of Polysaccharide Analysis

3.4.1 Polysaccharide Extraction

Two aqueous extractants were used on the Pinus radiata hypocotyl tissue for examining the cell wall constituents: ammonium oxalate and potassium hydroxide.

In the cell wall Ca^{++} neutralizes the negative charges and allows association of acidic polymer chains. Aqueous ammonium oxalate is a Ca^{++} chelating agent and therefore causes mutual coulombic repulsion of adjacent galacturonic acid containing polymers resulting in their solubilisation. Using ammonium oxalate causes 'salt' contamination of the residue and therefore extracts in this work were dialysed exhaustively when quantitating material. The ammonium oxalate fraction contains mostly pectic material. Solutions of oxalic acid, ammonium citrate, fluorides, arsenates, phosphates and ethylenediaminetetraacetic acid have all been employed as extractants for pectic substances.

Alkaline extraction solubilises carbohydrates in what is usually termed the hemicellulose fraction. Alkali is known to cause degradation of hemicelluloses (Whistler and BeMiller, 1958). One reaction, that of alkaline hydrolysis of glycosidic bonds, was found to be only significant at elevated temperatures (Hansson and Hartler, 1968). The more important reaction is degradation at the reducing end of the carbohydrate chain i.e. "peeling reaction". This is prevented by the addition of sodium borohydride which will convert the reducing end monomer to an alditol (Zinbo and Timell, 1965). All alkaline extracting solutions used in this work contained sodium borohydride for this reason.

This extraction scheme using mild extractants was used to isolate the structural polymers with the minimum of modification or degradation. In order to avoid oxidation and the onset of 'horniness', the residues after each extraction step were not air-dried. Instead a solvent-exchange procedure was used (section 2.2.1).

3.4.2 Polysaccharide Hydrolysis

Analysis of the various polysaccharide extracts were carried out as described in Materials and Methods. The hydrolytic step has been considered the main source of loss in carbohydrate analysis. Since glycosidic bonds vary greatly in their stability to acid hydrolysis (e.g. Conrad et al. (1966) calculated that the L-fucopyranosyl bond is hydrolysed 300x faster than the D-glucosyluronic bond in 0.5M sulphuric at 100°C) and different monosaccharides are destroyed at different rates, the conditions chosen for the hydrolysis of complex polysaccharides are inevitably a compromise between destruction of the less stable sugars and incomplete hydrolysis of the more resistant glysosidic bonds.

The acid hydrolysis of intact cell walls presents particularly severe problems, which have led to the development of combined enzymic-acid hydrolysis procedures (Jones and Albersheim, 1972) and methods using concentrated mineral acid at low temperatures (Mares and Stone, 1973). They are reported to be efficient procedures although D. R. Fenemor found them inefficient on Pinus radiata hypocotyl tissue (personal communication). Dilute acid can be used provided that correction factors are established for the decomposition of each monosaccharide under the conditions of hydrolysis used (see Dutton, 1973).

When polysaccharides containing uronic acids are hydrolysed, a further ambiguity is introduced, in that only partial cleavage of the glycosiduronic acid linkage may occur. For this thesis, nitric acid in combination with urea was used. Nitric acid and urea has been reported to give nearly quantitative conversion to monosaccharides for soluble neutral monosaccharides.

A degradation correction factor was worked out for each monosaccharide found, under the hydrolysis conditions used (see Table 3). The problem of glycosiduronic acid hydrolysis was not of any major significance in this thesis because (1) uronic acids could be adequately quantitated in the

unhydrolysed polysaccharides by a spectrophotometric assay using m-hydroxydiphenyl (section 2.3.2), and (2) the xyloglucan fraction, with which a structural investigation was carried out, contained very little, if any, uronic acid.

3.4.3 Gas Liquid Chromatography

3.4.3.1 Preparation Of Volatile Derivatives

The successful separation by gas-liquid chromatography of the monosaccharides formed from polysaccharide hydrolysis depends on two major techniques. In one, the monosaccharides are converted to their trimethylsilylated derivatives quantitatively (Sweeley et al., 1963). However two drawbacks to the use of silyl ethers are their reactivity with water (Holligan, 1971) and the fact that each aldose will give the α and β anomers of the pyranose and possible furanose forms. This means that where complex polysaccharides or polysaccharide fractions are involved there is going to be a large number of peaks with some not resolved. In the second approach monosaccharides are converted quantitatively to their alditol acetate forms. This method removes the anomeric centre and therefore gives only one alditol for each pyranose or furanose form enabling complex carbohydrate fractions to be easily analysed.

As the polysaccharide fractions analysed in this thesis were very complex, monosaccharides were converted to their alditol acetates for analysis. The disaccharides, separated by paper chromatography after partial hydrolysis (section 2.3.6), were converted to their silyl ethers as the double peaks formed gave greater accuracy in identification.

After acetylation, water was added to decompose the excess acetic anhydride (which may give a peak close to that of mannose) and both pentitol and hexitol acetates extracted into methylene chloride quantitatively (Borchardt and Piper, 1970).

3.4.3.2 Monosaccharide Quantitation

As a stationary phase, OV-225 (a cyano silicone polymer) was used rather than ECNSS-M because of its greater stability with high temperatures. Later, SP2340 became available and was used to effect separation of rhamnitol and fucitol peracetates which could not be separated on OV-225.

The most desirable property required for the internal standard in g.l.c. is that it is a sugar not in the sample, capable of being reduced and acetylated, and having a chromatographic mobility between those of the pentose and hexose derivatives under the g.l.c. conditions employed. With the OV-225 column, 2-deoxy-D-glucitol-pentaacetate meets these criteria exactly (see Fig. 12) and was therefore used in this thesis.

The main errors with quantitative g.l.c., other than from losses during hydrolysis, are in measuring amount of sample, evaluating the peak size and determining molar (or weight) response factors (Boček et al., 1969). In this work to minimise the errors in sample measuring, samples were dried to constant weight in vacuo over potassium hydroxide or phosphorous pentoxide. Of the seven methods available in determining peak areas (Mefferd et al., 1968) the rectangular method was used for this thesis. This method was simple and accurate without the need for an integrator. Weight-response factors were obtained for each derivative using the exact experimental conditions to be employed for subsequent analyses.

3.4.4 Starch Analysis

As the primary aim of the thesis was to investigate a primary cell wall gymnosperm 'glucan' it was very important to monitor the proportion of glucose derived from starch. The simplest, most accurate methods of starch analysis rely on enzymic hydrolysis (Dekker and Richards, 1971) and this was the technique adopted in this work (section 2.3.3). Amyloglucosidase hydrolyses starch to its component glucose and

glucose oxidase (specific for β-D-glucose) converts this to gluconic acid and hydrogen peroxide. A chromogen is oxidised in the presence of both peroxidase and hydrogen peroxide (Fales and Seligson, 1963). During the amyloglucosidase treatment of starch, the β-D-glucose produced is mutarotated to α-D-glucose which is not a substrate for glucose oxidase. Although its mutarotation to β-D-glucose at pH 7.0 is slow, this is not a problem as commercial glucose oxidase preparations contain a mutarotase as an impurity. In the presence of the mutarotase α- and β-D-glucose are oxidised at the same rate (Dahlqvist, 1961). Solubilisation or gelatinisation of the starch prior to hydrolysis either by boiling (MacRae, 1971) or by treatment with alkali (Dekker and Richards, 1971) can cause inhibition of the glucose oxidase in subsequent analysis. Inhibitors are removed with a charcoal wash (Dekker and Richards, 1971) as in section 2.3.3.

In this work the starch was only in very minor amounts in most xyloglucan fractions (less than 5% of the total glucose (see Table 9)). Fractionations or enzyme treatment to remove it were therefore not required.

CHAPTER 4

PURIFICATION OF THE XYLOGLUCAN

4.1 Introduction

In Chapter 3 the alkali-extracted hemicellulose B fractions of etiolated Pinus radiata hypocotyl cell walls, were found to be very high in a non-starch, non-cellulosic glucan. In order to characterise this hemicellulose glucan component, it was necessary to purify it as much as possible The fractionation procedures used, are described in this chapter.

Most hemicelluloses extracted from plant tissues are mixtures of different polysaccharides which are usually resolved into their components by fractional precipitation from an aqueous solution. Several such methods were tried in this work, initially to give a separation of the secondary wall component containing mannose from the primary wall 'glucan'.

Barium hydroxide and copper (Fehling solution) are widely used for fractionating and purifying polysaccharides by precipitation as an insoluble metal complex. Barium hydroxide has been found especially valuable for the precipitation of polysaccharides containing β-(1,4)-linked D-mannose residues due to the reaction of the barium ions with the vicinal cis-hydroxyl groups on carbon atoms 2 and 3 of the mannose units (Meier, 1958). As well, gymnosperm arabinogalactans and arabino-(4-O-methyl glucurono)-xylans are not precipitated (Meier, 1965) enabling a separation of the mannose polymers from the xylans (Timell, 1961). This was particularly important in relation to this work. The use of copper as a precipitating agent (Jones and Stoodley, 1965) did not seem to offer this same degree of specificity and was therefore not used.

Cetyl trimethyl ammonium bromide (Cetavlon) was used in conjunction with borate for separation of neutral polysaccharides from those polysaccharides with high proportions of uronic acid residues. Cetavlon is a quaternary ammonium

salt with a long lipophilic tail which forms insoluble salts with acidic polysaccharides (Jones, 1953). Neutral polysaccharides do not react except as borate complexes. The precipitation of the neutral polysaccharides as borate complexes is dependent on pH and the configuration of the glycol group. Cis-glycols precipitate at a pH < 8.5 (Scott, 1965).

Gaillard (1961) showed that when hemicellulose B was dissolved in aqueous calcium chloride and then iodine/potassium iodide solution added, a precipitate resulted which contains all those linear species including glucans, which did not enter the hemicellulose A fraction. The supernatant contains all the short chain or branched polysaccharides. Results from angiosperm cell wall fractionation showed most of the uronic acid was unprecipitated (Gaillard, 1965). This procedure was adopted on the hypocotyl fractions extracted by procedure B because of their already low mannose content (see section 3.2.3).

The homogeneity of polysaccharide mixtures can be determined by such methods as ultracentrifugation (Adams, 1960) and zone electrophoresis (Northcote, 1965) or by continual fractionation until no further change in composition occurs. In this work, two electrophoresis methods were tried to monitor homogeneity of purified fractions. If the polysaccharide is homogeneous, ethanol fractionation precipitates it as a single peak over a relatively narrow ethanol concentration range. Ethanol fractionation, carried out at pH 7.0 where the polysaccharides are most stable and the carboxyl groups, present in the hemicelluloses containing uronic acids, are in the form of ionised salts, was used here to test the glucan homogeneity as well as a general fractionation procedure.

Hemicellulose extracts to be fractionated were selected for their high glucan and low starch content. Alkali extracts were divided into two fractions by neutralization; hemicellulose A and B. Hemicellulose A are those polysaccharides precipitated by acidification of the alkali extract. They are usually long chain xylans presumably precipitated due to protonation of the uronic acid side groups allowing intra- and inter- chain alignment. No major hemicellulose A fraction

was obtained with Pinus radiata hypocotyl in any of the work described here. This may be related to the lack of secondary wall development. Hemicellulose B was that carbohydrate which remained in solution. All further fractionations were performed on hemicellulose B extracts.

4.2 Results

Pinus radiata hypocotyl hemicellulose B fractions contained predominantly glucose and xylose as neutral components (Table 7) with galacturonic acid the dominating acidic sugar (I. G. Andrew, personal communication).

