STRUCTURAL STUDIES ON CELL WALLS
OF <u>PINUS RADIATA</u> WITH PARTICULAR
REFERENCE TO CALLUS CULTURED CELLS

A THESIS PRESENTED IN PARTIAL FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

AT

MASSEY UNIVERSITY

NEW ZEALAND

DALLAS R. FENEMOR

1982

ABSTRACT

A study of the composition of the primary cell wall of <u>Pinus radiata</u> has been undertaken.

1. Preliminary work with hypocotyl tissue showed that hemicelluloses of hypocotyl consisted of a xylan (probably (4-O-methylglucurono)xylan) and a xyloglucan. Acidic sugars examined, showed that galacturonic acid was the main component, and that 4-O-MeGlcA was present.

2. Cell walls were prepared from callus tissue either by wet sieving in 80% ethanol (Batch 1) or by disruption in a French Pressure cell and washing with aqueous potassium phosphate buffer; (Batch 2).

    Each batch was submitted to a series of extractions with different reagents in order to investigate the mode of bonding of polymers within the walls. The polysaccharide and protein components of each fraction were studied by monosaccharide and amino acid analysis.

    Fractions of Batch 1 were assayed for lignin and selected fractions from both batches were studied by methylation analysis.

3. The results of investigations led to the following major conclusions. The non-cellulosic components recognised in the wall preparations were: -

    a) A(1→3)-linked galactan and an arabino-3,6-galactan which were largely extractable from the cell walls by hot water and may be only loosely bound in the cell wall.

    b) The pectic components consisting of;
        i) pectin, a galacturonate polymer containing a linear (1→4)-galacturonan back bone interspersed with branched rhamnose residues,
        ii) branched (1→5)-arabinan and
        iii) linear (1→4)-galactan,

which occurred together in cell wall fractions and
were not all extracted by classical extractants (such
as hot aqueous EDTA), some being tightly bound in the
cellulosic residue after alkali extraction.

c) A fucogalactoxyloglucan some of which was extracted by
water or EDTA, but the majority was extracted by
subsequent treatment with either alkali or in part by
a strong chaotropic reagent (6M GTC). Thus the
fucogalactoxyloglucan was probably bound in the cell
wall by strong hydrogen bonding. Some other bonding
may be involved in the GTC-resistant fraction.

d) A branched xylan which was removed by GTC and alkali,
the larger level being removed by GTC.

e) A galactoglucomannan, identified only by 4-linked
mannose residues in hot water extracts and strong
alkali fractions.

f) Hydroxyproline-containing protein which was extracted
from the cell wall by a variety of reagents but
hydroxyproline-rich protein remains tighly bound after
alkali extraction.

g) Lignin which was tentatively identified in the cell
wall. It appeared likely that cross-linking with
lignin would be responsible for the non-extractability
of some polysaccharides and protein from the walls. A
mild acid chlorite treatment followed by alkali
extraction removed most of the residual pectic
components, xylan and protein from the walls.

A basis has been laid for the further investigation of the
wall structure and isolation of polysaccharides.

ACKNOWLEDGEMENTS

CONTENTS

Page

TABLE OF CONTENTS CONTINUED   Page

TABLE OF CONTENTS CONTINUED   Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES   Page

LIST OF FIGURES   Page

LIST OF FIGURES   Page

ABBREVIATIONS

COMMON ABBREVIATIONS USED:

EDTA	Ethylene diaminetetra-acetic acid
TFA	Trifluoroacetic acid
TFA-enz	Trifluoroacetic acid -<u>Sclerotium rolfsii</u> enzyme treatment
Dimsyl	Dimethyl sulphinyl anion ($CH_3-S-CH_2^-$)
PEG	Polyethylene glycol
Amino Acids	Standard abbreviations are used
g.c.	Gas chromatography
g.c.-m.s. (G.C.-M.S.)	Gas chromatography-mass spectrometry
f.i.d./F.I.D.	Flame ionisation detection
m/e	Mass/charge ratio of ion fragments in mass spectrometry
mass spec.	Mass spectrometry
R_{xyl}, R_{xylose}	Coefficient of diffusion relative to xylose in paper chromatography
Inos	Inositol
2dglc - 2 deoxyglucose	2-Deoxy-D-glucose
Monocots	Monocotyledons
Dicots	Dicotyledons
Polysacch	Polysaccharide (only seldom used)
F.R.I.	Forest Research Institute (New Zealand)

SUGARS IN PLANT CELL WALLS:

Rha, L-Rha, L-Rha p	L-rhamnose,	L-rhamnopyranose
Fuc, L-Fuc, L-Fuc p	L-fucose,	L-fucopyranose
Ara, L-Ara, L-Ara f	L-arabinose,	L-arabinofuranose
Xyl, D-Xyl, D-Xyl p	D-xylose,	D-xylopyranose
Man, D-Man, D-Man p	D-mannose,	D-mannopyranose
Gal, D-Gal, D-Gal p	D-galactose,	D-galactopyranose
Glc, D-Glc, D-Glc p	D-glucose,	D-glucopyranose

OLIGOSACCHARIDES

GalA, D-GalA	D-galacturonic acid
D-GalA p	D-galactopyranosyl uronic acid
GlcA, D-GlcA	D-glucuronic acid
D-GlcA p	D-glucopyranosyl uronic acid
4-O-MeGlcA	4-O-methylglucuronic acid
4-O-MeGlcA-Xyl 4-O-MeGlcA-(1→2)-Xyl	4-O-methylglucuronosyl-(1→2)-xylose
GalA-GalA GalA-(1→4)-GalA	Galacturonosyl-(1→4)-galacturonic acid
GlcA-GalA GlcA-(1→4)-Gal	Glucuronosyl-(1→4)-galactose
GalA-Rha GalA-(1→2)-Rha	Galacturonosyl-(1→2)-rhamnose
GlcA-Xyl GlcA-(1→4)-Xyl	Glucuronosyl-(1→4)-xylose
MeGlcA-Xyl-Xyl	Methylglucuronosyl-(1→2)-xylose-(1→2) -xylose

POLYSACCHARIDES:

Named in text according to backbone structure. The general descriptions of polysaccharides are given in Chapter 1.

e.g.

β-(1→4)-mannan:	mannose (1→4)-linked in β configuration
Arabino-3,6-galactan:	polymer of (1→3) and (1→6)-linked β-D-galactopyranose units, to which are attached L-arabinofuranose units.

Arabino-(4-O-methylglucurono)xylan:

(1→4)-linked xylose backbone carrying arabinose, and 4-O-MeGlcA substituents.

METHYLATION DATA:

a) Sugar residues, with position of methyl groups,

e.g. 2,3,6-Tri-O-methyl-D-glucose

= 2,3,6-Me$_3$-glucose , or 2,3,6-Me$_3$Glc

b) Sugar residues indicating linkage positions,
    e.g. 4,6-linked glucose, are sometimes abbreviated
        to, e.g. 4,6-Glc.

CHAPTER 1

INTRODUCTION

1.1   PLANT CELL WALLS, MERIT FOR STUDY

The plant cell wall is regarded as a growing, constantly changing, composite material, consisting of a dispersed phase of microfibrils, within a complex continuous matrix of essentially polymeric material. In higher plants, cellulose exists as elementary fibrils visible with negative staining, several microns in length and approximately $35A^O$ in diameter, which from X-ray crystal lattice dimensions should correspond to a relatively small number of glucose chains in an associated arrangement, not clearly elucidated (1).

The cell wall imposes shape on the protoplast and determines expansion of the cell, yet knowledge of the structure and molecular mechanism behind this function is limited (2, 3). Though the understanding of the molecular nature of matrix polysaccharides has progressed, organisation and function of constituents in the 3-Dimensional structure is uncertain (1, 2, 3, 4, 5), and investigations of bonding between wall lignin, protein and polysaccharides is growing in importance (2, 6, 7).

Elucidation of the structure of the primary cell wall in particular, remains a centrally important step towards under-standing elongation, cessation of elongation and differentiation of the cell. Knowledge concerning the mechanism of the auxin induced response, the nature of the bonds undergoing change, and polymers involved in "stretching" must enhance an under-standing of growth and development processes. Hence the primary plant cell wall has been, and is still, the object of extensive degradative study (2, 6, 8, 56, 64, 67).

Secondly, wall polysaccharide composition is important economically in assessing nutritive value of various forage crops, towards understanding ripening of fruits, and chemical industrial uses, such as pulping for paper production.

Intercellular adhesion, a function of the primary wall important in the pulping process, has been studied by Kibblewhite et al (168, 169). Much is known of wood and bark polysaccharides (mainly those of gymnosperms and some woody angiosperms), yet relatively nothing on gymnosperm primary cell walls.

The process of differentiation of callus tissue is important in the forest industry, since the parenchymatous tissue can be used to study the changes that occur, in wood formation from the cambium, in mature trees. Tracheid length and wood quality is dependent on the growth and cessation of elongation of the primary cells of the cambium. Therefore the primary wall structure, the polymers, and polymer cross-linking which may affect growth and elongation, are important in providing a basis for improving wood quality through environmental and hormone regulation. The turnover of sugars in actively growing needles and shoots is also important in governing growth. This cannot be fully understood until the polysaccharides of the primary wall and their turnover have also been well characterised.

Albersheim (9) has surveyed the principal features of primary cell walls, explaining how cellulose fibres may be linked to each other by low molecular weight matrix hemicellulosic polysaccharides. In this way cellulose fibres may be indirectly linked together by covalent and non-covalent bonds (probably hydrogen bonds) resulting in a rigid network. His recent model (8, 9) though widely accepted for the primary wall is disputed by some workers (2, 3).

An alternative view expressed by Rees and Wight (43), and Knee (45), is that "non-covalent bonds are crucial to the overall strength and texture, because the walls can in principle be completely dispersed without breaking covalent bonds".

Additional compounds such as pentose-bound ferulic acid, may also be present (11) as well as proteins (12) and many active enzymes capable of metabolising cell wall constituents (13).

1.2   WALL ONTOGENY

Early electron microscope studies showed (26) that, in
metaphase of cell division, parts of the endoplasmic reticulum
penetrate the mitotic spindle of the equator producing a mesh
of tubules and vesicles, fusing in telophase to produce the
phragmoplast, and giving rise to the initial cell plate. This
grows laterally via a proliferation of tubules and vesicles at
the edges and establishes the plane of cell division. Thus the
plane of division, synthesis of material and its transport to
the cell plate is associated with the endoplasmic reticulum
system. It is thought that part of the cell plate materials
and even some of the cell wall substances are either transported
in and/or formed by the membranes and vesicles of the endo-
plasmic reticulum and golgi apparatus.

The middle lamella could result through cell plate modification,
via addition of material during development. The two plasma-
lemmas of the cell plate are formed possibly from the
membrane of golgi vesicles which is similar to that of the
plasmalemma. Fulcher et al (172) found evidence for the
transient formation of callose, a β-(1→3) glucan, associated with
the early stages of cell wall formation and cytokinesis.

After cell plate formation the primary cell wall is formed
during surface area increments, and subsequently the secondary
wall, during which the cell wall thickens.

1.3   CELL WALL CONSTITUENTS

The major classical cell wall fractions have been described
by Preston (65) as follows: -

a)   The Pectic substances, extracted by boiling water and
     hot ammonium oxalate.
b)   Hemicelluloses, extracted usually by 4N KOH, at room
     temperature.
c)   Cellulose, residual after Pectin and Hemicellulose
     extraction and often extracted with 72% sulphuric acid.

d)   Protein, often removed with polysaccharide fractions.

e)   Lignin, removed by acid-chlorite treatment.

Cellulose, pectic substances, hemicelluloses and protein constitute the major structural components of the primary cell wall. Secondary cell walls have increased levels of cellulose together with hemicellulose and lignin.

It is appropriate to discuss polysaccharides more specifically related to the primary wall since this work is concerned with a study of the primary wall. Most, if not all, primary cell walls studied contain pectic polysaccharides and have significant levels of uronosyl residues. Differences have been found between the hemicellulose present however. Determination of polysaccharides associated just with the primary wall have been hampered by the ability to obtain preparations free from secondary cell wall contamination. Nevertheless much work has been done to study the primary wall particularly by Albersheim (8, 9, 10), Monro and Bailey (2, 132, 133), Labavitch and Ray (19, 20), Northcote (26, 27, 41, 130, 131), Wilkie (57, 66), Joseleau (173), Selvendran (68), Stone (59, 60, 63), Rees et al (43), Siddiqui and Wood (48), Simson and Timell (141, 144, 153, 166), and Mollard et al (154, 155). Much of this has been done with hypocotyl and endosperm tissue, which is essentially primary walled. Advances in callus and suspension culture technology have enabled a systematic investigation of primary walls of the dicots, tomato, Red Kidney bean, soybean, and sycamore (53), and all have been found to be similar in polysaccharide composition. Rose callus has also been investigated by Mollard and Barnoud (154, 155). Also the primary tissues of the monocots wheat, rice, oats, sugar cane and brome grass cells, have been cultured in suspension from either the root, embryo or internode tissues, and their wall hemicelluloses surveyed (54).

1.3.1 Cellulose

Microfibrils are a constant feature of all walls of green
plants and in nearly all species the microfibrils consist of
cellulose, long chains of β-(1→4)-linked glucose residues,
with a degree of polymerisation of about 14000 in secondary
walls and 2000-6000 in primary walls (202). Within the
cellobiose units of the individual chains there are intra-
molecular hydrogen bonds which suggest that the cellobiose
units must have a bent conformation along the chain. Inter-
molecular hydrogen bonds help hold the chains together (27),
but there is still discussion (3) on whether the chains are
parallel or antiparallel, the arrangement affecting the
hydrogen bonding sequence, and therefore the mechanical
properties. The surface of the crystallite is closely covered
by OH groups, hence compatible wall polysaccharides can bond
strongly to the crystallite by hydrogen bonds.

1.3.2 Hemicelluloses

Hemicellulose refers to a broad group of heteroglycan
polymers, so named because of their close association with
cellulose and because they were once thought to be cellulose
precursors. They have been isolated mainly from mature
tissues, but also in later years from many primary walls. As
matrix polysaccharides they were thought to play a part in
bonding and growth of the wall. Wilkie considers poly-
saccharides are best described by using chemical terms
relating to their main structural features (66), but in so
far as hemicellulose refers to a total isolated fraction it
enables comparisons to be made between similarly obtained
polysaccharides from various different sources. Hemicelluloses
of the secondary wall have been divided into two broad groups:
the glucomannans and the xylans. (Northcote 27, Timell 49, 50,
Bailey 51, Aspinall 52). Those of primary walls may be
xyloglucans or mixed linked glucans, according to source,
often with a xylan as well.

Variations on basic structure render it valuable to discuss particular hemicellulose types individually.

a) Gluco-and galactoglucomannans usually form the bulk of the hemicellulose fractions of gymnosperm woods and high levels are also found in seeds. They occur in close association with cellulose in coniferous woods, and to a lesser extent in some hardwoods. They consist of chains of randomly arranged D-glucose and D-mannose residues linked β-(1→4) in the main chain. In gymnosperms, glucomannans can be divided into two groups according to solubility.

Galactoglucomannans once isolated, tend to be soluble in water (27); D-galactose/glucose/mannose ratio is commonly about 1:1:3. The molecules are small, usually with a Degree of Polymerisation (D.P) of about 100, and are probably O-acetylated at C-2, and C-3, of the main chain mannose residues.

The glucomannans tend to be insoluble in water and if resistant to weak alkaline extraction they can be extracted with alkali-borate solution (27), and subsequently fractionated with $Ba(OH)_2$. When prepared from gymnosperms, they always contain some D-galactose joined to the main chain as α-(1→6)-linked terminal units. The mannose/glucose ratio is approximately 3:1 and the D.P is usually greater than 200; acetylation may occur at C-2 or C-3 of mannose. Glucomannans occur to about 3-5% of the total cell wall material in angiosperms. Their general structure is indicated in Figure 1.1.

b) Xylans are laid down throughout the growth of the wall and form the bulk of the hemicellulose fraction (alkali soluble polysaccharides) of mature tissues in angiosperms. The general structure of xylans is that of a main chain of D-xylopyranose joined by β-(1→4) links. Xylans have a D.P. of about 150-200. Attached to these chains are L-arabinofuranose, D-glucuronic acid, or its 4-O-methyl ether, D-galactose and possibly D-glucose. L-arabinosyl units are generally furanosyl, (1→3)-linked to the xylan,

$$\longrightarrow 4)\text{-}\beta\text{-D-Xyl p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Xyl p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Xyl p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Xyl p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Xyl p -(1}\longrightarrow$$

$$\begin{array}{cc} 2\uparrow & 3\uparrow \\ 1 & 1 \\ \text{40Me-}\alpha\text{-D-Glc p A} & \text{L-Ara f} \end{array}$$
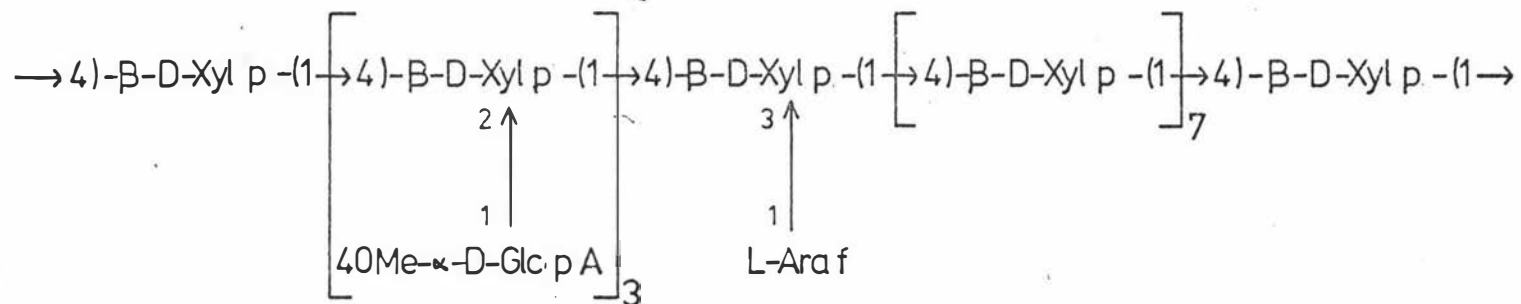
Figure 1.2 : Basic structure of a representative Arabino-(4-0-methylglucurono)xylan.
Timell (50)

$$\begin{array}{ccc} \text{D-Gal p} & & \text{D-Gal p} \\ 1 \downarrow & & 1 \downarrow \\ 6\downarrow & & 6\downarrow \end{array}$$

$$\longrightarrow 4)\text{-}\beta\text{-D-Man p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Glc p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Man p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Glc p -(1}\longrightarrow 4)\text{-}\beta\text{-D-Man p -(1}\longrightarrow$$
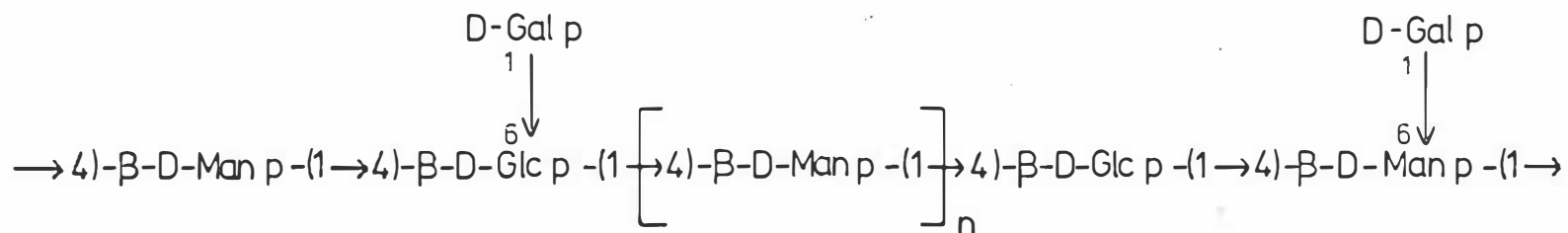
Figure 1.1 : Basic structure of a Galactoglucomannan from gymnosperms.
Timell (50)

while uronic acid residues may be linked through (1→2),
(1→3), or (1→4) links to the main chain. The (1→2)
linkage is the usual form. The grasses (monocotyledons)
have large amounts of xylans,α-L-arabinofuranose is
usually linked (1→3) to xylose, while the 4-O-methyl-D-
glucuronic acid is linked through the 2-positions (52).
The cereal gum arabinoxylans can have single side chain
units usually through position 3, but other xylose residues
have double branch points. Arabino-(4-O-methylglucurono)
xylans are also present in the cell walls of gymnosperms
but in smaller amounts than the xylans of angiosperms.
They have the same basic structure as that of the mono-
cotyledon xylans (30, 52). The Xyl/Ara ratio is approx-
imately 7:1 to 12:1 and the xylose/uronic acid ratio is
approximately 5:1 to 6:1 (27). Arabino-(4-O-methyl-
glucurono)xylans of gymnosperms are not considered to be
acetylated in vivo in contrast to the acetylated
(4-O-methylglucurono)xylans of woody dicotyledons (49,
50, 124). The general structure of xylans is indicated
in Figure 1.2.

Arabinoxylans.   Methylation data on alkali extracted
fractions from cultured ryegrass endosperm (Lolium multi-
florum) cell walls, indicate the presence of (1→4)-linked
xylans with most of the arabinosyl residues appearing to
be terminal, but the methylation data indicates that more
complex hetero or homo oligosaccharide substituents may
also be present (59). Xylans substituted with arabino-
furanosyl residues and oligosaccharides composed of
galactose, arabinose, xylose, glucuronic acid and its
4-O-methyl ether are characteristically found in cell
walls of stem, leaf, root, and husks of monocots (57, 58)
and in the suspension cultured cells studied, derived
from vegative monocotyledon tissues (54). Commonly,
heteroxylans from the Gramineae have low proportions of
L-arabinofuranosyl residues on C-3 positions, and either
4-O-methylglucuronic acid or glucuronic acid, or both on
C-2 positions. More complex side chains such as:
Galp-(1→4)-D-Xylp-(1→2)-L-Araf-1→C-3 of xylose in the

main chain are possible (58) as in the galactoarabinoxylan studied by Wilkie et al (58).

Glucuronoarabinoxylans were a major component found by Ray in oat (<u>Avena sativa</u>) coleoptile cell walls, and the GlcA was thought to be linked to C-2 of xylose in a β-(1→4) xylan backbone (19, 20). Non-starchy polysaccharides from monocotyledon endosperm, other than ryegrass are also rich in heteroxylans. (Mares and Stone - wheat, ref. 60, Fincher - barley, ref. 61 and others mentioned in ref. 59). The arabinose/xylose ratio is variable and uronic acids represent usually only a minor proportion. Glucuronoarabinoxylans have also been demonstrated in dicotyledon primary cell walls. The xylan of sycamore (171) has a linear β-(1→4)-linked D-xylopyranosyl backbone with neutral and acidic side chains attached at intervals along its length. The acidic side chains are terminated with glucuronosyl or 4-O-methylglucuronosyl residues, and the neutral side chains are composed of arabinosyl and/or xylosyl residues.

c) Xyloglucans are major hemicelluloses of dicot primary cell walls (8, 46), and they are structurally similar to the so called seed "amyloids" (deriving their name from the fact that they form coloured complexes with I_2 as does amylose), Aspinall (55). The structure consists basically of a cellulose-like β-(1→4)-linked glucan backbone with frequent xylosyl side chains attached to C-6 of backbone glucosyl residues and in addition further substituents may be linked to the 2 position of the xylose residues. Their general structure is as shown in Figure 1.3. Fucogalactoxyloglucans have been extracted from polygalacturonase-treated sycamore cell walls (8) by 8M urea, and analogous structures have been found in the extracellular polysaccharides secreted by suspension-cultured sycamore cell walls. More recently they have been demonstrated also in <u>Pinus radiata</u> primary walls by J.W. Little (145). As well as the fucogalactoxyloglucans observed (8, 48), galactoxyloglucans have been observed

$\rightarrow$ 4)-β-D- Glc p -(1$\rightarrow$4)-β -D-Glc p - (1$\rightarrow$4)-β-D-Glc p -(1$\rightarrow$4)-β-D-Glc p -(1$\rightarrow$

```
        6↑                    6↑                    6↑

        1                     1                     1
    α-D-Xyl p             α-D-Xyl p             α-D-Xyl p
        2↑                    2↑

        1                     1
    β-D-Gal p             β-D-Gal p
        2↑

        1
    L -Fuc p
```
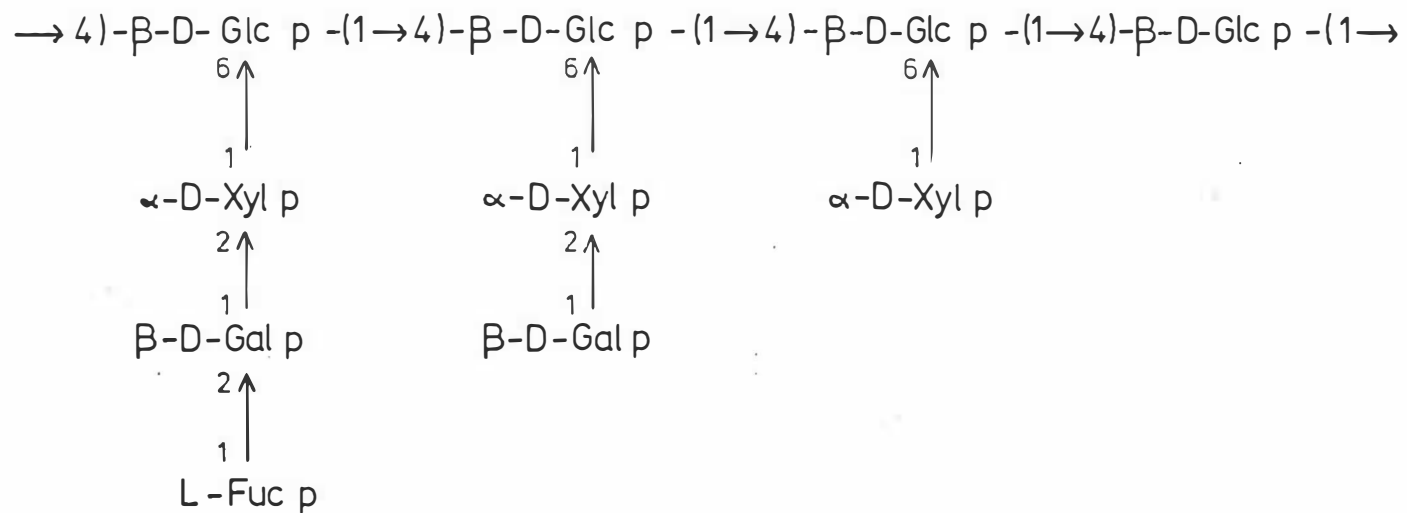
Figure 1.3 : General structure of a Fuco-galactoxyloglucan.
Albersheim (46), Little (145).

in dicot seeds and in gymnosperms (48, 54, 138), and also
an arabinoxyloglucan in <u>Nicotiana</u> leaves (201). In rape-
seed, both galactoxyloglucans and fucogalactoxyloglucans
appear to be present (48). Albersheim (46) has found that
xyloglucans will bind to cellulose at neutral pH and has
suggested that they may bind to the microfibrils, having
a role in elongation growth. Valent and Albersheim have
since disputed this (174).

d) Mixed linked glucans. Glucans containing varying
proportions of β-(1→3), and β-(1→4)-links have been
observed in primary walls of several plants, predominantly
monocotyledons, and are thought to be important
structural matrix components. Burke et al (54) observed
β-(1→3); β-(1→4)-D-glucans in ryegrass (<u>Lolium perenne</u>)
endosperm cell wall preparations but not in walls from
cultures of five other monocotyledon vegetative tissues,
(Bromegrass, sugar cane, rice, oat, and wheat), and they
suggested a nutritive rather than a structural role for
these polysaccharides. However, studies by Anderson and
Stone (59) on ryegrass, as well as by Forrest and Wain-
wright (62) and Fincher (61) on barley suggest the
presence of covalent interactions between β-glucans and
other wall polymers, in these monocots. Fincher found
the glucan in both the water and alkali-soluble fractions
of barley endosperm cell walls as did Anderson and Stone
(59), yet a portion of the mixed-linked glucan of the
wall is readily extractable with 8M urea indicating an
absence of covalent association with other cell wall
polymers. Forrest and Wainwright (62) showed that the
barley endosperm β-glucan existed in three forms, one hot
water-soluble, another hot water-insoluble but alkali-
soluble, and a third, insoluble in both, but which could
be solubilised by proteolytic enzymes such as thermolysin
or by hydrazinolysis, thus confirming that some of the
β-glucan is of true cell wall origin. This also reinforces
the involvement of protein in the organisation of cell wall
polymers. β-(1→3), β-(1→4)-glucans are missing from the
walls of suspension cultured gymnosperm - (Douglas fir)
cells (54).

1.3.3 Arabinogalactans

Type II of Aspinall (42).   Though once tentatively designated
as "pectic" polysaccharides (42) these are now more commonly
considered as a separate group.   Type II arabinogalactans,
as distinct from the pectic Type I linear β-(1→4)-galactan,
are characterised by (1→3) and (1→6)-linked β-D-galactopyranose
units containing terminal L-arabinofuranose and sometimes
D-glucuronic acid residues (42, 48).   They are most common in
coniferous woods, particularly larches (49, 50) where they are
present in the lumen of tracheids and ray cells, rather than
as a true wall component.   There is no evidence for the
existence of 3,6-linked arabinogalactans in monocotyledonous
wheat and ryegrass endosperm cell walls, isolated in 70%
aqueous ethanol (59 - 63) - although they are present in the
isolation and purification filtrate (63).   This suggests
that either they do not form part of the wall, or they readily
dissociate from it.   Galactose, if present in some monocot
primary walls, could be associated more with the major
hemicellulose arabinoxylan component (54), though the 3-, 6-,
and 3,6-linked galactosyl residues present in monocotyledon
walls surveyed by Albersheim's group (54), would suggest that
arabinogalactans are the types of polymers that may be found
in monocotyledonous primary walls.   Minor amounts of terminal,
3- and 3,6-linked galactose and minor amounts of terminal, 3-
and 2,5-linked arabinose in the polygalacturonase-released
fraction from cultured sycamore cell walls (47) were
suggested to be due to a small amount of the Type II
arabinogalactan.   Albersheim, who found a very similar
extra-cellular polysaccharide in the cell suspension with a
link to hydroxyproline protein, suggested that a 3,6-linked
arabinogalactan, though a minor polysaccharide, could be a
link between pectic polymers and protein in the wall.   This
link features in his original model for dicotyledon primary
walls (see Section 1.4).   The arabinogalactan proteins
secreted by cell suspension cultures have since been shown
to be clearly different from the cell wall hydroxyproline-
rich protein, with different amino acid composition and
different glycopeptide linkage; Gal-Hyp in the

arabinogalactan proteins and Ara-Hyp in the cell wall protein (5). These arabinogalactan proteins are widespread in plants, but do not appear to be cell wall constituents. Most of the Type II arabinogalactans may be arabinogalactan proteins (5). While polyuronide and associated galactan and arabinan require higher temperature, chelation and/or stronger alkali conditions for extraction, 3,6-galactans by virtue of their solubility in water are mostly only tentatively identified as true cell wall components.

1.3.4 Pectic Substances

This is a group designation for complex colloidal substances, containing polyuronic acids, and for polysaccharides associated and removed with uronic acid-containing poly-saccharides. Classically, pectic substances are those removed with boiling water (through β-elimination of uronic acid residues (143)), hot 70°C EDTA (42) or ammonium oxalate (43). Pectic polysaccharides of higher plants are a distinct component of middle lamella and primary cell wall region, formed when the active period of cell enlargement occurs (ref. 77, p. 205, 225). While their metabolism may be related to growth of the cell wall, plasticity cannot be solely explained by pectin metabolism, which has a specific role in new wall development. Pectic polysaccharides have been divided into three polymer types (8, 27, 42).

a) Acidic galacturonans and rhamnogalacturonans, are major constituents of pectic substances, consisting of chains of (1→4)-α-D-galacturonopyranosyl units, in which L-rhamnopyranosyl units occur linked as shown in Figure 1.4 in interior chains (8, 27). Typically rhamno-galacturonans have the structure shown in Figure 1.4, (3, 42) but variations of this basic structure may exist as shown in Figure 1.5 (27). The branching at rhamnose is of fairly normal occurrence; branching at GalA is less frequently encountered. A variable number of carboxyl groups are esterified as methyl esters, up to approximately 70% depending on the source and method of

$$\rightarrow 4)-\alpha\text{-D-GalA p}-(1\rightarrow\left[4)-\alpha\text{-D-GalA p}\right]_n(1\rightarrow 2)\text{-L-Rhap}-(1\rightarrow 4)-\alpha\text{-D-Gal A p}-(1\rightarrow$$
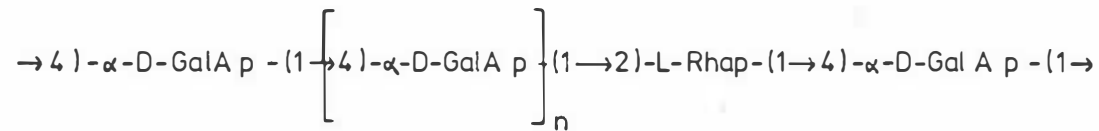
Figure 1·4 : Basic structure of a Rhamnogalacturonan.
reference (3)

There may exist variations on the basic structure as in fig 1·5   reference (27)
Branching at Gal A is less common than at Rhamnose.

$\rightarrow 4)-\alpha\text{-D-Gal A p}-(1\rightarrow 4)-\alpha\text{-D-Gal A p}-(1\rightarrow 2)\text{-L-Rhap}-(1\rightarrow 4)-\alpha\text{-D-Gal A p}-(1\rightarrow$

$\beta\text{-D-Xyl p}$ $\beta\text{-D-Xyl p}$ $\beta\text{-D-Xyl p}$

$\alpha\text{-L-Fuc p}$ $\beta\text{-D-Gal p}$
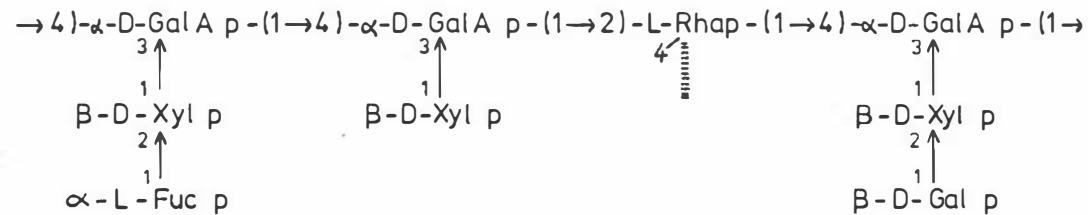
other side branches   i) β-D-GlcA p-(1$\rightarrow$4)-L-Fuc p
                                ii) β-D-GlcA p-(1$\rightarrow$6)-D-Gal p
                                iii) substitution at C_4 of L-Rhap of main chain (53)

Figure 1·5 : Variations of Rhamnogalacturonan structure.

extraction of the pectin (42). Pure rhamnogalacturonans seldom occur and main chains may have side chains containing L-fucose, D-xylose, and D-galactose. D-glucuronic acid is sometimes present in the poly-saccharide and is found attached to β-(1$\to$4)-links to fucose and by β-(1$\to$6)-links to galactose where it may terminate some of the side chains (27).

According to Rees and Wight (43), the interaction of unbranched galacturonan chains should provide a strong cohesive force within the cell walls. Galacturonans with low rhamnose content and little branching have been characterised from mature tissues such as rapeseed hull (198), as well as from actively growing cells from a sycamore cell-suspension culture (8). Pectins of mustard cotyledons (43) and other tissues with potential for rapid enlargement such as Pollen (Pinus mugo) and soybean cotyledons (43) have galacturonan backbones so interrupted with rhamnose residues, and branched on the GalA residues as well as rhamnose residues that scope for packing and cohesion is minimal (43). These polysaccharides would have a lubricating function which might be necessary for slippage of structural elements within the wall or between walls. Detailed studies of pectic acid and pectic arabinan of white mustard seed before and during germination (213) showed that a similarity existed between the arabinan and neutral portion of the pectic acid. There were complex changes in the arabinan during germination, related to changes in wall properties.

Results suggested that the arabinan and pectic acid may have some metabolic relation to each other. The arabinan in these studies was shown not to be a β-elimination product of pectin (203). Stoddart and Northcote (44) found two distinct types of pectic acid in sycamore, one which contained large blocks of arabinose and galactose, and the work indicated that the blocks are related to a separate neutral pectic arabinan-galactan fraction.

The level of 2-linked rhamnose in polygalacturonic acid
chains is higher in elongating tissues (43), causing
"kinking" and a break away from ordered chain
conformations. Side chains, "kinking" and de-esterificat-
ion in elongating tissue could prevent formation of
microcrystallite pectic gels (43), by disallowing align-
ment of polyuronide chains where the tertiary structures
may provide cohesion and strength within the wall.

b) Neutral Arabinans, are highly branched polymers of
L-arabinose linked α-(1→3) and α-(1→5). The arabinan
found in Pinus by Roudier and Eberhard (140) was
predominantly 5-linked with branching principally at
C-3 but also at C-2. Side branches were both single
residues and Ara-(1→3)-Ara→, disaccharides. A similar
arabinan was observed in the walls of phloem of Scots
Pine by Fu and Timell (137). Thus these arabinans
resemble the typical dicot pectic arabinans such as rapeseed
arabinan (204). Similar polysaccharides may be covalently
linked to other pectic substances and may be liberated
during isolation by β-elimination of pectin, but an
arabinan from white mustard cotyledons was evidently not
a product of such a degradation (203). The role of
arabinans in seeds and plant cell walls is not known with
certainty, but supposedly is related to cohesion and
bonding (27, 42 , 53).

c) Galactans Type I. (Aspinall 1973, 42). Galactans and
arabinogalactans that are often found associated with
pectic material consist of a basic framework of
β-(1→4)-linked D-galactopyranosyl residues (42, 47).
Similar polysaccharides have been found in the compression
wood cell walls of conifers, for example the β-(1→4)-
galactan isolated from the compression wood of Red Spruce
(138), which had a branch point only approximately once
every 50 galactose residues.

1.3.5 Callose and Laricinan

Callose: a linear β-(1→3)-D-glucan. In gymnosperms and angiosperms it fills the sieve pores of non functioning or dead sieve cells of tree barks (176). The β-(1→3)-glucan can be isolated from the phloem. It can be isolated from the wall by chlorite extraction and purified from starch by treatment with α-amylase, and its insolubility in hot water (176). A closely similar polysaccharide structurally has been isolated from compression wood of several conifers (175). The glucan, "Laricinan", (named because of its isolation from Larix laricina), consists of a minimum of 200 β-D-glucose residues, most of which are joined by β-(1→3) glucosidic bonds, with only approximately 6-7% by (1→4) linkages. A small number of glucuronic and galacturonic acid residues are present as for callose.

1.3.6 Comparison of Monocotyledon and Dicotyledon Primary Walls - Polysaccharides

Like dicots, monocot primary walls have cellulose in an amorphous matrix, however the most noticeable difference is that arabinoxylans are the principal components in monocots, replacing the major hemicellulosic xyloglucan in dicots. However arabinoxylans have been found in dicot primary walls in lower levels than the xyloglucan and vice versa (56, 177). As in sycamore, monocot walls contain a variety of possible polymers containing arabinose and galactose or both and it is not known whether these are similar to sycamore. They also contain uronosyl and rhamnosyl residues suggesting that pectic polymers are an integral part of both types of wall.