Barium hydroxide: Hemicellulose B extracts '107' and '127' (Table 7) were pooled to give fraction 'A' and fractionated with barium hydroxide (section 2.6.1). Most of the carbohydrate was precipitated (84%) with only 16% isolated from the supernatant (Table 9). There was an accumulation of xylose and arabinose in the supernatant, while the precipitate contained predominantly glucose and xylose. As expected most of the mannose (> 96%) was precipitated. Of special note was the finding that 96% of the non-starch glucose precipitated with 70% of the total xylose. As the arabino-(4-O-methyl glucurono)-xylan from gymnosperm wood is not precipitated by barium hydroxide and the high xylose content (67%) together with a fairly high arabinose content (14%) in the supernatant indicated that any hypocotyl xylan present remained in solution, the results suggest the 'glucan' may be a xyloglucan. Evidently the xyloglucan precipitates with barium hydroxide just as glucomannan does and is therefore not separable. In the literature (see Barker et al., 1957), some glucans and mannans are separable as their borate complexes by selective cetavlon precipitation therefore this method was used on a further hemicellulose B extract.

Cetavlon and borate: Hemicellulose B cotyledon extract '129' (Table 7) referred to as Fraction 'B' was fractionated with the use of cetavlon and borate (section 2.6.2). Cetavlon, which forms insoluble salts with acidic polysaccharides,

Table 9 : Composition Of Hemicellulose B Fractions

Fraction	(Mole %) Sample Rha.	Fuc.	Ara.	Xyl.	Man.	Gal.	Glc.	Uronic acid'	Starch'	% Dist. Neutral CHO
A1	4.7		3.9	32.2	13.0	9.4	36.8	-	0.9	84
A2	(+)		(+)	(+)	(+)	(+)	(+)	-	-	0
A3	4.8		13.7	66.9	2.5	5.3	6.7	-	4.8	16
A1-1	2.0	1.9	13.3	27.0	15.2	13.0	27.6	1.6	0.4	9
A1-2	0.5	3.7	4.3	33.2	12.9	9.4	36.0	3.6	0.9	26
A1-3	1.2	3.5	1.7	30.7	14.3	9.3	39.3	2.1	0.6	52
A1-4	0.0	3.4	4.1	45.9	6.8	6.4	33.4	1.5	0.3	13
B1	5.7		27.9	14.3	12.1	16.1	15.3	36.6	1.0	13
B2	6.0		4.6	27.1	13.9	12.6	35.7	3.5	0.8	62
B3	4.1		9.1	45.9	4.1	9.8	27.0	5.4	6.7	25
C1	1.2	1.5	39.9	18.7	13.8	10.1	14.9	6.2	1.2	9
C2	0.3	4.7	6.6	42.8	2.8	9.9	32.9	3.9	(+)	71
C3	1.3	1.4	15.1	56.7	2.8	8.3	14.3	17.2	0.6	20
C2-1	2	3	19	31	3	12	30	12	(+)	2
C2-2	1	4	15	32	5	14	29	11	(+)	6
C2-3	0	6	3	36	4	11	40	2	0	69
C2-4a	0	4	2	43	1	6	44	-	-	4
C2-4b	0	2	7	72	1	4	15	6	0	12
C2-4c	0	1	11	70	1	3	13	4	0	7
C2-3a	0	8	2	37	4	10	40	-	0	86
C2-3b	0	7	4	38	3	11	37	-	0	14

' Expressed as % of neutral carbohydrate.

(+) Negligible values.

- Not determined.

Scheme 1 : Fractionation Of Hemicelluloses

			Dominant** Component	Other Major** Components
Fraction 'A' (46.6mg)*				
+ $Ba(OH)_2$	A1	(27.2mg)	Xyloglucan	Mannan
+ HOAc — pH 5	A2	(nil)		
+ EtOH — 80%	A3	(4.8mg)	Xylan	
Fraction 'A1' (20.9mg)				
+ Cetavlon	A1-1	(1.8mg)		
+ Borate — pH 8	A1-2	(4.5mg)	Xyloglucan	Mannan
+ Borate — pH 10	A1-3	(9.0mg)	Xyloglucan	Mannan
+ EtOH — 80%	A1-4	(2.2mg)	Xyloglucan	Xylan
Fraction 'B' (20.4mg)				
+ Cetavlon	B1	(3.5mg)	Galacturonan	Arabinan
+ Borate — pH 8		(nil)		
+ Borate — pH 10	B2	(9.1mg)	Xyloglucan	Mannan
+ EtOH — 80%	B3	(5.0mg)	Xyloglucan	Xylan
Fraction 'C' (89mg)				
Insoluble $CaCl_2$	C1	(7.9mg)	Arabinan	Miscellaneous
+ I_2/KI	C2	(59.9mg)	Xyloglucan	Xylan
+ EtOH — 80%	C3	(19.4mg)	Xylan	
Fraction 'C2' (59.9mg)				
+ Cetavlon	C2-1	(1.3mg)	Xyloglucan	{ Pectic
+ Borate — pH 8	C2-2	(3.3mg)	Xyloglucan	{ Components
+ Borate — pH 10	C2-3	(32.7mg)	Xyloglucan	
+ EtOH — 40%	C2-4a	(1.9mg)	Xyloglucan	
+ EtOH — 50%	C2-4b	(5.7mg)	Xylan	
+ EtOH — 80%	C2-4c	(3.2mg)	Xylan	
Fraction 'C2-3' (28.7mg)				
+ EtOH — 40%	C2-3a	(24.0mg)	Xyloglucan	
+ EtOH — 80%	C2-3b	(3.9mg)	Xyloglucan	

* Yield of total non-starch carbohydrate shown in parenthesis.

** Major polysaccharides deduced from monosaccharide analysis.

precipitated 70% of the uronic acid from fraction 'B' (Table 9). This high uronic acid fraction (B1) was analysed and shown to be predominantly galacturonic acid (I.G. Andrew, personal communication). The cotyledons were still elongating when extracted and some pectic components would be expected to appear in the alkali fraction, possibly due to covalent bonding to hemicellulose polymers (Talmadge et al., 1973). Both the borate precipitate (B2), with 62% of the neutral carbohydrate, and the ethanol precipitate (B3) had high contents of glucose and xylose, although the respective ratios were quite different. The high glucose and xylose content of B2 suggests again that the 'glucan' shown in the hemicellulose extracts of elongating hypocotyl is a xyloglucan. The high glucose and xylose content of B3 with a ratio of glucose to xylose of 0.6 : 1 indicates the presence of both xyloglucan and xylan. The xyloglucan has again coprecipitated with most of the mannose (77%). However, results do show that most of the glucose (68%) was precipitated in a fraction separate from most of the galacturonic acid, arabinose, starch and xylan.

Owing to the limited success of this fractionation, the barium hydroxide precipitated carbohydrate (A1) was subjected to a cetavlon/borate fractionation (Scheme 1). However, fractionation of the borate precipitate between pH 8 and pH 10 could not achieve any further separation of the mannan and glucan polymers (Table 9).

At this stage of the project, hemicellulose B extracts had been obtained from the whole hypocotyl by procedure B and found to contain low mannose contents. Thus a specific fractionation procedure to separate the mannose component was not as imperative. The procedure of Gaillard and Bailey (1968) offered a very specific separation by iodine-complex precipitation of any linear 1,4-linked polymers from the hemicellulose B extracts. If the Pinus radiata hypocotyl xyloglucan was indeed like those isolated from other species it would be precipitated, with highly branched polymers and possibly uronic acid containing polysaccharides remaining in solution (see results of Gaillard, 1965).

Iodine and potassium iodide: Hemicellulose B obtained from whole hypocotyls (fraction 'C', Table 8) was fractionated with iodine and potassium iodide (section 2.6.3). Results presented in Table 9 show that the xyloglucan was precipitated as a dark blue precipitate leaving most of the xylan component in solution together with a large proportion of uronic acid. Ratios of glucose : xylose in precipitate C2 suggest that some xylan has also coprecipitated with the xyloglucan, together with most of the mannose. Starch contamination was very minor, with most remaining in the supernatant (C3) with a negligible amount present with the xyloglucan.

The material insoluble in aqueous calcium chloride, containing 9% of the total carbohydrate, had a large proportion of arabinose. As arabinans are very soluble polymers, it is possible that this precipitated arabinose arises from arabinose oligosaccharides bound to a hydroxyproline containing protein similar to that found in many higher plants and known to accumulate in cell walls which have ceased elongating (Sadava et al., 1973). D. R. Fenemor (personal communication) has found a high content of hydroxyproline in purified cell walls from Pinus radiata callus tissue-cultures.

Ethanol: Further fractionation to achieve an homogeneous xyloglucan was carried out on C2 using the cetavlon-borate method in combination with ethanol precipitation (Scheme 1). Fraction C2 gave six precipitates on fractionation (Table 9). The first two precipitates contained all the rhamnose and high quantities of arabinose and uronic acid, indicating the precipitation of mostly pectic material. Fairly high proportions of xylose and glucose show some glucan coprecipitation. Fractions C2-3 and C2-4a both show the precipitation of a glucan with a composition closely resembling that expected from a purified xyloglucan. The absence of rhamnose and negligible amounts of mannose and arabinose provide evidence for a fucogalactoxyloglucan. The two remaining ethanol precipitates contained the xylan found in most hemicellulose B extracts.

To test the homogeneity of the xyloglucan isolated in

fraction C2-3, further fractionation was carried out by ethanol precipitation (Scheme 1). Most of the polysaccharide precipitated over a narrow ethanol concentration (<40% ethanol) with little change in monosaccharide composition occurring. The higher ratio of xylose to glucose in fraction C2-3b still indicates the presence of a xylan but the content is very small.

Electrophoresis: Electrophoresis was carried out to monitor polysaccharide homogeneity during the glucan purification. Although standard polysaccharides such as an arabinoxylan (wheat flour), a galactomannan (carob bean) and laminarin were separated into separate bands no extracted Pinus radiata material showed separation into definable bands. A single wide diffuse band was usually observed. As results gave no indication of a quick reliable method of monitoring the polysaccharides during their fractionation, the electrophoresis procedures were abandoned.

4.3 Discussion

Results presented in this chapter suggest that the major hemicellulosic primary cell wall 'glucan' of etiolated Pinus radiata seedlings is a xyloglucan rather than a mixed-link glucan. The presence of high contents of both fucose and galactose coprecipitating throughout the purification procedure with the xyloglucan suggest a fucogalactoxyloglucan similar to that isolated from dicot tissue such as Phaseolus aureus hypocotyl (Kato and Matsuda, 1976).

The evidence so far available for the homogeneous nature of the xyloglucan rests on its constancy of composition on repeated fractionation, an admittedly unsatisfactory situation. However, the ratios of the sugars fucose, galactose, xylose and glucose remain fairly high and constant under all the different fractionation procedures. The other sugars vary constantly in ratio and in the more purified fractions either disappear (as with rhamnose) or are only present in minor amounts (such as arabinose, mannose and uronic acid),

* ose and β-D-glucopyranose residues. Probably owing to this structural feature, it has meant little separation has been achieved between the xyloglucan, with a linear backbone of (1-4)-linked β-D-glucopyranose

indicating they are probably not associated with the xyloglucan.