1.3.7 Protein

The glycoprotein of the wall contains hydroxyproline. Only traces of this amino acid are found in the cytoplasm (27). It seems probable that the glycoprotein is attached in some way to wall polysaccharides, and is of structural significance to the wall (3, 28), yet there is no complete agreement
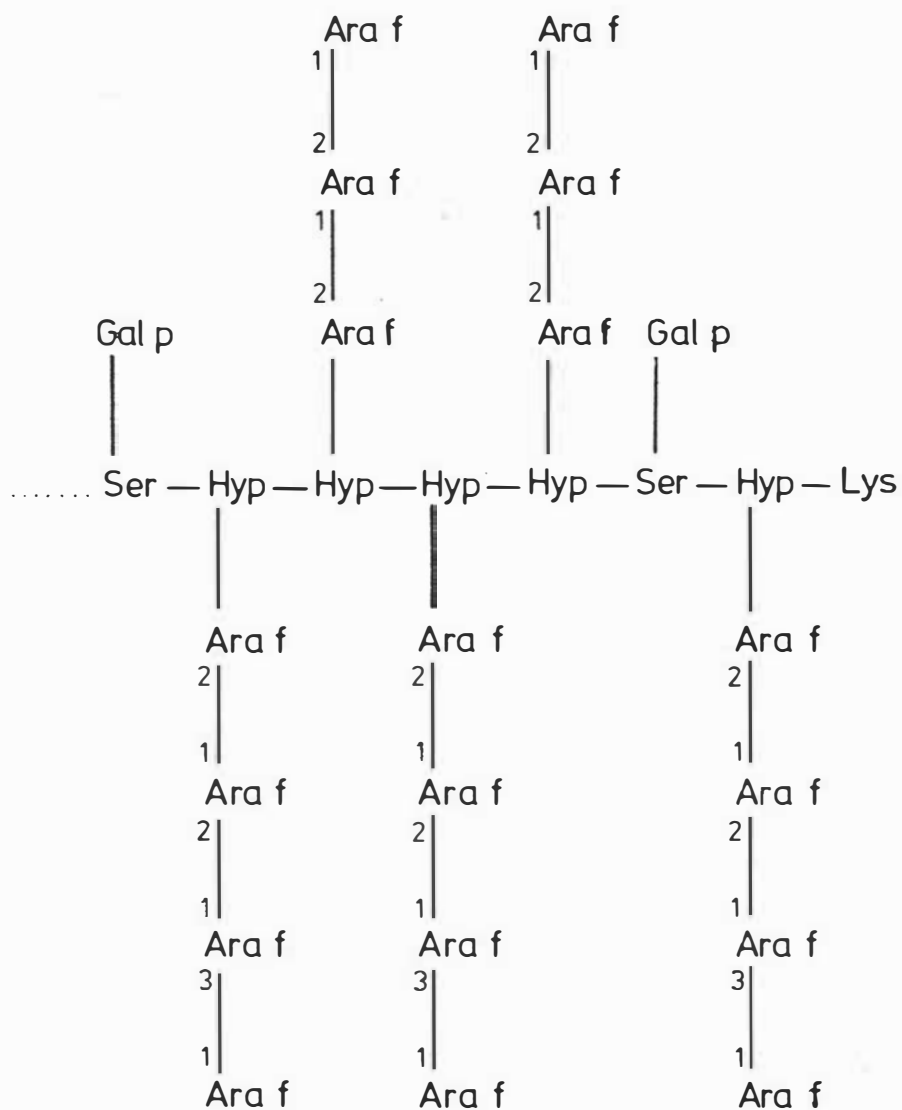
Figure 1.6 : Possible structure of cell wall glycoprotein
segment.
From Clarke et. al. (3, 5).

concerning the structure of the linkage region in the glycoprotein, the polysaccharides to which the glycoprotein is bound or whether one or more distinct proteins exists (3). Lamport's suggestion that the hydroxyproline residues carry arabinose side chains of known length while the serine carries galactose (28, 29) has tended to stand (3), but the linkage to polysaccharides through the galactose-serine (5, 8, 9, 29) has never been demonstrated. The name "extensin" was used to imply involvement in elongation growth. Extensin is thought to be functionally distinct from the water soluble arabinogalactan-proteins. A possible structure of cell wall glycoprotein segment is given in Figure 1.6 taken from Clarke et al (5).

Selvendran and co-workers (32, 33) demonstrated the presence of similar glycoproteins in Phaseolus coccineus wall preparations, and glycoproteins rich in hydroxyproline, arabinose and galactose have also been found to be major cell wall components of the green alga Chlamydomonas reinhardtii (5) and are present in wall preparations of other green algae (34, 35). Selvendran also found a hydroxyproline-"poor" protein fraction associated with the hemicellulose "A" fraction, which was not extracted with acid-chlorite as is the hydroxyproline-rich fraction (32, 33, 67). The arabino-galactan-protein secreted by cells grown in suspension culture and found to be widespread in plant tissues is also a hydroxyproline-protein, but appears to be not normally a cell wall component, (5).

1.3.8 Lignin

Lignin is a three dimensional polymer formed by random oxidative coupling of phenylpropane derivatives biosynthesised from phenylalanine. The phenylpropane units are linked in the polymer by C-O-C and C-C bonds. In softwoods most phenylpropane units have a methoxyl group plus a phenolic oxygen. Lignin is essentially a characteristic of secondarily thickened walls, penetrating the wall from the outside (primary wall and middle lamella), inwards at an early stage

of secondary thickening (27). As a hydrophobic filler it
encrusts the microfibrils and matrix polysaccharides so the
wall may become thicker. In addition, covalent bonds may
form, cross-linking polysaccharides and lignin (27)
(evidenced by the isolation of lignin carbohydrate complexes)
and possibly wall protein and lignin. Lau et al (36) found
in developing Pinus elliotti hypocotyls, that lignin formation
commenced only after cessation of cell wall elongation. How-
ever, there is evidence suggesting that even in essentially
primary walled tissue small lignins or lignin precursors can
exist, which will form strong linkages to carbohydrate, (6,
37, 38) and their role is further implied in regulating
growth by Fry (39). Whitmore has also found evidence (7) that
an early stage of lignification may be cross-linking of protein
of the primary wall, during polymerisation of lignin monomers.
In mature softwood, lignin reaches about the same content as
cellulose, but it is only about one half to two thirds this
level in hardwoods (130).

1.3.9 Tannins

Condensed tannins (proanthocyanidins or flavalones) form in
cell vacuoles, depositing towards the outside of the vacuole
near the cell wall (178) in Pinus elliotii. Being insoluble
in aqueous solvents, tannins may often be included in cell
wall preparations. Their effective removal from a protein-
tannin complex is described by Jones and Mangan (40) by
solubilisation with polyethylene glycol.

1.3.10 The Gymnosperm Primary Wall - Polysaccharides

Though much information is available on isolation and structure
of polysaccharides from xylem and phloem of gymnosperms, it is
almost completely lacking on polysacchs or polysaccharide
fractions of the primary wall. Early work by Meier (167) on
the distribution of polysaccharides in wood fibres showed
that in young fibres (middle lamella and primary wall) of
Birch, Spruce and Pine, there were high proportions of
pectic acid, arabinan and galactan, and cellulose content was

lowest in these walls. The glucomannan content was higher in
secondarily thickened walls as was the glucuronorabinoxylan,
and there appeared to be no arabinan in the secondary walls
of spruce and pine. Thornber and Northcote (130, 131) found
the pine cambium contained large amounts of pectic substances
(uronic anhydride, galactan, arabinan). Glucans, mannans, and
xylans were essentially features of secondarily thickened walls.
The small increase in galactans and arabinans may have been
due to formation of galactoglucomannans and arabinogalactans.
Meier and Wilkie (148) summarised the structures of polysac-
charides of pine and other coniferous woods and estimated the
proportions of these in the middle lamella and primary wall
regions. In these early studies the polysaccharides present
were inferred from the sugar compositions of the cell wall
regions. Numerous primary cell wall polysacchs of gymnosperms
have been characterised at least partially from wood and or
bark.

General procedures for isolation of polysaccharides from
wood and bark have been discussed by Timell (49, 50, 128,
129), by extraction of the holocellulose after chlorite
treatment successively with water, ammonium oxalate, and
alkali and with alkali $-BO_3^{3-}$. Polysaccharides are obtained
by fractionation and purification of the extracts, and the
final residue contains cellulose. Examples of the types of
polysaccharides that may be associated with the primary wall
in gymnosperms are demonstrated by the arabinans found by
Timell (137) and Roudier and Eberhard (140), xylans such as
arabino-(4-O-methylglucurono)xylans (125), galactans, (127,
138, 139) and pectic acid (54). Pinus arabinans are composed
of (1→5)-linked α-L-arabinofuranose residues many of which
are branched at C-3, and some at C-2 and C-3. The xylans,
contain backbones of (1→4)-linked xylose residues, (49, 50,
125) with branching at C-2 and C-3. In gymnosperms the
predominant xylan is the arabino-(4-O-methylglucurono)xylan,
(isolated from both wood and bark (50, 125)). To the main
chain are attached terminal side chains of (1→2)-linked 4-O-
methylglucuronic acid, and (1→3)-linked L-arabinofuranose
units. The uronic acid/arabinose/xylose ratio varies, but it

is usually round 1:1:6.

Gymnosperm galactans characteristically have backbones of
β-(1→4)-linked galactose residues. The example in Red Spruce
compression wood, (138) has a framework of approximately 300
(1→4)-β-D-galactose residues, with 5 to 8 branches occurring
per average molecule. The slightly acidic galactan from
compression wood of Tamarack (139), is slightly branched at
C-6, by a terminal galacturonic acid. The main chain has
200-300 (1→4)-linked β-D-galactose residues, and a branch
occurs only about every 20 galactose units (139).

The only other galactans occurring in wood of gymnosperms in
large quantities are the arabinogalactans, discussed in
Section 1.3.3. These are highly branched, H_2O-soluble and not
considered to be integral polymers of the wall.

A study by Albersheim (54) with suspension cultured Douglas-
fir cells, showed these to be rich in cellulose, protein and
uronic acid compared to monocot primary cell walls and
relatively deficient in non cellulosic sugars. A pre-
dominance of 5-linked and the occurrence of 3,5- and 2,3,5-linked
arabinose indicated a highly branched arabinan to be present.
Hydroxyproline was found in higher levels than for monocots,
but lower than in dicots and the absence of 2-linked
arabinose led to the conclusion that hydroxyproline arabino-
sides if present must be structurally different to arabino-
sides elsewhere (8, 28, 29, 31). The presence of 4,6-linked
glucosyl, terminal and 2-linked xylosyl and terminal
galactosyl residues were thought to indicate the presence of
a galactoxyloglucan, something like that of sycamore but with
many xylosyl residues substituted at C-2 with galactose
residues, many of these which should be terminal. Galacto-
xyloglucans have been found in compression wood (138) with a
Xyl/Gal/Glc ratio of 4/1/5 resembling amyloids. Methylation
data was not obtained on the sugar linkages. The first
evidence in gymnosperms was tentative, (200) but they have
been demonstrated fairly conclusively by Ramalingham and
Timell, who found a galactoxyloglucan in bark (183) and

Schreuder et al (138) who observed a galactoxyloglucan also
in compression wood of Red Spruce. A galactoxyloglucan from
Spruce bark (183) whose Gal/Glc/Xyl ratio was 1/4/3, was
branched at C-6 of some of the β-(1→4)-linked glucose residues.
Most xylose residues were also β-(1→4)-linked and the majority
of the galactose occurred as non-reducing end groups. This
seems to differ from the xyloglucan found by Albersheim and
the xyloglucan partially characterised by J.W. Little (145)
from Pinus radiata hypocotyl, in the mode of linkage between
xylose residues. The xyloglucan observed by J.W. Little
carries terminal fucosyl attached to C-2 galactose which is
attached to C-2 of xylose residues. Much of the xylose
exists as single side branches at C-6 of glucose. Fucose
does not appear to be present in the other xyloglucans
mentioned above.

1.4   WALL MODELS

The most recent comprehensive model of primary cell walls has
been that of Albersheim (64) who obtained fairly precise cell
wall fragments by using purified hydrolytic enzymes, then
methylating these fractions to deduce their polysaccharide
composition. From the overlap of certain polymer types in
different fragments the structural arrangement of polysac-
charides was inferred. Pronase digestion of walls pretreated
successively with endopolygalacturonase-endoglucanase,
released a fraction richer in sugar than if the pretreatment
had not been used. The protein of the fraction was 12%
Hydroxyproline and the carbohydrate composition was similar
to both the neutral sugar rich pectic fragments released by
endopolygalacturonase, and the pectic fragments in the acidic
endoglucanase product. The fraction of pronase products
consisted predominantly of pectic fragments, and results
indicated that the wall glycoprotein is attached in some way
to wall polysaccharides. Albersheim suggested that this
linkage may be a short 3,6-linked arabinogalactan. His only
characterisation of such a polymer was on the extracellular
3,6-arabinogalactan-protein that he found for sycamore. His

Figure 1·7 : Possible structural organisation of Primary Cell Wall modified after Keegstra et al 1973, Albersheim 1975.
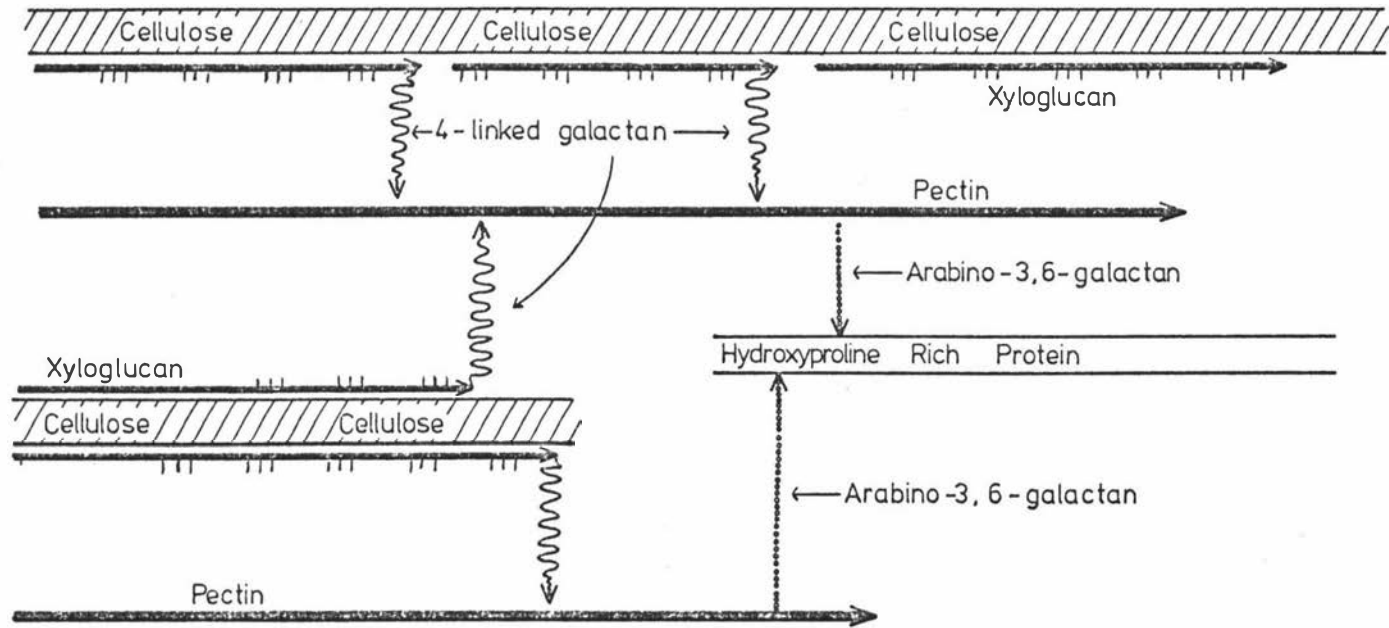
reasoning that extracellular polysaccharides are character-
istically representative of wall polymers has been shown to
be not generally valid (see Section 1.3.3). Realising the
tenuous nature of the evidence for a structural role of
protein in the wall, Albersheim put forward an alternative
model in which protein was omitted (9). The essential
elements of both of Albersheim's models are combined in
Figure 1.7, with all polymers linked into a single macro-
molecule. From a study of the hemicellulose of bean and
sycamore and from comparisons of methylated alditol acetates
from a variety of plant cell walls, he concluded that all
these walls were composed of similar polymers. He proposed
that his model could apply to dicotyledons generally. He
considered that since monocotyledon cell walls are all
similar to each other, but dissimilar to dicots in that
xyloglucan is replaced by arabinoxylan and mixed-linked
glucan, it is possible that monocots follow a similar
architectural plan. Preliminary evidence with Douglas fir
suspension-cultured cells suggested a similar wall structure
to that of dicots (54).

Monro et al (2) working with chemical extraction methods on
mung bean hypocotyl have proposed several differences from
the results of Albersheim et al (8). Treatment with alkali
(10% KOH at 20-24°C) removed hemicellulose without extracting
polyuronide (2, 133). Extractions of hemicellulose A
(including xyloglucan) should be accompanied by release of
extensin and polyuronide according to the model of Albersheim.
The complete insolubility of the pectin polysaccharide in the
alkali extraction system (133) which dissolves most of the
hemicellulose and glycoprotein, suggested that these latter
compounds were either not covalently linked to the pectin or
only through the alkali-labile ester links. At neutral pH
however guanidinium-thiocyanate (GTC) which presumably does
not split ester links (133) solubilised some of the
glycoprotein especially from the pectin-rich upper segments,
but none of the pectic substance was removed. Results
suggested that for mung bean hypocotyl, much of the pectin,

glycoprotein and hemicellulose was not involved in the type of cell wall structure established by covalent links as proposed by Keegstra et al (8). 6M GTC which is a powerful chaotropic reagent will remove about one third of the 10% KOH soluble hemicellulose (including xylan), from depectinated hypocotyl. If the only linkage between hemicellulose and the microfibrils is by hydrogen bonding then reagents such as 8M urea or 6M GTC should extract most of the hemicellulose and protein, but further hemicellulose could be extracted by 10% KOH at 0°C (2), which was rich in xylose. With depectinated hypocotyl, 10% KOH at 0°C extracted 60% of the wall hemicellulose, (including that extracted by GTC) without extracting the wall protein, but Monro et al report at 0°C that there was no loss of serine in extraction, though this did occur at room temperature with 10% KOH. They concluded that β-elimination of galactosyl-serine links was not necessary for the extraction of the bulk of wall hemicellulose, which would then not be dependent on links to serine for its covalent association in the wall (132). At 18-22°C, 10% KOH released most of the hemicelluloses not soluble at 0°C with the wall protein (measured by hydroxyproline extraction) (135).

Galactose-rich polymers were released first, and after 4 hours arabinose-rich polymers were extracted. It is unlikely that the arabinose was part of a serine-linked arabinogalactan, and a linkage of the protein in addition to that involving a galactan at 18-22°C was suggested (2). Not all of the wall protein or non-glucose polysaccharides were removed from the wall by 10% KOH in Monro's work. There was suggestive evidence for further alkali resistant linkages or some physical entanglement of polymers that rendered their chemical extraction impossible. Monro et al (2) suggested a more direct association of the protein with the cellulose, rather than through arabinogalactan, polyuronide, polyuronide side chains and xyloglucan.

Monro and Albersheim's results may be partially reconciled in that Albersheim used suspension-cultured cells, and that these lost some of their hemicellulose (including arabino-xylan) into the medium. These might be comparable to the $0^{\circ}C$ KOH - hemicellulose of Monro et al. But it is also likely that walls of suspension-cultured cells are considerably different from those of hypocotyls, despite the overall similarity in methylation analyses. It is possible that a situation may exist where pectin does not link all the extensin or hemicellulose to cellulose microfibrils, as suggested by Monro et al (2). Unfortunately much of the covalent bonding between polymers in the Monro model is unspecified.

1.5   WALL ELONGATION

Far from being an inert material, the wall can be regarded more as the result of a balanced turnover, with polysaccharides being continuously metabolised during the life span of the cell (146, 164). Elongation is triggered by Indole acetic acid (1AA) and giberellic acid (GA.3). Extrusion of H^+ into the wall is dependent on 1AA concentration, and stimulates wall expansion (15). Elongation growth inhibitors such as abscissic acid and cycloheximide prevent H^+ secretion (14, 69), suggesting a role for H^+ in elongation. Optimal growth is usually reached at pH 5.0 (13, 16). Albersheim suggested elongation for angiosperms may be by creep of H-bonded xyloglucans along cellulose at high H^+ ion concentration. This was demonstrated to be unlikely by Valent and Albersheim (174) and doubted by Monro et al (2, 132, 134, 135), who believe polymer creep should be at right angles to the direction of elongation (2), if it is involved and not along microfibrils.

An enzymatically regulated turnover at lower pH of a hemi-cellulose connecting pectin with cellulose could be responsible. When pea stem sections were incubated in the presence of radioactive glucose, a polysaccharide containing

xylose and glucose was shown to turnover (64), this polysaccharide was shown to be a xyloglucan structurally similar to the one studied by Albersheim et al (64). The hypothesis that expansion growth depends on IAA - induced H^+ - extrusion (14, 15, 16) was supported by the observation that IAA and lowered pH both promote appearance of water soluble xyloglucans in dicotyledon walls of pea stem tissue (16). Neutral pH abolishes both expansion growth and xyloglucan formation (13, 16). Though liberated xyloglucans appear important for elongation in dicots, the primary mode of action of H^+ or IAA is uncertain (13). IAA-induced growth and plasticity changes have been regarded as metabolic events, correlated with ATP formation and respiration (23). Roland (1) suggested that an auxin-plasmalemma interaction, causing a conformational change in the membrane, might alter activities of membrane-associated enzymes. Hager et al (24) postulated that auxin activated a membrane-bound anisotropic ATPase or proton pump to raise H^+ concentration to activate wall "softening" enzymes.

Transcriptional factors released by auxin activated plasma-lemmas translating interactions into wall extensibility have been postulated (25), but the change is too rapid for this explanation alone. The 10 minute lag with IAA-induced H^+ extension, is not observed with fusicoccin which acts directly on the plasmalemma ATPase (180) causing H^+ extrusion. Ray (181) suggested that an endoplasmic reticulum IAA-activated ATPase H^+ pump, allowing H^+ extrusion to the outside of the cell, could explain the time lag due to the lengthy route of transport. Pope (182) suggested that IAA- and H^+-stimulated elongation are separate events, and IAA can promote short term growth by a mechanism independent of acidification. Whatever the precise mechanism, IAA induces loss of β-glucans (20, 184) and solubilisation of xyloglucans (16). If bonding between hemicelluloses and cellulose is important in expansion, there appears to be some uncertainty from the literature (10, 19, 20, 56) as to the hemicellulose involved for monocots compared to dicots.

Cessation of elongation has been associated with accumulation of hydroxyproline-rich proteins (12, 18, 22) in pea epicotyls.

1.6   AIM OF THIS WORK

In view of the small amount of work that has been done directly on the primary wall of gymnosperms, this is an area that needs completion before primary cell wall structures can be fully assessed. It is also relevant before postulating any new wall models or making any further assumptions about the applicability of any existing models.

The work has set out to study the structure of the primary cell wall of Pinus radiata, a gymnosperm, with emphasis on the polysaccharide composition in order to compare it with the model proposed for angiosperms. Such a study would provide insight into the mechanism of certain physiological processes, viz.

1) extension growth and wall turnover, during seedling, shoot and needle development.

2) differentiation of callus cultures.

3) wood development from cambium.

4) invasion of tissues by pathogenic fungi (e.g. Dothistroma pini).

Pinus radiata is economically important to New Zealand both as a building material and to the pulp and paper industry and any further knowledge gained on the polysaccharide composition of tissues in pine is advantageous. The properties of the primary wall determine cell adhesion and separation, important to pulping. A knowledge of primary wall polysaccharides and their turnover, is important in understanding the total sugar balance in actively growing regions, before factors which affect this and hence the growth of the tree can be fully appreciated.

The principal source of primary cell walls has been callus
tissue, though some work has been done on the seedling
hypocotyl also. Thus it has been possible to make some
comparisons between hypocotyl and callus fractions. Some
data already existed on the xyloglucan found in hypocotyl
(145) which increased in abundance as elongation proceeded.

Calluses can be maintained in a relatively undifferentiated
state if maintained on the correct medium and therefore their
cell walls might present a reasonable model for primary walls.
Working with callus tissue may enable some comparison with
work with suspension cultured cells (in this regard it is
unfortunate that cells could not be grown in suspension) and
also with work on callus tissue (e.g. rose cell wall poly-
saccharides - Mollard and Barnoud (154, 155), compositional
studies on tobacco callus (208), and studies on pine callus
(6, 7, 11, 178)). This work lends itself to forming a basis
for the study of differentiation in callus.

Though suspension cultured cells do provide a reasonably
uniform type of cell wall for study, especially valuable for
comparison with work of Albersheim et al (8, 54), they are
not a perfect model for primary cell wall, since much material
may be lost into the culture medium. Callus tissue of Pinus
radiata was studied for the following reasons.

1) It is easier to grow, by comparison with suspension
    cultures.

2) It is relatively undifferentiated if hormone balance
    is correct (185).

3) It is important for an understanding of callus
    differentiation processes (6, 7, 11).

4) It is more suitable than suspension cultures, with
    regard to loss of cell wall material.

5) It enables a comparison with hypocotyl work.

6) Further work on the cambium is intended for a comparison with the integrated state in mature trees.

7) It should enable a comparison with results on other types of callus cell walls such as those of rose (154).

8) There is interest in early stages of lignification such as occurs in callus and early cambium derivatives, and the role this plays in bonding, strength and elongation of the wall (6, 7, 11).

CHAPTER 2

MATERIALS AND GENERAL ANALYTICAL METHODS

2.1   MATERIALS

Ion Exchange Resins

Amberlite IR-120 (H$^+$). Lab. Reagent. BDH
Dowex 1-X 8, (200-400) Dry mesh. Sigma
DEAE-Cellulose, (Diethylaminoethyl cellulose). Whatman
    DE-32

Gel Filtration Reagents

Sephadex G-25 Fine, Bead size 20-80μ. Pharmacia.
Sephadex LH-20,100(Lipophilic), Bead Size 25-100μ.
Sigma

Paper Chromatography

Whatman Paper No 1

Chemicals used in Chromatographic Developing Reagents

Silver Nitrate. Pure. BDH
Aniline. Analytic Reagent grade. BDH
Phthalic Acid. 99% pure. Lab. Reagent. BDH

Indicators and Stains

Bromothymol Blue. May and Baker
Phenolphthalein, (1% Soln made); Congo Red, and
    Phloroglucinol. BDH
Triphenylmethane. Art 821195 Merck

Colorimetry Reagents

Phenol. Analytical Reagent. BDH
meta-Hydroxydiphenyl. Eastman Kodak. USA
Iodine/Potassium iodide. Lab. Reagent. BDH
Chloramine T. Lab. Reagent. May and Baker

para-Dimethylaminobenzaldehyde (Ehrlichs Reagent (83)).
  Analytic Reagent. Sigma.

ortho-Dianisidine. Lab. Reagent. BDH

Acetylbromide. Analytical Reagent grade. BDH

Enzymes

1. Glucose oxidase, from Aspergillus niger Type II.
   Sigma. 26,000 units/gm Solid. (One unit to oxidise
   1.0μMole of β-D-Glucose to D-Gluconic acid and H_2O_2
   per minute at pH 5.1 at 35°C). Enzyme was stored
   desiccated below 0°C.

2. Peroxidase from Horse radish Type II. Sigma.
   approx. 200 Purpurogallin units per mg Solid.
   One unit forms one mg Purpurogallin in 20 secs at
   pH 6.0 at 20°C. Enzyme was stored desiccated below
   0°C.

3. Amyloglucosidase Grade II (from Rhizopus genus mould).
   Sigma. 9660 units/gm Solid. One unit liberates,
   1.0mg of Glucose from Soluble Starch in 3.0 min at
   pH 4.5 at 55°C. Enzyme was stored desiccated at
   0-5°C.

4. α-Amylase; from Hog Pancreas. Type IV A. Sigma.
   α-Amylase activity: 1mg will liberate approximately
   11mg of maltose from starch in 3 min at pH 6.9 at
   20°C. Enzyme was stored desiccated at 0-5°C.

Hydrolytic Acids

1. Formic acid. Analytical grade. Ajax Chemicals,
   Australia. (redistilled for use in methylation work).

2. Trifluoroacetic acid (TFA). Analytical grade. Sigma.
   (redistilled before use).

3. Concentrated Sulphuric and Nitric acids were
   of high purity.

Reagents in Methylation Analysis

1. All bulk solvents (n-Hexane, dimethylsulphoxide, pyridine, acetic anhydride, dichloromethane, chloroform, methanol) were redistilled before use. DMSO and n-hexane were subsequently stored over Molecular Sieve Linde 4A drying agent.

2. Methyl iodide. Analytical Reagent grade. BDH

3. Sodium borohydride and sodium borodeuteride -98 atom % of Deuterium. Sigma.

Amino Acids

Hydroxyproline, Cysteic acid,   BDH and
α-Amino adipic acid, Ornithine.   Sigma Biochemicals

Sugars

Standard sugars were obtained from BDH, May and Baker, Sigma.
Inositol. Eastman Kodak (recrystallised before use).
D-Ribose, -Pure, Sigma.
2-Deoxyglucose - grade II. Sigma.
1,4-Mannonolactone. Pure, Analytical Reagent. Sigma.

Polysaccharides

Partly Purified, Wheat Flour Arabinoxylan and Carob Bean Galactomannan, were kindly provided by Dr I.G. Andrew, Massey University.

Growth Nutrients

All chemicals used were as pure as could be obtained (Source of supply, BDH or May and Baker, Laboratory Reagent or Analytical Grade, including Ascorbic and Citric acid).
Difco Purified Agar: Difco Laboratories, USA.

All Other Reagents

    (BDH, May and Baker, Sigma): were either denoted as
pure, or had a high level of purity by their reported
analyses.

Buffers

    These were made up as in Preparation of Buffers, in
Methods in Enzymology Vol 1 (1955) p. 138 for the required pH,
and diluted as required. The concentration is given as
the sum of concentrations of the forms of partly
ionised species for the buffer solution.

Miscellaneous

    Polyethylene Glycol (PEG) mw 5000. Koch Light.
Guanidinium thiocyanate (GTC). Eastman Kodak USA

    Sclerotium rolfsii fungus - Botany Department, Massey
University.
Pine sawdust, Tiritea sawmill. P.N., N.Z.
Phebalium xylan. Courtesy Dr I.G. Andrew.
Reduced (4-O-methylglucurono)xylan. Courtesy Dr V.
  Harwood, F.R.I., N.Z.

GENERAL ANALYTICAL METHODS

2.2   ESTIMATION OF MAIN COMPONENTS

2.2.1   Total Carbohydrate

a)   Spectrophotometric Assay.   Total carbohydrate was
estimated by the phenol-sulphuric reaction (78) modified
by Immers (79), since it was found to be simpler and more
reliable than the Anthrone assay (179) for total sugar.

To 0.5ml of sample containing up to 60μg of carbohydrate,
was added 0.5ml 5% aqueous phenol (Anal·R grade) and 3.0ml
concentrated sulphuric acid (Anal·R), was added rapidly
from a Zipette dispenser, then tubes were spun on a vortex
mixer and allowed to cool for 5 minutes.   Assays were
done in duplicate and standards contained between 20 and
60μg of glucose from stock solutions which could be
stored frozen over a period of months.   The absorbance
was read at 490nm.   All solutions including blanks were
read against distilled water and blank values subtracted
from sample readings.

b)   Summation Method.   This estimated the total carbohydrate
present by simply adding the results for neutral sugars
determined from gas-liquid chromatography with uronic
acid values, determined from spectrophotometric assay.
Uronic acid is expressed as mg equivalents of
galacturonic acid.

c)   Correction applied to spectrophotometric assay for total
sugar.   In the spectrophotometric assay (2.2.1a) each
sugar has a different absorbance at the wavelength read.
The standard curves obtained for sugars are shown in
Figure 2.1.   The Anthrone assay (179) was also found to
give different absorbances for different sugars at a
particular wavelength.   This limits the usefulness of
these assays in determining accurately the amount of
total sugar present in a hydrolysate or fraction.   It
was therefore necessary to determine a "theoretical

Figure 2.1: Phenol-Sulphuric Colorimetric Standard Curves (reproducible points) for Total Sugars
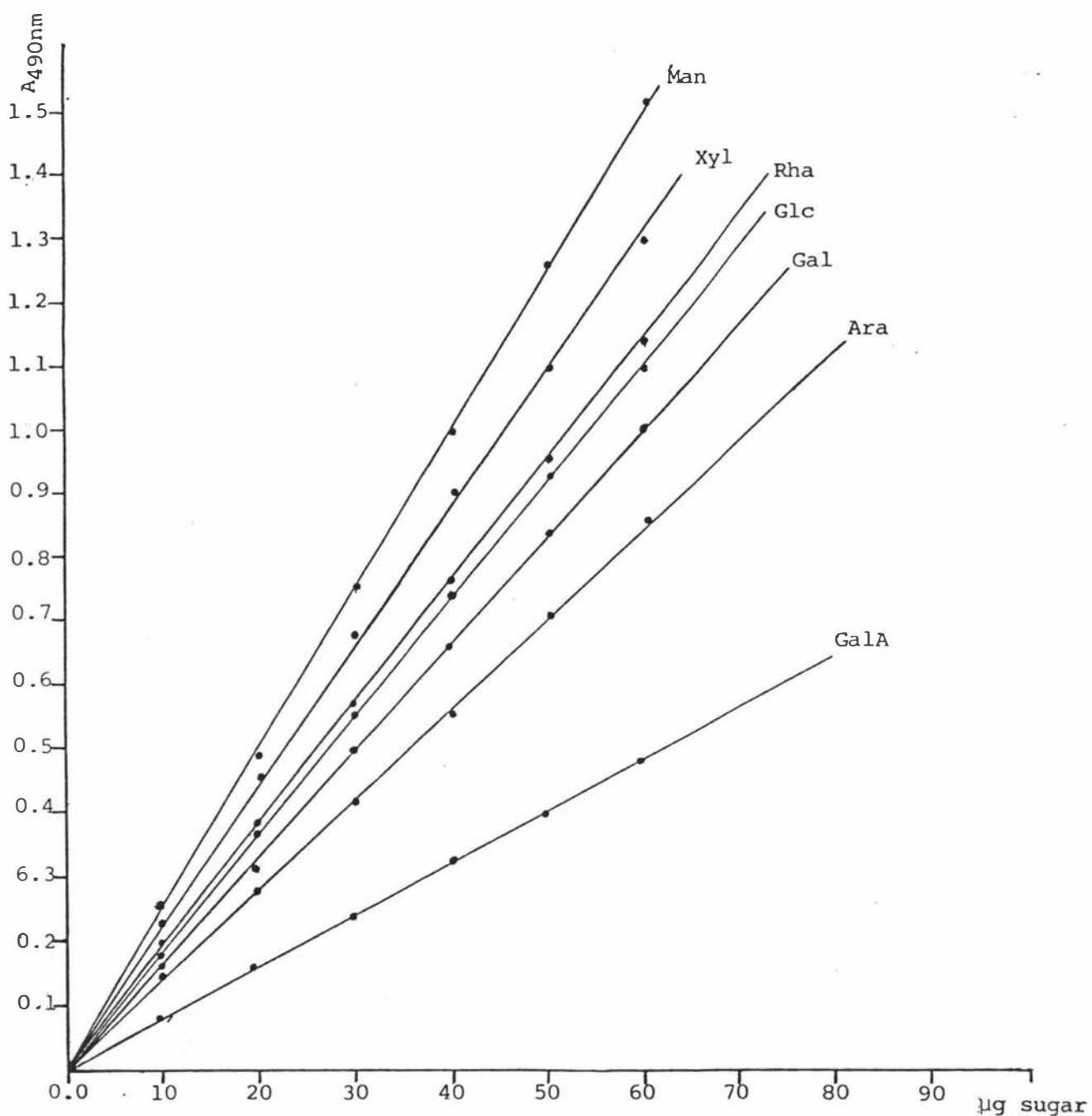
Standard absorbances at 490nm for 50μg of standard sugars in the Phenol-Sulphuric acid reaction.

Rhamnose, 0.95, Fucose 0.95, Arabinose 0.72, Xylose 1.1, Mannose 1.26, Galactose 0.84, Glucose 0.93, Galacturonic acid 0.4

standard absorbance" dependent on the composition of the
sample. The sugar composition was based on the neutral
sugar analysis by gas chromatography, summed with total
uronic acid (Section 2.2.2) and each sugar was expressed
as a percentage of the total thus determined. The
"Theoretical Absorbance" of e.g. 50μg polysaccharide was
then determined by multiplying the absorbance of 50μg of
each pure sugar by its % composition value. The sum of
absorbances for each sugar present gave the expected
"theoretical absorbance" for 50μg of sample carbohydrate.
By using this value for a standard absorbance, with the
actual absorbances obtained in the assay for aliquots of
fractions, a closer agreement was obtained between
colourimetry and results for the sugar content of a
fraction based on combined g.c. and uronic acid results,
than if glucose only was used as the standard in the
spectrophotometric assay.

2.2.2 Uronic Acid

Uronic acids were estimated spectrophotometrically as
galacturonic acid by the method of Blumenkrantz and Asboe-
Hansen (81). To 0.5ml sample containing up to 50μg uronic
acid, was added 3ml of a solution of sodium tetraborate in
concentrated sulphuric acid, (0.0125M sodium tetraborate in
A.R conc H_2SO_4). The tubes were refrigerated in crushed ice,
then shaken on a vortex mixer, and heated in a water bath (100°C)
for 5 minutes. After cooling in an ice/water bath, 50μl of
m-hydroxydiphenyl reagent (a 0.15% solution of m-hydroxydiphenyl
in 0.5% NaOH) was added, the tubes were shaken and the
absorbances read after 8 minutes at 520nm. As carbohydrates
produce a pinkish chromogen with H_2SO_4/tetraborate at 100°C
a blank sample was run without addition of the m-hydroxydiphenyl
reagent, which was replaced with 5.0 μl 0.5% NaOH. The
absorbance of the blank sample was subtracted from the total
absorbance. The pink colour produced was stable for at least
12 hours. Results were duplicated and galacturonic acid
standards were used between 20 and 50μg. Standard curves are
shown in Figure 2.2.

Figure 2.2: Meta-hydroxydiphenyl Colorimetric Standard Curves
for Uronic Acids (reproducible plots)
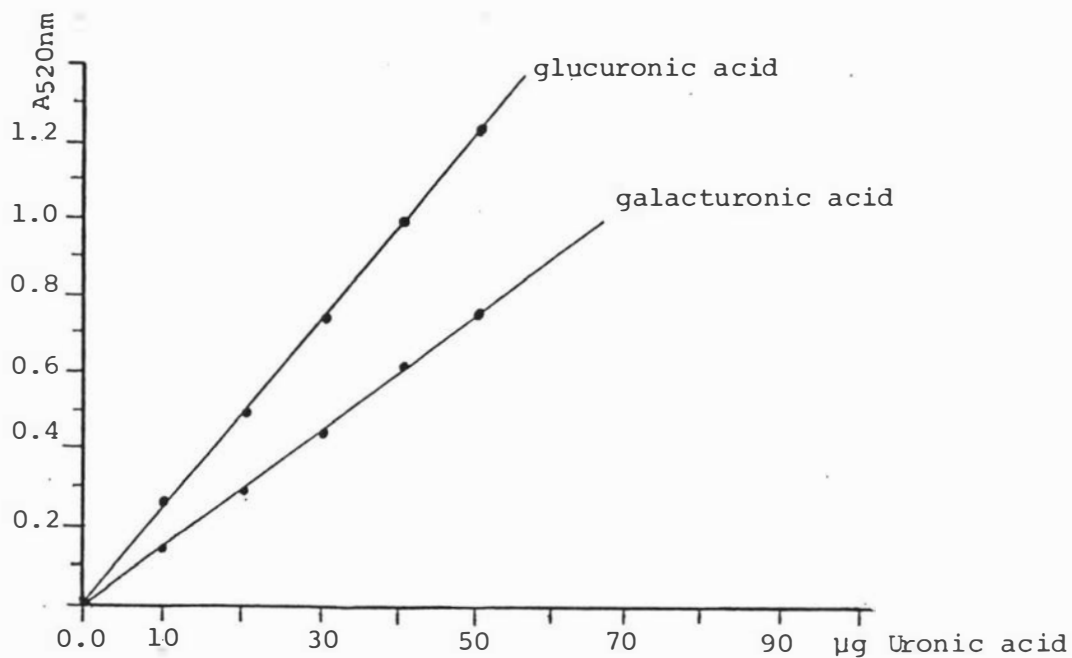
Figure 2.3: Standard Curve for Spectrophotometric Assay of
Hydroxyproline (reproducible plot)
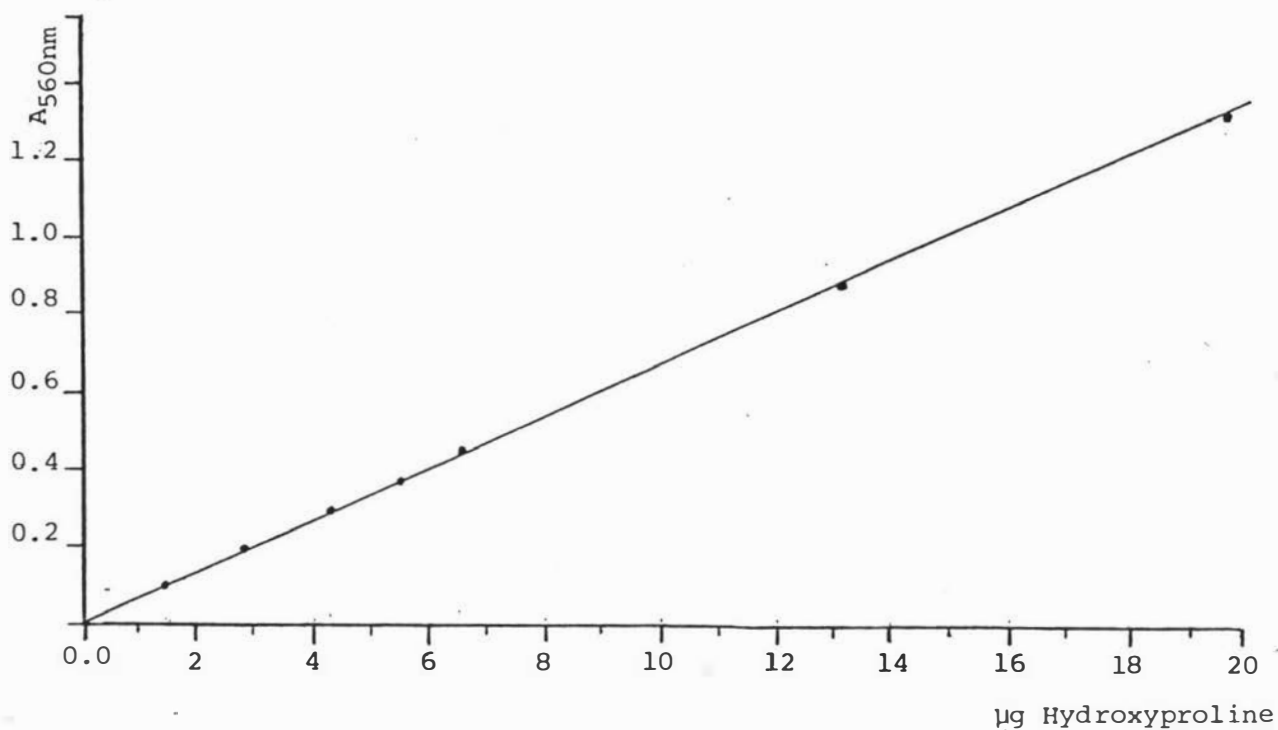
2.2.3 Amino Acid Analysis

Amino acid analyses of whole wall and wall fractions were obtained in order to:

a) determine the percentage of protein in the primary cell wall, and

b) determine the characteristics of proteins removed in the chemically extracted wall fractions.