Although the nitric acid hydrolysis procedure used here enables the quantitative determination of the monosaccharide composition of isolated carbohydrate fractions, there is a contribution from contaminating polysaccharides hard to separate from the xyloglucan. This results partly from the presence of both primary and secondary cell wall hemicelluloses (which appear to be different polymers) and partly from the fractionation techniques used. The results in Table 9 indicate the presence of at least three hemicellulosic polysaccharides, one the xyloglucan and the other two, similar polymers to those found in mature Pinus radiata wood; a xylan and a 'mannan'. The xyloglucan is easily separated from the xylan and from pectic components (methylation analysis also gave a separation between an arabinan and the xyloglucan (see Chapter 5)), but less easily separated from the 'mannan' component.

Mannose is a known secondary wall component and as such, the mannose residues are probably dirived from either a water-soluble galactoglucomannan or an alkali-soluble glucomannan (Harwood, 1973). The glucomannans in the xylem of all gymnosperms consist of a framework of (1-4)-linked β-D-mannopyran-* residues (see Chapter 5), and the mannan component. Further, Gould et al., (1971) have suggested that xyloglucan-iodine complex formation involves the interaction of iodine within the interstices between aggregated xyloglucan chains and xyloglucans have been demonstrated to hydrogen-bond with cellulose (Valent and Albersheim, 1974). Cellulose and glucomannan have very similar structures. It seems logical to expect glucomannan to also interact with the xyloglucan by hydrogen-bonding and therefore aggregate, leading to difficulties in separation when using fractional precipitation from aqueous solvents. It is of note that Perila and Bishop (1961) on their enzymic hydrolysis of a purified glucomannan from Jackpine obtained presumed xyloglucan fragments in the products.

Possibly due to the above reasons, in this project the xyloglucan and mannan were both precipitated with barium

hydroxide - a compound known to form an insoluble metal complex with gymnosperm glucomannan. Similarly, a 'galactoxyloglucan' was precipitated by Schreuder et al. (1966) in their fractionation with barium hydroxide of an alkali extract from compression wood of red spruce (Picea rubens Sarg.).

It was also found that the Pinus radiata xyloglucan and 'mannan' could not be separated as a borate complex over two pH values, that both formed an insoluble complex with iodine/potassium iodide and that both precipitated together over a narrow ethanol concentration range. Ramalingam and Timell (1964) in an attempt to effect a better resolution of their glucan from Picea engelmanni bark, used numerous fractionation methods similar to those used in these studies. While the composition of galactose, xylose and glucose remained remarkably constant, contaminants such as mannose, arabinose and uronic acid were always present.

Whatever the reason for the lack of success in separation, one fraction (C2-4a) shows a very low mannose content strongly suggesting that mannose is associated with a different polymer and is not an integral part of the xyloglucan.

As it turned out, the use of extraction procedure B, initially used to increase the carbohydrate purity of samples, led to a good separation of the xyloglucan from the mannose component, no longer making it imperative to separate the two polymers. No further techniques were attempted to give a separation because (a) time did not permit, and (b) the small mannose content would not interfere too much in the structural determination.

Realising that the Pinus radiata primary wall hemicellulose is a xyloglucan, future work could incorporate further fractionation on hemicellulose B extracts by ion-exchange chromatography on DEAE-sephadex (see Bauer et al., 1973) in order to separate the neutral xyloglucan from those polysaccharides which contain significant amounts of uronic acid. (The cetavlon procedure, used here for the same purpose, does not seem to be as selective.) The xyloglucan thus obtained would be further purified by column chromatography on cellulose

(to make specific use of its hydrogen-bonding ability) as described by Aspinall et al., (1969).

Monosaccharide analysis of the Pinus radiata hemicellulose material also demonstrated the presence of a xylan in several fractions similar to that isolated by Harwood (1972) and Haslemore (1974) from mature Pinus radiata wood. Evidence for a similarity between the supernatant hypocotyl xylan and the wood xylan comes from the xylose to arabinose ratios. R. Haslemore and V. Harwood obtained ratios of 4.8 and 5.3 respectively, which is in good agreement with the ratio of approximately 5 obtained from the hypocotyl xylan (Table 10). Similarly, as found with the hypocotyl xylan, the arabino-(4-O-methyl glucurono)-xylan from mature wood is not precipitated with barium hydroxide. The xylose to uronic acid ratio would not give such a reliable comparison as Thornber and Northcote (1961b) found the xylans formed during secondary thickening of Pinus ponderosa appeared to contain greater amounts of uronic anhydride than that deposited in the primary cell wall.

It is of note that the xylan precipitated by iodine/potassium iodide (C2-4b and C2-4c) with low arabinose : xylose ratios, was evidently more linear (i.e.with fewer arabinose sidechains) than the 'average' xylan from the hypocotyl. Wilkie et al., (1979) and others, have found that the arabinoxylan from monocot primary cell walls can be separated into fractions with wideranging arabinose to xylose ratios.

The lack of good separation found with electrophoresis is not inconsistent with a purified xyloglucan. Wide and continuous variation in the structure of plant polysaccharides is an established fact, even if in many cases it results from partial degradation during the extraction process. This possibly was the underlying reason for unsatisfactory results i.e. a wide diffuse band.

Analysis of the hemicellulose B fractions from whole hypocotyls (C1, C2 ...etc) has shown that greater than 60% of the hemicellulose carbohydrate is composed of xyloglucan. This makes it a major hemicellulosic polymer in the Pinus radiata elongating primary cell wall.

Table 10: Mole Ratios Of Xylan Fractions

Fraction	Rha.	Fuc.	Ara.	Xyl.	Man.	Gal.	Glc.	Uronic Acid
A3	7		20	100	4	9	10	nd
C3	2	3	27	100	5	15	24	25
C2-4b	0	2	9	100	1	5	21	7
C2-4c	0	2	15	100	2	5	19	5

Arabino-(4-O-methyl glucurono)-xylan from Pinus radiata wood.

Literature	Rha.	Fuc.	Ara.	Xyl.	Man.	Gal.	Glc.	Uronic Acid
Harwood (1972)			19	100				17
Haslemore (1974)			21	100				17

nd Not determined.

CHAPTER 5

CHARACTERISATION OF THE XYLOGLUCAN

5.1 Introduction

Structural studies on the xyloglucan from etiolated Pinus radiata hypocotyl cell-walls have involved four major methods: quantitative monosaccharide analysis, partial hydrolysis, methylation analysis and chromium trioxide oxidation.

(1) Monosaccharide analysis has involved quantitation of the neutral monosaccharides in purified xyloglucan fractions. The method gives accurate quantitation combined with quick analysis of isolated fractions.

(2) Partial hydrolysis is a strong tool in analysing monosaccharide linkage within a polysaccharide. The main effect of partial acid hydrolysis of a polysaccharide is to randomly cleave a few of the glycosidic bonds to produce low molecular weight oligomeric fragments. If these oligomers can be isolated and identified, the information derived from the procedure is a series of building bricks contributing to a major structural feature. For fine detailed structural analysis, the use of specific hydrolytic enzymes is profitable (Talmadge et al., 1973).

(3) Methylation analysis is probably the most important method for polysaccharide structural analysis. The process involves exhaustive methylation of polysaccharides by etherification of the hydroxyl groups. The methylated polymer is then hydrolysed, and the methylated monomers separated, identified and quantitatively analysed commonly after conversion to alditol acetates. Free hydroxyl groups in the partially methylated sugars are acetylated and correspond to linkage positions of glycosidic bonds in the original polysaccharide. For example, full methylation of amylose will give 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-glucitol indicating linkages through carbon atoms 1 and 4.

The technique contributes no information as to anomeric configurations or their order along a polymeric chain but does determine the proportions of constituent sugar residues with various glycosidic linkages.

The method of Hakomori (1964) involving the use of the strongly basic sodium methyl sulphinyl carbanion for carbohydrate methylation has been used in the present work. The conjugate base of dimethylsulphoxide is prepared by the action of sodium hydride on dimethyl sulphoxide under nitrogen at 65° - 70°C.

$$CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3 + NaH \rightarrow (CH_3\text{-}\overset{O^-}{\overset{|}{S}}{=}CH_2 \leftrightarrow CH_3\text{-}\overset{O}{\overset{\|}{S}}\text{-}CH_2^-)Na^+ + H_2$$

The strong base catalyses the formation of polysaccharide alkoxides and pushes the equilibrium in the following reaction almost completely to the right.

$$R\text{-}OH + CH_3\text{-}SO\text{-}CH_2^-Na^+ \rightarrow R\text{-}O^-Na^+ + CH_3\text{-}SO\text{-}CH_3$$

The advantage of this procedure over other methods is that the methyl sulphinyl carbanion in dimethylsulphoxide is a much stronger base than aqueous hydroxyl ions. Once alkoxide formation is complete, rapid alkylation follows with methyl iodide forming the methylated product.

$$R\text{-}O^-Na^+ + CH_3I \rightarrow R\text{-}OCH_3 + NaI$$

The review of Bjorndal et al., (1970) notes the possibility of undesirable side reactions with uronic acid residues. Small proportions of uronic acid were found in the purified xyloglucan fractions from Pinus radiata hypocotyl and these would not contribute significantly to any error in quantitation.

The impurities: non-polysaccharide materials such as salts, condensation products and lignin and also part of the polysaccharide that is less completely methylated, can be removed by fractionation between chloroform and water. Non-methylated products extract into the aqueous phase.

(4) Chromium trioxide oxidation enables investigation of the anomeric configuration of the glycosidic linkages in polysaccharides. Those residues oxidized will be where the aglycone (in a fully acetylated aldopyranoside) is equatorially disposed in the most stable chair form - generally the β-anomer. The α-form is only slowly oxidized. Comparison of oxidised polysaccharide with the unoxidised shows which sugar residues are α or β.

5.2 Results

5.2.1 Monosaccharide Analysis

Mole ratios of all fractions with greater then 30% glucose are presented in Table 11. All fractions contain major proportions of glucose and xylose together with constant minor proportions of fucose and galactose, suggesting a fucogalactoxyloglucan is present in Pinus radiata primary cell walls.

In those fractions where the xylose content is higher than the glucose content, it appears that a xylan is present along with the xyloglucan. This has been confirmed in some instances by further purification which separates out a xylan component and the ratio of xylose to glucose in the subsequent xyloglucan fraction drops below 1.0. The xylan has an arabinose to xylose ratio of around 5 (Table 10) which compares favourably with that reported by Harwood (1972) and Haslemore (1974) on the arabino-(4-O-methyl glucurono)-xylan from Pinus radiata wood.

The mannose content probably arises from a glucomannan similar to that isolated by Pee (1973) and Harwood (1973) from Pinus radiata wood. An estimate of the glucose derived from this polymer can be made if it is assumed the Pinus radiata hypocotyl and wood glucomannans are similar. Using an estimated glucose : mannose ratio of 1 : 3.5 (A. K. Pee and V. Harwood obtained 1 : 3.33 and 1 : 3.7 respectively), the glucose residues arising from the glucomannan would be 30% of the mannose content. Table 12 thus gives the monosaccharide ratios of all the xyloglucan fractions corrected for their glucomannan content.

Table 11: Mole Ratios Of Xyloglucan Fractions

Fraction	Rha.	Fuc.	Gal.	Xyl.	Glc.	Ara.	Man.	Uronic Acid
B2*	16		35	75	100	13	38	9
A1	9		25	74	100	9	34	7
A1-2	1	11	27	95	100	12	37	12
A1-3	3	9	24	79	100	4	37	6
A1-4	0	11	19	140	100	13	21	9
C2**	1	14	30	131	100	20	9	10
C2-3	0	16	27	90	100	7	9	4
C2-3a	0	20	25	93	100	5	10	nd
C2-3b	0	18	30	102	100	11	9	nd
C2-4a	0	9	13	99	100	4	2	nd

* B2 apparently contained arabinan + glucomannan (see methylation analysis).