Dry samples (between 1 and 3mg) were hydrolysed in 6.0M HCl overnight at 100°C.   The sample was then frozen in liquid air under vacuum and the HCl removed under vacuum in the presence of P_2O_5.   The dry samples were taken up in 2ml solution (buffers required for Beckman analyses) and aliquots of samples were run on a single column program on a Beckman 120C automatic amino acid analyser.   The chart read out from the analyser shows absorbance of the ninhydrin reaction product at 570nm (red scale) and at 440nm (blue scale).   The blue scale gives greater sensitivity for proline and hydroxyproline; the red scale for all other amino acids.

Amino acids were quantitated by peak areas (integrated manually by the height x width at half height method) converting to micromoles by the use of average calibration factors for each amino acid.   Calibration was carried out using Beckman standard amino acid calibrant containing 0.05 µmole each amino acid. Some additional standards (Section 2.1) were also run.

Hydroxyproline is associated with wall structural protein. The determination of hydroxyproline proved difficult by amino acid analysis runs, since by running standards it was found to chromatograph close to aspartic acid, often only resolving as a shoulder on the blue scale on which it was more intense.

To obtain accurately the levels of hydroxyproline in fractions this was analysed spectrophotometrically (Section 2.2.4).

2.2.4 <u>Determination of Hydroxyproline</u>

Hydroxyproline was assayed by the method of Switzer and Summer (82) as modified by Monro (21). Samples containing 0.3 to 13µg of hydroxyproline were diluted with distilled water to 1ml, 1 drop of 1% phenolphthalein in ethanol was added and the pH of the solution was adjusted to a faint pink colour by dropwise additions of dilute potassium hydroxide. Distilled water was again added to make the volume up to 2.5ml. 1.0ml, 0.05M sodium borate buffer pH 8.7 was then added, which maintains its pH during the sample oxidation so that addition of a second buffer is not necessary, for the subsequent toluene extractions. The samples were then oxidised with 2ml 0.2M Chloramine-T solution at room temperature for exactly 25 minutes. The oxidation was stopped by the addition of 1.2 ml 3.6M sodium thiosulphate with thorough mixing for 10 seconds. The solution was then saturated with 1.5g potassium chloride and 2.5ml toluene was added to the samples. The proline oxidation product was extracted by shaking the sample for ca. 5 minutes, centrifuging at 600g for about 1 minute to separate the layers and then completely removing the toluene phase. This extract was discarded.

The aqueous layer in a tightly stoppered glass tube was heated in a bath of vigorously boiling water for 30 minutes, and after cooling the sample to room temperature, the aqueous layer was extracted again with 3ml toluene and a 2ml aliquot was mixed with 0.8ml Ehrlichs reagent as described by Prockop and Udenfriend, for the colorimetric assay (83). After allowing the colour to develop at room temperature, for 30 minutes, the absorbance was measured against a reagent blank at 560nm. The amount of hydroxyproline was determined from a standard curve over the range 0.3 to 19.5µg hydroxyproline. Linearity of the curve was found at hydroxyproline levels up to 19.5µg,-refer to Figure 2.3

2.2.5 Determination of Lignin

Lignin was assayed spectrophotometrically, by a modification
of the methods as outlined by Martin (89), Morrison (90),
Johnson et al (84), and Bagby et al (91). The method was
adopted here to the following conditions. To 0.5 to 3.0mg of
dry sample in stoppered centrifuge tubes, was added 1ml of 25%
acetyl-bromide in glacial acetic acid (freshly prepared). The
tubes were then heated for 30 minutes at $70^{\circ}C$ with intermittent
shaking. The solution was cooled and 8.9ml ethanol was added
with swirling, followed by 0.1ml 7.5M hydroxylamine-hydro-
chloride (aq). The spectra of samples were read against a
reagent blank, and the absorbance was recorded at 280nm. A
standard lignin value was deduced for the acetyl bromide
method from absorbance and absorptivity values on standard
lignins recorded in the literature (84, 89, 90, 91). (0.2mg
of lignin had A_{280} of ca. 1.0 in 5.0ml of total assay volume.)
Data to lignin assays are recorded for wet sieved walls in
Chapter 5. The spectral curve obtained for the samples is
indicated in the diagram of Figure 2.4.

2.2.6 Determination of Starch - Spectrophotometric Assays

a) I_2/KI Assay. A modification of the starch assay using
    I_2/KI (70) was used to assay wall preparations and the
    starch content extracted, in fractions of callus
    Fractionation Scheme 1, Batch 1, Figure 5.1. To test
    tubes containing 0.1 to 0.5ml 10mg/100ml Pinus radiata
    starch stock solution (purified in the wet sieving of
    Batch 1 walls, Section 3.6.1) was added 0.2ml 0.1M NaOH,
    and the mixture warmed 3 minutes in a boiling water
    bath. Samples were cooled, and 0.1ml 1M HCl, 0.2ml
    5mg/ml potassium hydrogen tartrate buffer, pH 7.0, and
    0.01ml I_2/KI reagent (2mg iodine/ml; 20mgKI/ml in water)
    were added and the total volume of each sample was made
    up to 1.0ml with distilled water. All absorbances were
    read at 680nm using a 1cm cell in a microcell in a
    Hitachi 101 spectrophotometer.

Figure 2.4: Spectral Curves for Lignin
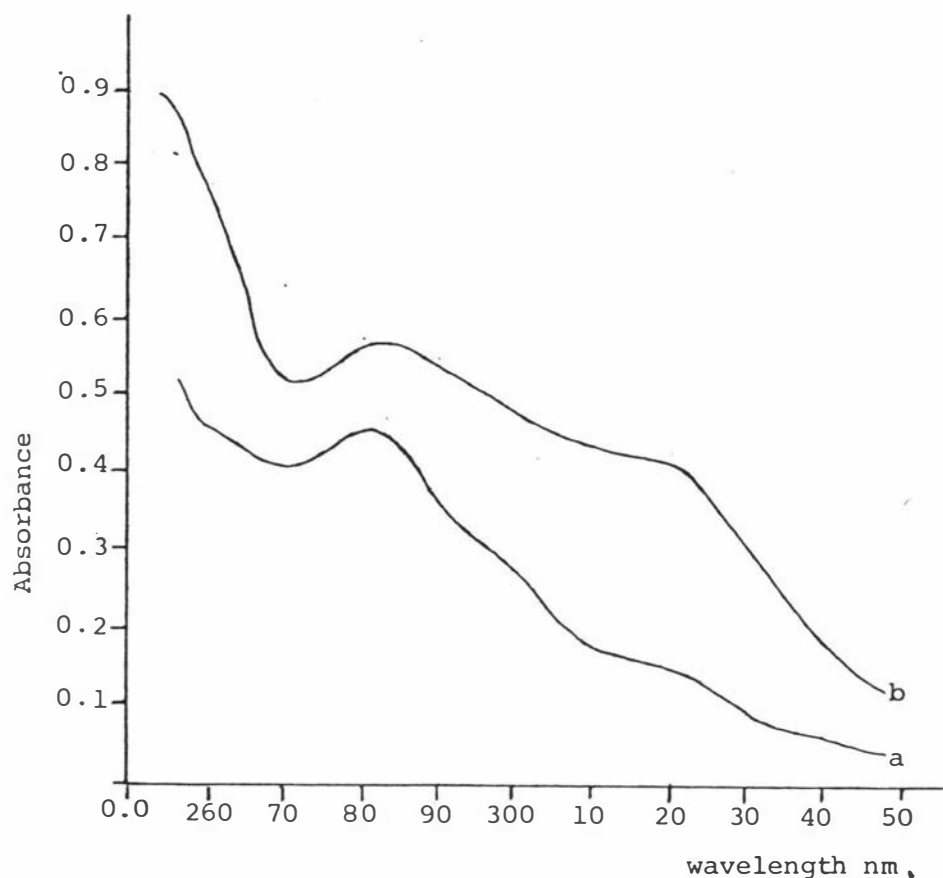
Curve a)   Ultraviolet spectra of wood lignin solubilised
           by acetyl bromide treatment, (84).
           (0.025g lignin per litre)

Curve b)   Ultraviolet spectra of <u>Pinus</u> <u>radiata</u> callus
           lignin solubilised by acetyl bromide treatment
           (ca 0.022g lignin per litre)

           The sample here was solubilised from the residue
           after alkali-borate extraction in Path B, Batch 1
           Walls.

The standard curve is linear up to 40μg of <u>Pinus radiata</u>
<u>starch</u>. A standard curve was repeated each time the assay
was performed, and a reagent blank was included with I_2/KI
reagent. All samples were read against distilled water,
and the blank was subtracted. The standard curve is as
shown in Figure 2.5

b) Amyloglucosidase Assay. A modification of the assay with
glucose oxidase (71, 80) was used for assaying the starch
content of cell wall preparations. Samples containing
up to 0.25mg starch were heated for 60 minutes in a
boiling water bath, to solubilise starch and then cooled.
Then 2ml of 0.2M sodium acetate buffer pH 4.5, and 1ml of
amyloglucosidase (1mg/ml) was added and the solution was
incubated at 55°C for 60 minutes, in a shaking water bath.
The total volume was 5ml at this stage. Samples were then
centrifuged and 1ml of the solution was removed and added
to 1ml peroxidase-glucose oxidase (PGO) reagent. The
PGO reagent consisted of 9.8ml 0.2M sodium phosphate
buffer pH 6.0 to which had been added 0.1ml 1%
orthodianisidine in 95% ethanol, and 0.1ml horse radish
peroxidase solution, 0.1% in 0.2M sodium phosphate
buffer pH 6.0, and 2mg glucose oxidase. The total
volume of the PGO reagent thus made up was 10ml. On
addition of the PGO reagent the 2ml samples were incubated
at 39°C for 30 minutes, then 1ml 50% (w/v) H_2SO_4 was added
and the absorbance read at 530nm. The pink colour was
stable for approximately 18 hours.

Standards using 0.25-0.5mg <u>Pinus radiata</u> starch and a
reagent blank were included in the assay, and all samples
read against distilled water and the blank subtracted.
The standard curve for <u>Pinus radiata</u> starch was linear
over the range shown in Figure 2.6.

Figure 2.5: Standard Curve for Spectrophotometric Assay of _Pinus_ _radiata_ Starch by the I_2/KI Method
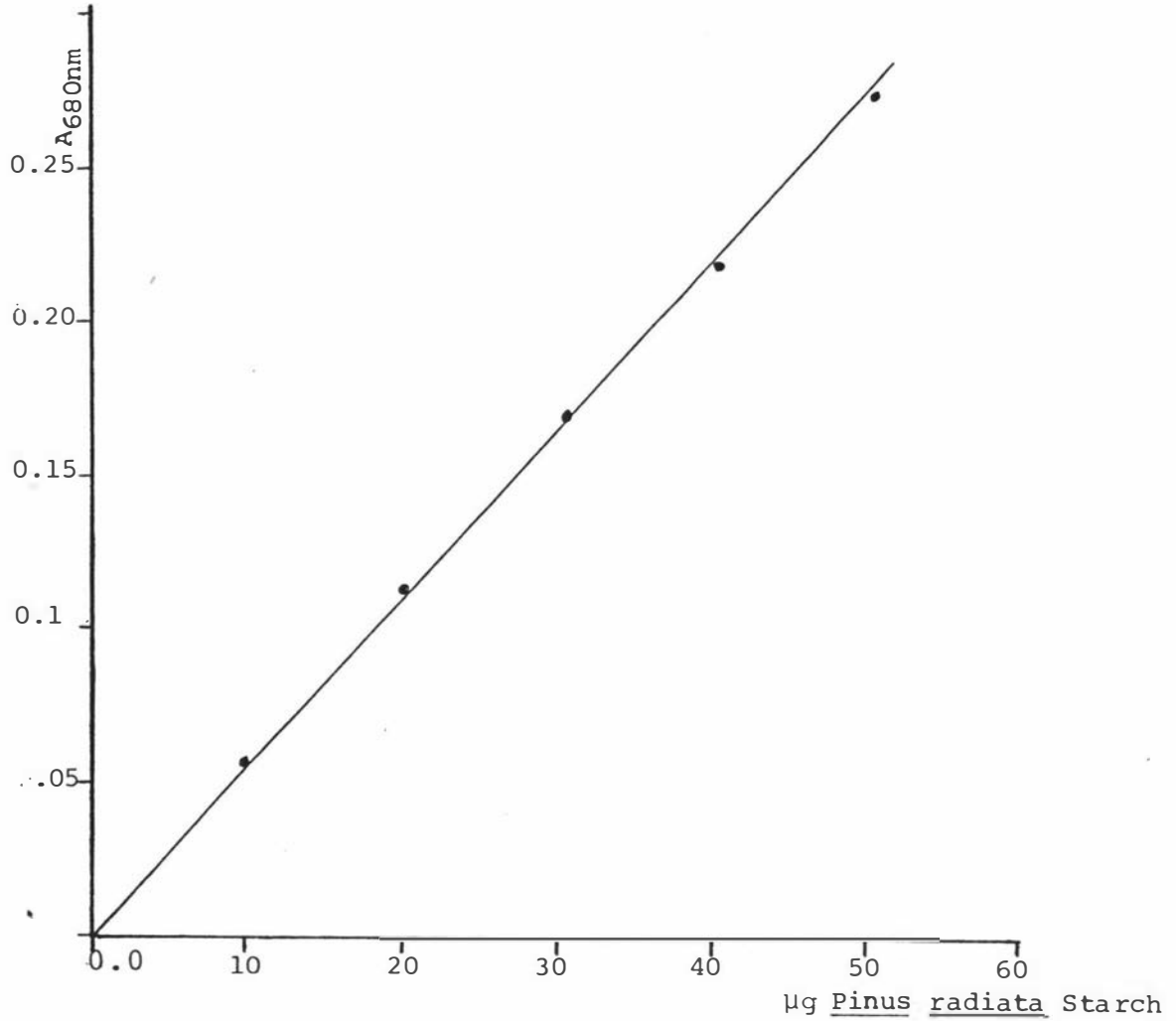
Figure 2.6: Standard Curve for Spectrophotometric Assay of _Pinus_ _radiata_ Starch by the Amyloglucosidase Method
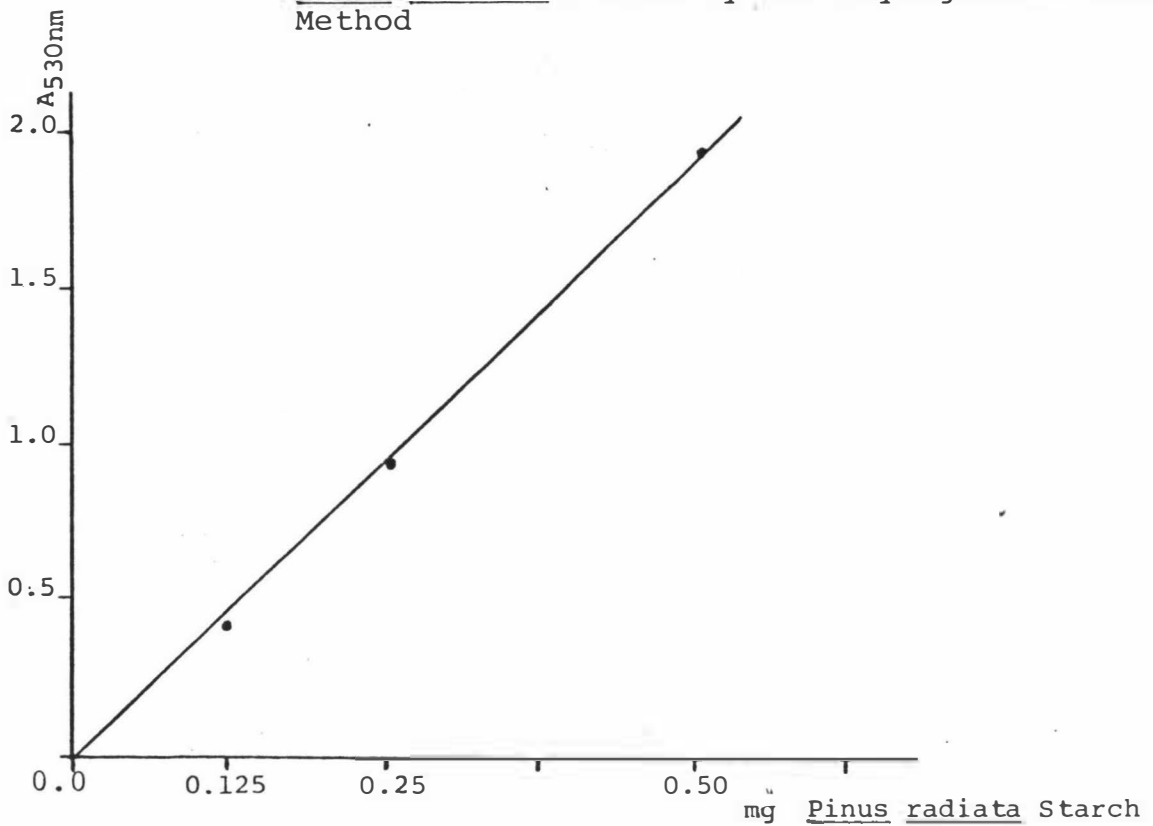
2.3 CARBOHYDRATE COMPOSITION ANALYSIS

2.3.1 <u>Neutral Monosaccharide Analysis</u>

2.3.1.1 <u>Total Hydrolysis</u>

The most convenient way of analysing the polysaccharide composition of fractions from the plant cell wall was by hydrolysis of the glycosidic linkages to produce the component monosaccharides, which could then be analysed by paper or gas chromatographic techniques (2, 8, 59, 60, 85, 86, 92). Hydrolysis has been used to study the sugars present in the native polysaccharide of fractions, and also to cleave methylated polysaccharides to analyse the linkage of components (47, 93, 94, 95, 96). Partial acid hydrolysis of polysaccharides to monosaccharides and oligosaccharides often affords further information on linkages existing in the poly-saccharides. Of primary interest here, is the complete hydrolysis to monosaccharides of a fraction, and their derivatisation and analysis.

a) Hydrolysis with Nitric Acid and Urea.

The bulk of the hydrolyses in this thesis have been performed with 0.5M HNO_3/0.5% urea (Stone et al 59, 60, 87) as this produces very little degradation of sugars and results in a high degree of depolymerisation of polysaccharide fractions (59, 60, 87) (see Section 2.3.3). Samples (in the range 1-5mg, and accurately known) were hydrolysed with 0.5M HNO_3 containing 0.5% urea, freshly prepared, in stoppered tubes at $100^{O}C$ for 4 hours. Samples were then cooled, and neutralised with 2.5M NaOH using bromothymol blue indicator, (60, 99) before reduction and addition of internal standards.

b) Hydrolysis by Sulphuric Acid

It was found that hydrolysis was essentially complete on some test polysaccharides, (galactomannans) with 0.5M H_2SO_4 at $100^{O}C$ for 2 hours (analysed by g.c. columns <u>5</u>-<u>8</u>) as used by Bailey (92), but degradation was greater

than for the HNO_3/urea method. Hydrolyates of 0.5M
H_2SO_4 were neutralised with $BaCO_3$ (97, 98) before
reduction and derivatisation.

Hydrolysis with 0.5M H_2SO_4 was carried out on a "sawdust"
sample to obtain markers for some neutral and acidic
oligosaccharides. The procedure was essentially that
employed by Brasch and Wise (108, 109). After neutralis-
ation, IR-120 (H^+) form was used to remove Ba^{2+} ions
before the hydrolysate could be ion exchanged on Dowex
1-X8, to separate neutral and acidic components.

c) Hydrolysis with 2.0M Trifluoroacetic Acid (TFA).
Hydrolysis with 2M TFA at $100^{\circ}C$ for 1 hour in sealed tubes
was carried out on some preliminary fractions obtained
from callus and on the Phebalium xylan. Degradation of
sugars was also observed with 2M TFA. Samples were
neutralised by evaporation of the TFA (85, 86). This
produced some lactonisation of acidic sugars on
concentration of the acid. Samples hydrolysed in 2M TFA,
after evaporation of the acid were therefore buffered with
a small volume of 1.0M $NaHCO_3$/Na_2CO_3 solution pH 8.5 for
30 minutes, to hydrolyse the lactones (17), after which
they were neutralised with IR-120 (H^+) resin before
reduction and addition of internal standards.

2.3.1.2 Reduction to Alditol Acetates

After neutralisation of the hydrolysates (as above) the free
sugars were reduced to the alditols by addition of 1ml
sodium borohydride (500mg/10ml) in 1M ammonia (60, 61, 85,
86). This was sufficient for sugar levels in all analyses
(level of 50mg $NaBH_4$ for 1-20mg of polysaccharide) and
reduction was allowed to proceed for 4 hours. Complete
reduction was observed after 2 hours. Excess $NaBH_4$ remaining
after reduction had finished, was discharged by addition of
glacial acetic acid until all effervescence had stopped.
Additions of internal standards (myo-inositol or 2deoxyglucose)
were made after neutralisation and before reduction. Myo

inositol was used in some earlier experiments with 2M TFA, on some preliminary callus fractions. But for all of the callus work of Batch 1 and Batch 2 walls, 2deoxyglucose was used as the internal standard. (ca. 1mg 2deoxyglucose standard/5mg polysaccharide fraction). This was then reduced to 2deoxyglucitol by the $NaBH_4$ treatment. After excess $NaBH_4$ had been converted to borate by acetic acid, samples were evaporated to dryness at 40-45°C and all boric acid was evaporated as methyl borate by addition and evaporation of five, 2ml lots of redistilled methanol, (10% in glacial acetic acid) (85).

2.3.1.3 Acetylation

Samples were then acetylated with 1ml of acetic anlydride/ 1ml pyridine at 80°C for 1 hour in an oven. Upon cooling the alditol acetates were extracted by partitioning with 1ml H_2O/1ml dichloromethane. The dichloromethane layer was extracted and this partitioning was repeated five times. Dichloromethane extracts were pooled, blown off at 40°C under a stream of N_2, and the alditol acetates redissolved in approximately 100µl of dichloromethane. Alditol acetates were injected onto the gas chromatography columns in dichloromethane (CH_2Cl_2) (85, 94, 100).

2.3.1.4 Gas Chromatography of Alditol Acetates

a) Columns. Hydrolysed sugars were analysed as their alditol acetates by gas chromatography. Three stationary phases were used in the analysis.

OV225;   3% OV225 100/120 mesh on Varaport 30
     OV225 - 75% Phenyl, 25% Cyanopropyl, methyl silicone copolymer

ECNSSM;   3% ECNSSM on 100/120 Gas Chrome Q,
     ECNSSM-Ethyl succinate and Cyanoethyl silicone copolymer

SP2340;   3% SP-2340 on 100/120 Supelcoport
     SP2340 - 75% Cyanopropyl methyl silicone

The following columns were used for gas chromatography in this thesis:-

Column 1; glass 2.5m x 3mm,i.d. containing SP2340 stationary phase

Column 2; glass 3.4m x 3mm,i.d. containing OV225 stationary phase*

Column 3; glass 1.8m x 2mm,i.d. containing SP2340 stationary phase#

Column 4; glass 1.8m x 2mm,i.d. containing OV225 stationary phase#

Column 5; steel 2.0m x 2mm,i.d. containing OV225

Column 6; glass 2.0m x 2mm,i.d. containing OV225

Column 7; steel 2.0m x 2mm,i.d. containing ECNSSM

Column 8; glass 2.0m x 2mm,i.d. containing ECNSSM

Columns used in early work

* Column 2 was used extensively for separation of permethylated alditol acetates.

Used for g.c.-mass spectrometry

b)   Identification of Alditol Acetates

These were identified by their retention times isothermally at 180°C or 210°C on 3% OV225, Columns 2, 5, 6; at 190°C on ECNSSM columns 7, 8, and at 230°C on SP2340 Column 1 (85,97).   Nitrogen flow rates were 40ml/min for Column 2 and approximately 30ml/min for the others.

Standard sugar mixtures each containing 1mg of each sugar and 1mg of 2deoxyglucose or Inositol from stock solutions, reduced and derivatised to alditol acetates, were run on the same columns to check on the identification of sugars. These standards were run each day that samples were run, since the exact running positions of standard sugars under the conditions of the analysis were prone to alter slightly from day to day.   Separations on Columns 2, 4-8 were long, of 1 hour+ duration.   Separations on Columns 1, 3 were quicker, approximately 20 minutes.   Relative retention times are given in Table 2.1.

c)   Relative Responses of Alditol Acetates

For the bulk of alditol acetate analysis, peak height response factors were used between sugars and standards, and analyses were on Column 1 - SP2340.

Table 2.1:   Relative Retention Times of Alditol Acetates

Sugar	Glycitol Acetate	Relative Retention Time for Stationary Phase Used			
		OV225	ECNSSM	SP2340	
L-Rhamnose	Rhamnitol penta-acetate	0.48	0.37	0.40	0.44
L-Fucose	Fucitol penta-acetate	0.48	0.37	0.44	0.48
L-Arabinose	Arabinitol penta-acetate	0.61	0.60	0.66	0.68
D-Xylose	Xylitol penta-acetate	0.80	0.82	0.92	0.92
2-Deoxy-D-glucose	2-Deoxyglucitol penta-acetate	1.00	1.00	1.00	1.00
D-Mannose	Mannitol hexa-acetate	1.62	1.28	1.42	1.37
D-Galactose	Galactitol hexa-acetate	1.79	1.87	1.61	1.54
D-Glucose	Glucitol hexa-acetate	2.00	2.33	1.90	1.75
myo-Inositol	my Inositol hexa-acetate	2.20	2.78	2.30	2.00
		*180-210°C	*$\sim$ 190°C	* 210°C	* 230°C

* column operating temperatures

Peak height response factors were determined (60, 85) for
alditol acetates on SP2340 Column 1, from samples in which
1.00mg of each of the sugars rhamnose, fucose, arabinose,
xylose, mannose, galactose and glucose and 2deoxyglucose
had been added from stock solutions. Each sample was
reduced with 1ml of 500mg/10ml sodium borohydride in
1.0M NH$_3$ for 4 hours, then excess borohydride was
discharged with glacial acetic acid, and the sample was
evaporated to dryness 5 times with 1-2ml methanol (10%
in acetic acid). Acetylation was as described in
Section 2.3.1.3., and the alditol acetates were injected
onto SP2340 Column 1 in dichloromethane as described.
The average peak height response parameter (2deoxyglucitol
= 1.00) from 3 separate analyses (variation $\pm$ 1%) is
given in the Table (Table 2.2).

Table 2.2: Peak Height Responses for Sugars

Sugar	Peak Height Response factor
Rhamnose	1.71
Fucose	1.80
Arabinose	1.32
Xylose	1.01
Mannose	0.70
Galactose	0.64
Glucose	0.57
2-Deoxyglucose	1.00

d)   Quantitation of monosaccharide residues from HNO$_3$/urea
     hydrolysis.

For accurate quantitation of monosaccharide residues in
polysaccharides, it was necessary to use a combined
response factor, incorporating parameters for degradation
of monosaccharides and incomplete depolymerisation of

polysaccharides as well as the peak height response factor. This is described in Section 2.3.3.1.b.

The ratio of peak height of sugar/peak height 2deoxy-glucitol standard on the chromatogram x mg 2deoxyglucose added, (usually 100µg) ÷ Peak Height Response Factor (weight response) = weights free sugars (unadjusted for degradation or depolymerisation).

The ratio of peak height of sugar/peak height 2deoxyglucitol standard on the chromatogram x mg 2deoxyglucose added, x Combined Response Factor (weight response) = weight free sugar (adjusted for minor levels of degradation and depolymerisation).

The residue weights of sugars in native polysaccharide were calculated from free sugar weights by allowing for loss of water in condensation.

In early work with 0.5M H_2SO_4 on hypocotyl and 2M TFA on preliminary callus fractions, alditol acetate levels were estimated by area measurements and responses between area of the sugars and the standard were taken as 1.0.

2.3.2   Total Monosaccharide Analysis.   TFA - Enzyme Hydrolysis

In order to obtain information on the identity of acidic residues that may exist in the Pinus radiata primary wall, a hydrolytic method that would release both neutral and acidic sugars from the wall was required.   Preliminary hydrolysis of cell wall material with 0.2M TFA - followed by incubation with an enzyme preparation containing endopolygalacturonase, extracted from Sclerotium rolfsii fungus was used by Albersheim et al (86) for the analysis of uronic acids and was adopted in this work.   The hydrolysates have been separated by ion exchange (Dowex 1-X8, acetate form) (85, 86) into neutral and acidic fractions for either work up and quantitative analysis by gas chromatography or qualitative analysis by paper chromatography.   Use of 0.2M TFA at 120°C for 1 hour

causes little degradation (85, 86) and lactonisation of
uronic acids ceases to be a problem since hydrolysis back to
the free acid has time to occur in the enzyme buffer of the
next step.

2.3.2.1 Hydrolysis Procedure

Cell wall (10-20mg) preparations (EDTA residue - See Chapter
4) were hydrolysed in sealed test tubes with 2ml, 0.2M TFA
containing 1mg recrystallised myo inositol (prepared from
10.0mg inositol and 2ml 2M TFA and 18ml distilled H_2O), for
1 hour in a pressure cooker at $\sim120^{o}C$. Samples were then
opened, TFA was evaporated with an air stream at $40^{o}C$, and
samples were dried in vacuo over KOH pellets for 12 hours to
remove residual TFA. Enzyme preparation (2ml in 0.01M Na
acetate buffer pH4.5) was added and samples were incubated
for 6 hours at $30^{o}C$, then removed to the refrigerator (86).
Allonolactone, (or mannonolactone) 9.1mg in 5ml 1M NH_3(aq),
was evaporated to dryness at $45-55^{o}C$ on a Rotavap, which
converts the lactone quantitatively to the acid. This was
used as an internal standard in samples for analysis (see
below). This standard was first taken back up in 5ml 1M
NH_3(aq) containing 30-60mg of $NaBH_4$. To the cell wall sample
in the enzyme solution was then added 0.5ml allonic (or
mannonic) acid standard (prepared as above), and 3-6mg $NaBH_4$.
Reduction of neutral sugars to alditols and uronic acids to
aldonic acids proceeded for 1 hour, after which glacial
acetic acid (few drops) was added to destroy excess
borohydride, methanol (1ml) was added and the sample was
centrifuged. The supernatant was collected and the residue
was washed with 2 x 0.5ml 70% ethanol (v/v) and washings
were added to the supernatant. After 5 successive additions
of methanol with evaporations each time to remove borate,
samples were dissolved in 3ml distilled water, and passed
over small "pasteur pipette" anion exchange columns containing
2ml Dowex 1-X 8,200-400 mesh resin in the acetate form.
Eluant and washings containing the alditols from the neutral
sugars of the hydrolysate were collected and dried over P_2O_5.
The aldonic acids and allonic acid standard were eluted from

the washed resin with approximately 2.0ml of 1.0M HCl.
Evaporation of the HCl converted the aldonic acids to the
aldono 1,4-lactones and the residue was stored 12 hours in
vacuo over KOH, to remove residual HCl. Aldonolactones were
reduced to the corresponding alditols by the addition to
each sample of 10mg $NaBH_4$ in 0.5ml sodium borate buffer, 0.01M,
pH 7.5. Excess borohydride was then removed with acetic
acid and co-distillation with methanol, and the dry residues
were acetylated as for neutral sugars (86).

Acetylation: Alditols formed from the neutral sugar fraction
and from the uronic acid portion were separately converted
to their peracetyl derivatives with 0.5ml of acetic
anhydride in sealed tubes at 121°C for 3 hours. Sodium
acetate remaining after removal of borate serves as a
catalyst. Samples were injected directly into the gas
chromatograph in acetic anhydride. Column 6 was used in the
gas chromatograph. Results from these hydrolyses are
summarised in Chapter 4.

2.3.2.2 Enzyme Preparation

The fungus Sclerotium rolfsii was used to produce enzymes
capable of degrading cell wall polysaccharide linkages.
Enzymes produced contain an endopolygalacturonase, capable
of liberating uronic acids associated with pectic and hemi-
cellulose polysaccharides (86). The fungus was grown on
autoclaved 10 day old Bean and Lupin hypocotyls for 10 days
at 30°C. Enzymes were extracted by blending the cultures
with 1 volume (w/v) of water for 1-2 minutes in a Waring
Blender (120). Hyphal cells were not significantly disrupted.
The liquid fraction was filtered through several layers of
cheesecloth and centrifuged at 20,000 g. for 15 minutes at
4°C. The supernatant was lyophilised and stored frozen.
Lupin enzyme preparation (100mg) and Bean prep (100mg) were
each dissolved in 10ml of distilled H_2O and dialysed over-
night against 0.01M sodium acetate buffer pH 4.5. After
dialysis, phenol-sulphuric "reduction assays" (Section 2.3.3.2)
showed that all the carbohydrate was present in oligomeric

form; there were no free sugars and that the carbohydrate was only approximately 5% of the enzyme preparation. Both preparations were diluted to 1mg/ml with 0.01M sodium acetate buffer pH 4.5.

2.3.2.3 Synthesis of Allonic Acid

Mannonolactone was used as the standard for quantitating the acidic sugars after ion exchange chromatography in the 1st TFA-enzyme run. Because this runs as for mannose after lactonisation and reduction in the vicinity expected for 4-O-methylglucitol penta-acetate, for the subsequent analysis, allonolactone was used as standard. Allonolactone was prepared as in Methods in Carbohydrate Chemistry (121), by a Kiliani synthesis. A solution of 50g (0.33 moles) of D-ribose in 50ml of water was cooled to 3^OC and to this was added a solution, also at 3^OC of 43.5g of NaCN in 150ml of water. The reaction flask still surrounded by an ice bath was allowed to stand 24 hours in a refrigerator at 4^OC. The clear pale yellow solution was heated 2 hours in a steam bath, then boiled gently an additional 6 hours to hydrolyse the nitriles and to expel ammonia. The hot solution was passed through a Dowex 50W-X 100-200 mesh cation exchange resin (130g) in the Ca^{2+} form. The Na^+ salts of the two aldonic acids formed in the cyanohydrin step were converted to the Ca^{2+} salts. The solution upon standing in the cold deposited the calcium-D-altronate hydrate, yield 43%. Reconcentration of mother liquors and washings yielded some further amounts. When no more altronate would crystallise, the remaining brown solution was passed over the Dowex 50W-X in H^+ form; the brown solution was reconcentrated under reduced pressure to a thick syrup and refrigerated. After extended time the allonolactone crystallised out. The sample was recrystallised from methanol. Its purity was tested by gas chromatography. A 9mg sample was dissolved in 0.01M $Na_2B_4O_7$ buffer pH 7.5, reduced with $NaBH_4$ (10mg) in 0.5ml of borate buffer, and excess $NaBH_4$ was decomposed with MeOH (10% in HOAc). The sample was then acetylated with

acetic anhydride at 121°C for 3 hours and chromatographed on OV225 column <u>6</u> at 210°C, with a carrier gas flow of 30ml/min. The alditol hexaacetate was chromatographically pure. The M.P. of the 1,4-allonolactone, was 125°C (literature M.P. allonic acid 120°C).

2.3.3 Determination of Extent of Hydrolysis

Optimum hydrolysis conditions for monosaccharide analysis required that maximum hydrolysis be accompanied by minimum degradation. Literature reports suggest that two frequently used acids for hydrolysis, - 0.5M H_2SO_4 (97, V. Harwood pers. comm) and 2M TFA (85, 97) resulted in more degradation than 0.5M HNO_3/0.5% urea as used by Jermyn and Isherwood (87, and also refs 59, 60, 97). The original users of the technique (87), in observing little degradation on hydrolysis, did not attempt to deduce the individual degradation parameters for each sugar. Two methods were used here to monitor the extent of hydrolysis of polysaccharides.

2.3.3.1 Degradation and Depolymerisation

The yield of monosaccharides from hydrolysis of a fixed amount of polysaccharide mixture under different conditions was monitored by gas chromatography. The method is described below for 0.5M HNO_3/0.5% urea (cf refs 54, 60) which proved to give better yields of monosaccharides than other acids tried.

The optimal conditions established from this work, (0.5M HNO_3/0.5% urea, 4 hours at 100°C, see sections below and Figure 2.7) were used routinely for monosaccharide analysis. In order to quantitate the monosaccharides in any mixture (see Section 2.3.1.4.d), it was necessary to derive a combined response factor, incorporating depolymerisation, degradation and peak height response factors. The derivation of the depolymerisation and degradation parameters is described following: -

a)   Degradation Parameters.

To three stoppered tubes each containing 1mg each of
rhamnose, fucose, arabinose, xylose, mannose, galactose,
and glucose from a stock solution (100mg each sugar/100mls)
was added 1ml 1.0M HNO_3/1% urea (freshly made up) and
then the acid soln (0.5M HNO_3/0.5% urea) was neutralised
with 2.5M NaOH (99), and 1mg 2deoxyglucose standard was
added from a stock solution (100mg/100ml). To a further
three stoppered tubes each containing 1mg of the above
sugars was added 1ml of 1.0M HNO_3/1% urea and the samples
were hydrolysed at $100^{O}C$ for 4 hours. The samples were
then cooled, and neutralised with 2.5M NaOH, using
bromothymol blue indicator. Both sets of tubes were
reduced after neutralisation with 1ml $NaBH_4$ (500mg/10ml
1M NH_3) in 1M NH_3, for 4 hours. The excess borohydride
was then discharged with glacial acetic acid, and tubes
were evaporated to dryness 5 times by codistillation with
1-2ml methanol (10% in glacial acetic acid). Samples
were acetylated with acetic anhydride/pyridine (1ml:1ml),
extracted with dichloromethane/H_2O, and injected onto
SP2340. Results calculated using the Peak Height
Response Factors (Section 2.3.1.4.c) are summarised in
Table 2.3, for the recovery obtained.

Table 2.3:   Degradation of Standard Sugars by 0.5M
             HNO_3/0.5% Urea

Sugar	Hydrolysis Recovery Factors Average of 3 Results	
	Time 0hr	4 hr
Rhamnose	1.0	0.98
Fucose	1.0	0.98
Arabinose	1.0	0.96
Xylose	1.0	0.90
Mannose	1.0	0.96
Galactose	1.0	0.95
Glucose	1.0	0.95

From these results it was concluded, in accordance with
the reports of Mares and Stone (60, 87), that little
degradation occurred over the 4 hour period in the acid
solution for each of the sugars being measured. However,
allowance was made for degradation of each sugar in the
final factor.

b)   Combined Depolymerisation and Degradation Factor

A slight factor was considered for each sugar to allow
for the undermeasurement of the sugar in the hydrolysate
due to incomplete depolymerisation (102, 104) of the
polysaccharides present.   To estimate these factors, a
polysaccharide fraction extracted from callus walls by
0.5% ammonium oxalate was chosen.   (Fraction described
in preliminary analyses of callus, Section 5.2.1a.) This
fraction contained all the major sugar residues found in
the cell walls.   The hydrolytic release of each of the
sugars rhamnose, fucose, arabinose, xylose, mannose,
galactose, and glucose, was followed by derivatisation to
alditol acetates and gas chromatography on SP2340
Column $\underline{1}$, as previously described, using peak height
response factors to 2deoxyglucitol, for sugars.

The results of the hydrolytic plots for three of the
sugars are summarised on the plots shown in Figure 2.7.
The combined depolymerisation and degradation factor,
by which to multiply each sugar, was obtained by
extrapolating the linear part of the curve to time zero.
The extrapolated curve approximates to the situation
where all the sugar is released and degradation after an
extended period of time is then proceeding at a steady
rate.   The ratio of the height of the extrapolated curve
to the height of the observed hydrolysis curve at time
= 4 hours, gives a measure of the extent of depolymerisa-
tion, and the small factor to allow for it.

Degradation was estimated from the curves by the ratio of
the value of the height of the extrapolated curve at time
zero, to the height at 4 hours, for the 0.5M HNO_3/0.5% urea
method.