** C2 contained a xylan (separated by further fractionation).

nd Not determined.

Table 12: Mole Ratios Of Hypocotyl Xyloglucan* Compared With Literature Xyloglucans

Fraction	Rha.	Fuc.	Gal.	Xyl.	Glc.	Ara.	Uronic Acid
B2	18		39	84	100	15	10
A1	10		28	82	100	10	8
A1-2	1	12	30	106	100	13	13
A1-3	3	10	27	88	100	4	7
A1-4	0	12	20	149	100	14	10
C2	1	14	31	134	100	21	10
C2-3	0	16	28	92	100	7	4
C2-3a	0	20	26	95	100	5	nd
C2-3b	0	18	31	105	100	11	nd
C2-4a	0	9	13	99	100	4	nd
Estimated Value	0	15	25	85	100	?	?

Literature	Fuc.	Gal.	Xyl.	Glc.	Ara.	References
Gymnosperm						
Red spruce compression wood	nd	21	92	100		Schreuder et al., 1966
Engelmann spruce bark	nd	25	75	100		Ramalingham and Timell, 1964
Cell-culture						
Sycamore	13	15	85	100	3	Bauer et al., 1973
Sycamore	12	28	56	100	4	Aspinall et al., 1977
Rosa glauca	14	17	95	100		Barnaud et al., 1977
Hypocotyl						
Phaseolus aureus	10	25	70	100		Kato & Matsuda, 1976
Seeds						
Rapeseed meal	14	21	71	100		Siddiqui & Wood, 1977
Rapeseed meal	19	23	86	100	3	Theander & Aman, 1978
Rapeseed meal	13	26	91	100	3	Theander & Aman, 1978
Rapeseed meal	14	26	81	100	5	Theander & Aman, 1978
Rapeseed meal	14	16	54	100		Aspinall et al., 1977
Rapeseed hull	15	25	48	100	4	Aspinall et al., 1977
Nasturtium	0	40	69	100		Aspinall et al., 1977
Nasturtium	0	32	48	100		Hsu & Reeves, 1967
Tamarindus	0	33	75	100		Kooiman, 1961
Tamarindus	0	25	50	100	12	Srivastava & Singh, 1967

* Values have been corrected for the possible presence of a glucomannan (see text).

nd Not determined.

Rhamnose has disappeared in several xyloglucan fractions, ruling it out as a part of the xyloglucan.

The arabinose component has not been separated off completely but in several fractions is of minor content. Further fractionation is needed to analyse whether it is a minor component of the xyloglucan. Methylation analysis (section 5.2.3) has suggested an arabinan is present in fraction B2. Most of the arabinose in xyloglucan fractions therefore, would arise from the arabinan and/or arabinoxylan present.

These results indicate the presence of a fucogalactoxyloglucan with a ratio of fucose : galactose : xylose : glucose of 15 : 25 : 85 : 100.

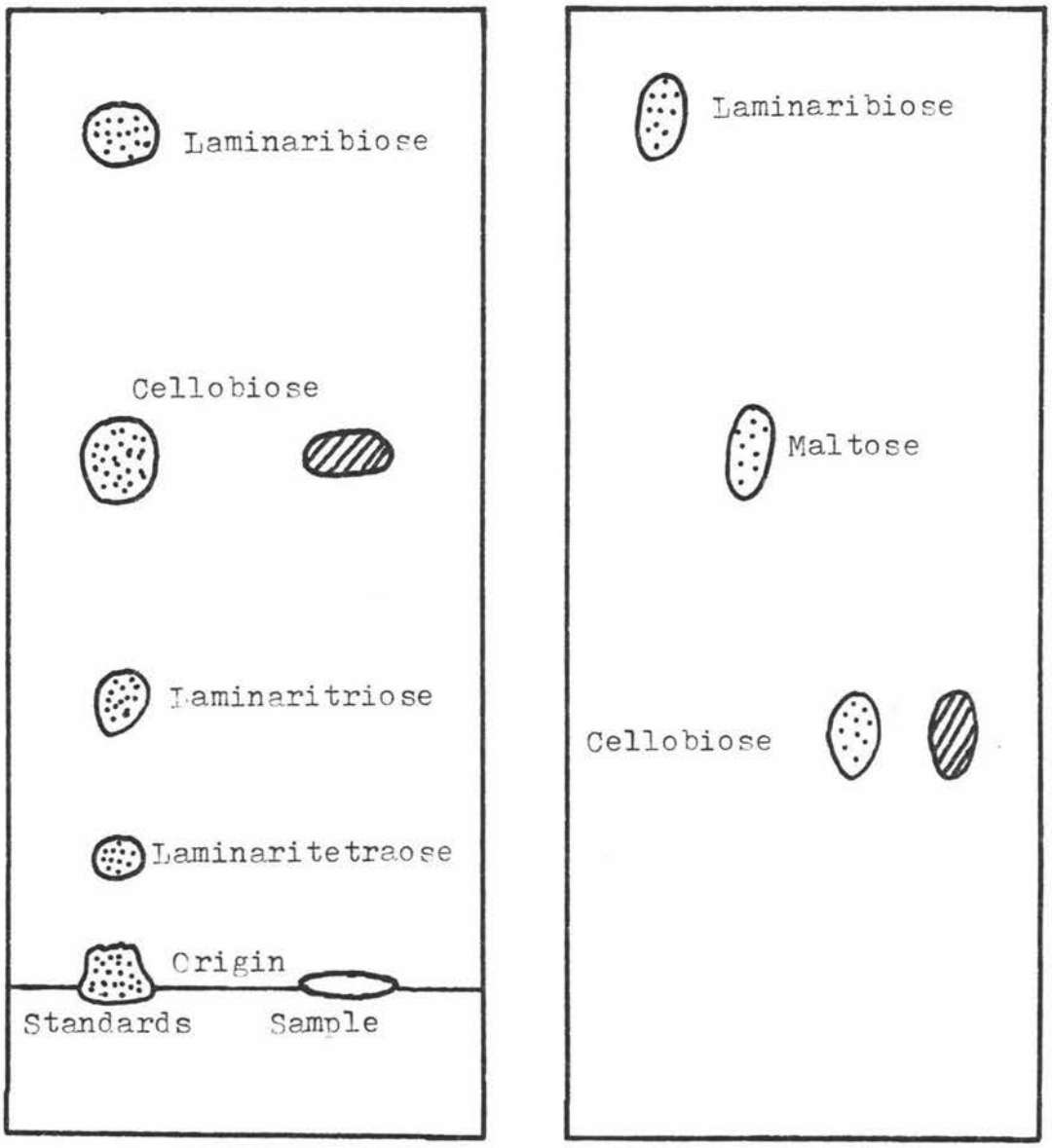
5.2.2 Partial Hydrolysis

Hydrolysis of fractions A1 and B3 (Table 9) with trifluoroacetic acid for 1 hour, 100°C, gave quantitative recovery of all monosaccharides except glucose. Separation of the trifluoroacetic acid hydrolysis products by paper chromatography indicated the presence of a disaccharide. Co-chromatography with standard glucose disaccharides showed this disaccharide to be cellobiose (Fig. 18). To confirm this, the disaccharide products of hydrolysis were eluted from Whatman Number I paper and trimethylsilylated (see section 2.3.6.3). Separation by gas chromatography of the pertrimethylsilyl ethers (Fig. 19) gave two peaks with relative retention times identical to the α and β anomer peaks of authentic cellobiose. No other disaccharides were found either by paper or gas chromatography. Especially important is the absence of laminaribiose. Cellobiose is composed of two glucose residues linked by a $\beta(1\text{-}4)$ glycosidic bond. This suggests there are β-1,4-linked glucose chain components as part of the xyloglucan.

5.2.3 Determination Of Sugar Linkage Compositions

A preliminary single Hakomori methylation was carried out on a sample of soluble starch. The resulting chromatogram showed several peaks including tri-O-methyl and di-O-methyl and

Figure 18: Separation Of Disaccharides By Paper Chromatography.

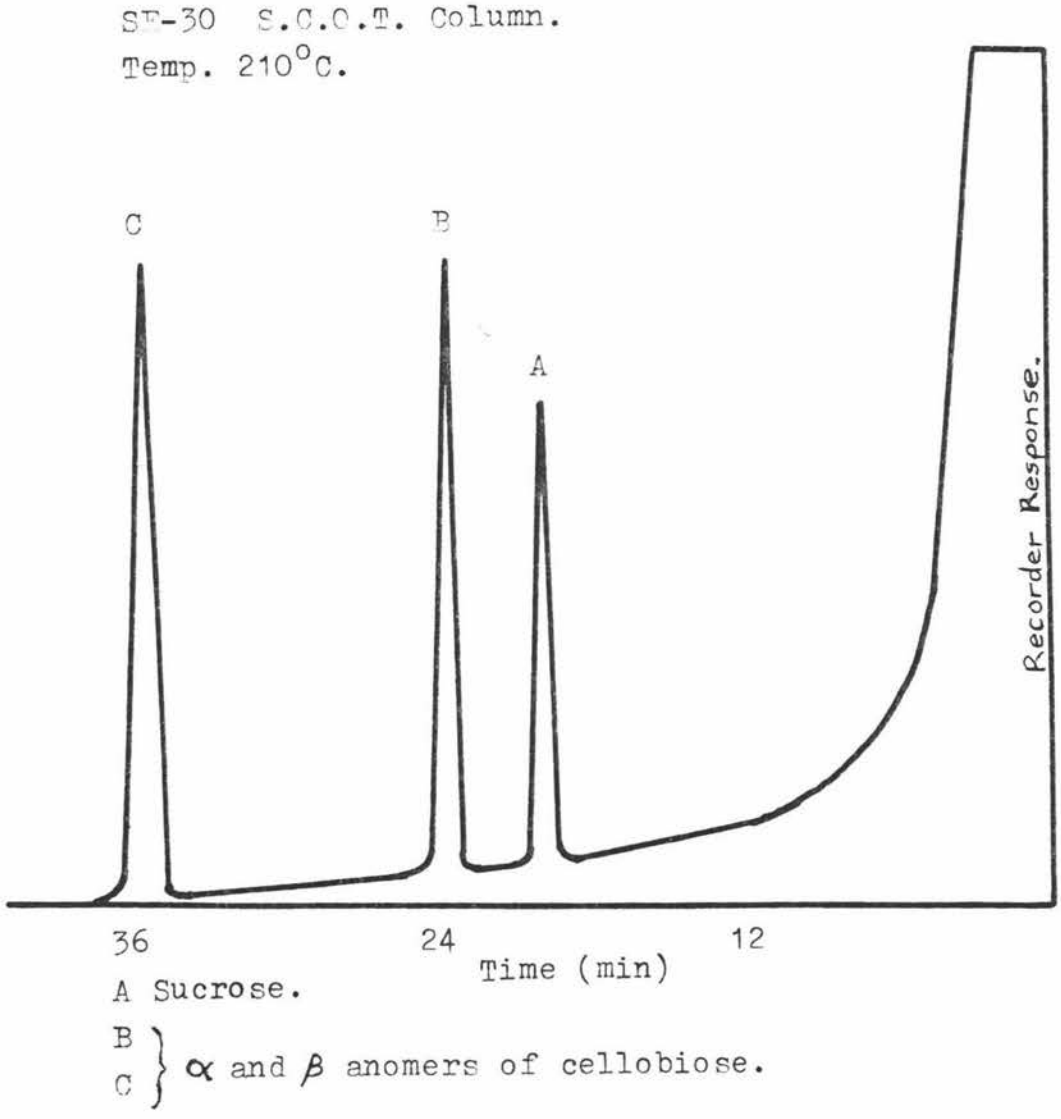


Solvent 1.
45 hours room temp.