Figure 2.7: Sugar Recovered from an Oxalate Fraction with
Time for Hydrolysis in 0.5M HNO_3/0.5% Urea
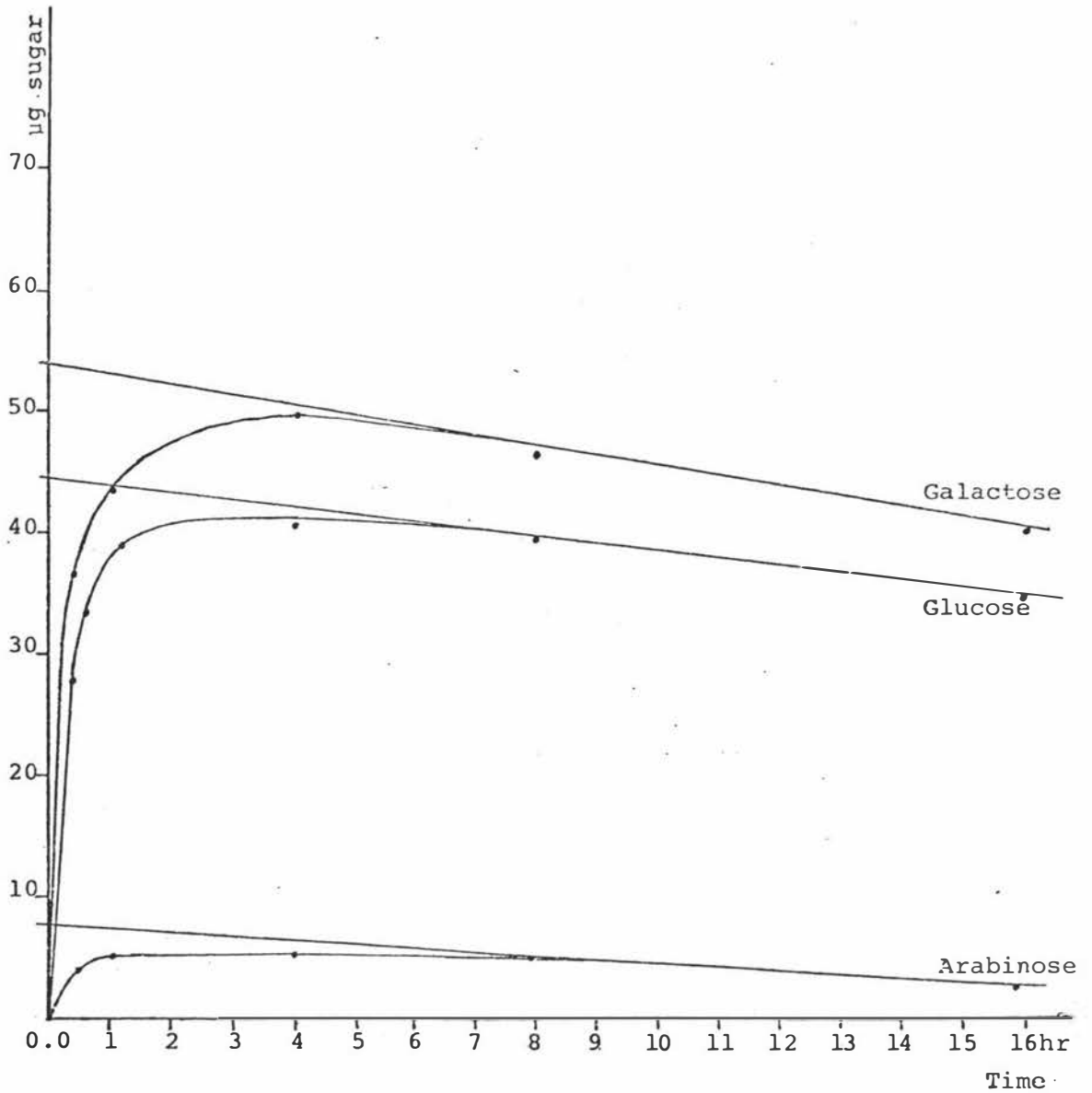
Reproducible recovery plots are shown for three sugars.

The factors observed and incorporated in calculating alditol acetate data on SP2340 are as listed in Table 2.4.

The extent of degradation in 4 hours was small and for each sugar the results obtained with the polysaccharide substrate were very close to those obtained with standard sugars (Subsection (a) above).

The depolymerisation factor for arabinose seems anomalously high, in view of the known lability of arabinose in the furanosidic linkage (the dominant linkage in plant cell walls), nevertheless this high value is used in the calculation of data in this thesis.

Table 2.4: Factors Involved in Quantitative Recovery of Monosaccharides by Gas Chromatography

Sugar	Peak Height Response	Degradation Factor	Depolymerisation Factor	Combined Factor
Rhamnose	1.71	1.07	1.08	0.68
Fucose	1.80	1.08	1.00	0.60
Arabinose	1.32	1.08	1.30	1.06
Xylose	1.01	1.09	1.16	1.25
Mannose	0.70	1.06	1.00	1.52
Galactose	0.64	1.06	1.03	1.73
Glucose	0.57	1.05	1.05	1.93

2.3.3.2 Assay for Non-Reducing Sugar Residues

Colorimetric "reduction assays" were developed for the phenol - H_2SO_4 (Total carbohydrate) and meta-hydroxydiphenyl (uronic acid) assays, to determine the amount of sugar residues still remaining, glycosidically-linked in oligosaccharides after hydrolysis (Timell 101). Such oligosaccharides would not be recovered as alditol acetates when the sample was worked up for gas chromatography.

These assays were applied to 2M TFA and 0.2M TFA-enzyme hydrolyses, described in Chapter 4. (Colorimetric phenol - H_2SO_4 assays were not operable with HNO_3/urea hydrolysates due to the presence of urea which inhibited formation of the chromophore.)

On addition of NaBH$_4$, 0.1ml (100mg/10ml 5M NH$_3$) to the aliquot used for the phenol - H$_2$SO$_4$ assay (0.1 to 0.4ml), and equalising to a total volume of 0.5ml with distilled water if necessary prior to the addition of reagents, reducing ends of the free sugars and oligosaccharides are reduced to the unreactive alditols. Hence the assay only measures the non-reducing sugar residues. The borohydride added is converted by the H$_2$SO$_4$ to boric acid which depresses the colour. A control was therefore used in which the same amount of boric acid was generated by acidifying some of the NaBH$_4$ soln (100mg/10ml 5M NH$_3$) with a drop of conc H$_2$SO$_4$ until effervescence ceased, and 0.1ml was added to the assay in the place of NaBH$_4$. The borate is thus of the same concentration in the assay as the NaBH$_4$. Samples, standards and blanks were then made up to 0.5ml and phenol (0.5ml) and H$_2$SO$_4$ conc (3.0ml) were added and absorbances read at 490nm. Reducing and non-reducing, and total sugar residues were measured in the assay and the approximate degree of monomerisation was calculated.

Similarly, non-reducing uronic acid residues were estimated by the m-hydroxydiphenyl method, modified by the inclusion of 0.1ml borohydride as described for total non-reducing carbohydrate residues.

Using this approach it was possible to show:-

1. that hydrolysis was virtually complete under the conditions employed except in samples rich in uronic acids;

2. that non-reducing sugar residues were predominantly uronic acids.

This was born out by paper chromatography (Section 4.2.5).

2.3.4   Identification and Isolation of Uronic Acids and Oligosaccharides

Monomeric uronic acids were identified in some instances (Section 4.2) by gas chromatography (as described in Section 2.3.2.1). Acidic oligosaccharides and monosaccharides were also examined by paper chromatography.

2.3.4.1 Recovery of Acidic Sugars by Ion Exchange
         Chromatography

In order to separate uronic acids from neutral sugars it was
necessary to ensure first that any uronolactones were convert-
ed to the free acid form. Where necessary this was done by
incubating at pH 8.5 for 3 minutes at room temperature. The
separation of Acidic and Neutral sugars for either gas
chromatography (Section 2.3.2.1) or paper chromatography
(Section 2.3.4.2) was carried out with Dowex 1-X9-200 mesh
in the acetate form (86, 88). The Dowex was conditioned after
washing with 1M acetic acid, and 6M acetic acid was used to
elute acidic sugars (88). Whereas 1M HCl was used to elute
acidic sugars for subsequent lactonisation and derivation
for gas chromatography, elution with 6M acetic acid was used
for samples which were to be analysed by paper chromatography.
Acetic acid (6M) produced less lactonisation on concentration
than HCl. Only a faint lactone spot was observed when 10^{mg}
Glucuronic acid was evaporated in 6M acetic acid, taken back
up in 0.5ml of 6M HOAc and approximately 50µg of sugar was
applied to paper and run in solvent B (Section 2.3.4.2), and
developed with $AgNO_3$.

The prior separation of acidic and neutral components
assisted with the identification of Glucuronic acid, and
(4-O-methylglucuronosyl)xylose, in hypocotyl and callus by
subsequent paper chromatography.

2.3.4.2 Paper Chromatography

Qualitative paper chromatography was used to identify sugars
and oligosaccharides after separation of neutral and acidic
components by ion exchange chromatography (Section 2.3.4.1).
The principal solvent system used for the separation of
principal aldobiouronic and hexuronic acids and for the
identification of neutral sugars was Ethyl acetate-water-
acetic acid-formic acid (18:4:3:1 by volume) (Solvent A), as
described by Ray and Rottenberg (88).

Chromatograms were run 18 hours descending on Whatman No 1
paper (9" x 22") in sealed chromatography tanks. The solvent
was freshly prepared for each chromatographic run. Even so
there was some variation in R_{xylose} values.

Sugars were located by either of two procedures: -

(a) $AgNO_3$ dip using the reagents described by Bailey (105):
    Chromatograms were developed by immersing the paper in
    the $AgNO_3$ solution, allowing to dry, developing in 0.5M
    NaOH in ethanol solution, allowing to dry and fixing in
    a 0.5M sodium thiosulphate solution and allowing to dry
    (105, 123).

(b) Aniline hydrogen phthalate spray reagent prepared by
    addition of 0.93g of aniline and 1.66g phthalic acid in
    100ml water saturated n-butyl alchohol as in reference
    (106).

Chromatograms developed by spraying with Aniline hydrogen
phthalate, (106) were heated 10 minutes at $105^{O}-110^{O}C$.
Aldoses appeared as reddish spots. In visible light hexoses
appeared brownish, xylose and arabinose, reddish, rhamnose
and fucose orangey-brown and uronic acids (GalA and GlcA)
more orange. Under U.V. irradiation, hexoses (Glc, Gal and
Man) fluoresced yellow-green. Glucuronic acid had a lower
fluorescence but galacturonic acid did not appear to
fluoresce.

Standard sugars were run in Solvent A (Ethyl acetate/water/
acetic acid/formic acid 18:4:3:1 v/v), 2-10µg of each sugar
being applied to each spot from a solution containing 1mg/1ml
of each of the sugars rhamnose, fucose, arabinose, xylose,
mannose, galactose, glucose, galacturonic acid and glucuronic
acid, to characterise running positions for a variety of
sugars. R_{xylose} values for standards and 4-O-MeGlcA and
4-O-MeGlcA-xylose in Solvent A are reported (88) in Chapter 4
Section 4.2.5 (see Table 4.3).

Solvent B (Ethyl acetate/pyridine/H_2O/acetic acid, 5:5:3:1 v/v) (107) was used to test for the presence of lactones, in some cases (Sections 2.3.4.1, 4.2.2, 4.2.5.1).

Table 2.5: R_{xylose} Values for Some Sugars in Solvent B

Sugar	R_{xylose}
Xylose	1.00
Glucose	0.83
Glucuronic acid	0.39
Galacturonic acid	0.31
Glucuronic acid lactone	1.13
Galacturonic acid lactone	1.32

2.3.4.3 The Problem of Lactonisation (see p.105)

The presence of lactones on evaporation of acid solutions of sugar acids, or prolonged heating of sugar acids in various mineral acids were tested for by chromatography in Solvent A (88) and Solvent B (107). This showed lactonisation did occur on concentration of galacturonic acid and glucuronic acid in HCl or TFA.

However, upon concentration of 6M HOAc used for eluting acidic sugars and oligosaccharides from Dowex 1 - Columns (Section 2.3.4.1), very little lactone formation was observed, and none for 4-O-MeGlcA.

2.4   METHYLATION ANALYSIS OF POLYSACCHARIDE FRACTIONS

Methylation analysis was employed to elucidate the linkages of residues present in cell wall polysaccharides. Methylation products were analysed after hydrolysis as their partially methylated alditol acetates by gas chromatography and combined gas chromatography - mass spectrometry (8, 10, 46, 47, 57, 60, 68, 93, 95, 96, 103, 110).

2.4.1 Theory of Methylation

The Dimethylsulphinyl carbanion used here is a powerful base (112), capable of extracting protons from the ring hydroxyls of sugars, to form the alkoxide ions which nucleophilically displace iodine from methyl iodide so forming methoxyl groups on the sugar rings (93, 95, 96, 111).   The reaction may be written as follows where R = polysaccharide:-

$$R\text{-}OH + CH_3\text{-}\overset{\displaystyle O}{\overset{\|}{S}}\text{-}CH_2^-Na+ \ \rightleftharpoons \ R\text{-}O^- + CH_3\text{-}\overset{\displaystyle O}{\overset{\|}{S}}\text{-}CH_3 + Na^+$$

<p align="center">alkoxide</p>

$$R\text{-}O^- + CH_3I + Na^+ \ \rightleftharpoons \ R\text{-}O\cdot CH_3 + NaI$$

<p align="right">methylated polysaccharide</p>
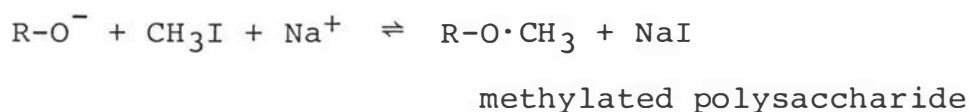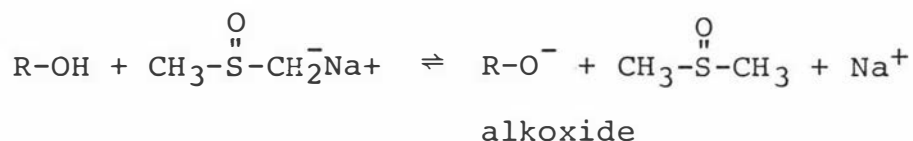
Reaction for preparation of dimethylsulphinyl (dimsyl) anion

$$NaH + CH_3\text{-}\underset{\underset{\displaystyle O}{\|}}{S}\text{-}CH_3 \ \rightleftharpoons \ CH_3\text{-}\underset{\underset{\displaystyle O}{\|}}{S}\text{-}CH_2^-Na^+ + H_2\uparrow$$
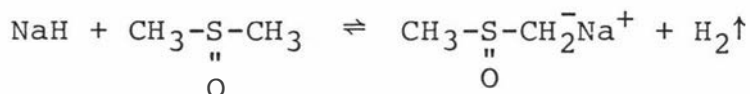
2.4.2 Preparation of Dimethylsulphinyl (dimsyl) anion

This was prepared according to Conrad (112); n-hexane (approximately 1 litre) was refluxed over $CaSO_4$ for 1 hour, then redistilled.   The liquid distilled over at $69^O C$ was collected.

Dimethyl sulphoxide (approximately 1 litre), was refluxed over CaH_2 for 30 minutes, then distilled, the fraction boiling between 189-190°C was collected.

Sodium hydride (5.0g) was washed, three times with 40 ml portions of dried n-hexane and residual n-hexane was evaporated off under nitrogen.   Redistilled, dried dimethyl-sulphoxide (DMSO) (50 ml) was then added with a syringe and stirred at approximately 50°C for 1 hour, after which most hydrogen evolution had stopped.   Continued stirring at 50°C for an additional 30 minutes produced a deep sea green

solution which was then transferred carefully with a pasteur pipette to glass vials, and stoppered under nitrogen with suba seals. Dimsyl anion was stored frozen for periods up to 4 months without significant loss of its alkalinity.

The concentration of the sodium dimethyl sulphinyl anion was determined by titrating 1 ml of the solution in 15 ml H_2O against 0.1M HCl with phenolphthalein indicator. All batches prepared were approximately 2M.

2.4.3 Methylation Procedure

2.4.3.1 Hakomori Procedure

Methylation of polysaccharides was carried out by the Hakomori procedure (93, 111). Freeze-dried polysaccharide fractions (2-20mg) were dried overnight in vacuo over P_2O_5 and were dissolved in 5ml of dry DMSO under nitrogen. Dissolution was facilitated in most cases by heating samples to 60°C and by brief periods (5 minutes) of sonication. Dimethyl sulphinyl anion (1 ml of 2M anion) was added slowly to form a gel which gradually dispersed. Alkoxide formation was allowed to proceed for 2-4 hours, with constant stirring, after which the presence of excess carbanion was tested for with Triphenyl methane (186). A positive test at the end of reaction indicated that there had been sufficient carbanion present in the reaction mixture throughout. Methyl iodide (0.2ml) was then added slowly with a 50 µl syringe over 30 minutes, taking care to maintain the temperature at 20-25°C by immersing the flask in a water bath. After all the methyl iodide had been added, the solution was stirred for an additional 30 minutes. Methylation was allowed to proceed for 1-2 hours during which the viscosity of the solution changed, and then 5ml chloroform/methanol (1:1 v/v) was added and the solutions were exhaustively dialysed against water or passed over Sephadex LH-20 (24 x 1.5cm) (47), to remove DMSO and any remaining methyl iodide and other monomers. Samples were passed over LH-20 only if after methylation they

appeared completely soluble in the chloroform/methanol.
Samples were freeze-dried. At this point remethylation of
samples which were not completely soluble initially in DMSO,
by the same procedure, was necessary for complete methylation.
A total of 3 successive methylations were usually performed
by this method after which methylation was normally complete
as indicated by the absence of undermethylated derivatives in
gas chromatography. The absence of undermethylated derivatives
at this stage could be confirmed (e.g. Section 2.4.8) by: -

(1) further methylations, which failed to produce any change
    in the pattern of methylated derivatives observed in gas
    chromatography,

(2) by a correct balance between derivatives corresponding
    to terminal and branch residues.

While complete methylation was eventually reached this method
was slow and laborious. Isolation of the material after each
methylation stage took a week for the complete process before
hydrolysis was commenced.

In light of reports by Albersheim (113, 114) that complete
methylation could be achieved with only one purification step
(and failure to detect undermethylation with first use of the
method here) a multiple methylation method was adopted for the
alkali-borate fraction (Section 6.2) and methylations of Section
6.3. This is described below.

2.4.3.2 Multiple Methylation Technique

About 100μl 2M Dimethylsulphinyl anion was added for each 3mg
of carbohydrate and the sample sonicated in DMSO as described
in Section 2.4.3.1, and cooled to room temperature. An amount
(slightly in excess of equimolar) of methyl iodide was added
and the solution stirred for 1 hour. This was repeated with
the same amount of anion but for this second methylation
stirring was overnight, with methyl iodide in a water bath
at room temperature. (The temperature often recommended is
20°C but this is not critical). The third methylation
commenced the second day. Dimsyl anion was added at 120μl

per 3mg of carbohydrate, (a slight excess over the previous 2 methylations), the sample was sonicated and stirred for 1-2 hours, then a 5 x excess of methyl iodide was added and the solution was stirred overnight. After 12 hours there was often some salt precipitation. The whole sample was now dialysed or run over an LH-20 Sephadex column in chloroform/methanol and the effluent monitored for methylated material by the phenol-sulphuric assay.

2.4.4   Methylation of Uronic Acid-Containing Polysaccharide

Materials with uronic acids will not tolerate multiple exposures to alkaline conditions, which cause β-elimination of uronic acid residues, as in the repeated standard methylation procedure, with loss of uronic acid, Figure 2.8.

If the uronic acid is already partly esterified as is often the case for native polysaccharide fractions (42, 117), β-elimination will occur even in the first single methylation. Therefore in fractions where pectin structure was of interest, polysaccharide in which uronic acid was methylated, was de-esterified first, as described by Aspinall et al (117).

It is preferable to convert uronic acids to neutral sugars and analyse them by gas chromatography - mass spectrometry of their alditol acetates. Therefore reduction of the carboxyl group is required, and an increase in neutral methylated sugars in gas chromatographic analysis of reduced methylated polysaccharides compared to the unreduced sample would correspond to those which had been derived from uronic acid residues by reduction of the carboxyl group. If this reduction is performed with an isotopic label such as deuterium the methylated sugars arising from uronic residues can be identified, and quantitated by their mass spectra (95, 114, 116) e.g. $2,3,6$-Me_3-galactitol-triacetate from a 1,4-linked galacturonic acid residue would carry a dideutero label, at C-6 giving it a distinct mass spectrum from that of the $2,3,6$-Me_3-galactitol-triacetate derived from 1,4-linked galactose.

In a single methylation with methyl iodide, uronic acid carboxyl groups are converted to their methyl esters which can then be reduced (93, 95) usually with lithium aluminium hydride (LiAlH$_4$) or lithium aluminium deuteride (LiAlD$_4$). Alternatively, the carbodiimide reaction product is prepared before methylation and reduced with NaBH$_4$ or NaBD$_4$ (116, 119). These techniques are difficult with small amounts of polysaccharide, as a high recovery of the polysaccharide after reduction is not easily attainable. Also with pectin, complete reduction normally requires several repeat treatments (43). Levels of reduction and recovery of polysaccharide with these techniques of reduction are discussed further in the results section (Section 6.1.a).

If a uronic acid residue was not completely methylated on all ring hydroxyls, these conditions would still be sufficient for methyl esterification of the carboxyl groups. The esterified groups could then be reduced after which it would be safe to remethylate the whole sample as β-elimination would no longer occur.

Procedure:

The procedure finally adopted was based on that of Albersheim et al (114). The polysaccharide fraction containing uronic acid was first de-esterified with 2.5M NaOH (Aspinall 117) in order to prevent possible fragmentation due to base-catalysed β-elimination during the next step. De-esterification was effected by adjusting the pH to 12.0 with 2.5M NaOH, and the residue was kept at 0°C for 2 hours. The pH was then adjusted to just acid pH 3.5, with 5M H$_2$SO$_4$, and the sample was dialysed and lyophilised before methylation. The de-esterified polysaccharide was then methylated in a single step (Section 2.4.3) before reduction.

About 100μl of 2M Dimethyl sulphinyl anion was used for each 3mg of carbohydrate. The mixture was sonicated for approximately 1 hour then stirred for 3 hours, cooled and a 5 times excess of methyl iodide was added. On further stirring the solution turned orange.
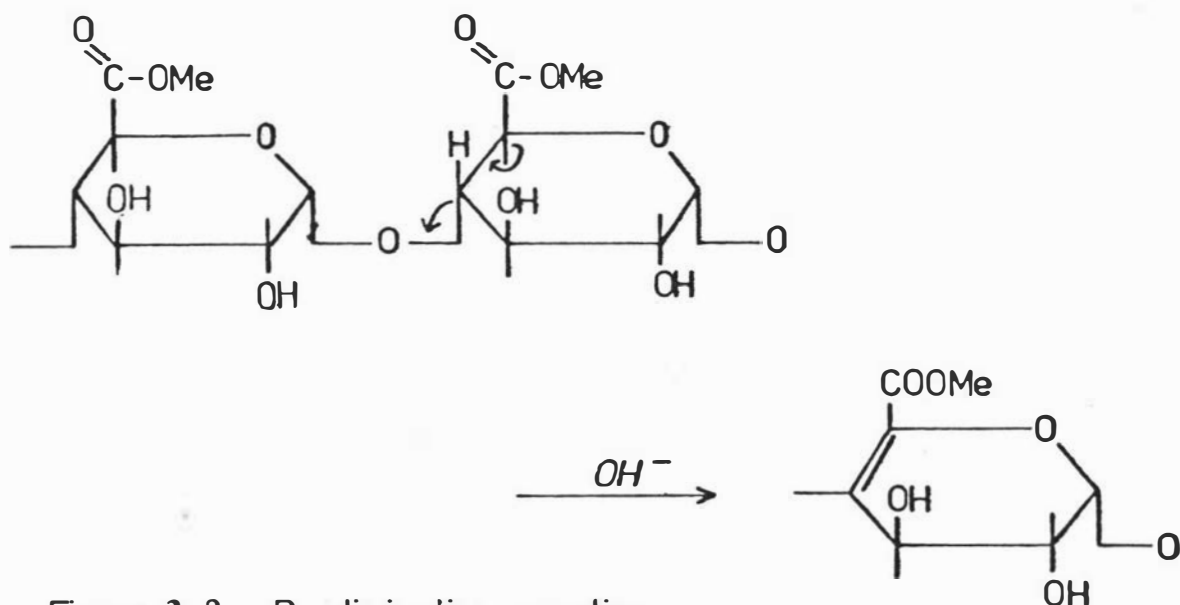
Figure 2.8 : β—elimination reaction
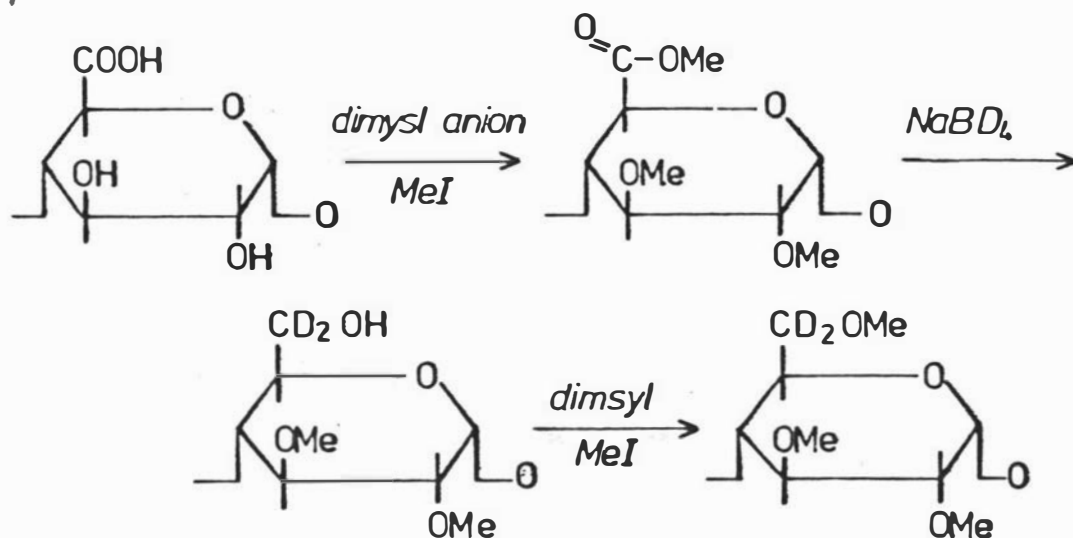
Figure 2.9 : Methylation-reduction reaction.

Stirring was continued for 4 hours. The sample was then dialysed and lyophilised. After methylation, the polysaccharide which was now methyl esterified was dissolved by sonication and warming in 95% ethanol/tetrahydrofuran 7:17 (v/v) (114) and reduced with sodium borodeuteride (ca 60mg NaBD$_4$/15mg polysaccharide) and incubated at room temperature for 18 hours and then heated for 1 hour at 70°C. After the reduction the sample was dialysed. A portion was remethylated with 120μl of anion per 3mg of carbohydrate for 1 hour and with an excess of methyl iodide. The samples were purified by dialysis and lyophilised.

A portion of this sample was dissolved in dioxane : ethanol 3:1 (v/v) (187) and reduced with NaBD$_4$ as described above.

The sample was dialysed and methylated a further two times, as for methylations 2 and 3 of the procedure in Section 2.4.3.2. The second methylation used a 5 times excess of methyl iodide. The sample was dialysed against deionised distilled water and lyophilised. By this stage the polysaccharide was completely methylated including the hydroxyl at the dideuterated position of carbon -6 as in Figure 2.9.

The summarised procedure is: -

1. De-esterification pH 12.0
2. Methylation, 5 x excess MeI
3. Reduction NaBD$_4$
4. Methylation, 5 x excess MeI
5. Reduction NaBD$_4$
6. 2 x methylation (Multiple methylation technique)

2.4.5 Hydrolysis and Derivatisation of Methylated Products

Methylated polysaccharides were hydrolysed by the method of Lindberg et al (93, 94). A sample of methylated polysaccharide (2-5mg) was heated with 2ml 90% (w/w) formic acid for 1 hour. This pretreatment is reported to cleave glycosidic linkages without causing significant demethylation (94, 110). Treat-

ment with HCl in water or methanol causes more demethylation than this method. The products at this stage were the formate esters of the partially methylated monomers. Formic acid was removed under a stream of nitrogen or by rotary evaporation at a temperature not exceeding $40^{\circ}C$, and the residue dissolved in 1ml 0.25M H_2SO_4 and held at $100^{\circ}C$ for 16 hours (93, 96), to cleave off the formate groups. The hydrolysate was neutralised for all methylations with barium carbonate and the insoluble barium sulphate removed by suction filtration on a glass filter. Care was taken after hydrolysis to keep temperatures to a minimum, otherwise loss of the more volatile methylated derivatives could occur (96, 110). Hydrolysates after filtration were reduced for 4 hours with 2-4ml 1% (w/v) $NaBH_4$ in 1M ammonia (aq). Excess $NaBH_4$ was removed by addition of 4M acetic acid until hydrogen evolution had ceased. Samples were then evaporated to dryness and methyl borate removed by 5 washes of 10ml methanol with evaporation to dryness each time. Methanol evaporations were carried out with a rotary evaporator at a temperature not exceeding $40^{\circ}C$. Samples were acetylated with acetic anhydride/pyridine (1:1) at $80-100^{\circ}C$ (95, 96) for 1 hour. Some early samples were acetylated with 1ml acetic anhydride at $100^{\circ}C$ for 1 hour but for some of these samples under acetylation was observed (see Chapter 9, Figure 9.6). Consequently the samples were reacetylated with acetic anhydride/pyridine at $100^{\circ}C$ for 1 hour and reanalysed.

2.4.6 Gas-Liquid Chromatography of Partially Methylated Alditol Acetates

The partially methylated alditol acetates were partitioned between CH_2Cl_2/H_2O; the dichloromethane extracts were pooled and concentrated under a stream of nitrogen at a temperature, not exceeding $35^{\circ}C$ (95). Samples were dissolved in approximately 100µl redistilled CH_2Cl_2 and analysed on OV225 Column 2 and SP2340 Column 1. The use of steel columns was avoided, since preliminary work showed that underacetylated derivatives could not be detected when steel columns were

used (Chapter 9, Appendix 1).

Methylated alditol acetates were identified by their running
positions ("T" values) with respect to 2,3,4,6-Me$_4$-glucitol acetate
(93, 115), and by comparing with the running positions on the
same columns of standard methylated alditol acetates from
reference polysaccharides (see Section 2.4.8). The identity
of permethylated alditol acetates was subsequently verified
by their mass spectra (95).

The running parameters as shown in the Appendix were compiled
for methylated derivatives on OV225 and SP2340 glass columns
(Columns 1 and 2), as the work progressed, more readily
facilitating the identity of peaks.

2.4.7   Quantitation of Results and Mass Spectrometry

2.4.7.1   Quantitation by Peak Areas

Partially methylated alditol acetates were primarily
quantitated from their peak areas on the gas charomatograms
using flame ionisation detection. Area response factors for
the compounds based on the Effective Carbon Response (ECR)
(Sweet et al (113)), were used to calculate molar
proportions of the variously-linked residues.

2.4.7.2   Quantitation by Mass Spectrometry

Quantitation by use of mass spectrometry was more indirect,
and is explained fully in Appendix 1 and 2. This was of use
where compounds may be inseparable on the normal g.c. trace,
but where scanning through the total peak width by mass
spectrometry allowed a view of the changing fragmentation
pattern. Mass spectra were recorded where essential, or
scanned across the whole chromatogram as the case necessitated.

Both OV225 and SP2340 columns were used in a Varian aerograph series 1700 gas chromatograph with a V.G. Micromass 12F. mass spectrometer.

With SP2340 a Helium gas flow rate of 20ml/minute and a program of 150 - 230°C at 2^{O}C/minute was used.

With OV225 a Helium gas flow rate of 20ml/minute and a program of 160 - 210°C at 1^{O}C/minute was used.

Some runs were also performed isothermally at 160°C on OV225.

2.4.7.3 Expression of Data

Mole ratios of permethylated derivatives were elucidated from the areas proportioned to each derivative (from the consideration of separations on the different columns [Columns 1 and 2] and area quantitations by mass spectrometry) and their adjustment by the E.C.R. factor (Section 2.4.7.1 and 2.4.7.2). Mole ratios for each derivative were converted to Mole % of the total methylated derivatives of the fraction. Neutral permethylated derivatives were expressed as µ moles of derivative in the fraction, as a proportion of the total neutral carbohydrate in the fraction (here referred to as total fraction sugar). Total fraction sugar was expressed as µ moles/g 70°C water residue, and the proportion of methyl derivative (also given as µ moles/g 70°C water residue) was obtained from this by taking the Mole % value from the composition of methyl derivatives as a percentage of the total fraction sugar.

In the case of uronic acid reduced fractions (Section 6.3) the total fraction sugar included µ moles of uronic acid, since the methylation data included derivatives derived from uronic acids. For these cases, the data were normalised relative to levels of total arabinose found in the neutral methylation (methylation 5 x M Table 6.2). This allows for under-reduction of the methylated uronic sample, which elevates the Mole % value of derivatives from neutral sugars, and hence produces slightly artificially high levels of most derivatives.

2.4.8 Trial of the Methylation Method

Before methylation of any sample, some partially purified
reference polysaccharides were methylated by the standard
Hakomori procedure. Carob bean galactomannan (20mg) and
Wheat flour arabinoxylan (20mg) (see Section 2.1) and an
arabinoxylan/galactomannan mixture (10mg each) were acetylated
overnight (118). Samples were dissolved in formamide (8ml)
and Ac_2O/pyridine (1:1 6ml total) was added and left at room
temperature overnight. The samples were then poured into
water, and the material was recovered by freeze drying.
Most of the polysaccharide was then dissolved in 5ml DMSO
and methylated as described in Section 2.4.3.1 to a 3rd
methylation stage.

The galactomannan/arabinoxylan was purified after each
methylation by dialysis. The arabinoxylan was purified after
each methylation by Sephadex LH-20 chromatography, as was the
galactomannan. The partially methylated alditol acetates
derived as in Sections 2.4.3 and 2.4.5 were analysed by gas
chromatography.

Portions of the samples purified by dialysis were hydrolysed
with 0.5M HNO_3/0.5% urea for 4 hours and the monosaccharide
composition calculated.

The ratios of the sums of the methylated derivatives for each
sugar, were compared with the ratios of the sugars determined
from their alditol acetate analyses (see Table 2.6).

The ratios of the sugars (sums of methylated derivatives)
from methylation data agreed quite closely with the sugar
ratios from alditol acetate analyses.

That methylation was essentially complete was indicated by
this and also that the appropriate number of terminal
residues were present to account for branch positions in the
polysaccharides.

Table 2.6: Methylation Data For Standard Polysaccharides

Table 2.6a: Galactomannan/Arabinoxylan Mixture Twice Methylated

Derivative	Mole %	Sugar Derivative Sums as Mole % Total	Sugars as % Total From Alditol Acetates (Mole Basis)	
$2,3,5\text{-Me}_3\text{Ara}$	12.9	<u>12.9</u>	<u>12.0</u>	<u>Ara</u>
$2,3\text{-Me}_2\text{Xyl}$	10.8	<u>16.9</u>	<u>17.6</u>	<u>Xyl</u>
2-MeXyl	6.1			
$2,3,6\text{-Me}_3\text{Man}$	22.1			
$2,6\text{-Me}_2\text{Man}$	1.4	<u>32.2</u>	<u>39.4</u>	Man
$2,3\text{-Me}_2\text{Man}$	8.6			
$2,3,4,6\text{-Me}_4\text{Gal}$	7.7	<u>7.7</u>	<u>12.4</u>	Gal
$2,3,4,6\text{-Me}_4\text{Glc*}$	3.5			
$2,4,6\text{-Me}_3\text{Glc}$	2.2	<u>30.3</u>	<u>18.6</u>	Glc
$2,3,6\text{-Me}_3\text{Glc}$	22.3			
$2,3\text{-Me}_2\text{Glc}$	2.2			

* Figure for $2,3,4,6\text{-Me}_4\text{Glc}$ includes $2,3,4,6\text{-Me}_4\text{Man}$.
  Glucose derivatives are from starch, also present in the sample; data suggest preferential
    methylation of starch.

Table 2.6b: Galactomannan, Two and Three Times Methylated

Derivative	Mole % Methylated		Sugar Derivative Sums Mole % Total (3 x Methylated)	Sugars as % Total From Alditol Acetates (Mole Basis)	
	(2x)	(3x)			
2,3,6-Me$_3$Man ⎞	63.1	63.5	82.5	80	Man
2,3-Me$_2$Man ⎠	21.2	19.0			
2,3,4,6-Me$_4$Gal	14.6	16.6	17.0	19	Gal
2,3,4,6-Me$_4$Glc *	1.0	0.9	1.0	1	Glc

Data for the 2nd and 3rd methylations on the galactomannan are closely comparable

* Includes 2,3,4,6-Me$_4$Man.

Table 2.6c: Arabinoxylan,* Once, Twice and Three Times Methylated

Derivative	Mole % Methylated			Sugar Derivative Sums Mole % Total (3 x Methylated)	Sugars as % Total From Alditol Acetates (Mole Basis)	
	(1x)	(2x)	(3x)			
2,3,5-Me$_3$Ara	20.2	18.0	27.0	27.8	27.7	Ara
3,5-Me$_2$Ara	0.5	0.7	0.75			
2,3,4-Me$_3$Xyl	0.75	0.8	0.94			
2,3-Me$_2$Xyl	21.5	24.0	23.7			
2-MeXyl	6.6	6.9	6.1	38.9	38.7	Xyl
Xyl	10.5	9.0	8.2			
2,3,4,6-Me$_4$Glc	5.3	6.5	5.4			
2,3,6-Me$_3$Glc	28.5	28.9	23.8	33.4	33.6	Glc
2,3-Me$_2$Glc	6.2	5.1	4.2			

Results show that there is little change in the mole % composition by the end of the 3rd methylation. Little further methylation occurs on the third treatment, and complete methylation is being achieved.

*The sample contains arabinoxylan and starch.

2.5   MICROSCOPIC EXAMINATION OF TISSUES AND WALLS

Structural cellular features of green callus tissue and the
nature of the primary wall prepared from it (Chapter 3,
Section 3.5) were examined by electron microscopy, and light
microscopy.

2.5.1   Transmission Electron Microscopy

Sections through whole green callus were prepared for
Transmission Electron Microscopy.

Small portions of callus were fixed at $40^{o}C$ for 4 hour in a
modified Karnovsky (72) fixative, consisting of 2% form-
aldehyde, 3% glutaraldehyde in 0.1M sodium phosphate buffer,
pH 7.2. After two buffer rinses they were post fixed for
1 hour in 1% osmium tetroxide in the same buffer.
Dehydration was carried out using a graded series of ethanol
washes (25%, 50%, 75%, 95% 100%) of 30 minute exposures,
with the exception of the 75% wash, which was left overnight.
The ethanol was replaced with propylene oxide (2 x 10 minute
changes) and infiltration was achieved, overnight with 30%
resin in propylene oxide in uncapped glass vials on a
mechanical stirrer in a fume cupboard. They were further
held in 100% resin for 8 hours and embedded in fresh resin
in butyl rubber moulds and polymerised at $60^{o}C$ over a period
of 48 hours. The embedding resin employed was Durcapan ACM
(Fluka Buchs A.G. Switzerland).

Pale gold (interference colour) sections were cut on glass
knives using an L.K.B. Ultramicrotome and picked up on
carbon stabilised Formvar support films on 200 mesh copper
grids. Such sections were post stained for 3 minutes in
saturated ethanolic uranyl acetate, washed with 50% ethanol,
followed by water, and subsequently stained for a further 3
minutes in lead citrate (73), and water washed. Sections
were examined in a Philips E.M. 200 electron microscope and
photographed on Kodak fine grain positive 35mm film.

2.5.2 Scanning Electron Microscopy

Dry wall material was sprinkled on to a thin layer of conductive silver paint glue, and excess particles were removed with an air stream. The particles were "sputter coated" with a $100A^{o}$ - thick layer of gold and then viewed under a scanning electron microscope.

2.5.3 Light Microscopy

Cell walls suspended in aqueous buffer (Batch 1 and 2 walls, see Chapter 3) were viewed under a Reichart Diapan light microscope fitted with a Nikon semi automatic camera and photomicrographs were recorded.

CHAPTER 3

GROWTH AND PREPARATION OF SAMPLE MATERIAL

3.1   GROWTH OF HYPOCOTYLS

Etiolated seedlings were grown from seeds in Opiki loam soil
in the dark under conditions of constant temperature (22-24°C)
and moisture (watering once every two days), in the Climate
Research Laboratory, Plant Physiology Division D.S.I.R., P.N.
The hypocotyls were harvested when they reached approximately
7cm in height. The hypocotyl segments were harvested approx-
imately 7mm above ground and immediately beneath the cotyledons.
They were then washed with distilled water and stored frozen
in plastic containers until required for use.

3.2   ORIGIN OF CALLUS CULTURES

Pinus radiata callus tissue were derived from two sources: -

a)   seedlings hypocotyls, and
b)   dissected embryos,

the latter by courtesy of the Forestry Research Institute,
New Zealand.

3.2.1   Derivation of Callus from Hypocotyl

Initially callus growth was investigated on a Murashige and
Skoog (74) salt medium. Growth on this medium was not easily
sustained, consequently a new medium was tried, of composition
based on Linsmaier and Skoog 1965 (75). Calluses were
generated from excised hypocotyl segments sterilised in 5%
hypochlorite solution and placed on the medium to produce
budding off, of callus tissue, on each end of the hypocotyl
segment (76).   Calluses were grown under controlled
conditions of light (30cms from fluorescent tubes of 40 watt
power) and temperature (22-24°C). Calluses were sub-cultured
at 3 weekly intervals, (when callus size had reached 1 - 1.5cm
in diameter) aseptically in a laminar flow hood onto fresh
media in sterile petri dishes. These were sealed with

"gladwrap" to prevent loss of moisture and drying out of the plates.

3.3   MAINTENANCE OF CALLUS CULTURES

Growth of callus received from the Forestry Research Institute was necessary for the continual supply of material for cell wall preparations. A few calluses were originally received to generate enough wall material for some preliminary analyses on browning callus (see Section 3.6.1 and 5.2.1). The bulk of the calluses maintained were from a subsequent large batch received from F.R.I., (see Section 3.5 and 3.6.2). Growth was sustained on a medium of composition based on Linsmaier and Skoog (75) but modified as by the Tissue Culture Group of the Forestry Research Institute, and as outlined in reference (142, 185) except that media used in this laboratory was supplemented with 75mg/litre of citric acid, and 50mg/ litre of ascorbic acid. Citric and ascorbic acids seemed necessary to sustain viable fresh green growth of calluses over an extended period of months (207).

Calluses which had been initiated at the Forestry Research Institute and then subcultured onto my Linsmaier, Skoog media grew more rapidly and survived subsequent subculturing better if citric and ascorbic acid supplements were used. Batches of growth media were prepared between 200mls to a litre as required with distilled water, and the pH adjusted to 5.6 - 5.8 with 1.0M NaOH. If the pH overshot this value, 0.1M HCl was used to bring it back down. Difco purified agar was added to a concentration of 0.6% and the mixture autoclaved in Erlenmeyer flasks (which were plugged with cotton wool filters) at 15 psi 15-20 minutes, then poured while still hot. The neck of the flask was sterilised by flaming before and at intervals during pouring. After transferral to new media, calluses were maintained under light, temperature and moisture conditions as mentioned in Section 3.2.1 before the next subculture.

Table 3.1: Linsmaier and Skoog Growth Medium

	Stock Solutions Concentration g/litre	Concentration in Medium mg/litre of medium
SOLUTION A: MAJOR ELEMENTS		
NH_4NO_3 (Ammonium nitrate)	33.0	1650.0
KNO_3 (Potassium nitrate)	38.0	1900.0
$MgSO_4 \cdot 7H_2O$ (Magnesium sulphate)	7.4	370.0
KH_2PO_4 (Potassium dihydrogen orthophosphate)	3.4	170.0
Na_2EDTA, disodium salt	0.746	37.3
$FeSO_4 \cdot 7H_2O$ (Ferrous sulphate)	0.556	27.8
SOLUTION B		
Sucrose	300.0	30,000
$CaCl_2 \cdot 2H_2O$ (Calcium chloride)	4.4	440
SOLUTION C: MICRONUTRIENTS		
$MnSO_4 \cdot 4H_2O$ (Manganous sulphate)	2.0	20.00
H_3BO_3 (Boric acid)	0.5	5.0
$ZnSO_4 \cdot 7H_2O$ (Zinc sulphate)	0.1	1.0
KI (Potassium iodide)	0.1	1.0
$CuSO_4 \cdot 5H_2O$ (Copper sulphate)	0.02	0.2
$NaMoO_4 \cdot 2H_2O$ (Sodium molybdate)	0.02	0.2
$CoCl_2 \cdot 6H_2O$ (Cobalt chloride)	0.02	0.2
Thiamine hydrochloride	0.04	0.4
Inositol	10.0	100.0
SOLUTION D: HORMONES		
IBA (Indole butyric acid)	0.50	5.0

3.4 ATTEMPTED GROWTH OF SUSPENSION CULTURES

Pieces of callus derived from hypocotyl in this laboratory as
in Section 3.2.1 were transferred to solutions of the same
nutrient media as used for growth of callus from hypocotyl
on agar (74, 75). Cultures were swirled at approximately 100
revolutions/minute in modified Erlenmyer flasks (with side
arms for absorbance and length measurements), at 25°C under
constant light conditions. The growth increment determined
by the cell density-packed cell volume, was monitored over a
period of 6 weeks, but no positive increment in growth was
observed at any stage and cells appeared to eventually all
die. Growth could not be sustained on transferral of callus
tissue to the liquid media.

3.5 CALLUS CULTURES FOR CELL WALL PREPARATION

The cell wall preparations described in Sections 3.6.1 and
3.6.2, were derived from cultures initially provided by the
Forestry Research Institute. Of 210g calluses received from
F.R.I., approximately 147g were harvested 10 days after
transfer (3 days after receipt of cultures) for preparation
of walls (Batch 1) by the wet sieving method, as described
in preparation of walls subsequently.

The remaining calluses were maintained as described above
with successive transfers and divisions until a further crop
of 320g was produced. This was then harvested for preparation
of a larger batch of walls (Batch 2).

3.5.1 Examination of Callus Cultures

a) Macroscopic appearance.
   Approximately one week after transferral to new media,
   calluses grown on the medium of Table 3.1, with IBA growth
   hormone were green and moist in appearance. Calluses which
   were let grow past the 3 week period, between subcultures,
   were observed to produce a crusting on their external
   surfaces, which developed a brown discolouring. The

viability of such calluses decreased in relation to loss
of greenness and appearance of external crusting.

b) Microscopic appearance.
Light and electron microscopy indicated that differentiation
had been kept to a minimum by the medium, but some
tracheids were found in wall preparations (see microscopy
results of Sections 3.7.1 and 3.7.2).   Sections through
green callus cells demonstrated that essentially these
were isodiametric, thin walled and highly vacuolated, as
described in following sections.

3.5.2   Electron Microscopic Examination

a) Callus material was prepared for transmission electron
microscopy as described in methods 2.5.1.

Figure 3.1 shows three cells, side to side, demonstrating
the thin primary walls and middle lamella region.   There
are large vacuoles in the two end cells with tannin
deposition at the edges of the vacuoles of the two end
cells.   Tannin deposition is normal in Pinus callus (178).

Figure 3.2 is a larger side view of the right of the
photograph in Figure 3.1.   The plasmalemma is visible.

In Figure 3.3, the microfibrillar appearance of the cell-
ulose is evident.   This photograph demonstrates the
uniformity of appearance right across the wall, showing
that morphologically there is only one main structure under
analysis, and little further differentiation of the wall
into a multi-structured organelle has occurred.

b) Examination of Possible Secretory Material.   Toward the
end of periods between sub-culturing, many of the calluses
were observed to possess milky edges around the base
region, which set in the agar.   This material was collected
and examined by light and electron microscopy.   Preliminary
light microscopic examination showed that this material
consisted of rod-like bodies, and bacterial contamination
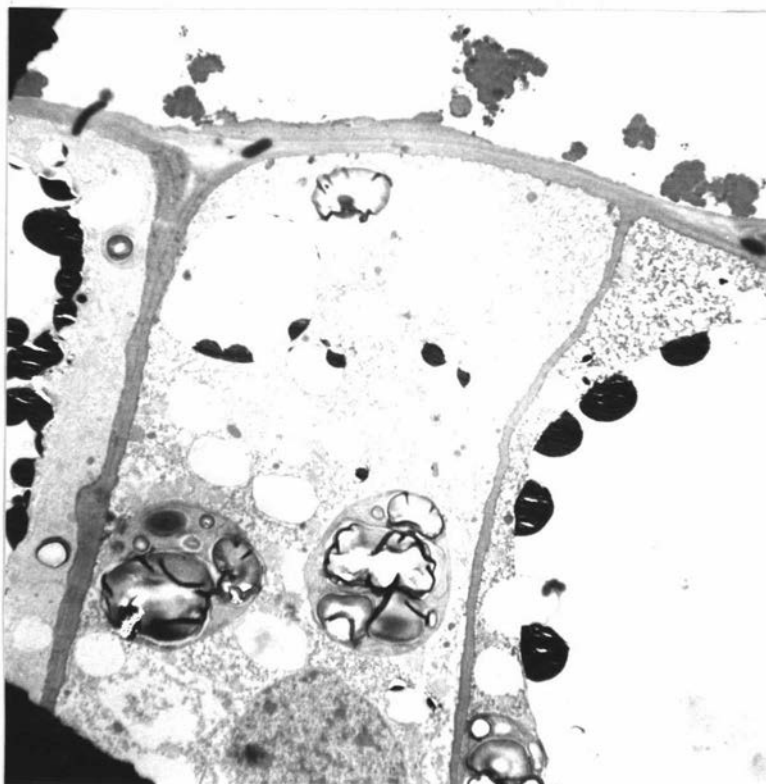
Figure 3.1 Section through green callus cells, 3,400x.
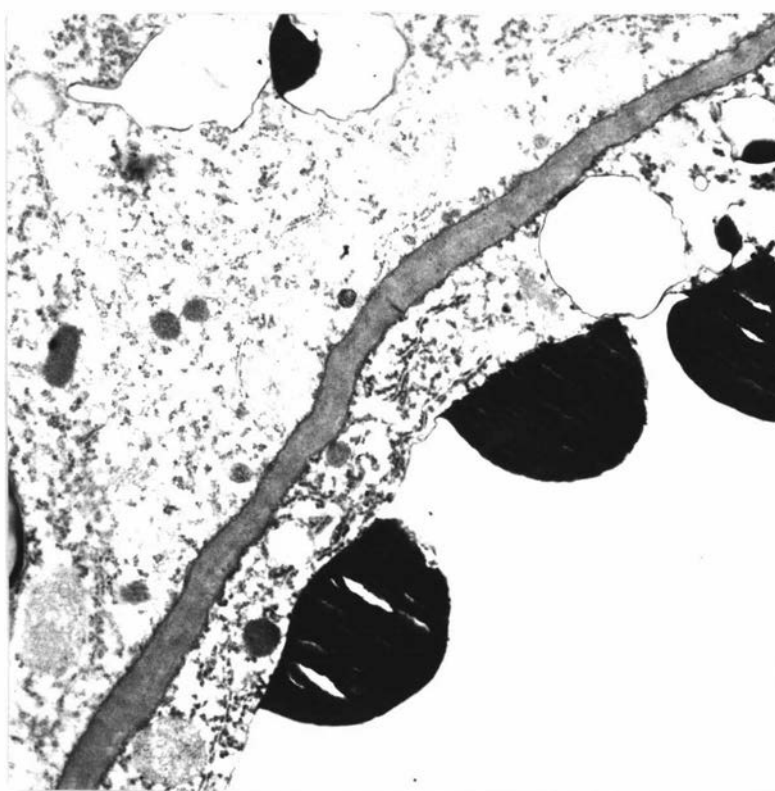
Figure 3.2 Section through the right of Photograph l,
            17,500x. Showing clearly distinguished plasmalemma.
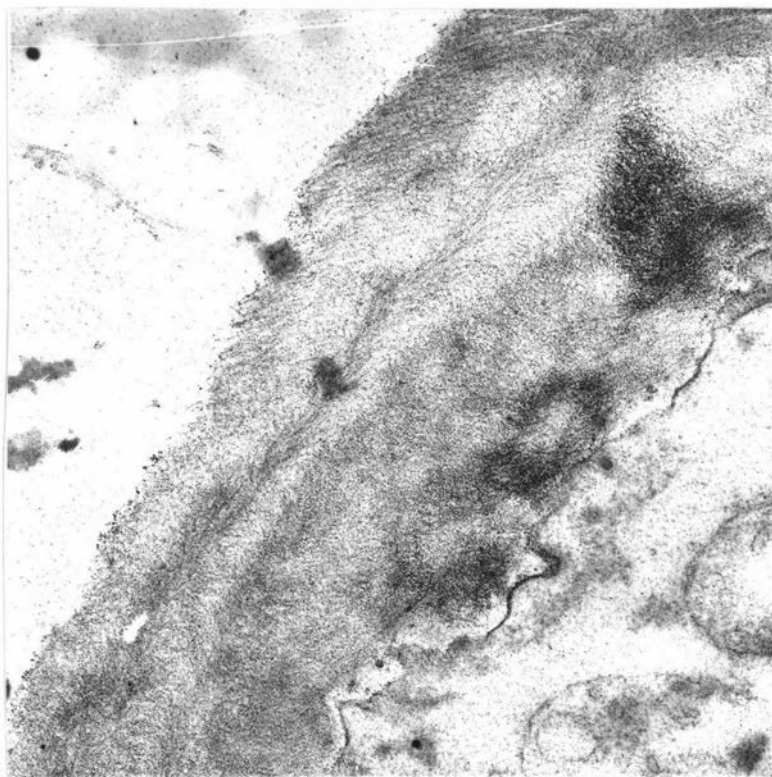
Figure 3.3 Cross section of the primary wall 41,400x.

Figure 3.4 Rod-like material from around callus bases 74,000x.

of the cultures was suspected.   However, the material
would not produce evidence of bacterial growth when it was
plated out on a variety of rich media.   Also use of anti-
biotics in the callus cultures did not suppress production
of this material.   It was hence demonstrated that the
cultures were uncontaminated.   The nature of the material
was examined further by transmission electron microscopy.

Figure 3.4 demonstrates the nature of the rod-like material
found around the base of many actively growing calluses.

The material appears to have a regular microcrystalline
repeating structure with 9nm longitudinal repeat, and 5nm
transverse repeat (Figure 3.4).   The dimensions suggest
that the material could be a protein of molecular weight
ca. 1-2x 10^5 daltons.   It is suggested that this
proteinacious material is extruded through the callus walls
as it was observed in electron microscopy to crystallise
from amorphous material still attached to the wall in some
cases.   It is possible that this extracellular material
which crystallises is related directly to wall glycoprotein.
An insufficient amount of it could be purified for amino
acid analysis.

3.6   PURIFICATION OF CALLUS WALLS

Two types of wall preparations were used:-

a) The non aqueous wet sieving method of Mares and Stone (60),
   as also used by Fincher (61).

b) The aqueous method of Albersheim (8, 46, 47) using cell
   disruption and suspension in potassium phosphate buffer.

3.6.1 Preparation of Walls by Wet Sieving

Calluses supplied by F.R.I. (147g wet weight) were harvested
carefully from the agar medium, briefly rinsed with distilled
water, and ground in a mortar with 80% aqueous ethanol.   The
ground callus material was sonicated in short bursts of

1-2 minutes to assist the freeing up of cytoplasmic contaminants and starch granules, and filtered on 25μm mesh nylon bolting cloth. Wall material was removed from the cloth and examined with the light microscope for size and purity. The procedure was repeated until a high degree of purity of wall fragments was observed microscopically, and the preparation was essentially free of starch (figures 3.9, 3.12 and 3.13). Some starch (145mg) was purified by slow centrifugation, from wall material that had passed the mesh. The pellet surface and supernatants contained almost pure starch.

The wall was collected and solvent exchange dried (60) by successive washing with absolute ethanol, methanol, and n-hexane. The wall was further dried over P_2O_5 and stored in vacuo over KOH pellets. Aqueous ethanol (80%) was used for suspension of the cells and walls throughout the procedure, since it solubilises lipids and most cytoplasmic components but has been observed to precipitate most polysaccharides and therefore provides for retention of water soluble poly-saccharides in the wall preparation (60, 61).

The yield by this technique was calculated as 700mg. This is equivalent to 0.5% of the wet weight of callus taken. This preparation is referred to as Batch 1.

A lesser amount of wall (600mg) was prepared by wet sieving of some "browning callus" (derived as in Section 3.3 from a few calluses supplied from F.R.I., to initiate some cultures for preliminary wall purifications and analyses). This wall was used for a time course of hydrolysis study, (see Section 2.3.3.1) and some preliminary experiments on extraction and paper chromatography of hydrolysed portions (Section 5.2.1).

For large amounts of callus the Albersheim method of wall preparation was adopted, since a large scale wall preparation was impractical by wet sieving. The wet sieving process has inherent problems of reduced filtration by clogging if a large amount is handled at once, and particle size reduction

if prolonged sonication has to be used to free up starch from
a larger mass. Consequently greater loss of wall than
necessary occurs.

3.6.2 Preparation of Wall by Disruption and Buffer Washing (Batch 2 Walls)

Green callus, grown as described in Section 3.5, from calluses
which were initiated at F.R.I., was harvested (320g wet weight)
10 days after transfer and washed twice with approximately 5
volumes (v/w) of cold (4°C) 0.1M potassium phosphate buffer
pH 7.0. The suspension was centrifuged and the cell mass
divided into ratio 3:1 by volume.

The cells of the larger batch (Batch 2, equivalent to 240g
wet weight callus), were then suspended in 1 volume (v/w) of
0.1M potassium phosphate buffer pH 7.0 at 2-4°C.

The cells of the smaller batch, (Batch 2P, equivalent to 80g
wet weight callus) were suspended in phosphate buffer, 10% in
polyethylene glycol, (mw 5000), to give a final volume of
cell suspension which was 5% in polyethylene glycol. Poly-
ethylene glycol was used to solubilise tannins from the
preparation (40). Cells of both batches were sheared by
passage through a French Pressure Cell at 2000-3000 psi and
the cell homogenate was passed directly into 4 volumes (v/w)
of potassium phosphate buffer (0.1M, pH 7.0, at 4°C) (5% in
polyethylene glycol for Batch 2P). Light microscope examin-
ation showed complete disruption of the cells. Both batches
were centrifuged, for 10 minutes at approximately 2000g in a
bucket sorval centrifuge, and the collected supernatant
solution for each batch was stored at approximately 10°C.
The pellets were then washed with 5 volumes (v/w) of 0.1M
potassium phosphate buffer, pH 7.0 at 4°C and centrifuged at
1000g for 5 minutes. The supernatants were collected and
added to the original supernatant to give a combined "cold
buffer extract", for each batch which was kept in a cool
storage (10°C) in 0.02% sodium azide.

Figure 3.5: Preparation of Batch 2 Walls

<u>Green Callus wet weight 320g</u>

Washed 2x with 5 volumes (v/w), 0·1M phosphate buffer

Suspension centrifuged and divided in ratio 3:1 by volume

Batch 2 | 240g equivalent   Batch 2P | 80g equivalent

Cells suspended in 1 Vol   Cells suspended in 1 Vol
(v/w) phosphate buffer   (v/w) phosphate buffer,
                                  5% in polyethylene glycol
                                  (PEG)

Both cell batches sheared by French Pressure Cell at 2000-3000 p.s.i.

Cell homogenate passed   Cell homogenate passed
directly into 4 Vols (v/w)   into 4 Vols (v/w) of phos-
of phosphate buffer.   phate buffer, 5% in PEG.

Both batches were centrifuged for 10 minutes at 2000g, separately.
Supernatants were stored at approximately 10°C. The two pellets were
washed with phosphate buffer and recentrifuged. The "cold buffer
extract" for each batch was made 0·02% in azide and stored at 4°C.

Distilled water washes of pellet (5x). Supernatants stored in cold in
azide.

Chloroform/methanol washes, and filtration to remove lipids; batch 2 and
2P.

Both samples resuspended in phosphate buffer, stirred at 70°C for 60
minutes. Both batches treated with α-amylase, 48 hours at 30°C.

Both samples filtered and washed, filtrate collected as 70°C water
extract.

Residues (Cell Wall) were stored frozen, for both batches.

The pellets were then washed with 5 volumes (v/w) distilled water, 5 times, and centrifuged after each washing in a sorval RC.3 centrifuge at 650g for 5 minutes. Distilled water washes containing most of the starch were pooled and stored in the cold in 0.02% azide.

The pooled 0.1M potassium phosphate buffer and cold water solutions represent the 4^OC water extract for each batch (2 and 2P), as for the major Fractionation Scheme for Batch 1 Walls, Figure 5.1. Both Batches 2 and 2P, were then suspended in 5 volumes of chloroform-methanol (1:1, v/v) and filtered and the residue washed 3 times with 10 volumes of chloroform/methanol, then 3 times 10 volumes of acetone, and before completely dry, suspended in 3 volumes of 70% aqueous acetone and left until α-amylase treatment. Both samples were filtered, to remove acetone and resuspended in 0.1M potassium phosphate buffer pH 7.0, separately and stirred at 70^OC for 60 minutes, then cooled.

α-Amylase Treatment. Porcine pancreatic α-amylase (20mg/200ml) (Sigma preparation) was added to the heat-treated suspension and stirred for 48 hours at approximately 30^OC. Under these conditions it was observed that starch levels were reduced to zero, on a test run with Pinus radiata starch (see Figure 3.6). Both samples were then filtered and washed and residues (cell wall) were stored frozen. Batch 2P was frozen in a total volume of 30ml of solution, and Batch 2 in 200ml of solution. The buffer filtrates after the 70^OC and subsequent α-amylase treatment were collected and kept in the cold in 0.20% azide as this represented the 70^OC water extraction of Batch 1 walls; Figure 5.1. Yields of Cell Walls, were: -

      Batch 2 - Calculated dry weight* 3.36g
               % Callus wet weight   1.4%

      Batch 2P - Calculated dry weight* 0.54g
               % Callus wet weight   0.7%

* A known portion of the preparations were dried and weighed. The procedure is summarised in Figure 3.5.

Figure 3.6: Solubilisation and Hydrolysis of Pinus Radiata Starch
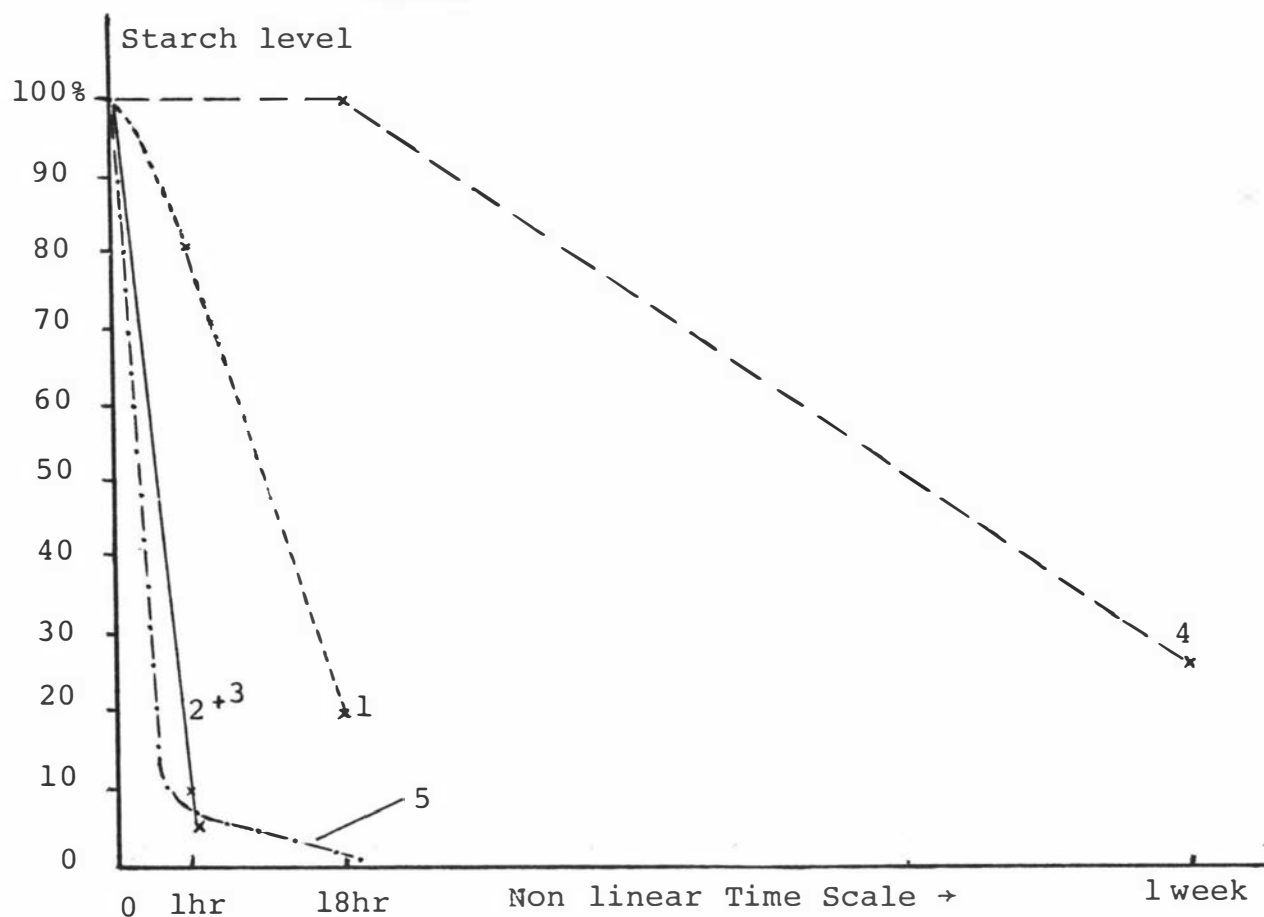
Pinus radiata starch purified from wet sieved walls as described in Section 3.6.1. was suspended in phosphate buffer and incubated with α-amylase. The extent of hydrolysis was determined by absorbance of aliquots with the KI/I_2 reaction (See Section 2.2.6.), when compared with the absorbance at zero time of hydrolysis, which was taken as 100% starch.

Porcine α-amylase concentration (Stock solution)
= 20mg/200ml of 0·1M potassium phosphate buffer.

Footnotes to the figure are listed on the following page.

Figure 3.6   Footnotes

1.   Pinus radiata starch (8·5mg), 1ml 0·1M phosphate buffer
     and 1ml α-amylase stock solution, incubated at 25°C.

2.   Pinus radiata starch (9·0 mg), 1ml 0·1M phosphate buffer,
     heated at 70°C for 1 hour, then 1ml α-amylase stock
     solution and incubated at 25°C.

3.   Pinus radiata starch (8·7mg), 1ml 0·1M phosphate buffer,
     heated at 95°C for 1 hour, then 1ml of α-amylase stock
     solution and incubated at 25°C.

4.   Pinus radiata starch (9·0mg), 1ml 70% ethanol, and 1ml
     α-amylase stock solution and incubated for 1 week at
     40°C.

5.   Pinus radiata starch (8.0mg), 1ml 0·1M phosphate buffer,
     heated at 70°C for 1 hour, then 0·8ml 0·1M phosphate
     buffer and 0·2ml (20μg α-amylase) of α-amylase stock;
     incubated at 25°C.

Albersheim (46, 47), recorded a cell wall yield of approximately 1% wet weight of callus by the same technique.

3.6.3 Starch Assays

Two methods were used for assaying starch contamination in wall preparations. These are described in methods, Section 2.2.6.

a) Wet Sieved Walls

Microscopy of wet sieved walls (Batch 1) showed very little starch, however starch was assayed as described in methods by the KI/I_2 technique (70) and a direct spectrophotometric assay, using purified Pinus radiata starch standards showed the level of starch in this preparation to be less than or equal to 0.5% of the whole wall dry weight.

b) Aqueous Buffer Wall Preparation

Aliquots were removed from Batch 2 and 2P samples after α-amylase treatment to give an equivalent of 5mg dry weight of wall and assayed by the amyloglucosidase procedure (71) as described in methods. The I_2/KI assay showed slightly higher levels of residual starch.

Samples	Starch Levels	
	Amyloglucosidase	KI/I_2
Batch 2P	0.0	0.3%
Batch 2	0.03%	0.5%

The amyloglucoside assay showed that near complete hydrolysis of the starch had occurred, but traces of monomeric glucose remained and had not all been washed out. However, this should all be removed in subsequent dialyses carried out for further fractionations on the wall. The reason for the difference in residual starch between the two methods of assay is not clear. That a small amount of starch was still present became apparent during fractionation of this wall, as indicated by methylation analysis of the extract removed with $100^\circ C$ water. Further treatment with α-amylase caused a further decrease in this starch level.

3.6.4 Tannin, Lignin and Phenolic Acids

Assays kindly performed by Dr I. Andrew on walls of Batch 1,
showed the preparation to contain approximately 2% (w/w)
tannin (for method used see reference (40)) 10% lignin
(method of (89, 90, 91) as in Section 2.2.5), and only traces
of phenolic acids such as ferulic acid (by paper chromatography
of ether extracts from reacidified alkali-treated samples).
Ferulic acid probably does not play a significant part in
polymer bridging in these walls (38, 39), but the presence of
lignin demonstrated by absorbance in acetyl-bromide and
fluorescence under U.V. is of significance since this is a
true cell wall component. The presence of tannins were not
considered important as a structural component, as these are
seen in the electron micrographs to be vacuolar components
before wall disruption and purification and since they are
deposited in the wall preparation, the use of polyethylene
glycol to solubilise the tannins in the preparation to produce
a "cleaner" wall preparation was investigated (40). Its use
here was shown to produce somewhat cleaner walls (Figures 3.8
cf 3.11).

3.7   EXAMINATION OF WALL PREPARATIONS

3.7.1 Light Microscopy

Wet sieved walls (Batch 1) were stained with congo-red
(Figure 3.9) to demonstrate wall material (59, 60, 61).
Staining with phloroglucinol (60, 77), showed darkening of
regions of the wall, possibly indicating lignification of
the walls. The walls were also observed to fluoresce on
irradiation at a wavelength of 300nm, Figure 3.7.

A low number of starch granules were detected by staining with
I_2/KI (60, 70) and by birefringence of these granules under
the polarising light microscope. From visual observations
starch was estimated at approximately 0.1→ 0.5% of the total
material.
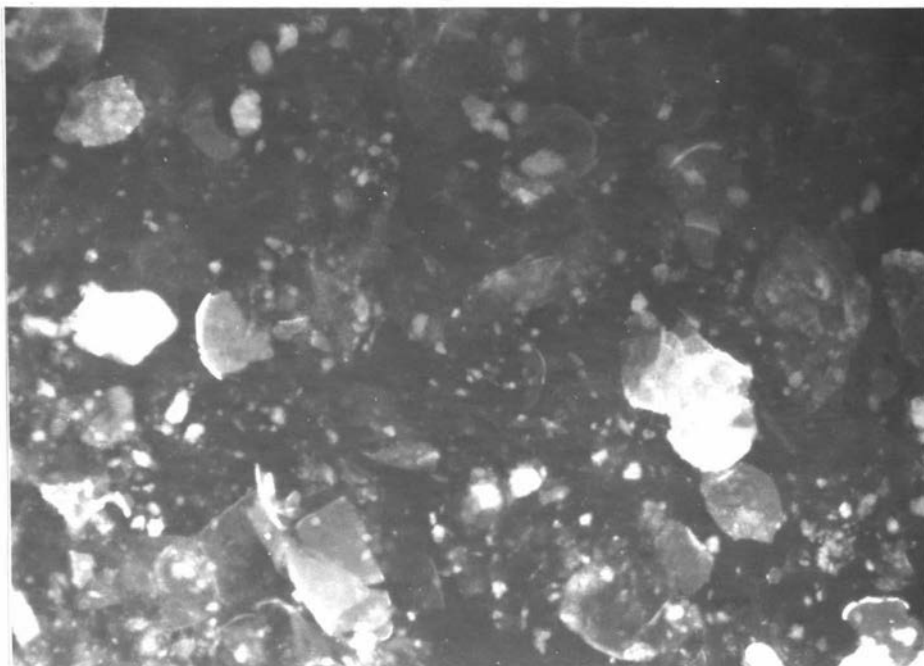
Figure 3.7: Wet sieved walls (Batch 1), showing fluorescence under light of 300nm wavelength, using a BG-2 exciting filter, with an FY-5 barrier filter. 100x
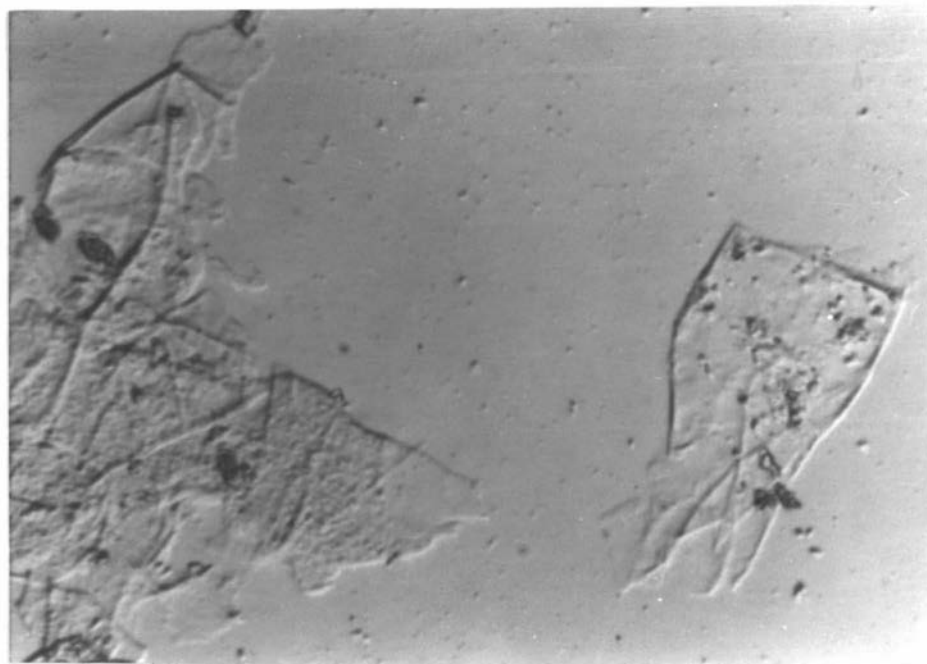
Figure 3.8: Walls prepared in phosphate buffer with polyethylene glycol treatment (Batch 2P). Walls appear cleaner than for Figure 3.10 and 3.11. 500x.

Figure 3.9   Wet sieved walls (Batch 1) stained with congo
             red, showing essentially clean walls, few starch
             granules.   125x

Figure 3.10 Walls prepared in phosphate buffer without
             polyethylene glycol treatment (Batch 2).   Stained
             with congo red.   Evidence of secondary thickened `
             wall exists, (bottom right), though not prominently.
             Wall pitting is better demonstrated in Figure 3.14.
             250x

Figure 3.11 Walls prepared in phosphate buffer without poly-
             ethylene glycol (Batch 2), viewed with Nomarski
             Differential Interference demonstrating the
             rough crusty nature of the walls, due largely to
             apparent precipitation of tannins in the cell
             vacuoles, but otherwise free of cytoplasmic
             contamination.   500x

Figure 3.9:
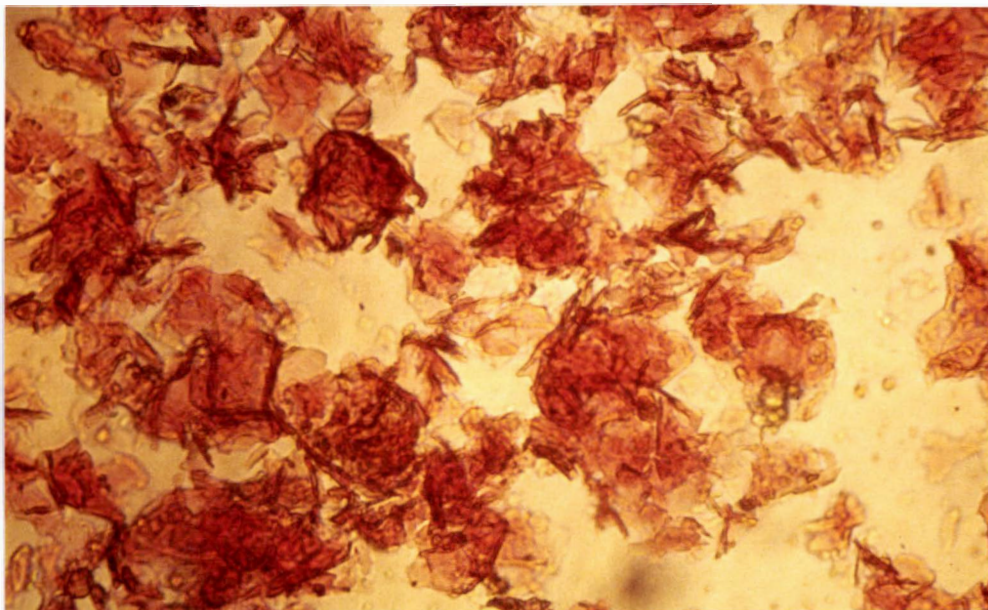
Figure 3.10:
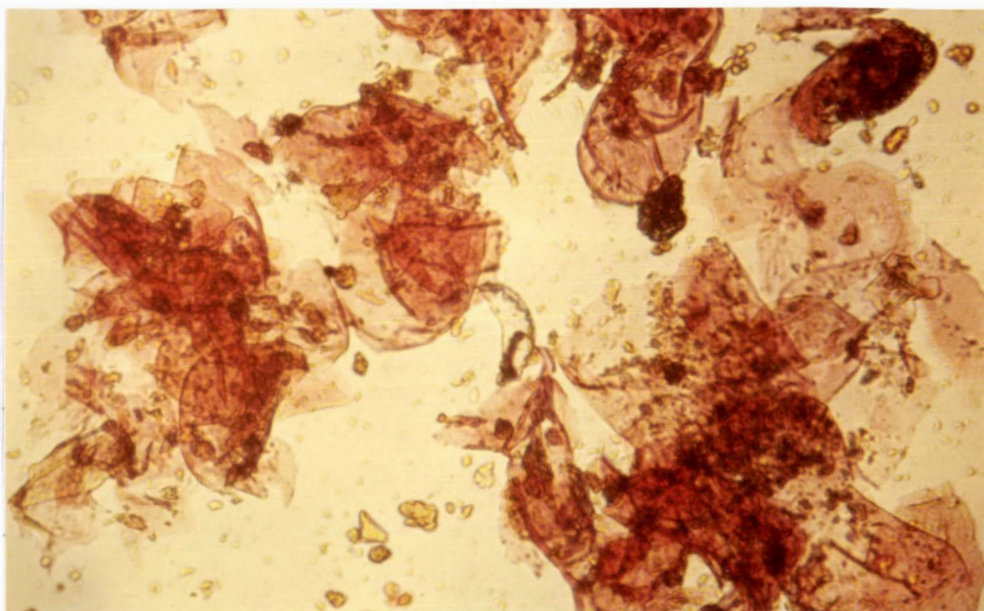
Figure 3.11:
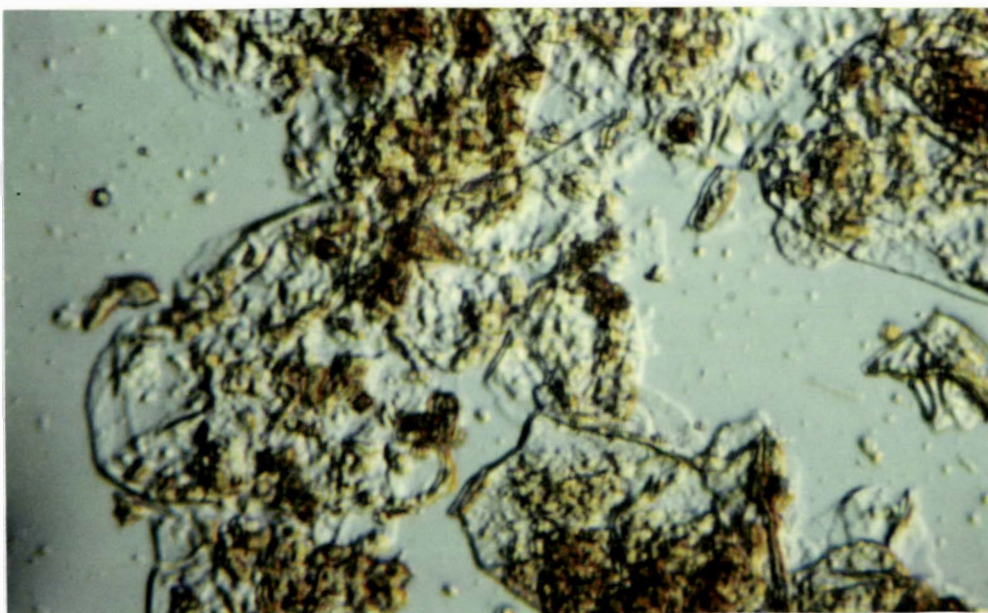
In all wall preparations the bulk of wall material consisted
of thin walled parenchyma, but tracheids with secondary
thickening were observed and some thick "lignified" walls.

Walls prepared in phosphate buffer which had not had treatment
with polyethylene glycol appeared somewhat rougher and as
though some extra material (perhaps tannins) could be partly
encrusting their surfaces. Walls treated with PEG appeared
slightly cleaner of surface encrustacions.

3.7.2   Electron Microscopy

Scanning electron micrographs were obtained (as described in
Methods Section 2.5.2) for samples of walls prepared by the
wet sieving method.