Solvent 2.
72 hours room temp.

Standard Oligosaccharides.
Hydrolysed Xyloglucan Disaccharide.

Figure 19: Cellobiose And Sucrose Separation As Their T.M.S. - Ethers.



A Sucrose.

B, C } α and β anomers of cellobiose.

mono-O-methyl-glucitol as well as the unmethylated glucitol-hexaacetate. These peaks, initially identified by relative retention times and confirmed by mass spectrometry, showed that a high degree of undermethylation had occurred with the one Hakomori starch methylation. Major peaks in order of decreasing size included 2,3,6-tri-O-methyl, 2,6-di-O-methyl, 2-mono-O-methyl, 3,6-di-O-methyl, 2,3-di-O-methyl, 6-mono-O-methyl, 2,3,4,6-tetra-O-methyl and the unmethylated glucitol (Fig. 20).

Fraction B2 (Table 9) was then chosen for methylation analysis of the Pinus radiata xyloglucan. This was initially to be a preliminary xyloglucan methylation but due to lack of time caused by undermethylation problems and the future unavailability of a mass spectrometer no further fractions were methylated. Fraction B2 was subjected to one Hakomori methylation (section 2.3.8). The chloroform-soluble portion was hydrolysed and derivatised and gave the chromatogram (on OV-225) as shown in Fig. 21. The presence of such peaks as unmethylated xylose, galactose and glucose showed that either undermethylation or demethylation or both were occurring. The chloroform-insoluble portion was subjected to a second Hakomori methylation, hydrolysed and derivatised and the resulting chromatograms on OV-225 and ECNSS-M are shown in Figs. 21 and 22, respectively. As these chromatograms had far fewer peaks than the single methylated chromatogram, it indicated that undermethylation rather than demethylation was causing most of the problems.

Separation of the B2 partially methylated alditol acetates showed 24 peaks. Twenty-three of these peaks were shown to contain carbohydrate derivatives by combined gas-liquid chromatography and mass spectrometry (GC-MS). Peak 24 was identified as a phthalate ester, probably present due to contamination in one of the solvents used. Identified peaks arising from OV-225 separation are given in Table 13.

Peak areas are reported in Table 13 on a semiquantitative basis only because of inaccuracies in reliable peak area determination (due especially to losses of volatile methylated derivatives and the large number of overlapping peaks).

FIGURE 20: Methylation Chromatogram of Starch

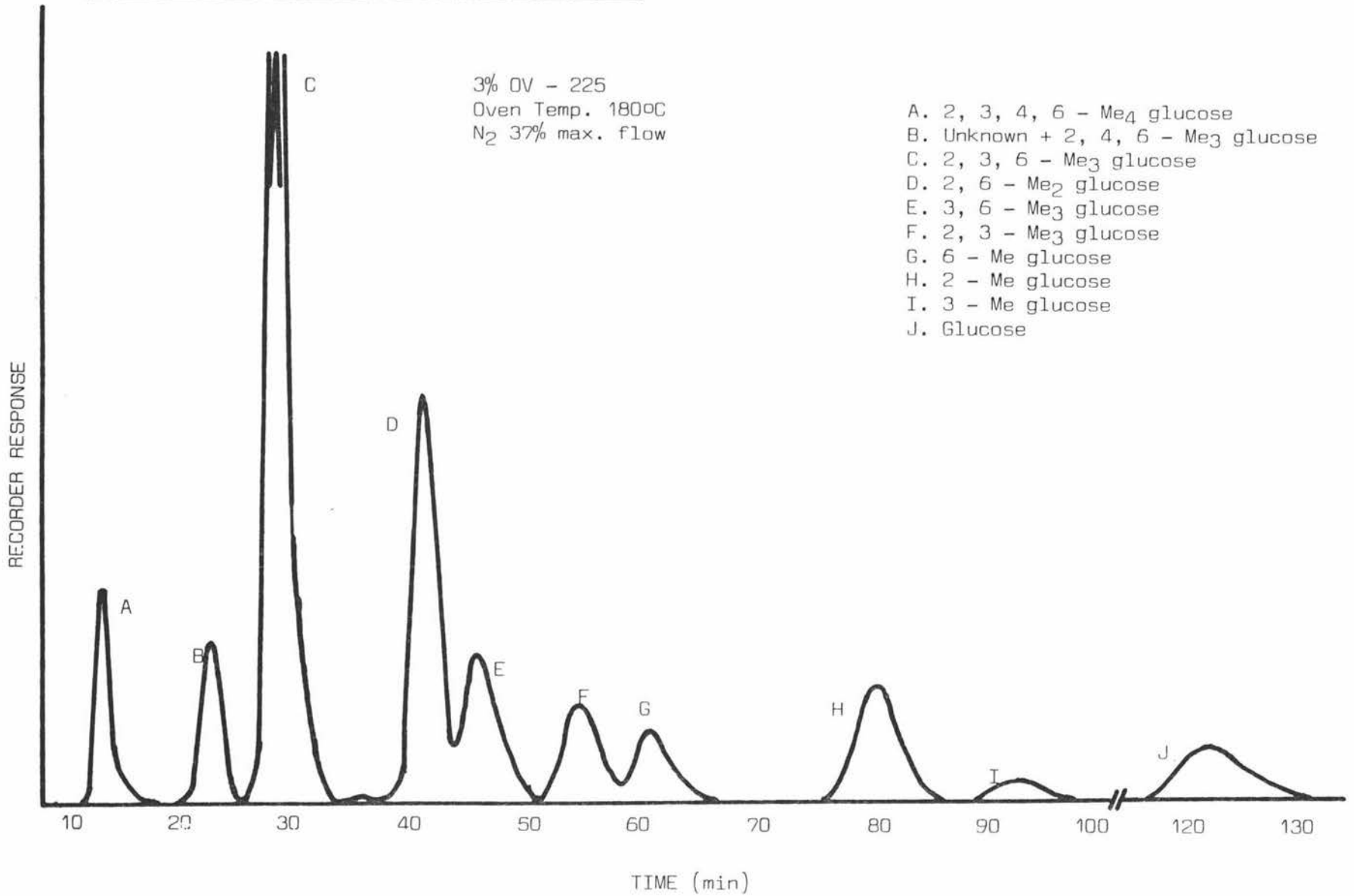
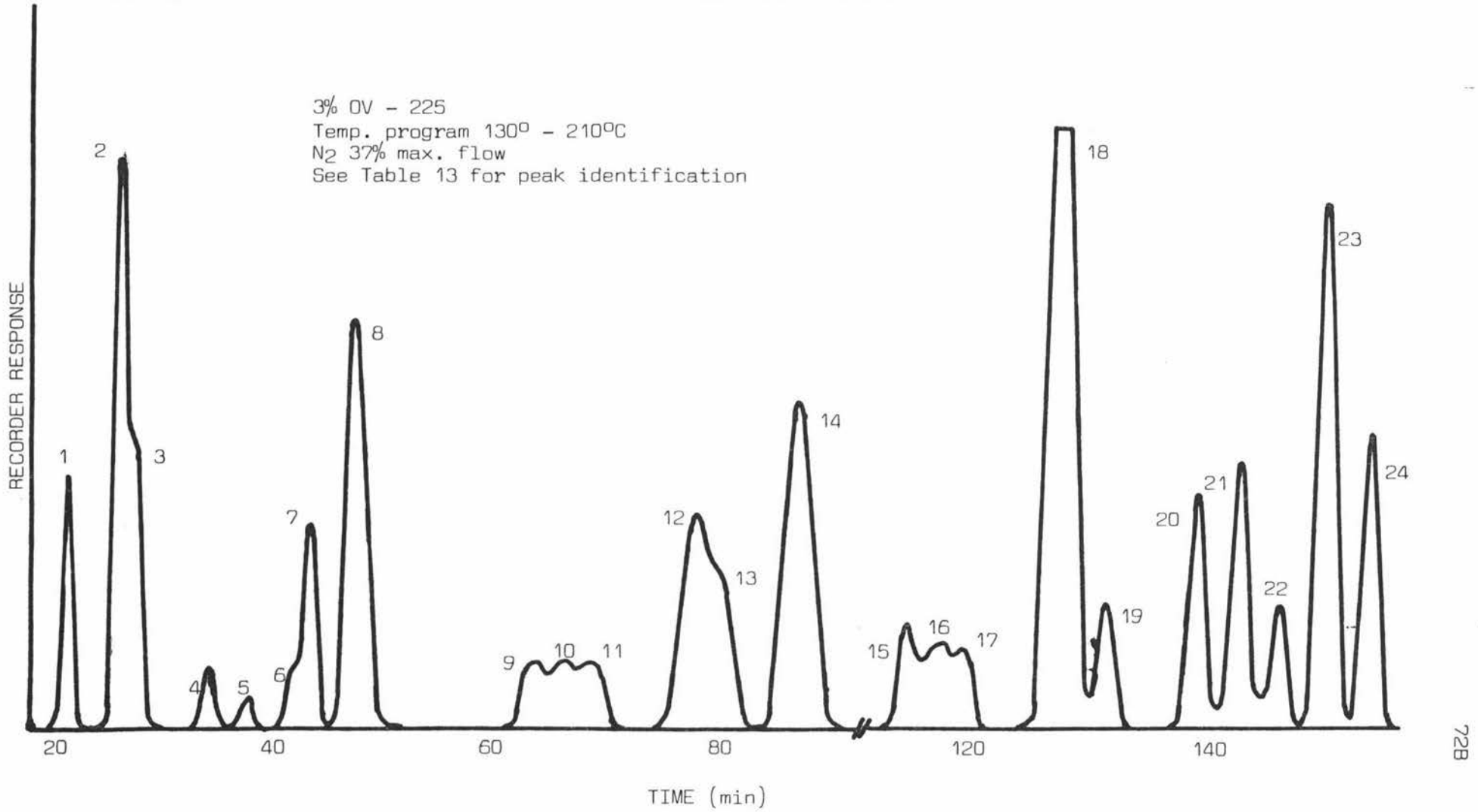