Figures 3.12 and 3.13 show walls at 1400x, and 1600x,
demonstrating the thin sheet like nature of the walls, and
the lack of cytoplasmic contaminants. The small number of
granular bodies lying on some of the wall surfaces, may be
due to a small amount of cytoplasmic protein.

Figure 3.14 shows an overview of a bordered pit in the wall
of a less frequently found differentiated cell, as discussed
in Section 3.5.1. Though numerous scans were performed, only
one such case was found.

3.8   SYNOPSIS

It has been possible to grow and prepare enough cell wall
material in an adequate state of purity to enable subsequent
analyses of whole wall and fractions of the wall.

A low degree of differentiation in the callus was observed by
light and electron microscopy. The uneven distribution of
lignin, presence of a few tracheids, and the lignin content,
was higher than expected from undifferentiated walls. Never-
theless the bulk of the wall material appeared to be primary

Figure 3.12: Wall prepared by wet sieving (Batch 1). Scanning E.M. 1400x

Figure 3.13: Wall prepared by wet sieving (Batch 1). Scanning E.M. 1600x Showing possible protein body, bottom right, which only rarely appeared throughout the wall preparation.
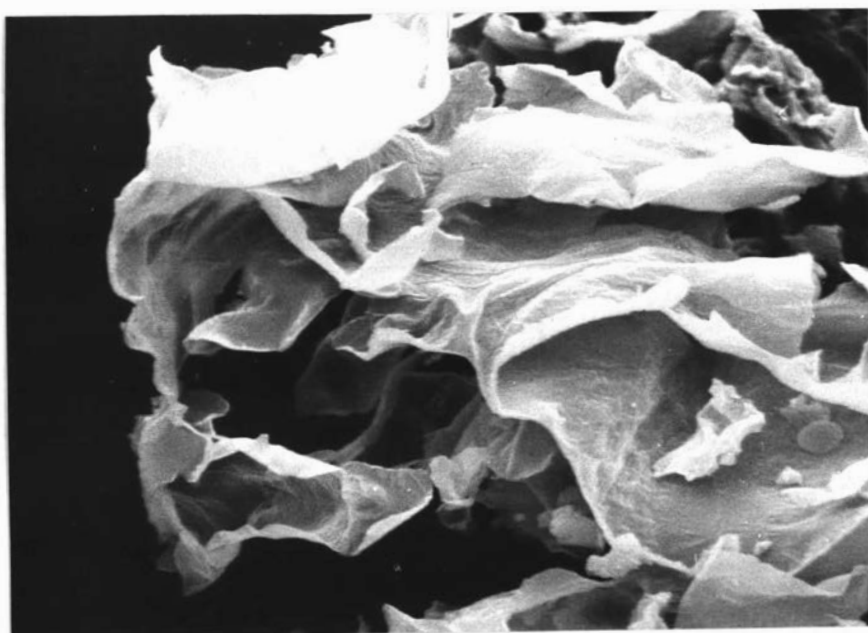
Figure 3.14: Wall prepared by wet sieving (Batch 1). Scanning E.M. 3200x Portion of a wall found containing a bordered pit region from a tracheid.
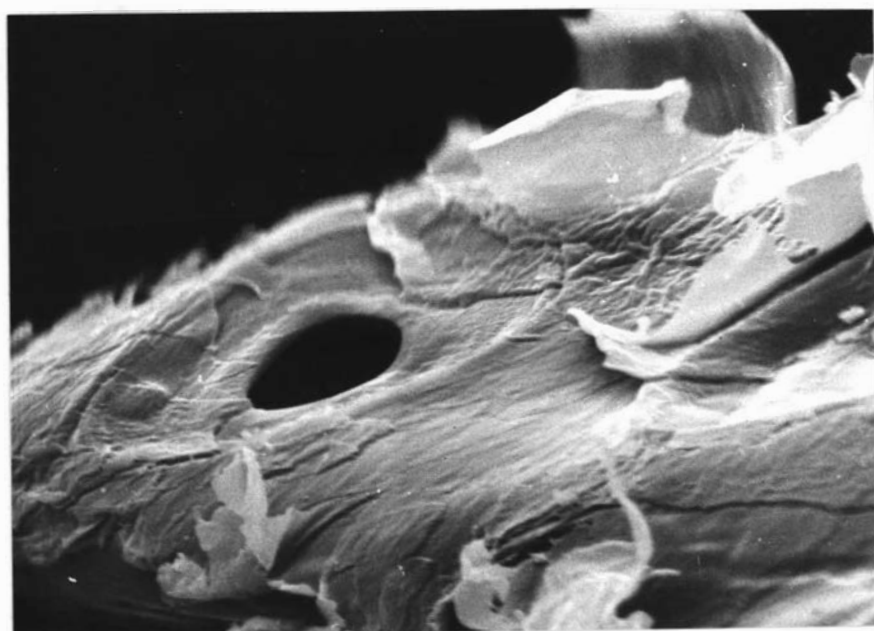
wall, and cell separation techniques, or complicated methods to separate walls after the purification step, were not employed.

CHAPTER 4
HYPOCOTYL STUDIES

4.1   INTRODUCTION

In the hypocotyl, the primary cell walled parenchymatous tissue is found in the in situ condition. Preliminary data on polysaccharides from hypocotyl tissue was produced (145) by J.W.L. Little. In that work, macerated hypocotyl tissue was purified of cytoplasmic contaminants, and lipids, fats and waxes were removed by acetone extraction. The resulting "wall" was extracted with hot ammonium oxalate, and 10% KOH (plus $NaBH_4$). The 10% KOH fraction in the hypocotyl extracts was found to be high in xylose and glucose, and the glucose was predominantly from a non starch glucan. The extract was fractionated with a series of well known reagents (50). Two polymers were purified, a xyloglucan, and an impure xylan. The structure of the xyloglucan was reported by methylation analysis, but the structure of the partially purified xylan was not investigated. The structure of the xylan has now been investigated in this work. To further the investigation of the types of polysaccharides that are present in hypocotyl primary walls, data is also presented on partial acid hydrolysis, and acidic components, of hemicellulosic poly- saccharides. The results suggest that the xylan may be a (4-O-methylglucurono)xylan.

4.2   STUDY OF ACIDIC COMPONENTS IN HYPOCOTYL CELL WALLS

4.2.1 Preparation of Hypocotyl Fraction

Hypocotyls were grown and harvested as described in Section 3.1.

Etiolated hypocotyl tissue (18g) frozen with liquid nitrogen, was ground in a precooled mortar with acetone. Cytoplasmic contents, pigments, waxes and lipids were removed by filtering off the acetone extract, and by washing several times with acetone. Subsequent extractions were carried out in the ratio for extractant to residue, of 100:1,(w/w).

Figure 4.1: Hypocotyl Extraction Scheme

Hypocotyl (wet weight 18.96g)

Acetone Extraction $\longrightarrow$ 17.97 g
(890 mg)

Hot (70°C) Water $\longrightarrow$ 471 mg
(419mg)

2% (Na)$_2$EDTA (70°C) pH 6.7 $\longrightarrow$ 71 mg
(348 mg)

Portions were used for
hydrolytic extraction

120mg   10mg 10mg   10mg   40mg

0.5M H$_2$SO$_4$, 100°C 2hr   0.2M TFA-Enzyme Extraction
and Extract hydrolysed
1hr further at 121°C.

Derivatised for   Sample for
Gas   Paper
Chromatography   Chromatography

Residues

6.44mg 6.13mg 6.65mg

The cell wall remaining after acetone extraction, was extracted
with hot (70°C) water for 1 hr, the suspension centrifuged,
and the supernatant was lyophilised. This extract contained
the water soluble polysaccharides, of the wall preparation.
The residue was again extracted with 2% (Na)$_2$EDTA, pH 6.7 at
70 - 75°C to remove classical "Pectins" (polygalacturonic acid)
as described in Section 5.1, 5.2.2.2. The extract was
dialysed exhaustively to remove the EDTA, and lyophilised.

The residue was then analysed for hemicellulosic components as
described here.

4.2.2 Acid Extraction of Sugars

After EDTA extraction to remove most of the "Pectin" a series
of mild hydrolyses were performed on the residue to investigate
the nature of the polysaccharides present in the hemicellulosic
fraction of the wall. Hemicellulosic and pectic poly-
saccharides were solubilised by this treatment and the extracts
were analysed for monosaccharides and oligosaccharides.

Extraction was carried out with 0.2M TFA at 120°C for 1hr,
followed by enzyme digestion as described in methods, Section
2.3.2. Lactonisation of uronic acids upon concentration of
TFA to dryness before enzyme digestion is not a problem (17)
as conversion of any lactones back to the acids has time to
occur in the enzyme buffer as described in Chapter 2 (2.3.2).
Lactones were tested for with solvent B. The carbohydrate of
the extracts was separated into neutral and acidic fractions
as described in Section 2.3.4 to assist in elucidating the
amounts of uronic acid present.

Neutral sugar data is compared between extracts removed by
hydrolysis with 0.5M H$_2$SO$_4$ (92, 122) and extracts obtained by
the TFA-enzyme hydrolysis method of Albersheim (86) to compare
extractability and the extent of hydrolysis of hemicellulose
by the two methods.

4.2.3   Monosaccharide Compositional Analysis by Gas
         Chromatography

4.2.3.1 Separation and Derivatisation of Neutral and Acidic
         Fractions for Gas Chromatography

Hydrolysates extracted with 0.2M TFA-enzyme, after addition
of standards and reduction with $NaBH_4$ to alditols and aldonic
acids as described in Methods, Section 2.3.2.1, were passed
over Dowex 1-X8 anion exchange resin columns, prepared in the
acetate form (ca. 2ml of resin) in small pasteur pipettes (86).
The column capacity was capable of exchanging 180 - 190mg of
uronic acid.   Solutions were passed over the resin, alditols
from neutral sugars collected with water washings and aldonic
acids from acidic sugars were eluted with 1M HCl, which on
evaporation produced the required lactones from the free
acids.   The lactones from acidic sugars collected from the
ion exchange eluant, were then reduced to alditols and
derivatised to alditol acetates, separately to the alditols
from neutral sugars.   The uronic acids were hence analysed as
their corresponding neutral alditol acetates, separately from
those of neutral sugars, by gas chromatography as outlined in
Section 2.3.2.1.

The 0.5M H_2SO_4 acid hydrolysates were neutralised with $BaCO_3$,
reduced and derivatised for gas chromatography as described
in Section 2.3.1.

4.2.3.2 Results from Gas Chromatography - Monosaccharide
         Composition

Results from alditol acetate analysis are presented for the
two hydrolytic methods; 0.5M H_2SO_4 (neutral mono-
saccharides) and 0.2M TFA-enzyme (neutral and acidic
monosaccharides) on OV-225 (Column 6).   The data from the
TFA-enzyme experiments are from three separate sets of analyses
of the alditol acetates after separation of neutral and acidic
sugars by ion exchange, and the acidic fraction has alditol
acetates derived only from acidic monomers.   Data for the two
hydrolytic methods is given in Table 4.1.

Table 4.1:   Relative Mole % of Sugars in Extracted Fractions of Hypocotyl EDTA Residue

| Sugar | TFA-ENZYME EXTRACTION | | | | | | $0.5M\ H_2SO_4$ HYDROLYSIS $100^{\circ}C$ 2hr, + $121^{\circ}C$ 1hr | |
| | Mole % of Total Monosaccharides. Hydrolysis | | | Mole % of Neutral Sugars. Hydrolysis | | | Mole % of Neutral Sugars. Hydrolysis | |
	1	2	3	1	2	3	1	2
Rhamnose	1.4	2.4	1.4	1.9	4.5	2.3	4.0	4.3
Fucose	1.4	1.3	1.2	1.9	2.4	1.9	2.4	2.8
Arabinose	24.0	18.0	23.6	33.0	33.6	38.3	30.1	35.7
Xylose	19.1	13.1	17.7	26.3	24.4	28.7	14.1	19.0
Mannose	7.0	4.4	4.7	9.6	8.2	7.6	8.1	7.1
Galactose	13.1	9.6	10.6	18.0	17.9	17.2	24.2	21.4
Glucose	6.6	4.8	2.4	9.1	9.0	3.9	16.8	9.5
GalA	23.0	43.8	35.4					
GlcA	4.8	0.0	1.2					
4-O-MeGlcA[c]	0.0	2.6	1.8					
*	16.2	20.2	15.0	11.6	10.8	9.2	6.9	5.8

* Total % recovery of EDTA Residue Weight

c This peak co-chromatographed with 4-O-MeGlc from a reduced xylan supplied by the Forestry Research Institute

It is evident from gas chromatography that the prominent sugars are arabinose, galactose, xylose and galacturonic acid. The high levels of arabinose and galactose suggest the presence of an arabinogalactan, or pectic type arabinan and galactan. Higher xylose than glucose is in accordance with the results of J.W.L. Little for the presence of a xyloglucan and a xylan as the dominant hemicelluloses, as for the primary tissue of other gymnosperms (54) and some angiosperms (46, 48, 56). The TFA-enzyme seems to be more efficient overall in liberation of monosaccharides, especially arabinose and xylose, and glucose is sometimes produced less by TFA-enzyme. This could be due to limited hydrolysis of the xyloglucan as demonstrated by J.W.L. Little.

The 4-O-methylglucuronic acid could be attached (1 → 2) to the main chain xylose in the xylan (48, 50, 51, 52, 124, 125, 127).

The uronic acid not extracted as "Pectin" by hot water and EDTA, but extractable by the TFA-enzyme treatment appears to consist almost entirely of galacturonic acid. Such uronic acid-containing polymer segments may be closely associated with the hemicellulose.

After hot EDTA extraction, which removes degraded polygalact-uronan by solubilisation and chelation (42, 43, 143), the hemicelluloses and uronic acid material still held in the wall by other links, as well as much of the wall protein and cellulose, are expected to be left unextracted. That most of the uronic acid remaining is still galacturonic acid (with a lesser amount of 4-O-methylglucuronic acid, and 4-O-MeGlcA-Xyl (see paper chromatography)), indicates that a polygalacturonic acid could account for nearly all of the uronic acid residues of the wall. This polymer may be typical of other pectins, with rhamnose interspersed in galacturonic acid chains. Paper chromatographic data suggest the existence of GalA-(1 → 2)-Rha. Reduction and isotopic labelling of methylated uronic acid residues (Section 6.3), demonstrates that GalA is the major uronic component of the wall pectic fraction of callus, and indicates the linkages between GalA residues. But the

existence of lower levels of uronic acid in a hemicellulosic fraction have been demonstrated here.

4.2.4 Extent of Hydrolysis with TFA - and Enzyme

Reduction assays were performed with borohydride for total sugar and uronic acid as described in Section 2.3.3.2 according to the technique of Timell (101), on the TFA-enzyme hydrolysate which was used for paper chromatography. Assays were performed on hydrolysates before ion exchange, and on neutral and acidic portions after ion exchange. The results are expressed in the next table.

Table 4.2: Summative Levels of Reducing and Non-Reducing Sugar Residues in TFA-enzyme Extracted Fractions (Expressed as % of Total Sugars)

	Total Sugars			Uronic Acids			Neutral Sugars		
	*Total	N.R.	R	Total	N.R.	R	Total	N.R.	R
Total Extract	100	14	86	32	7	25	68	7	61
Neutral Fraction	59	3	56	(2)	0	(1)	59	3	56
Acidic Fraction	40	8	32	33	7	26	7	1	6

* N.R. = Non Reducing Sugar Residues
*   R = Reducing Sugar Residues

The results indicated that there was near complete hydrolysis of neutral sugars to monosaccharides. Non-reducing sugar residues are predominantly uronic acids. Approximately 75% of the total uronic acid was present as monomers, the remaining 25% were in non-reducing positions as acidic oligosaccharides, such as have been determined by paper chromatography.

4.2.5   Paper Chromatography

4.2.5.1 Separation of Neutral and Acidic Fractions for Paper Chromatography

A sample of 0.2M TFA-enzyme extract from 40mg of EDTA residue was quickly titrated to pH 8.0 with 0.1M KOH, and 1.0 ml of 1.0M $NaHCO_3$/NaOH buffer to pH 8.5 was added. The sample

was left for 30 min. Lactones were absent from paper
chromatography in Solvent B. The sample was then deionised
on IR-120(H$^+$) and the effluent and washings brought back to
pH 8.5 with a small amount of 0.1M KOH. The 50 ml solution
was ion exchanged on Dowex 1-X8, OAc$^-$ form as described in
methods, Section 2.3.4.1. Neutral sugars and water washings
were collected, and acidic sugars were eluted with 6M HOAc.
Acidic sugars were concentrated under reduced pressure at 40°C
on a Rotary Evaporator.

4.2.5.2   Chromatographic Conditions

Approximately 200µg of sugar (acidic sugars in 6M HOAc) was
spotted on to the paper. The chromatograms were run in ethyl
acetate/water/acetic acid/formic acid (18:4:3:1) Solvent A
overnight as described in Section 2.3.4.2.

Xylose, galacturonic acid, and glucuronic acid markers (10µg
each) were also run. R_f and R_{xylose} values were compared
with literature values for sugars and oligosaccharides (88),
and with R_{xylose} values for 4-O-MeGlcA-Xyl from a xylan
fraction isolated from Phebalium wood (courtesy of Dr I.G.
Andrew).

4.2.5.3   Results

The TFA-enzyme extract from the hypocotyl EDTA residue, the
2M TFA hydrolysate of the xylan fraction isolated from
Phebalium and the acid extract from Pinus radiata sawdust
(from Tiritea Sawmill), were chromatographed on paper after
separation of acidic and neutral components in this work as
described in Section 4.2.5.1. Results from the paper
chromatography suggested the presence of 4-O-methylglucuronic
acid and 4-O-methylglucuronosyl -(1 → 2)-xylose in both the
Phebalium and Pine sawdust (Table 4.3, Figure 4.2). The spots
identified as 4-O-methylglucuronic acid and (4-O-methyl-
glucuronosyl)xylose in the Pinus radiata sawdust hydrolysate
gave a red-orange colour reaction on a chromatogram developed
with aniline hydrogen phthalate, and the spots fluoresced in U.V.,

Table 4.3: R_{xylose} Values of Mono and Oligosaccharides

Literature (88) Compound and R_{xylose}		Aniline Phthalate Colour	Phebalium Standards (S)	Sample	Pine Sawdust (S)	Sample	TFA-Enzymes (S)	Sample
GalA-(1→4)-GalA	0.18	Orange				0.28?		
GlcA-(1→4)-Gal	0.18	Brown						
GlcA-(1→4)-Xyl	0.38	Red				0.43?		0.36
MeGlcA-Xyl-Xyl	0.38	Reddish						
GalA-(1→2)-Rha	0.53	Orange-brown						0.59
* Glc		Brown	0.65(S)					
* GalA	0.63	Orange	0.71(S) GalA ⎤		0.70(S) ⎤		0.75(S) ⎤	
* GlcA	0.68	Orange	0.72(S) GlcA ⎦ —— 0.68		0.68(S) ⎦ — 0.65		0.83(S) ⎦ — 0.75	
4-O-MeGlcA-(1→2)-Xyl	0.84	Red-orange		0.87		0.82		0.92
Xyl	1.00	Red	1.00(S)	1.08	1.0 (S)	1.00	1.0 (S)	1.07
4-O-MeGlcA	1.37	Red-orange		1.20		1.31		1.32

* Fluorescence with Aniline

Hydrogen Phthalate & U.V. Glucose fluoresced yellow, GalA pinky-yellow

Chromatograms are not drawn and GlcA fluoresces greeny-yellow (lower intensity)
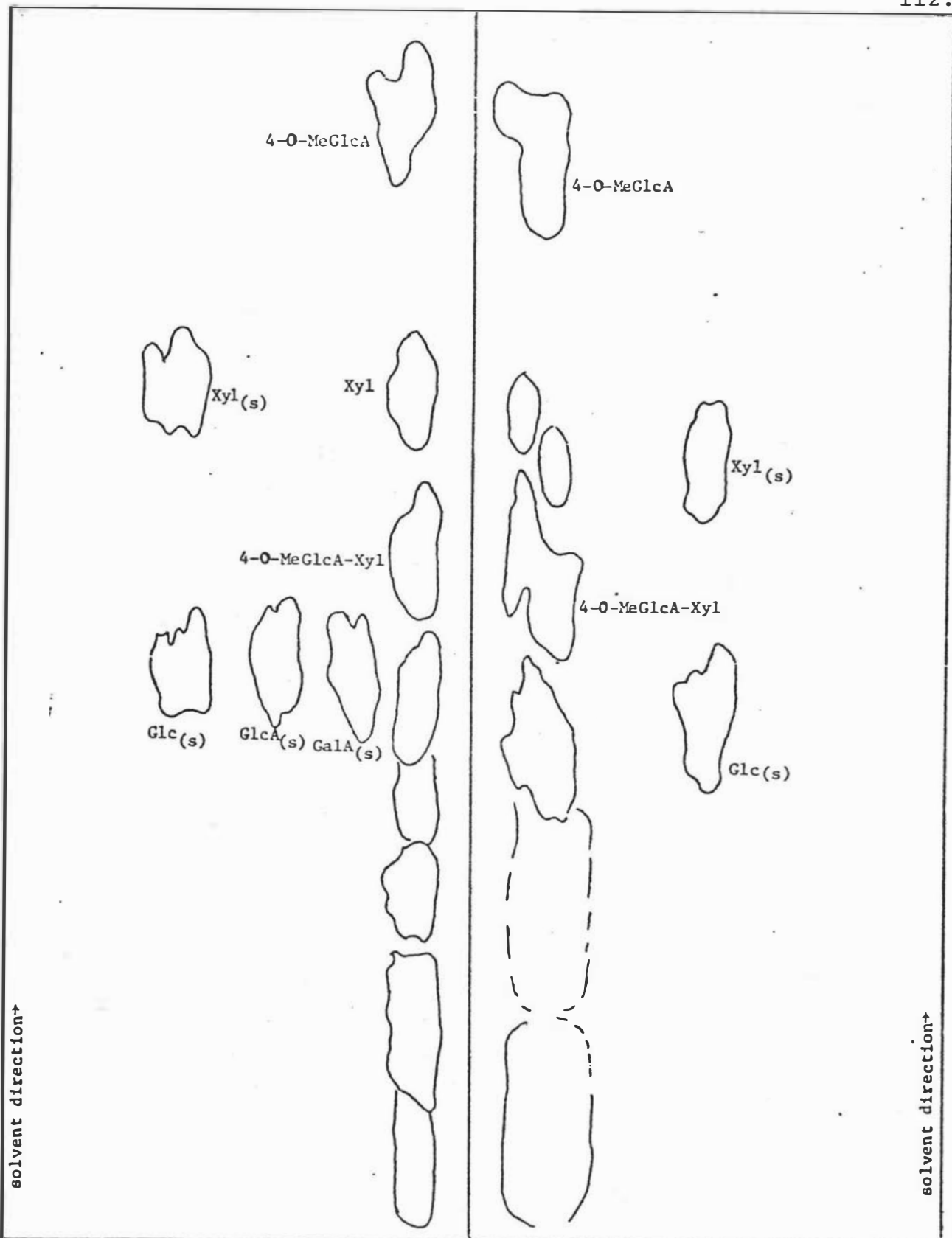
Hydrolysate of _Pinus radiata_ sawdust.   Hydrolysate of Xylan from _Phebalium_.
Acidic sugars.   Acidic Sugars.

Figure 4.2:   Paper chromatography of Acid Hydrolysates of _Pinus
              radiata_ sawdust, and the _Phebalium_ Xylan, after
              Dowex 1-X8 separation into acidic and neutral
              sugars. Solvent: EtOAc/H_2O/HOAc/Formic acid
              (18:4:3:1). Developed with $AgNO_3$.

              Chromatograms include standards-(s).
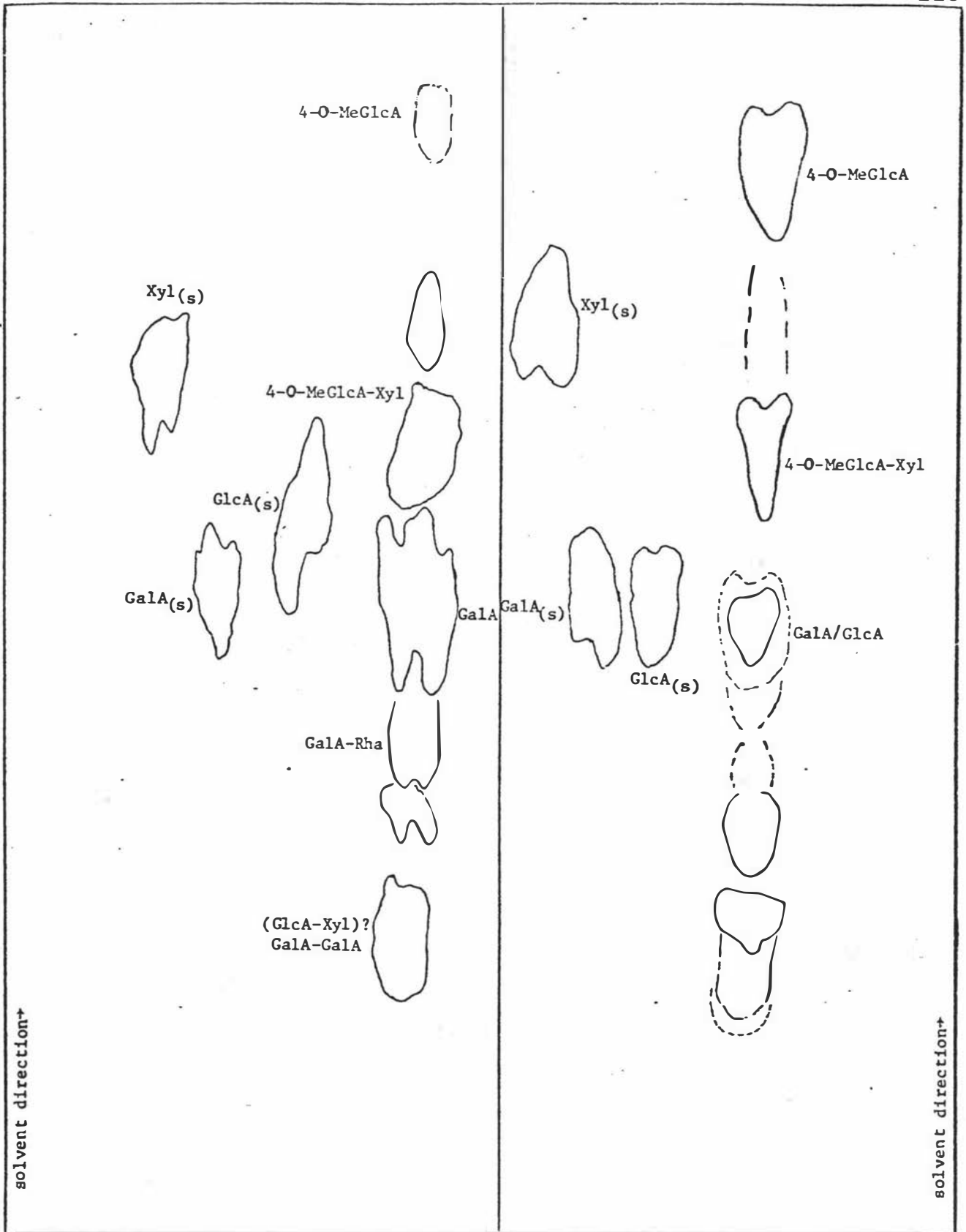
Hydrolysate with 0.2M TFA-enzyme of
Pinus hypocotyl EDTA residue. Acidic
Sugars.

Hydrolysate of Xylan from
Phebalium. Acidic Sugars.

Figure 4.3: Paper chromatography of Acid Hydrolysates of
Hypocotyl EDTA residue and of the Xylan preparation
from Phebalium (with standard sugars) after
separation on Dowex 1-X8 into acidic and neutral
sugars. Solvent EtOAc/H_2O/HOAc/Formic Acid
(18:4:3:1). Developed with $AgNO_3$.
Chromatograms include standards-(s).

greeny-yellow. The approximate quantitation indicated the ratio of 4-O-MeGlcA/4-O-MeGlcA-Xyl to be 2:1. Brasch and Wise (108, 109) obtained analogous results for the observation of these uronic and aldobiouronic molecules in Pinus radiata sawdust. Other compounds in Pine hemicellulose were identified by R_{xyl} values and colour reactions with phthalate, as GalA, GlcA, and possibly some GlcA-Xyl(R_{xyl} = .433). Other compounds on the Phebalium chromatograms were identified by R_{xyl} values as GalA and GlcA and xylose. From the intensity of the spots 4-O-methylglucuronic acid and (4-O-methylglucuronosyl) xylose represent nearly all of the acidic sugars in the Phebalium hydrolysate. Table and Figure 4.3 show TFA-enzyme data.

Compounds detected in the TFA-enzyme extract had R_{xyl} values corresponding to GalA, GalA-Rha, as well as 4-O-methylglucuronic acid, and (4-O-methylglucuronosyl)xylose according to the literature values and the comparison made with the xylan, and pine sawdust acidic sugars and oligosaccharides .

The relative intensities of the spots indicated that GalA was the predominant uronic acid, with (4-O-methylglucuronosyl) xylose about 1/20 this amount and other oligosaccharides (perhaps GalA-Rha, and GalA-GalA) less still.

The detection of 4-O-methylglucuronic acid and (4-O-methyl-glucuronosyl)xylose in the TFA-enzyme extract is significant (48, 50, 109, 124, 126), as many plant hemicellulose xylans, including that from Pinus radiata wood (30, 108, 109), contain 4-O-methylglucuronic acid as a constituent of (4-O-methylglucurono)xylans (48, 50, 51, 52, 125, 127). The methylation data for the xylan partially purified from hypocotyl, is reported in the next section. The presence of 4-O-methylglucuronic acid in the xylan was not established. The presence of 4-O-methylglucuronic acid and (4-O-methyl-glucuronosyl)xylose in TFA-enzyme extracts tends to indicate that the xylan observed by J.W. Little in the hemicellulosic fraction of primary cell walls of Pinus radiata is a (4-O-methylglucurono)xylan (or an Arabino-(4-O-methylglucurono) xylan).

Table 4.4: Composition of the Xylan.

Mole % Composition of Methylated Polysaccharide Sugar
Residues

Monosaccharide, O-Methyl ether	Mole %
Arabinose	
*2,3,5-	8.0
3,5-	1.1
2,5-	3.1
2,3-	4.5
2-	0.8
3-	1.4
Xylose —	0.9
*2,3,4-	21.8
2,3-/3,4-	27.6
2-/3-	13.4#
Fucose	
*2,3,4-	1.2
Galactose *2,3,4,6-	1.9
3,4,6-	tr
2,3,4-	0.7
2,4-	0.9
Glucose	
*2,3,4,6-	0.4
2,3,6-	4.1
2,3-	8.1
Rhamnose	
*2,3,4-	0.4
3,4-	0.5

Mainly 3-MeXyl by mass spectrometry; m/e fragments as in
  Appendix 1.

* Derivatives subject to some loss through volatility.

tr = trace

4.3   STRUCTURE OF HEMICELLULOSIC XYLAN, BY METHYLATION
       ANALYSIS

The methylation of the hypocotyl xylan was carried out in this
work as described in methods: Section 2.4.3.1. The following
partially methylated derivatives (Table 4.4) were identified by gas-
chromatography and mass-spectrometry. The results indicate
that the major constituent of the fraction is a 4-linked
xylan, from the high mole % of 2,3-Me$_2$-xylose. Branching of
xylose residues is indicated by the high level of 2-/3-MeXyl.
The presence of terminal arabinose also in relatively high
yields could suggest that much of the branched xylose is
substituted with arabinose residues, as in many primary cell
wall arabinoxylans (49, 50, 125). Xylose could also be
found as branch residues in the xylan. The data is also
consistent with the presence of some xyloglucan. The 3-MeXyl
implies 2-branched xylose as is typical of (4-O-methyl-
glucurono)xylans. The lower levels of galactose are probably
due to contamination from an arabinogalactan.

4.4   CONCLUSIONS

Several types of polysaccharides appear to be part of the
primary tissue wall in hypocotyl. Arabinose and galactose
containing polysaccharides are probably associated largely
with the polygalacturonic fraction of the wall. Xylose and
glucose containing polysaccharides may be more directly
associated with cellulose, and less directly with the pectic
acid component. The predominant pectic material observed is
polygalacturonic acid. Evidence from partial acid hydrolysis
would suggest that the xylan is a (4-O-methylglucurono)xylan.
The results confirm the presence of two major hemicelluloses
in these walls, and provide some initial basis for comparison
with data on walls of callus.

CHAPTER 5

ANALYSIS OF CALLUS CELL WALLS: MAIN COMPONENTS

5.1   INTRODUCTION

As discussed in Chapter 1, various models of the molecular organisation in cell walls are possible. The evaluation of these models in respect to Pinus radiata callus cell walls is one aim of the studies reported in this chapter. Evidence for the existance of polysaccharides, proteins and lignin, and their possible arrangement in the wall was obtained by fractionating the wall by chemical means, and analysing the fragments. A comparison was made of the composition of the fractions of the two major wall preparations and an overall perspective of the composition and arrangement of the callus wall in terms of polysaccharide, protein and lignin was obtained.

In order to retain any water soluble polysaccharides that might be integrally associated with the cell wall, the first major batch of walls (Batch 1) prepared for polysaccharide analysis was prepared in 80% ethanol (59, 60, 61).

Cell walls of Batch 1 were subjected to a series of extractions with different reagents in order to dissociate polysaccharide components, according to their mode of bonding within the cell wall.

Extraction with water was used first to extract any water soluble components followed by calcium-binding or chelating agents (ammonium oxalate, EDTA), in an attempt to solubilise pectin. A series of alkali extractions was then employed to extract hemicelluloses and any components bound in the walls by ester linkages. This procedure was based on the extraction sequence used by Fu et al (176).

A delignification step, employed by Fu and Timell in their extraction sequence was not used in this series of extractions

as it was considered that some damage to protein and also
perhaps to hemicelluloses might occur through such treatment
(2, 49, 50, 135). This could invalidate any attempt at
reconstructing the intermolecular bonding within the wall.
Delignification has been shown to be an unnecessary step in
the extraction of hemicelluloses from tissues with low lignin
content such as lupin and mung bean (2, 132, 135). However
a tentative estimate of the lignin content of the cell wall
preparation used here was about 10% (Section 3.6.4), a value
about one-third of that in mature pine wood. This level of
lignin might hinder the extraction of hemicelluloses by
covalent cross-linking of these with lignin. Lignin-poly-
saccharide complexes are well known, (6, 90, 161), although
the nature of the linkage between lignin and polysaccharide
is not clear. In the second batch of walls studies in this
chapter (Batch 2), the alkali extraction sequence was followed
by a mild chlorite delignification and a further alkali
extraction, in order to assess the role of lignin in the
wall structure.

Many workers including Albersheim et al (8) have prepared
walls in aqueous media but Mares and Stone (59, 60) have shown
that some walls prepared in aqueous ethanol solution contain
cold water extractable polysaccharides. This has been
supported by the work of Fincher(61) and by analyses of water
extracted fractions here. In order to investigate the nature
of such cold water extractable polysaccharides in Pinus
radiata callus cell walls, Batch 1 walls were prepared in 80%
ethanol and then extracted with cold water.

An extraction with hot water has commonly been used in
preparing cell walls (190, 192). Preliminary experiments
with small scale preparations showed that the temperature of
water extraction had a profound effect on the amount and
nature of polysaccharides extracted. This effect was most
noticeable between 70°C and 100°C. From the results of
Albersheim et al (189, 191) it would be expected that water
at 100°C would cause extensive degradation of pectin (see
also ref. 143). The result of the treatment might therefore

be similar to that obtainable by treatment with poly-
galacturonase. Thus any component bound in the wall by
covalent linkage to pectin might be either released or
rendered more susceptible to subsequent extractions.
Talmadge et al (47) observed such an affect on treatment of
suspension-cultured sycamore cell walls with polygalacturonase.
Xyloglucans were relatively resistant to extraction by 8M
urea, unless the wall had been pretreated with polygalacturonase.

The difference between $70^{O}C$ and $100^{O}C$ water treatments with
regard to amount of polysaccharide extracted observed in
preliminary experiments was profound. Knee 1973 (192)
observed a similar difference between extraction at $75^{O}C$ and
$95^{O}C$ with aqueous EDTA. Waite and Gorrod (190), adopted
$60^{O}C$ as a suitable temperature for aqueous extraction of non-
wall components. Higher temperatures extracted appreciable
non ionic material.

In the current study a temperature of $70 - 75^{O}C$ was used for a
preliminary hot water extraction of Batch 1 walls. This
provided a residue comparable to the Batch 2 wall preparation.
Analytical data on the wall preparations are generally
expressed on the basis of the $70^{O}C$ water residue.

The $70^{O}C$ water extracted walls of Batch 1 were extracted in
two portions and submitted to different treatment.

The first portion (Batch 1A) was treated with ammonium oxalate
at $75^{O}C$ as described by Waite and Gorrod (190) and used
by Fu et al (176) and many others for the extraction of
pectin. This was followed by EDTA, at $70^{O}C$ as described
by Aspinall and McGrath (193) and others (42, 43). EDTA is
acknowledged by some workers (132-136, 157, 158) to extract
pectin more effectively than ammonium oxalate. The previous
exhaustive treatment with $70^{O}C$ water, ensured that any
extraction of polysaccharides by oxalate and EDTA at $70-75^{O}C$
was not simply an effect of hot water, but was due to the
presence of oxalate or EDTA. These compounds presumably
extract pectin by breaking of ionic calcium bridges within

the wall (21, 51).   Extraction with l,2-diaminocyclohexane
N,N,N',N'-tetraacetic acid (CDTA) has recently (1981) (205)
also been shown to have the same effect.

The walls treated as above were then submitted to a series of
alkali extractions (cf. Fu et al (176)) beginning with 0.1M
Na_2CO_3 (149) and ending with 25% NaOH-borate (49, 50, 176).
The Na_2CO_3 was expected to cleave ester links and extract any
polysaccharides that were bound solely by this type of bond
(149).   Hemicelluloses generally require strong alkali for
extraction (49, 50, 128, 129) and glucomannans require the
addition of borate for efficient extraction (49, 50, 159, 160,
176).   Strong alkali will further β-eliminate pectin, break
ester links and disrupt hydrogen bonding.   Sodium hydroxide
is more efficient for extraction of glucomannans than KOH (50)
and borate is believed to complex with vicinal cis hydroxyls
on mannose pyranose rings (50).   The alkali reagents used
were supplemented with sodium borohydride (0.5%) to prevent
degradation of polysaccharides through the "peeling reaction".
Reduction of ester linkages by $NaBH_4$ would be unlikely, since
these should all be hydrolysed by prior treatments.

The second portion of 70°C water extracted walls (Batch 1B)
was exhaustively extracted with water at 100°C in order to
increase the yield of pectin through β-elimination (143, 191).
This was followed by treatment with oxalate and EDTA to
remove Ca^{2+}-bridges and extract further pectin as for Batch
1A walls.   A treatment with 6M urea was then used before the
alkali extraction (Na_2CO_3 0.1M followed by 25% NaOH-borate).
Extraction of polysaccharide with chaotropic reagents has
been reported (2, 46, 132).

Urea is a denaturing agent, widely used for separating non-
covalently linked protein subunits but also used for denaturing
nucleic acids and some polysaccharides (194).   Grant et al
(195) have shown that urea will dissociate a polyuronide-glucan
complex from white mustard seeds.   Monro et al showed
that 8M urea as well as 6M guanidinium thiocyanate (GTC)

extracted a portion of the hemicellulose from lupin hypocotyl cell walls (2, 132). The urea treatment was less effective than GTC. Other reagents that have been used for extracting non-covalently linked polysaccharides (192) include guanidine hydrochloride, dimethyl formamide, dimethyl suphoxide, ethylene diamine, chloral hydrate, and simply high salt concentrations. Since urea is an efficient protein denaturant at relatively low concentrations, it was considered that 6M urea might extract a major portion of the non-covalently linked protein from the walls, without extracting much polysaccharide. Hence its use with Batch 1B walls. The extraction of polysaccharides with 8M urea and 6M GTC was tested with Batch 2 walls.

The first wall preparation (Batch 1) was effected in 80% ethanol - in order to retain water-soluble wall polysaccharides. These could then be studied in water extracts of the walls.

A second batch of walls (Batch 2) was prepared by an aqueous procedure, as described by Talmadge, Albersheim et al (47) except that they were treated with alkali at $70^{\circ}C$ to gelatinise the starch before treatment with α-amylase. This method of starch removal contrasted with the wet sieving procedure used for Batch 1 walls. The Batch 2 wall preparation obtained could be compared directly with the $70^{\circ}C$ water residue of Batch 1 walls. It was thus possible to compare three different extraction sequences, commencing from the $70^{\circ}C$ water residues of 1A, 1B and 2; respectively. It should be noted however that Batch 2 walls were derived from a different callus preparation from Batch 1 walls (see Chapter 3), although both were from the same stock. Thus differences between Batch 1 and Batch 2 walls might be expected according to the state of growth or differentiation at the time of harvesting.