FIGURE 21 A: Single Methylated Chloroform Soluble Palysaccharide



728

FIGURE 21 B: Remethylated Chloroform Insoluble Polysaccharide

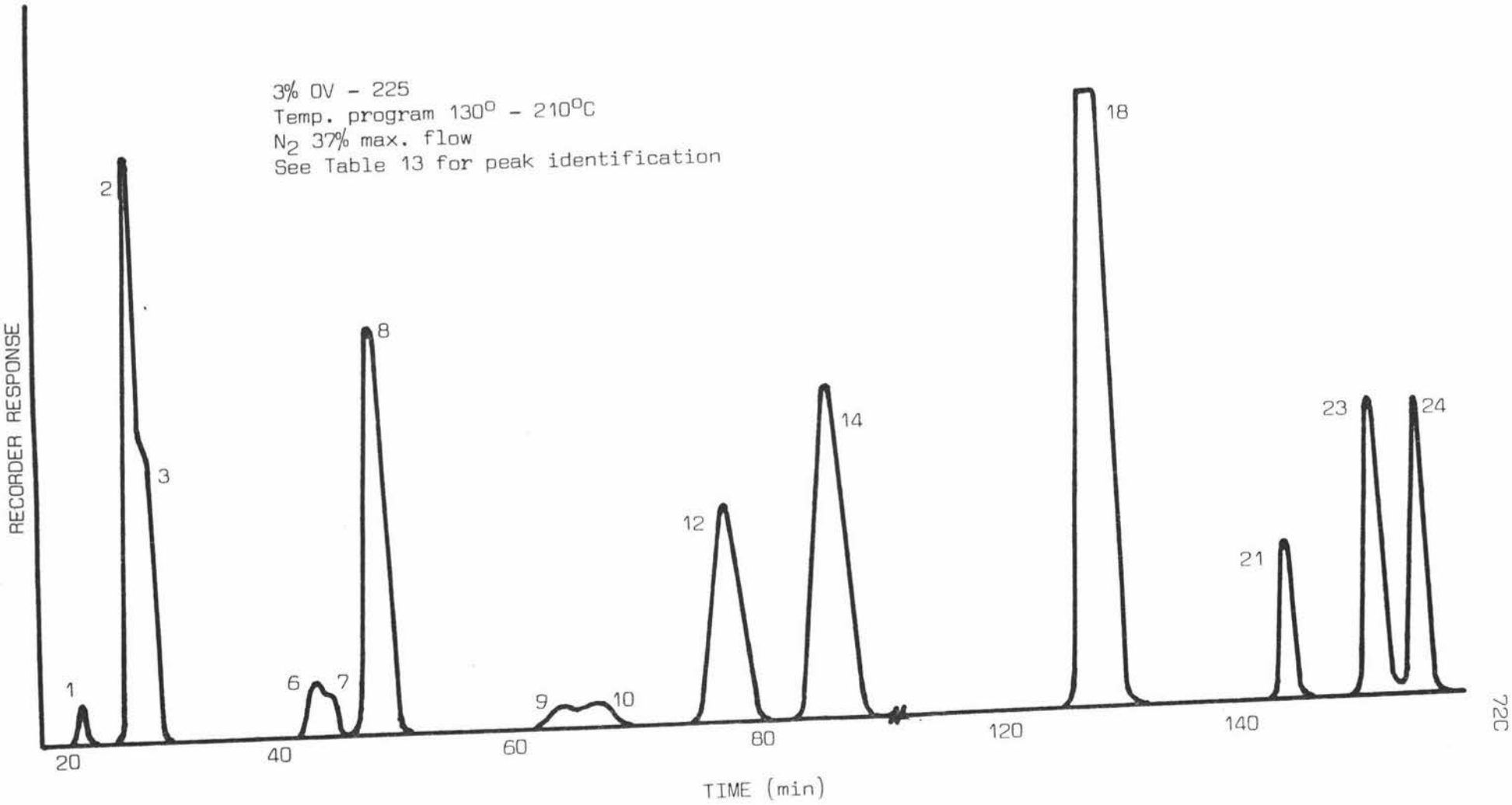


FIGURE 22: Separation of Chloroform Insoluble Polysaccharide

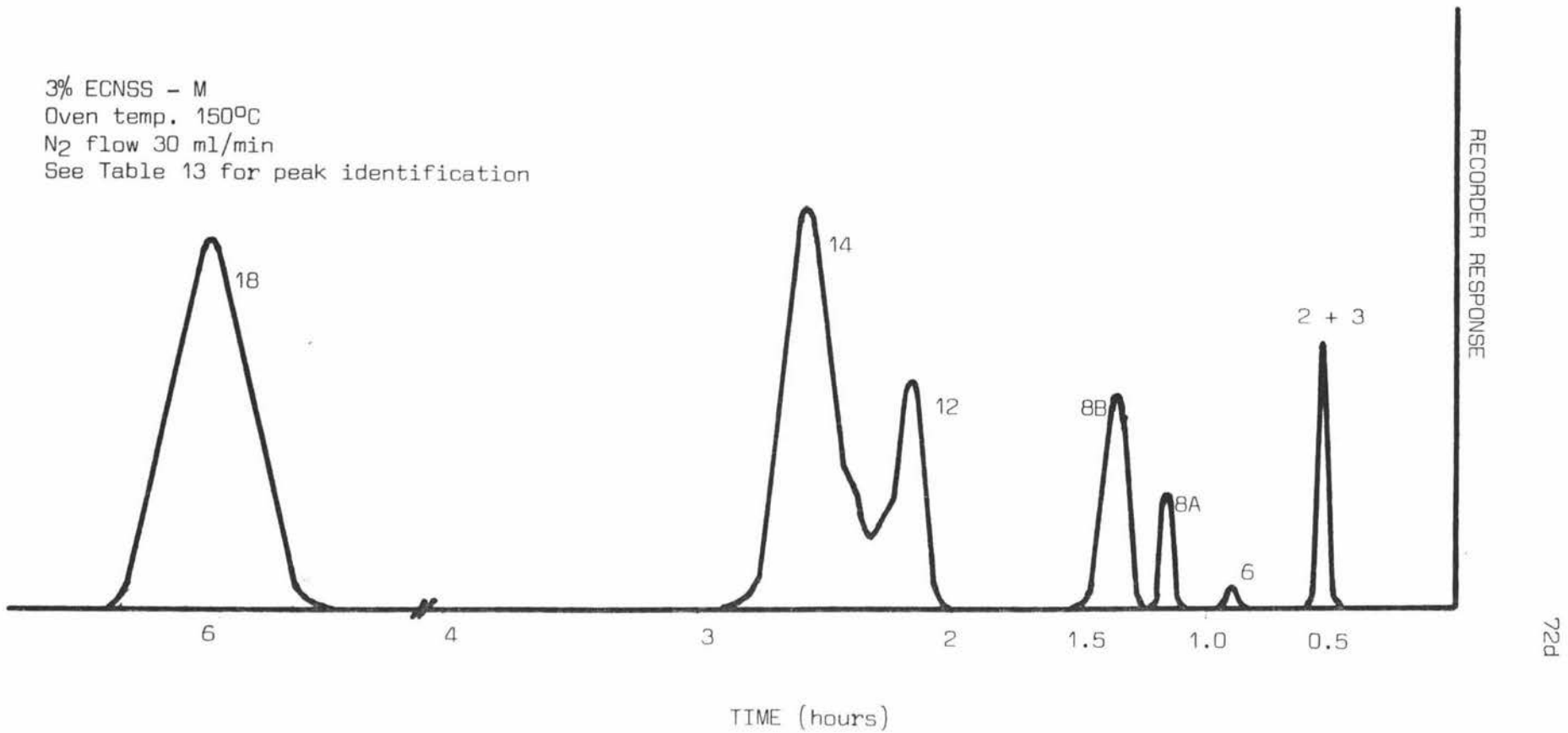


Table 13: Identified Partially Methylated Alditol Acetates #

Peak	Monosaccharide	Chloroform Soluble*	Chloroform Insoluble**
1	2,3,5-tri-O-methyl arabinose	+++	tr
2	2,3,4-tri-O-methyl xylose	+++	+++
3	2,3,4-tri-O-methyl fucose	++	++
4	3,5-di-O-methyl arabinose	+	-
5	2,5-di-O-methyl arabinose	+	-
6	2,3,4,6-tetra-O-methyl glucose	+	+
7	2,3-di-O-methyl arabinose	++	tr
8A	2,3,4,6-tetra-O-methyl galactose	++	++
8B	3,4-(or 2,3-)di-O-methyl xylose	+++	+++
9	Unknown	++	tr
10	Unknown	++	tr
11	2-O-methyl arabinose?	++	-
12	2,3,6-tri-O-methyl mannose	++	++
13	mono-methyl xylose	+	-
14	3,4,6-tri-O-methyl galactose	+++	+++
14	2,3,6-tri-O-methyl glucose	+++	+++
15	2,6-di-O-methyl glucose	+	-
16	xylose	+	-
17	3,6-di-O-methyl glucose	+	-
18	2,3-di-O-methyl glucose	++++	++++
19	6-O-methyl glucose	+	-
20	2-O-methyl glucose	+	-
21	3-O-methyl glucose	+	+
22	galactose?	+	-
23	glucose	+	+
24	phthalate ester	+	+

Separated on column A (OV225).

* Single methylated carbohydrate.

** Carbohydrate methylated twice.

- Negative.

tr Trace.

+ Relative quantity of monosaccharide.

However, it is clear from Fig. 21 that after a single Hakomori methylation, the amount of arabinose derivatives relative to other sugar derivatives (compare arabinose and xylose), is higher compared with the neutral monosaccharide analysis of Table 9, whereas the twice-methylated material shows almost a total absence of arabinose derivatives. These arabinose derivatives consist of two major peaks; 2,3,5-tri-O-methyl and 2,3-di-O-methyl arabinose, and two minor peaks; 3,5-di-O-methyl and 2,5-di-O-methyl arabinose. A fifth quite large peak was tentatively identified as 2-O-methyl arabinose but due to the close proximity of other peaks and the small amount of material available GC-MS could not confirm the identification*. These results suggest an arabinan, highly methylated after a single methylation due to its greater solubility, has almost totally fractionated out with the chloroform-soluble material. Hence the twice-methylated material is largely purified free of arabinan.

Mannose was found only as the 4-linked derivative as expected, assuming it to be a constituent of a contaminating glucomannan or galactoglucomannan.

Although 6-O-methyl and 2-O-methyl glucose were only present in the material subjected to a single methylation both 3-O-methyl and the unmethylated glucose were found in similar proportions in both methylated samples (Table 13). Glucose probably derived from either undermethylation or demethylation and similarly the 3-O-methyl glucose. However the 3-O-methyl glucose could also arise from a small proportion of doubly branched glucose residues. The 2,4,6-linked glucose (3-O-methyl glucose) has been found as a component of the sycamore xyloglucan (Bauer et al., 1973) and Courtois et al., (1976) also found evidence for a linkage through C-2 of the D-glucosyl units of Balsamina xyloglucan.

* In the GC-MS used, the column effluent could not be split, therefore there was no simultaneous flame ionisation detection and ion measurement. The use of total ion current to show peaks is much more difficult to enable pinpointing of a small obscured peak.

A comparison of the methylation results with the data from neutral monosaccharide analysis suggest that the volatile tri-O-methyl pentoses and tetra-O-methyl hexoses have been lost during workup of the hydrolysed partially methylated monomers. This unfortunately has meant accurate quantitation cannot be done. To correct for the losses, the assumption may be made that partially methylated alditol acetates with similar retention times (as for example the 2,3,4-tri-O-methyl xylose and fucose derivatives) undergo similar losses during workup. As well, the bulk of the evidence presented in this thesis suggests that the structure of the Pinus radiata hypocotyl xyloglucan is is similar to that of other species. Thus there should be equal moles of: 4,6-linked glucose and total xylose; 2-linked xylose and total galactose; and 2-linked galactose and terminal fucose. Using such assumptions, the ratios of the major methylated monomers can be estimated for the twice methylated sample (Table 14). Application of this type of analysis gives a ratio of fucose : galactose : xylose : glucose of approximately 20 : 40 : 70 : 100.

5.2.4 Analysis Of Anomeric Configuration

A preliminary investigation was carried out using a standard galactomannan (Carob bean). Three hours of chromium trioxide treatment on a sample decreased the mannose content to 10% of the original while the galactose only decreased slightly to 74% of the original value (Table 15). This agreed with the known anomeric configurations: β-linked mannose and α-linked galactose.

Fractions C2-4a and C2-3b, containing a fairly pure xyloglucan, were combined and analysed. Results presented in Table 15 are slightly ambiguous. Fucose has undergone no oxidation implying an α-configuration while galactose has been 80% degraded implying a β-configuration. Analysis of the other results do not give any clear-cut conclusions. The low level of glucose oxidation agrees with slow oxidation of β-linked glucose reported by Woolard et al., (1976) in certain glucans. The anomeric configuration of arabinosyl residues in these

Table 14: Prominent Peaks In Mass Spectra Of Hypocotyl Fucogalactoxyloglucan

Peak	2	3	6	8A	8B	14	14	18
Sugar Linkage	t-xyl	t-fuc	t-glc	t-gal	2-xyl (or 4-)	2-gal	4-glc	4,6-glc
Relative Moles*	30	20	trace	20	40	20	30	70
m/e 43	+	+	+	+	+	+	+	+
45			+	+		+	+	
71			+	+				
87	+		+	+	+	+	+	
89		+						
99						+	+	+
101	+	+	+	+	+	+	+	+
113							+	
115		+						
117	+	+	+	+	+		+	+
127								+
129			+	+	+	+		
131		+						
145			+	+				
161	+		+	+		+		
175		+						
189				+		+		
205			+	+				
233							+	
261								+

* Obtained as described in text.

\+ Primary fragments.

Table 15: Chromium Trioxide Oxidation

Sample	Time (hours)	Relative Monosaccharide Areas*					
		Fuc.	Xyl.	Gal.	Glc.	Man.	Myo-inositol
Galactomannan	0			162		543	100
	3			143		58	100
Anomeric Configuration				α		β	
Xyloglucan	0	46	284	120	270	74	100
	4	52	140	26	224	39	100
Anomeric Configuration		α	?	β	?		

* Not corrected for response factors.

polymers was not determined since this method degrades both α- and β-linked furanosides equally.

5.3 Discussion

Results presented in this thesis show that the primary cell wall of etiolated Pinus radiata seedlings contain a fucogalactoxyloglucan as a major hemicellulose polymer. The absence of both 3-linked glucose in the methylation analysis and laminaribiose in the partial hydrolysate indicate that no mixed-link glucan is present.

It is important to realise that the purity of the original cell wall preparation is often questionable. The fractions obtained are thus ill-defined, and there is considerable overlap, especially between pectins and hemicelluloses, depending on which variation in extraction procedure is followed. Pectic material was often separated out from hemicellulose B extracts by further fractionation (Scheme 1). Methylation analysis has also shown a possible pectic arabinan to be present, together with the hemicellulosic xyloglucan. Thus in each polysaccharide fraction there may coexist molecules of different structural type and data analysis must always take into account this possibility.

The non-cellulosic neutral sugars in the compositional analyses in these studies were analysed as their alditol acetates after hydrolysis in 0.5M nitric acid for 3.5 hours at 100°C. This allowed quantitative determination of the monosaccharide composition of isolated Pinus radiata hypocotyl cell wall hemicellulose B extracts which showed a major 'glucan' to be present (Chapter 3). Data from fractionation of this 'glucan' has suggested it is a fucogalactoxyloglucan similar to that found in sycamore (Bauer et al., 1973) and other dicot tissues (Chapter 4). These observations were borne out by detailed characterisation studies. Methylation analysis has been the major tool from which the structure has been built although undermethylation has been a major problem with this process. This problem has been encountered by other workers;

Seymour et al. (1979) showed that with cellulose and glucose dextrans, this undermethylation is not random but selectively occurs at the 3-hydroxyl group. Sonication emphasizes this effect, apparently by promoting methylation at non-3-hydroxyl groups. This same effect has been observed with starch in this work as shown by the largest peak after 2,3,6-tri-O-methyl glucose being 2,6-di-O-methyl glucose (Fig. 20). The 3,6-di-O-methyl and 2,3-di-O-methyl glucose derivatives are slightly less again. This agrees with an ease of methylation C-6 > C-2 > C-3 as found by Seymour et al. (1979).

Two hemicellulosic polymers shown to occur in Pinus radiata hypocotyl, are similar to those found in Pinus radiata wood. An arabinoxylan has been separated from the xyloglucan and shown to have similar arabinose : xylose ratios to the wood arabino-(4-O-methyl glucurono)-xylan (see Chapter 4). Mannose, although not shown to, probably arises from a gluco-mannan (or galactoglucomannan) similar to that isolated from Pinus radiata wood by Harwood (1973) and Pee (1973). Mannose is unlikely to be a component of the xyloglucan owing to the following observations:

(1) The changes of monosaccharides with tissue age (Table 7) showed mannose increased constantly with increasing age while the glucose increase did not continue after cessation of elongation.

(2) In several xyloglucan fractions the mannose content is very small, especially fraction C2-4a (Table 9).

(3) The percentage of mannose in samples could be varied by varying the extraction procedure (see section 3.2.3).

Furthermore in the literature:

(4) Mannose has only been found as a β-1,4-linked component of higher plant cell walls either as a homoglycan or as a heteroglycan with galactose and glucose but not with xylose. (Methylation analysis in this work reveals only 4-linked mannose.)

(5) Mannose is a characteristic component of secondary cell walls, not primary cell walls (see section 3.3).

(6) Xyloglucan fractions have been successfully isolated with no mannose component.

Any mannose present would therefore indicate the glucose derived from a possible glucomannan. Other workers when isolating xyloglucan polymers, have not had a similar problem as no gluco- or galactoglucomannan was present in the tissue. The xyloglucan and glucomannan are difficult to separate possibly because both hydrogen-bond well to each other due to their similar backbone structures (see section 4.3). Complete separation of the two polymers might have been achieved by DEAE-cellulose/borate and/or cellulose column chromatography or possibly by preacetylation of the polysaccharides to disrupt any hydrogen-bonds followed by chloroform/petroleum ether fractionation.

It is apparent that similar polysaccharides to both of the hemicellulose polymers found in mature Pinus radiata wood are already present in hypocotyl tissue, perhaps arising from secondary-thickened cell walls, and will need to be clearly separated from the xyloglucan in the future refinement of the xyloglucan structure.

Most arabinose is from polysaccharides other than xyloglucan. A preponderance of fractions would contain arabinose arising from the xylan present. As well, methylation analysis has shown the probable presence of an arabinan in fraction 'B2'. Most of the xylan would have been removed from this fraction as it is soluble in cetavlon-borate. The arabinan has been demonstrated by the greatly increased proportion of arabinose in the first methylation as would be expected for a highly soluble polymer. High contents of 2,3,5-tri-O-methyl and 2,3-di-O-methyl arabinose along with the tentative identification of fairly high 2-O-methyl arabinose, all indicate an arabinan. Although 2,5-di-O-methyl arabinose was found as a constituent of the arabinan from Pinus pinaster var. maritima (Roudier and Eberhard, 1965) the 3,5-di-O-methyl arabinose was

not. The 2-linked arabinose is a component of arabinans, such as from rapeseed (Larm et al., 1975) and could be a component of the Pinus radiata arabinan.

The presence of both 3,5-di-O-methyl and 2,5-di-O-methyl arabinose is associated in the literature with the presence of oligosaccharides (usually tetra) linked to the hydroxyproline of the cell wall protein 'extensin'. Hydroxyproline has been shown to be a major constituent of Pinus radiata callus cell wall protein (D. R. Fenemor, personal communication) and it is possible these methylated arabinose derivatives arise from similar oligosaccharides in Pinus radiata hypocotyl cell wall protein. The two arabinose derivatives may also arise from undermethylation.

There is a possibility that a small content of arabinose arises from the xyloglucan polymer. Siddiqui and Wood (1977) have isolated rapeseed meal xyloglucan from which arabinose is absent but no other workers have been able to do this with xyloglucans from other sources. Further investigations will be necessary to determine whether these arabinose residues arise from incomplete fractionation of the polysaccharide preparations or from residual, covalently attached stubs of another cell wall component as suggested in the model for plant cell wall structure proposed by Albersheim and his collaborators. Albersheim (1976) has proposed that the arabinose 'stubs' are due to covalent linkage between the xyloglucan and pectic arabinogalactan present in sycamore cell-suspension cultures. Although he quotes M. McNeil as having isolated a fragment of the wall which contains the xyloglucan attached to the pectic galactan, no literature has appeared to support this claim.

These results presented in this thesis, show that a polymer composed of fucose, galactose, xylose and glucose is present in the Pinus radiata hypocotyl cell wall. Taking into account the glucose derived from a possible gluco- or galactoglucomannan, Table 12 reports the found monosaccharide ratios of high xyloglucan fractions. In those fractions with low xylose content it is assumed the xyloglucan is free of the

more soluble xylan. This leaves a polymer with ratios of fucose : galactose : xylose : glucose of 15 : 25 : 85 : 100. A more accurate calculation would need the isolation of the xyloglcuan, free of other hemicelluloses, by methods involving a minimum of both degradation of monomers and hydrolysis of bonds.

The following results from the characterisation studies support the formulation of the fucogalactoxyloglucan as shown in Fig. 23.

(a) Evidence suggesting a linear β-(1-4)-glucan backbone include the following:

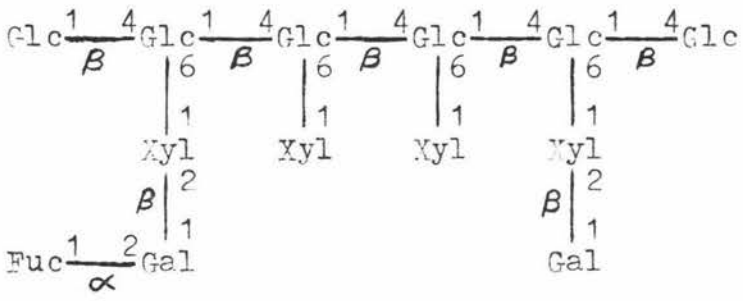
(1) The hydrolysis of Pinus radiata xyloglucan by the trifluoroacetic acid procedure gives quantitative recovery of all monosaccharides except glucose. The trifluoroacetic acid procedure also fails to hydrolyse the glycosidic linkages of cellulose (a linear β-(1-4)-glucan) and hydrolyse quantitatively the β-(1-4)-glucosyl linkages present in the backbone of the xyloglucan of sycamore (Bauer et al., 1973).

(2) Separation of the trifluoroacetic acid hydrolysis products by both paper chromatography and gas chromatography gave only one disaccharide, cellobiose. Cellobiose is a β-(1-4)-linked glucose disaccharide, indicating there are chains of linear β-(1-4)-linked glucose residues in the xyloglucan.

(3) The polysaccharide was precipitated as a dark blue iodine complex. Gaillard et al. (1969) have reported that a variety of polysaccharides having a sequence of (1-4)-linked glucose, xylose, or mannose residues, stained blue or black and precipitated with iodine in the presence of calcium chloride.

(4) The xyloglucan was very difficult to separate from the mannose-containing polysaccharide. The mannose probably derives from a linear β-1,4-linked glucomannan (in support of this, methylation analysis shows all the mannose 4-linked) which would aggregate with a similar linear β-(1,4)-glucose chain leading to difficult separation.

Figure 23: Possible Structure Of Pinus Radiata Xyloglucan



(5) All the glucose residues were shown to contain a C-4 linkage (except for trace amounts of terminal glucose) while all other sugar residues had large proportions of terminal derivatives, giving support to a linear 4-linked glucan chain.