The extraction sequence used for Batch 2 walls was based partly on experience from Batch 1 walls, but with additional features introduced to explore more fully the nature of the bonding between polysaccharides. The first extraction was with water at $100^{\circ}C$, because results with Batch 1 showed more efficient removal of pectin by this

procedure.   One aim of the extraction sequence was to determine how pectin that was not extracted by hot water might be bound in the walls.

The second extraction was with 2% EDTA at $100^{O}C$ to ensure disruption of Ca^{2+}-bridges.   Some workers (e.g. Bailey and Kauss, Monro et al (132-136), Knee (192)) have reported virtually complete removal of pectin under these conditions. It is therefore likely that pectin remaining after such treatment might be covalently linked to other wall polymers.

The next extractions were designed to disrupt non-covalent linkages as effectively as possible.   Urea is a denaturant widely used for such purposes.   Others include guanidinium hydrochloride, potassium thiocyanate, dimethyl formamide etc (194) but Von Hippel and Wong (196) showed that guanidinium thiocyanate (GTC) was a more potent chaotropic (disruptive) agent than any other tested.   Thus the independent action of the guanidinium and thiocyanate ions is combined in one reagent.   Monro et al showed that 6M GTC extracted hemicellulose more effectively from depectinated lupin hypocotyl cell walls than did 8M urea (132).   Apparently the xyloglucan was more quantitatively extracted by GTC but the xylan was not.   The latter was mostly extracted by subsequent mild alkali treatment.

After $100^{O}C$ EDTA treatment, the Batch 2 walls were divided into two portions.   One portion was extracted with 8M urea followed by 6M GTC; the other portion was extracted by the same two reagents in the reverse order.   The residues were combined.   Thus the relative potency of both reagents for extraction of cell wall polymers could be compared and yet both portions were exhaustively extracted with both reagents. The urea concentration used here was higher than with Batch 1 walls, the object being to extract more hemicellulose.

After exhaustive GTC and urea extraction, the wall residues were then treated with 0.1M Na_2CO_3 to cleave ester linkages as in Batch 1.   However, with Batch 2 walls, exhaustive

Na_2CO_3 extraction was followed by exhaustive extraction with 8M urea, and then aqueous EDTA at 100^OC.   This was done to ensure maximal extraction of those polymers that might be bound non-covalently in the wall after hydrolysis of ester linkages (8, 149).

The wall residue of Batch 2 walls was then subjected to the same alkali extraction sequence as for Batch 1 walls, for extraction of hemicelluloses, as for a variety of angiosperm monocots (19, 20, 59, 60, 61, 151), dicots (130, 131) and gymnosperm plants (49, 50, 128, 129, 148), but with a nitrogen atmosphere to inhibit alkaline degradation by the peeling reaction (150), rather than $NaBH_4$ as used in Batch 1 walls.

The cell wall residue after the alkaline extraction sequence may be compared with the final residues of Batch 1A and. Batch 1B extraction sequences.   Extraction by 25% NaOH-borate is commonly used to remove the more tenaciously bound hemi-celluloses from holocellulose, leaving an α-cellulose which is nearly pure cellulose.   However, the residues from extraction of callus walls, which had not been delignified, contained substantial amounts of lignin, protein and non-cellulosic carbohydrate (including pectic material).   The classical methods for removing lignin (151, 152) cause some degradation of polysaccharides and protein (2, 135), but Selvendran (32, 33, 67, 151) has shown that a brief treatment with acid-chlorite (1 hr at 70^OC instead of the usual 4-5 hr approx.) causes minimal protein destruction, the only significant changes being oxidation of Met, Cys, Tyr, Lys and Arginine side chains.   Moreover the bulk of the residual wall protein was extracted by this treatment.   Kibblewhite (188) has used an even milder acid-chlorite delignification procedure (3 days at room temperature) in order to minimise degradation.   His procedure was adopted here for treatment of the NaOH-borate residue.   The course of delignification was monitored by the bleaching of the residues.   This was an indication of the depolymerisation of the lignins.   Only 24 hr treatment was in fact used here.   A further alkali

extraction of the residue after chlorite treatment was deemed
necessary, in order to extract hemicelluloses rendered alkali
extractable by the delignification step.   While acid-chlorite
degrades the lignin by oxidation of C-C and C-O-C bonds, it
may not release polysaccharides linked to it which may also
be linked or hydrogen bonded still to other polymers such as
cellulose and therefore one further alkali fractionation was
included to assist solubilising any material which had at
least in part been held in the wall by cross-linking with
lignin.

Practical details concerning the extraction sequences used
are given in the next section.

5.2   EXTRACTION SEQUENCES

Chapter 4 has commented on the possibility of the existence
of arabinogalactans, or arabinan and galactan, arabinoxylans
and some xyloglucan in the primary hypocotyl tissue.   More
emphasis has been placed on the results from the callus
tissue; on which this and the following chapter concentrate.
A detailed fractionation scheme was carried out on each cell
wall preparation (Batch 1 and Batch 2 walls).

5.2.1 Preliminary Fractionations

a) The wall purification technique in 80% ethanol initially
    was tested on a small amount of callus grown as described
    in 3.2, from calluses originally supplied by F.R.I.   An
    oxalate extracted fraction from this wall preparation was
    used for hydrolysis studies to obtain some approximations
    to degradation and depolymerisation parameters as
    described in Methods Chapter 2.   The weight of callus wall
    prepared was 600mg.   Of this, 200mg was extracted with
    20ml 0.5% ammonium oxalate at $75^{O}C$ for 1 hr followed by
    18ml 10% KOH.

b)   Another portion (400mg) of the above wall was extracted
     with 0.5% ammonium oxalate to give a fraction 70mg, and
     the residue was again extracted with 10% KOH to give a
     fraction 61mg. Some of this oxalate fraction (40mg) was
     hydrolysed with 2$\underline{M}$ TFA and the hydrolysate used for
     qualitative paper chromatography, of sugars and
     oligosaccharides in the fraction. The results are
     discussed briefly in the following Section (5.3.1).

c)   A small amount of cell wall (20mg) from Batch 1 purified
     by the wet sieved method was extracted with;
     1) cold water,
     2) hot water (95°C)
     3) hot ammonium oxalate (70 - 75°C).
     4) 10% KOH at room temperature.

     Water soluble fractions were lyophilised and collected.
     Oxalate and KOH extracted fractions were passed over
     Sephadex G-25 and lyophilised. Samples of these fractions
     were hydrolysed with 2M TFA as described in Section 2.3.1.1.

d)   A larger amount of cell wall of Batch 1 was extracted
     with;
     1) Cold H_2O,
     2) 70°C hot water,
     3) 75°C hot oxalate
     4) 70°C hot EDTA pH 6.7
     5) 0.1M Na_2CO_3 pH 11.3
     6) 10% KOH, and
     7) 17% $NaOH-BO_3^{3-}$,
     to give a final residue. Water extracted fractions were
     lyophilised, and all other fractions were purified by
     passage over Sephadex G-25, then lyophilised. Samples
     of fractions were hydrolysed with 0.5M HNO_3/0.5% urea.

Analytical data for these preliminary fractionations are
referred to in Section 5.3.1. The fractionation schemes
Path A and Path B for Batch 1 walls were devised partly on
the basis of these preliminary results.

5.2.2 Fractionation of Batch 1 Walls

The major fractionation scheme for wet sieved walls, prepared
in 80% aqueous ethanol (Batch 1) was as described below; see
also Figure 5.1. As discussed in Chapter 3 the wall prepar-
ations were relatively free of starch so an α-amylase pre-
treatment step was not included. All extractions were
performed with a ratio of extractant to cell wall weight of
100:1(w/w). Each extraction was carried out with stirring
for 1 hour. All extracted material was collected by
centrifugation at approx. 1000g. The wall material (300mg
dry weight) was divided approximately in half and each portion
weighed. Effectively two fractionation schemes were carried
out on the two portions of 170 mg dry weight (Path A) and
130 mg dry weight (Path B).

5.2.2.1 Removal of Water Soluble Polysaccharides

Since sieving of the cell wall in 80% ethanol afforded
retention of the water soluble polysaccharides (60, 61) it
was desirable to recover these from the wall preparations
separately, not to wait until these are solubilised in a
subsequent aqueous extraction step. Direct extraction by
stirring the wall in $4^{O}C$ water was carried out twice for
both Path A and Path B. After each extraction samples were
centrifuged at ∿ 1000g for 5 minutes and supernatants were
collected.

Extracts from both paths were pooled to give a common fraction
at this point. The $70^{O}C$ water extraction was repeated 3x for
each path, and supernatants to both paths were pooled. All
walls to both paths were then washed and the supernatant
washings were added to the $70^{O}C$ water extract. The $4^{O}C$ and
$70^{O}C$ water extracts were lyophilised and stored in vacuo over
KOH pellets, before recording the dry weight of these
fractions.

5.2.2.2 Removal of Pectic Polysaccharides

Walls in Path A were extracted with 0.5% ammonium oxalate
at $75^{O}C$ and 2% (sodium)$_2$ EDTA pH 6.7 at $70^{O}C$ and each

extraction was repeated three times.   Extracts to each
fractionation were pooled, dialysed exhaustively and
lyophilised.   Residues to both paths were washed with warm
(70°C) water and added to the EDTA extracts.

Walls in Path B were fractionated with 100°C water, 0.5%
ammonium oxalate 75°C, and 2% (sodium)$_2$ EDTA pH 6.7 at 70°C.
The extraction with 100°C water was repeated three times and
supernatants after centrifugation were collected and
lyophilised.   Further pectin removal was assisted by
extraction with hot EDTA (removing metal ion links to pectin
carboxyl groups).   These extractions were also repeated three
times, and supernatants pooled, dialysed exhaustively against
distilled water, and lyophilised.

5.2.2.3   Removal of Hydrogen-bonded Components

The EDTA residue has been extracted in Path B with 6M urea
at room temperature.   Extraction was repeated three times.
Supernatants and washings (cold water) were dialysed
exhaustively and lyophilised.

5.2.2.4   Removal of Hemicellulosic Polysaccharides

Extraction with 0.1M sodium carbonate (pH adjusted to 10.0
with 1.0M HCl) was used in both paths.   Such weakly alkaline
conditions are just sufficient for hydrolysis of ester links
(149).   Walls of both paths were extracted three times with
the Na_2CO_3 (0.1M pH 10.0) at 4°C.   A graded series of
alkaline extractions have been used (0.5%, 10%, 24% KOH) here
for Path A, to observe polysaccharide removed with alkali
strength.   Sodium borohydride (0.5%) was included in the
extractions to remove reducing ends of extracted polysaccharides
to prevent degradation by the "peeling reaction" (150).   The
last fractionation in Path A was with 25% NaOH - 4% Boric acid.
For Path B only one alkali extraction was performed, with 25%
NaOH - 4% Boric acid.   All alkali extractions were repeated
three times, supernatants for each fraction pooled, and
neutralised with 6M acetic acid, dialysed exhaustively against
distilled water and lyophilised.   Residues were washed and
dried over P_2O_5 then stored in vacuo over KOH pellets before
weighing.

Figure 5.1: Major Fractionation Scheme for Cell Walls of Batch 1

Cell Walls, Batch 1 (300mg)
Prepared by wet sieving in 80% ethanol

*Cold (4°C) water

Residue

*Warm (70°C) water

Batch 1A: (Path A)   Residue   Batch 1B: (Path B)

0.5% $(NH_4)_2C_2O_4$, 75°C

Residue

*2% Na_2 (EDTA) pH 6.7, 70°C

Residue

*0.1M Na_2CO_3 pH 10, 4°C

Residue

*0.5% KOH (0.5% $NaBH_4$)

Residue

*10% KOH (0.5% $NaBH_4$)

Residue

24% KOH (0.5% $NaBH_4$)

Residue

25% NaOH–BO_3^{3-} (0.5% $NaBH_4$)

Final Residue

*Water 100°C

Residue

*0.5% $(NH_4)_2C_2O_4$, 75°C

Residue

*2% Na_2 (EDTA) pH 6.7, 70°C

Residue

6M Urea (Room Temp)

Residue

0.1M Na_2CO_3, pH 10.0, 4°C

Residue

*25% NaOH–BO_3^{3-} (0.5% $NaBH_4$)

Final Residue

* Fractions which were methylated (Chapter 6)

5.2.3 Fractionation of Batch 2 Walls

Detailed Procedure. The fraction scheme is summarised in Figure 5.2. All extractions on "Cell Wall" were carried out with extractant ratios and times as in Section 5.2.2. Extracted material (supernatant) was collected by centrifugation at approximately 1000g.

5.2.3.1 Water Soluble Polysaccharides

Walls were prepared as discussed in Chapter 3 Section 3.6.2. The two water extractions used on Batch 1 walls ($4^{O}C$ and $70^{O}C$ water fractions) correspond to the complete buffer extraction and α-amylase treatment used here in wall preparation. The material was not dried in between these and subsequent steps, as it was not desirable to alter the solubility properties of cytoplasmic substances (protein etc) which might contaminate the wall if not removed by buffer washing.

5.2.3.2 Removal of Hot Water-Soluble and Pectic Polysaccharides

As for Batch 1 walls Path B, a $100^{O}C$ water treatment was used to extract pectic polysaccharides by β–elimination (143). It was found that this removed a major component of the wall in terms of dry weights.

Subsequently to this the wall was treated with $100^{O}C$, 2% (sodium)$_2$ EDTA pH 6.7. The extraction was repeated 3 times and the extracted solution collected as supernatant after centrifugation. Extracts were pooled, dialysed exhaustively, and freeze dried. They were kept in vacuo over KOH before weighing.

5.2.3.3 Extraction of Hydrogen-Bonded Polymers

After extraction with hot EDTA the residue was divided into two equal portions by weight and both portions treated with chaotropic reagents to remove hydrogen bound polymers (as in the diagram). Portion B was extracted twice with 100ml, 8M urea (2, 46, 59) at room temperature overnight and again for 1 hr, and then with 6M guanidinium thiocyanate, for 12hr at

Figure 5.2: Fractionation Scheme for Batch 2 Walls

Buffer extractions, used
to prepare the wall.

Callus
4°C H_2O

Residue

70°C H_2O

Residue

Cell Wall   2.51g (Dry weight calculations)

100°C H_2O *

Residue

100°C EDTA

Residue
50/50
(w/w)

8M Urea (B_1)   6M GTC (A_1)*

Residue   Residue

6M GTC (B_2)   8M Urea (A_2)

Residue   Residue

0.1M Na_2CO_3, pH 10.0

Residue

8M Urea (C)

Residue

100°C EDTA

* Fractions which were
methylated, (Chapter 6).

Residue

0.5% KOH, N_2

Residue

10% KOH, N_2*

Residue

24% KOH, N_2

Residue

25% NaOH-4%H_3BO_3, N_2

Residue

50/50
(w/w) ClO_2^- /HOAc

Residue

Residue (1)   10% KOH, N_2

Residue (2)

room temperature (Volume GTC = 30 ml, wt sample approx. 1.0g
dry weight).

Portion A was extracted 3 times with 30 ml, 6M guanidinium
thiocyanate (2, 134) at room temperature overnight and twice
again for 1 hr and then with 8M urea (100 ml overnight), at
room temperature. Extracts to each fractionationwere pooled,
dialysed and lyophilised and stored over KOH in vacuo.

At the conclusion of the chaotropic extractions, the residues
to the two paths were recombined, and a single extraction
path was followed.

5.2.3.4 Cleavage of Ester Links Within the Walls

a) After removal of the bulk of pectic and non-covalent
    bound material, an extraction step was included with
    0.1M sodium carbonate pH 10.0, at 4^OC. The extraction
    was carried out 3 times at 4^OC and the supernatants after
    centrifugation were combined, dialysed and lyophilised.

b) A further three one hour extractions were carried out
    with 8M urea (100 mls, residue weight approximately 1800
    mg) at room temperature.

    Extracts were pooled, dialysed and lyophilised.

c) A further three one hour extractions with 2% EDTA pH 6.7
    (80 mls, residue weight approx. 1700 mg) at 100^OC were
    carried out.

5.2.3.5 Extraction of Residual Hemicelluloses

A sequence of alkali extractions was carried out on Batch 2
walls analagous to the alkali extracts of Batch 1. A series
of 0.5%, 10% and 24% KOH fractionations were carried out with
four, 1hr extractions for each fractionation. Nitrogen was
bubbled through the mixture during extraction (129) in the

place of 0.5% NaBH$_4$ as in extractions on Batch 1 walls.
Supernatants to each fractionation were pooled after
centrifugation, dialysed and lyophilised.

The residue was then extracted with 25% NaOH - 4% Boric
acid (3 x 1hr) in the presence of nitrogen. After
centrifugation, the supernatants were collected, pooled,
dialysed exhaustively and lyophilised.   The residue was
washed and washings were added to the supernatants to be
dialysed.

The residue (902 mg), was then divided in half by weight,
for subsequent analyses and some further fractionations.

5.2.3.6   Chlorite Extraction

The residue from above was stirred in 15ml of water,
0.7 gm of sodium chlorite added and approx 2.5 ml of
glacial acetic acid was added with stirring at room
temperature over 30 minutes.   The pH was kept constant,
pH 4 - 5, over the reaction period (24 hr) with small
additions of glacial acetic acid.   After the colour of
the mixture was reduced to a pale cream (24 hr) the
suspension was centrifuged at about 1000 g for 10 min,
and the supernatant and residue washings were collected
dialysed and lyophilised.

5.2.3.7 Post Chlorite Alkali Extraction

The residue after chlorite extraction was further extracted with 10% KOH (0.5% in $NaBH_4$) and the extract collected and purified as before.

5.2.3.8 The Final Residue

This was washed and freeze-dried, and dried to constant weight over KOH in vacuo. This was analysed for uronic acid, monosaccharide and amino acid content.

Division of the residue prior to chlorite extraction was as near as possible in equal parts by mass of the material. All extracts were exhaustively dialysed, and then lyophilised. The final residues were rigorously washed prior to drying.

5.3 ANALYTICAL DATA

Analyses performed. To indicate the nature of the structural polysaccharides of the wall each fraction was analysed for:

1) constituent neutral sugars by hydrolysis with 0.5M HNO_3/0.5% urea, derivatisation of neutral mono-saccharides to alditol acetates, and subsequent gas-chromatography, as described in Section 2.3.1;

2) the distribution of "pectin" (polygalacturonic acid) by spectrophotometric assay for uronic acid (Section 2.2.2).

The protein component of the wall was examined by the amino-acid analyses of fractions (Section 2.2.3 - 4).

The possible role of lignin in the wall was investigated by analysis for lignin in fractions by colorimetric assay (Section 2.2.5). The bonding of lignin to other wall components was investigated further by chlorite extraction of the alkali residue. The nature of polysaccharide and protein components removed with, or labilised by, the chlorite

extraction, have also been ascertained from the sugar and amino acid analyses of these fractions.

5.3.1   Results to Preliminary Wall Fractionations

The results of monosaccharide analysis on the oxalate fraction (Section 5.2.1a) are described in Chapter 2.

Qualitative paper chromatographic results on the hydrolysate of the oxalate fraction (Section 5.2.1b) were obtained after separation of neutral and acidic components (Section 2.3.4.1) and chromatography of the neutral and acidic fractions as in section 2.3.4.2 with Solvent A. That callus contained much GalA, some GalA-rhamnose and some (4-O-methylglucuronosyl) xylose in its acidic portion was demonstrated.

Data from monosaccharide analysis on fractionations of 5.2.1c and d, are given in Tables 5.1 and 5.2 respectively.

Table 5.1: Sugars Recovered as Percentages of Cell Wall Weights

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
FRACTION							
Cold H_2O ($20^{\circ}C$)	0.02	0.01	0.17	0.06	0.01	0.30	0.27
Hot H_2O ($95^{\circ}C$)	0.30	0.10	3.20	0.30	0.20	2.70	2.50
Hot Oxalate ($75^{\circ}C$)	0.20	0.10	3.40	0.41	0.10	2.30	0.70
10% KOH ($20^{\circ}C$)	0.10	0.10	2.20	0.90	0.12	2.20	1.30

The neutral sugar components of polysaccharides were seen to be present in approx. the same proportions for pectic and alkali extracts as in hypocotyl.

The data in Table 5.2 shows that less sugar as a percentage of total cell wall weight is removed by lower temperature aqueous extractions, than by $95^{\circ}C$ H_2O as shown in Table 5.1.

Table 5.2: Sugars Recovered as Percentages of Cell Wall
Weight

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
FRACTION							
Cold H_2O ($0^{O}C$)	0.01	0.01	0.37	0.10	0.04	0.21	0.10
Hot H_2O ($70^{O}C$)	0.04	0.01	0.28	0.07	0.03	0.90	0.99
Oxalate ($75^{O}C$)	0.03	0.01	0.37	0.06	0.05	0.20	0.31
EDTA pH6.7 ($70^{O}C$)	0.12	0.03	2.14	0.03	0.11	0.55	1.50
Na_2CO_3 (pH 11.3)	0.08	0.00	0.60	0.02	0.01	0.32	0.10
10% KOH	0.12	0.16	1.18	1.36	0.18	1.00	1.50
17% NaOH-borate	0.03	0.01	0.25	0.04	0.01	0.14	0.05

The sugars arabinose and galactose are dominant in both schemes,
suggesting callus walls are rich in pectic type polymers.
Higher xylose and glucose in alkali extracts suggest the
presence of a xyloglucan, (as observed for hypocotyl by
J.W.L. Little). The preliminary results here are further
born out in the results of the following large scale
extractions on callus.

5.3.2 Gross Summative Analysis of Main Components (Batch 1
and 2 Walls) An Overview of the Major Components of
Callus Walls

Tables 5.3 and 5.4 summarise the gross analytical features
of the two main wall preparations obtained from analysis for
individual neutral sugars, uronic acids, amino acids and
lignin.

For Batch 1 walls there is considerable neutral carbohydrate,
uronic acid and protein removed in all fractions. In both
pathways EDTA removed a significant amount of pectic material
that was not extracted by oxalate. A further substantial
amount was removed by Na_2CO_3 in both pathways.

Table 5.3: Gross Analysis of Cell Wall Fractions: Batch 1
Walls — mg/g 70°C Water Residue

	Neutral Sugar	Uronic Acid	Protein	Lignin
4°C H_2O	7.5	8.0	1.3	3.2
70°C H_2O	14.7	6.4	1.8	7.1
PATH A				
Oxalate	14.7	5.4	1.3	N.O
EDTA	9.5	16.9	1.0	L.A
Na_2CO_3	14.7	14.8	1.2	0.8
0.5% KOH	22.1	9.5	2.4	6.5
10% KOH	66.0	16.1	8.7	8.1
24% KOH	47.9	7.8	24.4	9.1
25% NaOH-BO_3^{3-}	25.0	7.1	10.6	5.2
Residue	80.0	27.1	7.9	3.8
PATH B				
100°C H_2O	88.3	50.8	4.8	9.1
Oxalate	9.5	11.6	1.5	L.A
EDTA	8.4	13.7	1.0	L.A
Urea	6.7	3.5	2.4	0.4
Na_2CO_3	10.3	12.3	1.1	L.A
NaOH-BO_3^{3-}	94.0	14.2	16.0	6.1
Residue	51.5	30.8	12.9	20.0

N.O.: a reading was not obtained

L.A.: a negligible absorbance implied a very low level of
lignin

The principal difference between Paths A and B is in the 100°C water extraction of Path B. This resulted in extraction of much pectic material which would otherwise mostly have appeared in the alkali fractions. Thus the alkali fractions of Path A, contained more uronic acid than the alkali fraction of Path B.

In addition to pectic material, 100°C water extracted much neutral carbohydrate in Path B. The levels of neutral carbohydrate in both the alkali fractions and the final residue were greater in Path A. It is noteworthy that the residues of both paths contained approximately equal amounts of uronic acid.

It is possible that the release of both neutral carbohydrate and uronic acid by water at 100°C was due largely to the β-eliminative cleavage of the polygalacturonic acid, although it could also be due in part to increased solubility of certain poorly soluble polysaccharides (see Chapter 7). It is therefore likely that much, though not all of the neutral carbohydrate in this fraction would have been covalently linked to pectic material in the unextracted wall. The nature of the neutral carbohydrate in this 100°C water fraction has been studied in some detail and is reported in subsequent sections of this thesis.

The retention of substantial amounts of non-cellulosic polysaccharide in the final residue may be due to cross-linking by lignin. The lignin assays were unfortunately subject to considerable error owing to solubility problems (see Section 2.2.5, 5.3.5).

In both paths, protein was somewhat resistant to extraction (Table 5.3). In Path A, about 10% of the total protein was extracted prior to alkali (10% KOH) and about 75% by the alkali sequence. In Path B, the corresponding figures were approximately 30% and 35% respectively. The higher first figure being due partly to the 100°C water treatment, and partly to the urea extraction. It is notable that 6M urea extracted

Table 5.4: Gross Analysis of Cell Wall Fractions: Batch 2
Walls — mg/g 70°C Water Residue

	Neutral Sugar	Uronic Acid	Protein	Hydroxyproline Mole % Protein
BUFFER EXTRACTS				
4°C H_2O	15.7	17.4	29.3	0.7
70°C H_2O	15.4	52.9	2.7	2.0
WALL FRACTIONS				
100°C H_2O	34.3	29.0	15.7	2.9
100°C EDTA	18.8	4.8	1.7	2.9
6M GTC (A$_1$)	24.4	1.3	2.1	3.8
8M Urea (A$_2$)	0.4	0.07	0.9	3.8
8M Urea (B$_1$)	7.9	0.7	2.7	4.7
6M GTC (B$_2$)	11.6	0.8	0.6	4.5
0.1M Na_2CO_3	0.2	0.3	0.3	2.7
8M Urea (C)	0.5	0.3	0.6	4.1
100°C EDTA	3.5	2.4	0.9	3.9
0.5% KOH	6.0	1.5	1.7	4.3
10% KOH	17.5	4.1	4.1	5.0
24% KOH	3.4	1.3	2.4	4.2
25% NaOH-BO_3^{3-}	8.4	2.1	5.0	4.5
ClO_2^-/HOAc	16.0	3.3	15.2	5.7
10% KOH	14.6	5.3	6.1	14.5
Residue	19.3	4.6	1.8	16.3

a higher proportion of the total protein, than of the total carbohydrate.

In Batch 2 walls (Table 5.4) again the content of both neutral carbohydrate and uronic acid was high in the 100°C water fraction but a much greater amount of uronic acid was lost in the 70°C water fraction (in Batch 2 walls). This may have been due to an inherent difference between Batch 1 walls and Batch 2 walls reflecting their different origins or it may have resulted from a change in wall structure brought about by the prolonged buffer washing that had to be used in the wall preparation.

The elevated levels of protein in the water fractions of the Batch 2 preparation would be cytoplasmic in origin.

The use of 6M GTC was shown to cause extraction of much more neutral carbohydrate (subsequently shown to be mostly hemi-cellulosic) than either 6M urea (Batch 1) or 8M urea (Batch 2).

The level of neutral carbohydrate remaining to be extracted by alkali was therefore lower in Batch 2 walls. On the other hand the level of protein extracted by chaotropic reagents in the Batch 2 sequence was only a little higher than that extracted from Batch 1 walls by 6M urea.

Acid chlorite treatment labilised most of the non-cellulosic carbohydrate and nearly all the remaining protein from the alkali residue. The labilised material appeared either in the chlorite extract or in the subsequent alkali extracts. Labilisation of polysaccharide by chlorite supports the possibility of cross-linking by lignin in the untreated wall. A cross-linkage through protein is also conceivable. This would be supported by the observation by Selvendran (151,32) that chlorite labilises most of the alkali-resistant wall protein from walls of a parenchymatous, and presumably non lignified, tissue of bean pods.

The composition of fractions in terms of individual neutral sugars and amino acids, has been examined to determine the nature and type of polysaccharides and protein components of these fractions. Polysaccharides inferred from neutral sugar compositional data were confirmed by methylation studies, Chapter 6. The protein components are described in this chapter.

5.3.3 Sugar Analysis. Batch 1 and Batch 2 Walls Results and Discussion

a) Batch 1 Walls (Tables 5.5, 5.7).

The sugar analysis here on the <u>Pinus radiata</u> callus wall demonstrates the presence of arabinose, xylose, mannose, galactose and glucose as well as the deoxy sugars rhamnose and fucose. Uronic acid is distributed through all the fractions obtained for extractions on Batch 1 walls. Although identification of galacturonic acid was confirmed in only a few fractions, the accompanying presence of rhamnose, and the linkage analysis for rhamnose, galactose and arabinose reported in Chapter 6, suggest that all fractions contained pectin (polygalacturonic acid). It was not completely removed from walls of either batch (see also Table 5.6) by the extractions which classically define the pectic substances ($100^{\circ}C$ H_2O, oxalate and EDTA), but considerable amounts were still being removed by the alkali extracts and high levels remained behind in the residue after all alkali extractions had been completed.

Proportions of different neutral sugars vary between fractions. Arabinose was the most abundant neutral sugar in most fractions. The galactose/arabinose ratios vary greatly and the galactose was greater than the arabinose in the $70^{\circ}C$ H_2O and oxalate fraction of Path A. Galactose and arabinose are typical pectic components but the varying ratio actually reflects the presence of a non-pectic galactan in certain fractions as demonstrated in Chapter 6, similar to the soluble arabinogalactans of the type found in coniferous woods (50).

Table 5.5: Sugar Residues in each Fraction of Green Callus
Batch 1 Walls — mg/g 70°C Water Residue

	Uronic Acid "GalA"	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
4°C H$_2$O	8.01	0.03	0.07	3.24	0.52	0.03	2.98	0.63
70°C H$_2$O	6.44	0.13	0.19	4.05	0.39	0.32	6.55	3.08
PATH A								
Oxalate	5.42	0.06	0.18	3.94	1.20	0.35	4.62	4.39
EDTA	16.94	0.46	0.12	5.37	0.34	0.13	2.11	0.88
Na$_2$CO$_3$	14.77	0.29	0.06	8.69	0.23	0.12	4.21	1.05
0.5% KOH	9.52	0.23	0.12	13.15	0.12	0.06	7.02	1.40
10% KOH	16.05	0.23	2.08	15.44	13.55	1.11	13.22	20.35
24% KOH	7.84	0.18	1.22	16.58	6.29	2.46	12.98	8.19
25% NaOH-BO$_3^{3-}$	7.08	0.18	0.24	12.01	1.20	1.11	8.07	2.22
Residue	27.12	0.81	0.70	43.91	3.94	0.23	7.78	22.63
PATH B								
100°C H$_2$O	50.80	0.38	0.45	52.56	0.75	0.46	26.38	7.34
Oxalate	11.64	0.15	0.08	3.96	0.08	0.46	2.68	2.14
EDTA	13.72	0.23	0.08	4.56	0.08	0.04	2.68	0.77
6M Urea	3.50	0.03	0.08	2.24	1.13	0.08	1.68	1.45
Na$_2$CO$_3$	12.26	0.31	0.03	7.63	0.08	0.03	1.76	0.46
NaOH-BO$_3^{3-}$	14.18	0.83	3.48	18.10	18.54	4.89	20.95	27.22
Residue	30.83	2.35	1.50	25.49	2.77	0.43	13.08	5.89

Table 5.6: Sugar Residues in Each Fraction of Green Callus
Batch 2 Walls - mg/g 70°C Water Residue

	Uronic Acid "GalA"	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
BUFFER EXTRACTS								
4°C H_2O	17.40	0.79	0.30	4.30	0.44	0.67	6.22	2.95
70°C H_2O	52.93	0.60	0.30	8.41	0.29	0.92	3.87	1.00
WALL FRACTIONS								
100°C H_2O	29.00	1.39	0.36	13.27	0.31	0.28	16.53	1.95
100°C EDTA	4.82	1.16	0.28	12.55	0.68	$\delta+$	3.84	0.27
6M GTC (A_1)	1.30	0.49	0.96	5.26	5.58	0.72	5.18	6.17
8M Urea (A_2)	0.07	0.01	0.02	0.17	0.03	0.04	0.08	0.02
8M Urea (B_1)	0.66	0.03	0.15	1.79	1.91	0.39	2.39	1.22
6M GTC (B_2)	0.80	0.20	0.45	1.91	2.55	0.38	2.79	3.37
0.1M Na_2CO_3	0.29	0.01	0.01	0.07	0.01	$\delta+$	0.05	0.01
8M Urea (C)	0.33	0.01	0.01	0.26	0.02	0.02	0.17	0.04
100°C EDTA	2.43	0.16	0.08	1.75	0.17	0.10	1.24	0.05
0.5% KOH	1.52	0.13	0.09	2.75	0.60	0.13	1.51	0.76
10% KOH	4.10	0.40	0.64	3.51	3.03	1.41	2.11	6.45
24% KOH	1.34	0.10	0.06	1.43	0.22	0.23	0.84	0.52
25% NaOH-BO_3^{3-}	2.10	0.37	0.14	2.83	0.72	1.16	1.87	1.28
Residue (1)	10.20	1.42	0.96	21.76	3.22	0.86	5.80	6.06
ClO_2^-/HOAc	3.26	0.40	0.26	11.20	0.70	0.22	3.02	0.24
10% KOH	5.3	0.52	0.34	8.12	0.96	0.24	3.74	0.56
Residue (2)	4.56	0.64	0.50	8.04	0.78	0.52	5.40	3.40

Table 5.7: Individual Sugar Residues in Each Fraction of Green Callus – Batch 1 Walls: μ moles/g 70°C Water Residue

	Uronic Acid "GalA"	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Total Neutral
4°C H_2O	45.5	0.2	0.5	24.5	3.9	0.2	18.4	3.9	51.6
70°C H_2O	36.5	0.9	1.3	30.6	3.0	2.0	40.4	19.0	97.2
PATH A									
Oxalate (75°C)	30.7	0.4	1.2	29.9	9.1	2.2	28.5	27.0	98.2
EDTA (70°C)	96.2	3.2	0.8	40.7	2.6	0.8	13.0	5.4	66.4
Na_2CO_3 (4°C)	83.9	2.0	0.4	65.8	1.7	0.7	26.0	6.5	103.1
0.5% KOH	54.0	1.6	0.8	99.5	0.9	0.4	43.3	8.7	155.1
10% KOH	91.1	1.6	14.2	116.8	102.6	6.9	81.5	125.5	449.1
24% KOH	44.5	1.2	8.3	125.5	47.6	15.2	80.1	50.5	328.3
25% $NaOH-BO_3^{3-}$	40.2	1.2	1.6	90.9	9.1	6.9	49.8	13.7	173.1
Residue	154.0	5.6	4.8	332.3	29.9	1.4	48.0	139.6	561.4
PATH B									
100°C H_2O	288.4	2.6	3.1	397.8	5.7	2.8	162.7	45.3	619.9
Oxalate (70-75°C)	66.1	1.1	0.5	30.0	0.6	2.8	16.5	13.2	64.6
EDTA (70-73°C)	77.9	1.6	0.5	34.5	0.6	0.3	16.5	4.7	58.7
6M Urea	19.9	0.2	0.5	17.0	8.6	0.5	10.4	9.0	46.1
Na_2CO_3 (4°C)	69.6	2.1	0.2	57.7	0.6	0.2	10.9	2.8	74.5
$NaOH-BO_3^{3-}$	80.5	5.7	23.8	137.0	140.3	30.2	129.1	167.9	633.9
Residue	175.0	16.0	10.3	192.9	21.0	2.6	80.6	36.3	359.8
Total Path A	594.6	16.7	32.1	901.3	203.4	34.3	370.0	376.8	1934.7
Total Path B	777.4	29.3	39.0	867.0	177.3	39.3	426.7	280.0	1857.5
Ave Totals	686.0	23.0	35.6	884.2	190.4	36.8	398.4	328.4	1896.1
(inc 4°,70°C H_2O)	768.0	24.1	37.4	939.3	197.3	39.0	457.2	351.3	2044.9

Table 5.8: Individual Sugar Residues in Each Fraction of Green Callus ⁻ Batch 2 Walls: µ moles/g 70°C Water Residue

	Uronic Acid "GalA"	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Total Neutral per path
4°C H_2O	98.8	5.4	2.1	32.6	3.3	4.1	38.3	18.2	103.9
70°C H_2O	300.5	4.1	2.0	63.6	2.2	5.7	23.9	6.2	107.7
WALL FRACTIONS									
100°C H_2O	164.6	9.5	2.5	100.4	2.4	1.7	102.0	12.1	230.5
100°C EDTA	27.4	7.9	1.9	95.0	5.1	δ+	23.7	1.7	135.2
6M GTC (A_1)	7.4	3.4	6.5	39.8	42.2	4.5	31.9	38.0	83.2 ⌉*
8M Urea (A_2)	0.4	0.1	0.1	1.3	0.2	0.3	0.5	0.2	1.3
8M Urea (B_1)	3.7	0.2	1.0	13.6	14.5	2.4	14.7	7.5	27.0
6M GTC (B_2)	4.5	1.4	3.1	14.5	19.3	2.4	17.2	20.8	39.2 ⌋
0.1M Na_2CO_3	1.7	0.1	0.1	0.5	0.1	δ+	0.3	0.1	1.3
8M Urea (C)	1.9	0.1	0.1	2.0	0.2	0.1	1.0	0.3	3.7
100° EDTA	13.8	1.1	0.5	13.3	1.3	0.6	7.6	0.3	24.7
0.5% KOH	8.6	0.9	0.6	20.8	4.5	0.8	9.3	4.7	41.7
10% KOH	23.3	2.7	4.4	26.5	22.9	8.7	13.0	39.8	118.1
24% KOH	7.6	0.7	0.4	10.9	1.7	1.4	5.2	3.2	23.4
25% NaOH-BO_3^{3-}	11.9	2.5	1.0	21.4	5.4	7.2	11.6	7.9	56.9
Residue (1)	57.9	9.6	6.6	164.7	24.4	5.3	35.8	37.4	141.9 ⌉*
ClO_2^-/HOAc	18.5	2.7	1.8	84.8	5.3	1.4	18.6	1.5	58.1
10% KOH	30.1	3.6	2.3	61.4	7.3	1.5	23.1	3.5	51.3
Residue (2)	25.9	4.4	3.4	60.8	5.9	3.2	33.3	21.0	66.0 ⌋
Totals from 70°C H_2O Residue	343.3	38.8	24.4	532.4	100.2	31.4	280.9	129.4	1137.0 *
Totals (inc 4° + 70°C H_2O)	742.6	48.3	28.5	628.6	105.7	41.2	343.1	153.8	1348.6 *

For Column Totals, "GalA"-Glc, values for chaotropic reagents $(A_1 - B_2)$ were halved (to give the total actually removed over this divergent part of the scheme) and, after the NaOH-BO_3^{3-} extraction, the values from the chlorite extracted path were used (i.e., Residue (1) values are not used).

]* Half values here, show the actual sugar levels removed where the 50/50 divisions by weight occur in the scheme. * The chlorite extracted path values were doubled to derive totals.

Glucose and xylose residues were high in alkali fractions and in Path B the alkali fraction has both glucose and xylose levels greater than arabinose. This reflects the hemicellulose content of the alkali fractions. The glucose level was somewhat higher in all fractions prior to the alkali fractionations. For the alkali fractions, xylose and glucose levels were approximately equal. The presence of these two sugars probably reflects the removal of xyloglucan of the type observed by J.W.L. Little (145) in _Pinus radiata_ seedling hypocotyls. It is demonstrated in Chapter 6 that both xylan and xyloglucan were present.

The presence of glucose and xylose in the 4^OC water fraction may indicate a xyloglucan present. Starch should not be significantly solubilised at 4^OC and xyloglucans extracted with cold water have been reported before in the literature (46). The glucose levels in hot aqueous extracts (oxalate, EDTA, and 100^OC water) may be due to residual starch solubilisation, and the glucose is higher than that expected for xyloglucan. These extracts gave positive results to the I_2/KI starch assay. The presence of starch was confirmed by methylation analysis (93, 95) in Chapter 6. Methylation analyses also confirm the presence of linkages characteristic of xyloglucan rather than starch in the cold water extracts (section 6.2).

Arabinose, galactose and rhamnose to some extent parallel uronic acid in their distribution and were present in all fractions. They are especially high in water fractions and in the residue. The possibility exists for both pectic type galactans and pectic arabinans to be present, of the type found in sycamore (47), rapeseed (48) and coniferous tissue (127, 137, 138, 139, 140).

The rhamnose/uronic acid ratio is low in water fractions ($\sim$ 1:100 or less) and this implies the presence of predominantly uninterrupted homogalacturonan backbones. The methylation results of Chapter 6 suggest that galacturonic acid is relatively unbranched, in components liberated by

water fractions (hot H_2O, oxalate, EDTA) by β-elimination and Ca^{2+} chelation.

The rhamnose/GalA ratio is somewhat lower in the hot aqueous extracts than noted for pectins observed by Albersheim (47) Ishii (206).

The rhamnose/uronic acid ratio is higher in later fractions becoming more comparable with, or slightly higher than some other pectins in the literature (8). The branching region of pectin is more resistant to extraction, and may involve covalent links including lignin cross linking. The high rhamnose in the residue is also noted in this regard.

Although most uronic acid in the water fractions has very little associated rhamnose, the galactose and arabinose polymers of these fractions may still be pectic fragments as discussed in Chapter 7.