(b) Evidence suggesting the fucogalactoxyloglucan consists of xylose, galactose and fucose attached as sidechains to a sequence of β-1,4-linked glucose residues through carbon-6 includes the following:

(6) Methylation analysis has shown that 70-80% of the total methylated glucose derivatives were 4,6-linked while the remaining 20-30% were only 4-linked. Kooiman (1961), using an enzyme preparation called 'luizym', was able to hydrolyse the tamarind xyloglucan so that almost all of the xylosyl residues of the polymer were recovered in the disaccharide 6-O-α-D-xylopyranosyl-D-glucopyranose. The essentially quantitative isolation of this disaccharide clearly demonstrated, that all of the xylosyl residues occur as monoxylosyl side-chains linked to the 6-position of glucosyl residues in the glucan backbone. A similar conclusion is suggested with Pinus radiata xyloglucan by the very similar ratios of total glucose to the 4,6-linked glucose (100 : 70-80) from methylation analysis (Table 14) and total glucose to total xylose (100 : 85) from monosaccharide analysis (Table 12).

(7) All the fucose was present as terminal fucose while the xylose and galactose had fairly equal proportions of both terminal and linked residues. The linked galactose was shown to be 2-linked. Aspinall et al., (1977), by acetolysis of rapeseed hull fucogalactoxyloglucan derived: cellobiose, 6-O-α-D-xylopyranosyl-D-glucose and 2-O-β-D-galactopyranosyl-D-xylose. As well, their analysis of nasturtium galactoxyloglucan showed only terminal galactose to be present. Taking into account these and other literature results, the presence of both terminal fucose and 2-linked galactose in this work, suggests all the fucose is bound in a 2-O-D-fucopyranosyl-D-galactose linkage. As all the xylose is bound to the glucose

and all the fucose to the galactose, it follows all the galactose must be bound to the xylose as found by Aspinall et al., (1977) and others. Such assumptions by no means proves the linkages as set out, but, as all fucogalactoxyloglucans found to date follow these linkages and no evidence in this work contradicts such a structure for the Pinus radiata xyloglucan, these deductions are valid.

(8) Ratios of the four sugars always show the content of glucose > xylose > galactose > fucose. An estimate of their ratios gave 100 : 85 : 25 : 15. After considering the fact that only glucose is all present as linked residues, indirect support for sidechains of fucose, galactose and xylose, linked as in Fig. 23, is shown by these ratios.

Table 12 shows the monosaccharide ratios found in isolated xyloglucans from other tissues. All have very similar ratios even though the tissue types vary considerably. The structure of the Pinus radiata xyloglucan suggested in Fig. 23, is the same as that found for all those xyloglucans isolated from dicots.

(c) The anomeric configuration of the glycosidic linkages of Pinus radiata xyloglucan were investigated.

(9) Results of chromium trioxide oxidation suggested that the fucosyl residues are linked in the α-configuration and that the galactosyl residues exist in the β-configuration. The oxidation of xylose and glucose were inconclusive. Talmadge et al., (1973) found chromium trioxide oxidation gave equivocal results with sycamore xyloglucan.

(10) Isolation of cellobiose from the partial hydrolysis products with no other glucose disaccharide indicate that the glucose residues are linked in the β-configuration.

From these results it may be concluded that the overall structural features of the Pinus radiata primary cell wall fucogalactoxyloglucan, are similar to those of fucogalactoxyloglucans isolated from dicots.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.0 General Discussion

The seed xyloglucans (amyloids) which have been isolated and investigated structurally, are highly branched polysaccharides, showing variations in many respects. The galactoxyloglucans from tamarind (Kooiman, 1961) and nasturtium seeds (Courtois and Le Dizet, 1974) are dominating components in these sources (20-50% of dry matter) and they contain no fucose residues. Another group are the fucogalactoxyloglucans isolated in smaller amounts from white mustard seeds (Gould *et al.*, 1971), turnip rapeseed meal (Siddiqui and Wood, 1977) and rapeseed hulls (Aspinall *et al.*, 1977). Smaller amounts of arabinose are found in this group of xyloglucans, but Siddiqui and Wood (1977) have isolated a small fraction where arabinose was absent.

Fucogalactoxyloglucans have now been found in the cell-suspension cultures of sycamore (Bauer *et al.*, 1973) Red Kidney bean (Wilder and Albersheim, 1973) and *Rosa glauca* (Barnoud *et al.*, 1977). These cultured cells possess primary, but no secondary, walls, (Albersheim, 1976). Seedling tissue (which would possess mainly primary cell-walls) of *Phaseolus aureus*, *Glycine max* and *Vigna sesquipedalis* (Kato and Matsuda, 1974) all contain a similar polymer. The xyloglucan which has shown exclusive turnover in peas (Labavitch and Ray, 1974) resembles that reported in other tissues (Albersheim, 1976).

Most recent work has involved the study of the angiosperm primary cell wall polysaccharides, and no reports of any xyloglucan from gymnosperm primary cell wall have been published. However, Ramalingham and Timell (1964), as well as Schreuder *et al.*, (1966), have presented results indicating the presence of a xyloglucan in the bark of *Picea engelmanni* and red spruce compression wood respectively. The *Picea* 'glucan' contained glucose, xylose and galactose residues in a ratio of 4 : 3 : 1. Fucose content was not reported.

Evidence was obtained for the presence of a sequence of β-(1-4)-linked glucose residues, frequently branched through C-6. The majority of the galactose occurred as non-reducing end groups. The red spruce 'glucan' gave, on hydrolysis, galactose, xylose and glucose in a ratio of 1 : 4 : 5.

It is also pertinent that Perila and Bishop (1961), on enzymic hydrolysis of a glucomannan from Pinus banksiana wood, obtained both 6-O-(α-D-xylopyranosyl)-D-glucose and 6-O-(α-D-xylopyranosyl)-cellobiose among the products.

The analysis of Douglas fir suspension-culture cell walls (Burke et al., 1974) gives a monosaccharide composition very similar to that of sycamore, perhaps also suggesting the possible presence of a xyloglucan in Douglas fir primary cell wall. Similarly, the high xylose and glucose contents of Pinus ponderosa cambium cell wall (Thornber and Northcote, 1961a and b), together with the large degrease in glucose from heartwood cell wall, suggest that a xyloglucan is present in the cambium cell wall.

All the major features, of the xyloglucans mentioned above and the Pinus radiata seedling xyloglucan, are similar. The features that these polysaccharides have in common include the β-1,4-linked glucan backbone, the xylosyl residues substituted along the glucan backbone, and the fucosyl-galactosyl side chains substituted onto xylose residues.

The fucosyl-1,2-galactosyl side chains, attached to the 2-position of the xylosyl residues fold back over the glucan chain in a manner that allows the xyloglucan to hydrogen-bond strongly to cellulose on only one side (Bauer et al., 1973). This most significant feature of the molecule may, according to Bauer et al., allow it to ensheath cellulose fibrils with only a single xyloglucan layer. The xyloglucan polymers surrounding a cellulose fibril would then cross-link covalently through the pectic polymers to xyloglucan monolayers surrounding other cellulose fibrils (Bauer et al., 1973).

Polysaccharides appearing in the hemicellulose fraction of secondary plant cell walls (xylans, mannans, glucomannans and galactoglucomannans) and the primary cell walls (arabinoxylan of the monocot and the xyloglucan of the dicot) all have

structures that are well suited to the formation of interchain hydrogen bonds with cellulose chains. Several such polysaccharides have, in fact, been reported to bind to cellulose *in-vitro* (Albersheim, 1976). It is possible that these polysaccharides may belong to a single class of polysaccharides with the structural function to interconnect the cellulose fibres and the pectic polysaccharides of the wall (Bauer *et al.*, 1973).

In view of the structural similarities between primay cell wall xyloglucans and the seed 'amyloid' xyloglucans, it would be of considerable interest to ascertain whether or not the 'amyloid' xyloglucans are incorporated into the structure of the primary cell walls of the germinating seedlings. The fate of the 'amyloid' xyloglucan from white mustard seeds has been studied by Gould et al., (1971). These investigations found that, before germination, two xyloglucan fractions could be obtained from the cotyledons : a soluble xyloglucan that could be extracted with hot EDTA solution and an insoluble xyloglucan that required further extraction with aqueous alkali. After germination the soluble xyloglucan fraction was not detected, but the insoluble xyloglucan was still present. Albersheim (1976) postulates that the difference between the soluble and insoluble xyloglucans is the binding of the latter to cellulose, and that the disappearance of the soluble xyloglucan after germination involves the binding of this fraction to cellulose or its incorporation into newly synthesized cell walls.

All the xyloglucans isolated derive from dicotyledons that are, in some cases, very distantly related (for example sycamore and Red Kidney bean) yet retain very similar molecular features. It can now be added that the primary cell wall of a gymnosperm, *Pinus radiata*, also contains a similar polymer. Evolution conserves those features of molecules that are most important to their function. The structure of the xyloglucan has remained relatively unchanged during the divergence of sycamore and bean, and possibly even between that of gymnosperm and angiosperms. This conservation of structure indicates the importance of this hemicellulose in cell wall structure and function.

6.1 Conclusion

The data presented in this thesis has resulted from an investigation of the composition of primary cell walls of elongating Pinus radiata hypocotyl. The turnover studies by Labavitch and Ray (1974) on a xyloglucan in dicot primary cell walls and the changes found in a mixed-link glucan of monocot walls by Buchala and others, suggested the initial approach. This was to study, in particular, the change with cell wall elongation, in glucose composition of hemicellulose B fractions. The non-starch, non-cellulosic, glucose was found to undergo a major increase in cell walls on cessation of elongation.

The identification of this glucose fraction as a fuco-galactoxyloglucan, similar to that isolated from dicot tissues, is reported here. Although this strongly suggests a role for this polysaccharide in the elongating gymnosperm primary cell wall as in the pea epicotyl, it must be noted that we are dealing with an increase with age in the proportion of xyloglucan, in contrast to the decrease associated with turnover in the pea epicotyl.

The increase in xyloglucan as well as other hemicellulose polysaccharides in the etiolated Pinus radiata hypocotyl, is clearly at the expense of starch. Whether the changes reported in Chapter 3 of this thesis bear any relation to those under normal growth conditions is unknown. The etiolated seedlings used here served the purpose of providing greater quantities of starting material, with much less secondary wall thickening and lignification, than in light-grown seedlings. They may thus provide a better model for primary cell-wall structure than light-grown seedlings, as well as providing more xyloglucan for structural characterisation, without any requirement for delignification of the tissues.

However, in order to evaluate the true role of the xyloglucan or any other polysaccharides in hypocotyl elongation, it will be necessary to use light-grown seedlings. Further, in order to assess the turnover of these polymers, as well as the possible hormonal control of such turnover, it will be

necessary to use labelled precursors.

The basic structure of the xyloglucan reported in this thesis has been elucidated as similar to that found for the dicot xyloglucans. Although some of the fine detail was not investigated, the important concept of a β-1,4-linked glucan backbone to which sidechains of xylose, galactose and fucose are linked, was proven fairly conclusively. Detailed partial hydrolysis studies, similar to those successfully employed by Aspinall *et al.*, (1977) on rapeseed xyloglucan, are needed to confirm the suggested structure.

Other significant aspects will also require future investigation; namely (i) the extraction of the radiata pine hypocotyl xyloglucan free of any glucomannan, and (ii) the linkage and anomeric configuration of the xylose residues. Reduction of the methylated xyloglucan with sodium borodeuteride with subsequent GC-MS analysis will confirm the xylose linkage as either 2- or 4- (see Lindberg, 1977).

Finally, the use of enzymes, such as the cellulase from *Penicillium notatum* (Courtois *et al.*, 1976) or the endoglucanase from *Trichoderma viride* (Bauer *et al.*, 1973) which cleave specific glycosidic bonds, used in conjunction with these other studies, will allow reconstruction of the polysaccharide fragments so that a knowledge of the whole polysaccharide may be determined.

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