The observation that galacturonan and rhamnogalacturonan appear to be widely distributed between wall fractions, as do arabinose and galactose and lower amounts of other sugars might be explained if these were bonded to another structural component not yet extracted. Lignin might be responsible for binding residual pectic substances (and lower amounts of hemicelluloses) in the cell wall (6, 7, 37, 161).

Mannose in the water and alkali fractions is indicative of the presence of some water soluble galactoglucomannan, and some alkali soluble glucomannan, the latter being more resistant to extraction than xylan or xyloglucan, since it requires 24% KOH for maximum extraction compared to 10% KOH for xylose and glucose. Some further mannan is removed on addition of borate which complexes with mannose. The level of glucomannan in this tissue is apparently much lower than that of xyloglucan.

The levels of fucose are very low throughout the fractions here except for the strong alkali fractions (10% and 24% KOH

Path A, and 25% NaOH-BO$_3^{3-}$ Path B), and there is some in the cold water fractions. This suggests that the xyloglucan indicated by the xylose and glucose in these fractions is a fucogalactoxyloglucan. This is discussed further in Chapter 6, where fucose is identified as terminal fucose, possibly associated with xyloglucan as for seed amyloids (46, 48).

The presence of fucose in the residue is not explained, but this may be present in a fucose-rich xyloglucan (Table 5.5, 5.7). Such an observation also applies to Batch 2 walls, particularly the chlorite extract and the final residue (Table 5.6, 5.8).

b)   Batch 2 Walls (Tables 5.6 and 5.8)

The data generally bear out the results from sugar analyses on Batch 1 walls. Significant arabinose and galactose in the phosphate buffer extracts (4°C and 70°C) may again indicate the presence of water soluble arabinogalactan (50). The lower levels of arabinose in these walls than in those of Batch 1 could be mainly due to lower levels of 100°C water-extractable arabinose.

The increased level of arabinose in the 70°C water fraction could be due to some pectic arabinan removed at the higher temperature. Arabinose and galactose are prominent sugars in the 100°C water extraction, probably in part signifying pectic arabinan and galactan removed by β-elimination. There is high arabinose in the 100°C EDTA fraction and this is likely to be pectic arabinan.

Uronic acid is more readily extracted by 70°C H$_2$O than for Batch 1. Therefore there is a lower level of uronic acid in the 70°C water residue of batch 2. The higher rhamnose levels in the water fractions than for the corresponding fractions in Batch 1 partly parallels the increased uronic acid in these fractions, but some fractions have a very high rhamnose/ uronic acid ratio (4°C water and 100°C EDTA).

Only low levels of xylose and glucose (or for Batch 1) are removed in the $100^{O}C$ water and $100^{O}C$ EDTA fractions, which appear to remove predominantly the pectic arabinan, galactan, rhamnogalacturonan and perhaps some non-pectic arabinogalactan.

Extraction with 6M GTC and 8M urea after degradation of pectin by $100^{O}C$ water and EDTA enabled an investigation of the chaotropic removal of polymers. Such removal is subsequent to some degradation of pectin, but before affecting further bonding with weak or strong alkali treatment.

8M urea and 6M GTC extract both hemicelluloses and arabinose-galactose polymers. Removal of approximately equal parts of xylose and glucose in the GTC and urea fractions suggests the presence of a xyloglucan, a trend similarly observed for the alkali-extracted fractions of Batch 1 walls.

The highest levels of hemicellulose were observed for the 6M GTC fraction, this being the strongest chaotropic reagent. The ratio of xylose/glucose is high in the 8M urea extract which implies possibly the predominance of a xylan rather than a xyloglucan. The xylose level is lower in the 6M GTC, where probably more efficient extraction of both xylan and xyloglucan has occurred. Levels of xyloglucan then fall until the alkali extractions are commenced. Highest levels are removed by 10% KOH, at room temperature and by 25% NaOH-borate which also removes some mannan. The xylose/glucose ratio is higher in GTC(A_1) than in 10% KOH. The amount of xylose in the two fractions is about equal, but glucose is twice as high in the 10% KOH fraction. In conjunction with results from methylation analysis (Chapter 6) it is confirmed that xylan is approx. 40% extracted by GTC, and xyloglucan approx. 30%. This contrasts with the observation of Monro et al (170) who observed that GTC extracted 90% of the xyloglucan from depectinated lupin hypocotyl walls, but very little xylan.

Albersheim (46) also noted that xyloglucan was extracted by 8M urea from depectinated walls of sycamore suspension cultured cells.

Mannose is again rather resistant to extraction as for Batch 1 walls. There are high mannose values in the 25% NaOH-borate fraction and in the final residue, but a lower value in the 24% KOH fraction than in the 10% KOH fraction is surprising. Mannose appears to reach a peak in the 24% KOH fraction, Path A of Batch 1 walls. It is possible that the 24% KOH extraction conditions were not as vigorous as in Batch 1.

The sodium carbonate extraction labilised some pectic material, much of which (arabinose, galactose, uronic acid and low rhamnose) was extracted by subsequent 8M urea and 100°C EDTA.

The chlorite treatment of the alkali residue, and subsequent 10% KOH extraction removes arabinose, galactose, rhamnose and uronic acid in both fractions at relative levels, showing little variation except in the arabinose/galactose ratio which implies the co-extraction of arabinan, galactan and rhamnogalacturonan as a pectic complex. But extraction is still not complete, and pectic material remains in the residue (Table 5.6, 5.8).

Both xylose and glucose are present in the NaOH-borate residue, although xylose is mostly extracted by chlorite and alkali whereas glucose is not. The high xylose/glucose ratio in chlorite and subsequent alkali fraction implies that some xylan may be cross linked by lignin and is released after ClO_2^- treatment.

The glucose in the final residue probably represents some xyloglucan, and cellulose which is not extracted by chlorite and alkali but may be sufficiently disordered by prior alkali treatments to be hydrolysed by nitric acid.

Thus it seems likely that some xylan (approx 25-30% of the total see Table 5.10, and Table 6.3) is lignin bound, but xyloglucan largely may not be.

Lignin-xylan complexes have been reported in the literature (161, 162).

Xyloglucan is approximately 90% extracted in prechlorite fractions, and about 5% is present in ClO_2^- and subsequent KOH fractions assuming the glucose is from a xyloglucan. Thus only 5-10% is lignin bound.

Conclusions

1) Xyloglucan is indicated in water fractions (4^oC water) some in GTC, 10% KOH, and lower amounts in stronger alkali extractions. Xyloglucan that can be removed by chaotropic reagents after cleavage of polygalacturonic acid is probably linked directly or indirectly to pectin and by hydrogen bonds to other polymers, such as cellulose. This would agree with Albersheim's model (8). However, the additional xyloglucan requiring alkali for removal (before the chlorite extract) as well as linkage to pectin and hydrogen bonding to cellulose may have links to other polymers such as lignin, the linkage (possibly esters (162)) breaking down under more rigorous alkali treatment. There appears to be very little xyloglucan in the alkali residue, at least, little extracted by chlorite and alkali. The presence of fucose in the final residue is not well understood, but could be due to a fucose-rich xlyoglucan.

2) Xylan possibly occurs to some extent in water fractions. The 100^oC EDTA fraction has a high xylose/glucose ratio, and extraction with 8M urea and 6M GTC reveals that xylan is more labile than xyloglucan. However, some xylan appears to be present in alkali fractions. That which is present in chlorite labilised fractions would appear to be lignin bound.

3) Mannans are seen to be extracted by water and alkali, which implies the presence of water-soluble glucogalacto-mannans and alkali-soluble glucomannans respectively. The glucomannans are more resistant to extraction than other hemicelluloses.
Mannans are not an important hemicellulose of these walls from the results of fractionations of Batch 1 and 2 walls.

Low mannan levels throughout, indicate the low levels of gluco or galactoglucomannans in these walls. The presence of mannose, together with partial lignin formation, suggests a low level of secondary thickening in the walls as mannans are usually present only in secondary cell walls (3, 27). J.W.L. Little observed an increase in the percentage of mannose with increasing age of hypocotyl tissue in Pinus radiata etiolated hypocotyls (145).

4) Pectin. The components of pectin are observed throughout the wall fractions. In the water fractions, low rhamnose implies a pectin with a high homogalacturonan backbone. The higher extraction of uronic acids in the water extracts of Batch 2 may be due to a different method of preparation of wall and, in part, to differences in sample material. In subsequent extracts (EDTA onwards) the rhamnose/galacturonic acid ratios vary between 1/10 to 1/30, suggesting a pectin interspersed with rhamnose (47, 206). The rhamnose/uronic ratio is very high for the first EDTA fraction in Batch 2.

Highest levels of the pectic polymers are removed by conditions which β-eliminate and degrade pectin. Substantial amounts of uronic acid were removed by strong alkali even after extraction by hot water, boiling EDTA and with chaotropic reagents. There seems no obvious reason why pectin should not be extracted by $100^{\circ}C$ EDTA treatment, unless it is linked to some other polymer in the wall. Pectin extracted by chaotropic reagents after the $100^{\circ}C$ EDTA treatment might be covalently linked to hemicelluloses. The same might apply to pectin subsequently extracted by alkali. The difference in extractability of the latter two pectin fractions would be due to a difference in the bonding of hemicellulose of the two fractions in the wall.

After extraction with 25% NaOH-borate, about 20% of the pectic components still remain locked in the residue. Acid chlorite liberates much of the remaining arabinose

Table 5.9: Polysaccharide Sugar Residues, Protein and Hydroxyproline, Batch 1 Walls
% of Totals Accounted for in 70°C Water Residue

	GalA	Gal	Ara	Rha	Glc	Xyl	Fuc	Man	Protein	Hydroxyproline
$4° + 70°C\ H_2O$	12.2	14.9	6.2	5.0	7.0	3.6	5.1	5.9	6.3	4.6
PATH A										
Oxalate & EDTA	21.3	11.2	7.8	21.2	8.6	5.8	6.2	8.5	3.9	1.7
Na_2CO_3	14.1	7.0	7.3	12.0	1.7	0.8	1.2	2.1	2.1	0.4
Alkali Extracts (KOH)	31.9	55.4	37.9	26.3	49.0	74.4	72.9	65.2	61.8	46.2
Alkali & Borate	6.7	13.5	10.1	7.2	3.6	4.5	5.0	20.0	18.5	15.7
Residue	25.9	13.0	36.9	33.3	37.1	14.7	14.9	4.2	13.7	35.9
PATH B										
100°C Water	37.0	38.1	45.9	8.9	16.2	3.2	7.9	7.2	12.1	7.9
Oxalate & EDTA	18.5	7.7	7.4	8.9	6.4	0.6	2.7	7.9	6.3	3.3
6M Urea	2.6	2.4	2.0	0.8	3.2	4.8	1.3	1.2	6.1	1.7
Na_2CO_3	9.0	2.5	6.7	7.1	1.0	0.3	0.6	0.4	2.7	2.0
Alkali & Borate	10.4	30.3	15.8	19.5	60.0	79.2	61.1	76.8	40.3	22.3
Residue	22.5	18.9	22.3	54.7	13.0	11.9	26.4	6.7	32.5	62.8

Table 5.10: Polysaccharide Sugar Residues; Protein and Hydroxyproline. Batch 2 Walls
           % of Totals Accounted for in 70°C Water Residue

	GalA	Gal	Ara	Rha	Glc	Xyl	Fuc	Man	Protein	Hydroxyproline
4° and 70°C H_2O	120.0	23.0	18.1	24.5	19.0	6.0	16.7	32.0	57.0	8.1
H_2O & EDTA 100°C	58.0	44.7	36.7	45.1	10.6	7.5	17.9	5.5	29.7	15.1
Chaotropic Reagents	2.4	11.5	6.5	6.5	25.6	38.3	22.0	15.2	5.4	4.1
Na_2CO_3 & Labilised	5.0	3.0	3.0	3.1	0.5	1.6	2.8	2.2	3.0	2.0
Alkali Extracted	15.0	14.0	14.9	17.6	43.0	34.7	26.2	57.6	23.0	18.2
ClO_2^- & Labilised	13.0	15.0	27.5	16.3	4.4	12.6	16.8	9.0	36.0	53.0
Residue	7.0	12.0	11.4	11.4	16.0	5.3	14.1	10.3	3.0	7.7

on degrading lignin, and further arabinose, galactose and galacturonic acid is released with 10% KOH. It is concluded that partial lignification of these walls is responsible for cross-linking with some of the pectin, as well as neutral pectic polymers, hemicelluloses and possibly protein.

5)   The non-pectic arabinogalactan, (see methylation analysis Chapter 6) is tentatively assigned as an integral component in these walls.

6)   Ester links to lignin (Labile to Na_2CO_3), might enable extraction of some polymers by sodium carbonate as seen here. Linkage of polysaccharides to lignin by ether bonds (161, 162) might render polymers unextractable under any of the conditions used here (except by acid chlorite) and would explain the occurrence of sugars in the residues after all alkali fractionations. It is possible that alkali may cleave some bonds to lignin (possibly ether bonds, see Section 5.3.5 regarding the possibility of alkali cleavage of ether bonds), and that hemicelluloses not released by GTC, urea, or Na_2CO_3 are then released with alkali.

However much carbohydrate may be linked by alkali resistant ether bonds, and it is suggested that the partial lignification is important in the interpretation of the results obtained on Batch 2 walls.

5.3.4   Amino Acid Analysis;   Batch 1 and Batch 2 Walls Results and Discussion

As discussed in Section 1.3.7, protein containing hydroxyproline has long been regarded as a cell wall component of land plants. In the primary cell wall it appears to be important structurally in cross-linking polysaccharides in some manner (28). Results for amino acid analyses are summarised in Tables 5.11 - 5.14. The distribution of total protein and hydroxyproline among fractions are detailed in Table 5.9, 5.10.

Table 5.11: Individual Amino Acid Residues in each Fraction of Batch 1 Walls as Mole % of Total Protein

Fraction	Aspartic Acid	Threonine	Serine	Glutamic Acid	Proline	Glycine	Alanine	Half Cystine	Valine	Methionine	Isoleucine	Leucine	Tyrosine	Phenylalanine	Lysine	Histidine	Arginine	Hydroxyproline
$4^{\circ}C$ H_2O	7.8	4.4	6.7	9.2	5.2	12.2	6.0	2.5	5.7	0.9	3.2	5.6	2.0	2.9	5.4	3.6	15.0	1.8
$70^{\circ}C$ H_2O	8.0	4.1	7.1	10.8	6.4	16.0	6.7	2.2	5.0	0.5	3.6	5.6	2.4	2.7	4.7	2.1	9.9	2.0
PATH A																		
Oxalate	7.5	4.4	13.3	14.0	3.6	20.3	7.5	0.4	3.8	0.5	2.9	5.0	2.9	2.6	3.4	2.8	2.7	2.4
EDTA	9.3	3.6	7.9	10.7	4.0	19.8	6.8	2.4	4.5	0.7	3.7	6.0	2.3	3.1	2.4	1.2	10.8	1.1
Na_2CO_3	8.2	4.9	7.5	9.6	5.4	18.1	6.5	1.4	5.9	0.5	3.8	7.6	3.2	4.6	2.0	0.7	9.2	1.0
0.5% KOH	11.8	6.0	8.4	10.9	6.1	16.3	7.8	1.8	5.2	0.4	3.1	5.7	2.2	3.0	1.4	1.3	5.8	2.7
10% KOH	12.2	5.2	6.7	12.2	3.9	9.5	9.0	0.7	6.8	0.8	4.9	7.8	3.7	4.4	1.5	2.1	4.8	3.7
24% KOH	11.1	4.7	5.3	12.9	6.2	6.9	9.0	0.1	8.0	1.0	5.3	8.7	4.1	4.5	2.1	3.5	3.1	3.2
25% NaOH-BO_3^{3-}	10.0	4.4	5.3	11.9	6.7	6.2	8.8	0.2	8.5	0.6	5.7	8.8	3.8	4.8	2.6	3.4	4.4	3.9
Residue	6.6	3.8	4.6	6.3	12.7	5.0	6.0	0.5	11.7	0.7	5.9	5.4	4.5	2.9	3.9	3.5	4.0	11.8
PATH B																		
$100^{\circ}C$ H_2O	9.4	4.0	7.0	10.2	6.6	17.3	7.4	1.3	6.6	<0.1	3.6	5.4	2.9	4.1	4.1	1.4	4.9	3.6
Oxalate	8.2	3.5	10.9	11.2	4.7	14.6	6.5	2.1	5.9	0.6	2.9	5.3	2.8	3.3	4.5	1.5	9.2	2.3
EDTA	6.6	4.1	10.9	11.4	1.4	16.0	5.6	2.5	4.9	0.1	3.4	6.1	3.1	3.3	3.5	2.5	11.0	3.6
Urea	8.5	4.6	11.9	14.4	4.1	15.3	8.3	0.7	5.4	0.6	3.4	6.1	2.5	3.3	3.0	1.2	5.2	1.4
Na_2CO_3	8.7	3.7	9.1	12.5	4.5	15.1	6.4	2.9	5.3	0.8	3.2	4.9	2.6	4.4	1.9	1.7	8.4	3.9
NaOH-BO_3^{3-}	14.4	4.6	6.3	13.0	4.9	6.7	8.3	0.5	7.5	1.1	5.4	8.4	3.3	4.1	2.3	1.7	4.5	2.9
Residue	8.3	4.6	5.3	10.6	9.1	5.7	8.6	1.7	8.5	1.0	4.5	7.6	3.2	3.0	3.2	1.5	3.5	10.1

Table 5.12: Individual Amino Acid Residues in Each Fraction of Batch 2 Walls as Mole % of Total Protein

	ASPARTIC ACID	THREONINE	SERINE	GLUTAMIC ACID	PROLINE	GLYCINE	ALANINE	HALF CYSTINE	VALINE	METHIONINE	ISOLEUCINE	LEUCINE	*TYROSINE	PHENYLALANINE	LYSINE	HISTIDINE	*ARGININE	HYDROXYPROLINE
4°C H₂O	10.0	4.9	17.2	13.1	6.1	13.9	7.9	1.9	5.4	1.1	3.0	6.5	3.2	3.4	3.6	1.8	6.2	0.7
70°C H₂O	12.8	5.3	7.6	14.5	6.1	12.9	9.8	0.3	5.7	0.8	3.5	5.6	3.3	2.5	3.5	1.6	2.1	2.0
100°C H₂O	9.7	5.0	6.9	10.0	6.7	9.9	8.8	0.6	6.6	1.4	4.3	8.4	3.3	4.1	5.0	2.4	4.1	2.9
100°C EDTA	9.3	5.4	8.2	10.4	7.4	9.7	9.5	0.6	6.2	1.4	4.4	7.9	3.6	4.2	3.6	2.0	3.3	2.9
6M GTC (A₁)	9.7	5.2	7.3	11.5	7.7	10.4	9.0	0.8	6.9	0.9	4.3	8.3	3.0	3.9	2.3	1.3	3.8	3.8
8M Urea (A₂)	8.2	5.4	8.1	10.8	6.8	10.2	9.6	0.8	6.9	1.6	4.5	8.5	3.3	3.5	2.6	1.4	3.9	3.8
8M Urea (B₁)	10.3	4.9	7.3	10.1	6.8	8.3	9.4	0.8	6.8	1.5	4.5	8.7	3.5	3.9	2.3	1.9	4.3	4.7
6M GTC (B₂)	10.3	5.1	8.5	11.1	6.5	10.3	9.3	0.3	6.2	1.4	4.1	7.3	3.4	3.8	2.5	1.7	3.8	4.5
0.1M Na₂CO₃	8.4	4.7	11.2	11.2	5.7	10.9	9.3	1.5	7.4	1.0	2.8	6.8	3.4	3.7	3.0	3.0	3.5	2.7
8M Urea (C)	9.1	5.2	6.4	10.4	7.7	9.6	9.6	0.7	6.8	1.5	4.6	7.7	3.9	4.3	2.5	1.8	4.1	4.1
100°C EDTA	9.8	5.6	7.9	10.3	6.3	9.5	9.3	0.8	6.1	1.2	4.1	8.0	3.7	4.3	2.8	2.2	4.1	3.9
0.5% KOH	8.5	6.2	7.8	11.1	6.8	9.6	8.9	0.4	10.1	1.3	3.9	8.0	2.9	3.9	1.9	1.2	3.0	4.3
10% KOH	11.1	5.0	8.3	12.4	5.8	7.3	9.4	0.5	6.6	1.0	4.5	8.5	4.1	3.9	1.6	2.0	3.1	5.0
24% KOH	9.8	4.6	7.3	11.0	6.7	8.7	10.8	0.7	7.1	1.2	4.4	8.8	3.2	4.2	2.4	1.5	3.4	4.2
25% NaOH-BO₃³⁻	10.9	4.3	6.8	13.0	7.1	6.3	9.2	0.8	7.4	0.4	4.8	9.1	3.5	4.6	2.6	1.5	3.3	4.5
*ClO₂⁻/HOAc	10.3	4.1	4.2	10.8	9.7	4.3	9.2	0.7	9.7	2.0	5.7	9.2	0.5	4.1	7.1	2.4	3.1	5.7
*10% KOH	7.3	3.5	5.4	7.2	15.3	4.4	5.6	0.8	11.7	1.0	4.9	5.7	1.0	2.6	5.8	2.9	1.9	14.5
*Residue(2)	6.7	3.8	5.9	5.2	16.3	4.0	5.8	0.7	11.8	0.8	5.4	4.7	2.1	2.4	6.5	2.6	1.9	14.5
Residue(1)	9.6	3.3	4.4	9.2	10.7	4.8	8.7	1.0	8.6	1.2	4.8	8.2	3.2	3.7	2.8	2.1	4.3	9.5

* ½ Cystine = cysteic acid, Methionine = methionine sulphone estimated

Tyrosine partly destroyed after oxidation by chlorite

Arginine, possibly converted to ornithine by chlorite treatment

Lysine totals include α·amino-adipic acid from lysine and may include ornithine which co-chromatographs with lysine

Table 5.13: Individual Amino Acid Residues in Each Fraction of Batch 1 Walls, as μ moles/g 70°C Water Residue

	Aspartic Acid	Threonine	Serine	Glutamic Acid	Proline	Glycine	Alanine	Half Cystine	Valine	Methionine	Isoleucine	Leucine	Tyrosine	Phenylalanine	Lysine	Histidine	Arginine	Hydroxyproline
4°C H_2O	0.89	0.50	0.77	1.05	0.60	1.39	0.70	0.28	0.64	0.10	0.37	0.65	0.23	0.33	0.62	0.42	1.72	0.20
70°C H_2O	1.36	0.69	1.22	1.84	1.09	2.73	1.14	0.38	0.86	0.08	0.61	0.97	0.40	0.47	0.79	0.36	1.69	0.34
PATH A																		
Oxalate	1.00	0.58	1.76	1.86	0.47	2.69	0.99	0.06	0.50	0.06	0.39	0.66	0.39	0.34	0.46	0.37	0.36	0.31
EDTA	0.85	0.33	0.72	0.97	0.37	1.81	0.62	0.22	0.41	0.06	0.34	0.55	0.21	0.28	0.22	0.11	0.98	0.10
Na_2CO_3	0.95	0.56	0.87	1.11	0.63	2.10	0.75	0.16	0.69	0.06	0.44	0.88	0.38	0.53	0.24	0.09	1.07	0.10
0.5% KOH	2.79	1.42	1.99	2.57	1.45	3.85	1.84	0.42	1.24	0.10	0.74	1.36	0.52	0.73	0.33	0.31	1.39	0.64
10% KOH	12.77	5.39	7.02	12.79	4.04	9.88	9.42	0.66	7.14	0.86	5.15	8.21	3.81	4.63	1.61	2.17	4.99	3.90
24% KOH	22.12	9.42	10.53	25.64	12.23	13.75	17.79	0.27	15.82	2.08	10.69	17.28	8.25	8.94	4.28	7.00	6.20	6.39
25% NaOH-BO_3^{3-}	9.58	4.26	5.13	11.46	6.42	5.96	8.44	0.26	8.14	0.53	5.43	8.50	3.66	4.59	2.51	3.23	4.27	3.71
Residue	4.73	2.69	3.34	4.52	9.12	3.62	4.36	0.32	8.44	0.50	4.23	3.93	3.23	2.06	2.84	2.49	2.86	8.50
PATH B																		
100°C H_2O	3.87	1.66	2.90	4.24	2.73	7.17	3.08	0.52	2.75	0.02	1.50	2.25	1.19	1.70	1.69	0.60	2.03	1.51
Oxalate	1.16	0.50	1.56	1.60	0.67	2.08	0.93	0.30	0.84	0.09	0.41	0.75	0.40	0.47	0.65	0.21	1.31	0.32
EDTA	0.59	0.37	0.98	1.02	0.12	1.44	0.50	0.23	0.44	0.01	0.31	0.55	0.28	0.30	0.31	0.23	0.99	0.32
Urea	1.97	1.06	2.75	3.32	0.96	3.54	1.92	0.16	1.25	0.14	0.78	1.41	0.59	0.77	0.69	0.28	1.21	0.32
Na_2CO_3	0.89	0.37	0.92	1.26	0.45	1.53	0.64	0.29	0.53	0.08	0.32	0.49	0.27	0.44	0.19	0.17	0.85	0.39
NaOH-BO_3^{3-}	20.94	6.69	9.10	18.92	7.15	9.25	12.12	0.77	10.87	1.66	7.86	12.18	4.74	6.00	3.37	2.45	6.56	4.28
Residue	9.90	5.43	6.27	12.58	10.80	6.78	10.15	2.03	10.11	1.20	5.29	8.99	3.84	3.59	3.77	1.74	4.13	12.03

Table 5.14: Individual Amino Acid Residues in Each Fraction of Batch 2 Walls as μ moles/g 70° Water Residue

	Aspartic Acid	Threonine	Serine	Glutamic Acid	Proline	Glycine	Alanine	*Half Cystine	Valine	*Methionine	Isoleucine	Leucine	*Tyrosine	Phenylalanine	*Lysine	Histidine	*Arginine	Hydroxyproline
4°C H_2O	27.54	13.51	19.71	35.97	16.87	38.35	21.65	5.33	14.94	3.07	8.22	18.00	8.90	9.24	10.02	4.84	17.00	2.01
70°C H_2O	3.26	1.35	1.94	3.71	1.55	3.30	2.50	0.88	1.45	0.21	0.90	1.42	0.85	0.64	0.90	0.41	0.55	0.51
100°C H_2O	14.12	7.33	9.98	14.57	9.75	14.46	12.81	0.84	9.60	2.03	6.27	12.19	4.75	5.95	7.34	3.49	5.96	4.19
100°C EDTA	1.49	0.86	1.32	1.66	1.18	1.55	1.53	0.09	0.98	0.22	0.71	1.27	0.58	0.67	0.57	0.31	0.52	0.47
6M GTC (A$_1$)	1.89	1.02	1.43	2.23	1.49	2.02	1.75	0.15	1.35	0.17	0.84	1.62	0.58	0.75	0.44	0.25	0.74	0.74
8M Urea (A$_2$)	0.69	0.45	0.68	0.90	0.57	0.84	0.80	0.07	0.57	0.14	0.37	0.71	0.27	0.29	0.22	0.12	0.33	0.32
8M Urea (B$_1$)	2.60	1.33	1.85	2.56	1.72	2.10	2.37	0.21	1.71	0.37	1.14	2.20	0.89	0.99	0.58	0.49	1.08	1.20
6M GTC (B$_2$)	0.60	0.29	0.49	0.65	0.37	0.60	0.54	0.02	0.36	0.08	0.24	0.42	0.20	0.22	0.14	0.10	0.22	0.26
0.1M Na$_2$CO$_3$	0.23	0.13	0.30	0.30	0.16	0.29	0.25	0.04	0.20	0.03	0.08	0.18	0.09	0.10	0.08	0.08	0.10	0.07
8M Urea (C)	0.54	0.30	0.37	0.61	0.45	0.57	0.57	0.04	0.40	0.09	0.27	0.45	0.23	0.25	0.15	0.10	0.24	0.24
100°C EDTA	0.78	0.45	0.64	0.83	0.51	0.76	0.75	0.07	0.49	0.10	0.33	0.65	0.30	0.35	0.22	0.18	0.33	0.31
0.5% KOH	1.40	1.02	1.31	1.82	1.12	1.57	1.46	0.07	1.67	0.21	0.64	1.31	0.48	0.63	0.31	0.19	0.50	0.71
10% KOH	4.23	1.91	3.15	4.72	2.20	2.80	3.58	0.19	2.53	0.37	1.72	3.24	1.55	1.47	0.61	0.76	1.19	1.90
24% KOH	2.22	1.06	1.67	2.50	1.51	1.98	2.47	0.15	1.62	0.28	1.00	2.00	0.72	0.95	0.56	0.35	0.78	0.95
25% NaOH-BO$_3^{3-}$	5.06	1.97	3.12	5.94	3.27	2.92	4.23	0.38	3.43	0.20	2.24	4.20	1.62	2.11	1.20	0.71	1.52	2.06
*ClO$_2^-$/HOAc	14.36	5.72	5.94	15.14	13.53	5.98	12.84	0.98	13.60	2.00	7.93	12.94	0.73	5.74	9.96	3.35	4.33	8.03
*10% KOH	4.15	1.97	3.08	4.07	8.69	2.53	3.20	0.44	6.61	0.70	2.80	3.23	0.59	1.45	3.27	1.62	1.08	8.24
*Residue (2)	1.09	0.61	0.96	0.85	2.66	0.66	0.96	0.11	1.94	0.16	0.88	0.76	0.34	0.40	1.06	0.42	0.32	2.37
Residue (1)	24.17	8.37	11.10	23.01	26.75	12.11	21.71	2.59	21.64	2.90	12.13	20.50	7.90	9.37	6.96	5.20	10.72	23.76

*   see Table 5.12

Reference to Table 5.9 reveals that, in Batch 1 walls protein is mostly removed by the alkali extracts but with a significant proportion found remaining in the residues. Although protein in the alkali extracts of Batch 1 walls contains hydroxy-proline, levels of hydroxyproline are offset by the relatively large levels of protein removed and the protein appears to be hydroxyproline-poor.

The distribution of hydroxyproline is somewhat different in that a higher proportion remains behind after all extractions. The residue appears to possess a hydroxyproline-rich protein (12% Hyp, Table 5.11) a result similar to that obtained by Selvendran with bean tissue (32, 33, 67, 147).

In Path B, the alkali extraction of protein (including hydroxyproline) is less complete, but the residue is still hydroxyproline-rich.

In Path B, a higher amount of hydroxyproline is observed in the $100^{O}C$ water extracted fraction, than for the other non-alkali extracts, and the fraction contains significant hydroxyproline-containing protein. This fraction also contains a high percentage of the total wall pectin (see Table 5.9). For subsequent fractions (after Na_2CO_3) in both paths, there is a parallel distribution of pectin and hydroxy-proline-protein. About 50% of the hydroxyproline is resistant to alkali in the walls of Batch 1, along with pectic components.

In Batch 2 walls, the trends are similar to those observed for Batch 1, but the slightly higher protein (and hydroxyproline) in the $100^{O}C$ water fraction, probably reflects the presence of cytoplasmic protein. Hydroxyproline is extracted mostly by $100^{O}C$ water, the stronger alkali fractionations, and the chlorite/acetic acid treatment in Batch 2 walls. Chaotropic reagents show little improvement over 6M urea at removing protein.

Treatment with chlorite/acetic acid, labilised most of the protein from the alkali residue. Approximately 70% of the remaining protein is extracted by the chlorite and this is hydroxyproline-poor. Approximately 30% is extracted by the subsequent alkali treatment but this is hydroxyproline-rich. These results contrast with those of Selvendran who found that the chlorite released fraction was rich in hydroxyproline-containing protein (33, 67, 147).

The minor residue after the chlorite and alkali treatment contains a small level of hydroxyproline-rich protein (about 8% of the total wall hydroxyproline (see Table 5.10)).

It can be seen that although hydroxyproline levels are high for hot water and alkali extracts, total protein is again elevated in these fractions of Batch 2 walls. In Batch 1 walls the highest hydroxyproline/protein ratio is found for material not extracted by any treatment. Similarly in Batch 2 walls the high hydroxyproline protein is not extracted by alkali.

It is concluded that there are three main protein fractions:-

1) the water fractions which, though containing hydroxy-proline, are loosely bound and hydroxyproline-poor.

2) the alkali and chlorite extracted proteins which are tightly bound but hydroxyproline-poor, but which may have properties consistent with a cross-linking role (212).

3) the protein released by chlorite and subsequent treatment with alkali, which is tightly bound and hydroxyproline-rich.

These conclusions are borne out by amino acid analyses (Tables 5.11 - 5.14).

It also appears that the pectic substances remaining in the wall after Na_2CO_3 treatment may be associated with protein. The protein of the pine callus wall could be linked to pectin, some of which could in turn be linked to other polysaccharides.

The association between pectin (galacturonic acid) and hydroxyproline-rich protein is highest in the residues. That hydroxyproline-rich protein is labilised following a chlorite pretreatment tends to suggest that the Hyp-rich protein is involved in cross-linking with lignin in these walls. Thus protein could be binding pectic substances in the wall and, to some extent, lignin may be binding both protein and pectin (6, 7) in the wall. (Tables 5.9 and 5.10)

A study of the amino acid composition of the cell wall fractions (Tables 5.11 and 5.12) supports the idea of three distinct protein fractions; a loosely-bound collection of proteins and two integral wall protein fractions (hydroxyproline-poor and hydroxyproline-rich). A significant observation is that the hydroxyproline-rich protein fractions are also high in proline (especially the post-chlorite alkali extract and final residue in Table 5.12 where total Pro and Hyp content is 30-31 mole %).

It is convenient to discuss the amino acid compositions of these fractions in relation to the results of analogous fractions reported by Selvendran (32, 33, 67, 147) and Monro et al (132-136). A summary comparison is presented in Figures 5.3 and 5.4.

Monro et al (132-136) extracted lupin and mung bean walls with 10% KOH at $0^{O}C$, without extracting protein. At $20^{O}C$ however, 10% KOH extracted most of the hydroxyproline and protein, and the $CaCl_2/I_2$ precipitate of this fraction resulted in a purified protein rich in hydroxyproline (Figures 5.3 and 5.4). The final supernatant contained a lower hydroxyproline protein (11% Pro and Hyp). Subsequent extraction with 24% KOH at $20^{O}C$, removed further protein, leaving a residue of 11% of the wall protein which was 40% in Pro and hydroxyproline.

Selvendran (32, 33, 67, 147), with walls purified in aqueous detergent buffer, found that 4N KOH at $27^{O}C$ extracted hydroxyproline-poor proteins. Extraction of the alkali

Figure 5.3:   Flow Diagram for Preparation of Fractions containing Protein, from _Pinus_, _Phaseolus_ and Lupin.
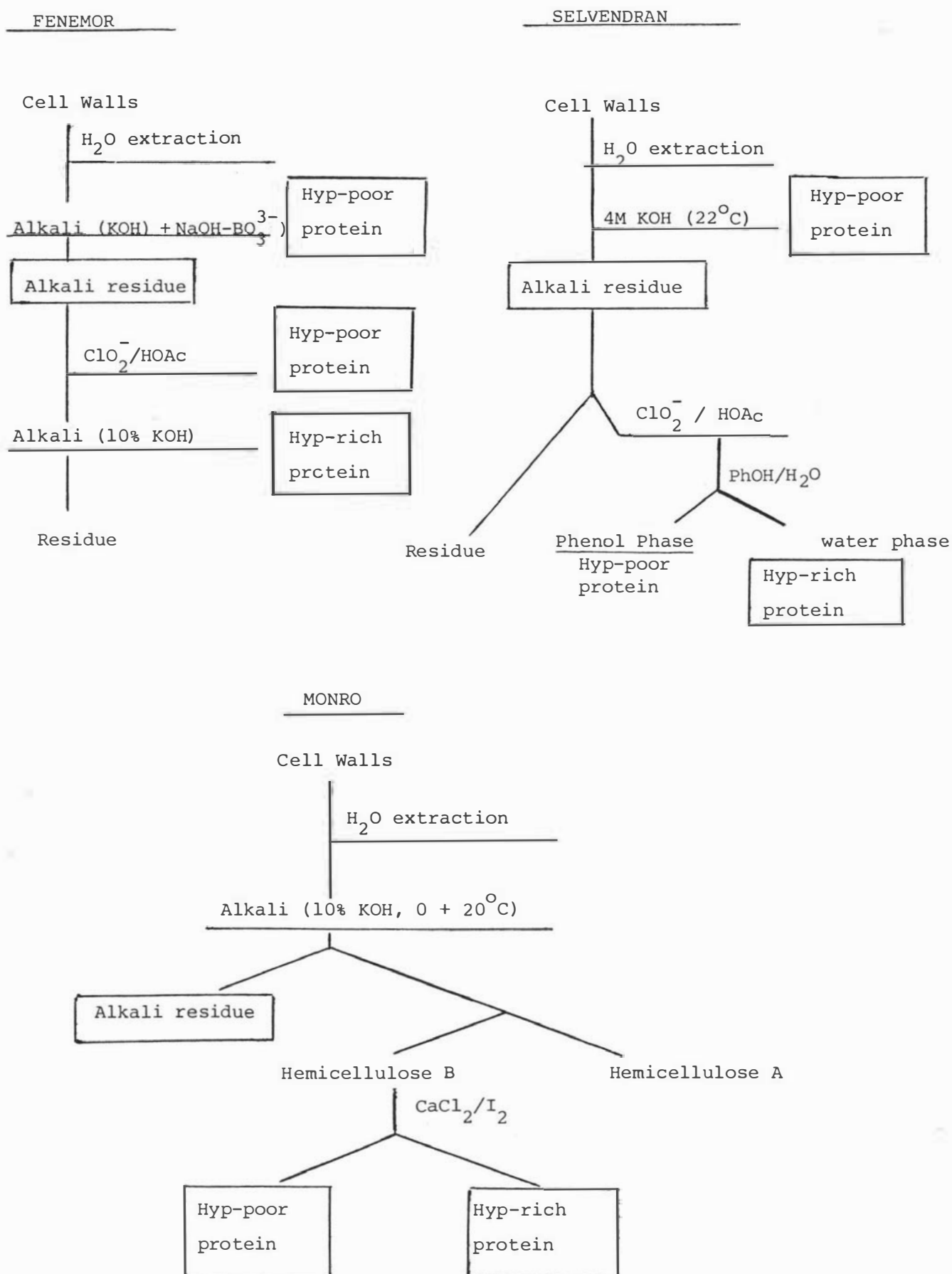
Figure 5.4: Protein Composition with Extractant for _Pinus_, _Phaseolus_ and Lupin.

FENEMOR

Pinus radiata callus. Pre-extracted with, cold, hot water, hot EDTA, 8M urea, and Na_2CO_3.

SELVENDRAN

Mature runner bean, part parenchyma (or whole). Ball milled. Pre-extracted with sodium deoxycholate and Phenol-acetic-water, then hot 2% hexametaphosphate.

MONRO et al

Lupin seedling hypocotyl. Pre-extracted with hot neutral detergent.

Alkali Extraction
Hyp-poor (11% Pro + Hyp), low Lys, Moderately high valine

Hyp-poor (9% Pro + Hyp), high Lys.

Hyp-rich (31% Pro + Hyp), high Ser, high Lys.

Alkali Residue
Hyp-rich (20% Pro + Hyp), Low in Ser, Lys, but high Val.

Hyp-rich (25% Pro + Hyp), high Ser, Lys;   Low Ile

Hyp-rich (31% Pro + Hyp) Low Ser, Asp, Glu, Ala; high Gly and Phe.

ClO_2^- Extraction
Hyp-poor (15% Pro + Hyp), Low Ser, Gly; high Val compares to Hyp-rich

Hyp-rich (33% Pro + Hyp) high Ser.

KOH Extraction
Hyp-rich (30% Pro + Hyp) Low Ser, Lys; high Val.

Final Residue
Hyp-rich (31% Pro + Hyp), Low Ser, Lys; High Val.

Hyp-rich Protein
Shows results for purification of protein from ClO_2^- and Alkali (Monro)

Phenol/water partitioning — H_2O phase with (43-50% Pro + Hyp). 11-16% Ser. 10-12% Lys.

$CaCl_2/I_2$ precipitation — Hyp-rich protein (45% Pro + Hyp). 10% Ser;   10% Lys.

+

Supernatant Hyp-poor

Table 5.15: Amino Acid Mole % Composition for Analogous Fractions of _Pinus_, _Phaseolus_ and Lupin

		FENEMOR						SELVENDRAN					MONRO et al		
		10% KOH Batch 2	NaOH--BO$_3^{3-}$ Residue Batch 2	NaOH--BO$_3^{3-}$ Residue Batch 1-Path A	ClO$_2^-$ Extract Batch 2	Final KOH Batch 2	Final Residue Batch 2	4M KOH	KOH Residue	ClO$_2^-$ Extract	Hyp-rich* Protein	Final Residue	Hyp-rich CaCl$_2$/I$_2$ Precipitate	Hyp-Poor* Fraction	Alkali Residue
Aspartic acid	Asp	11.1	9.6	6.6	10.3	7.3	6.7	11.2	7.7	8.7	5.2	9.3	2.8	8.0	4.4
Threonine	Thr	5.0	3.3	3.8	4.1	3.5	3.8	4.7	3.7	3.2	2.2	4.8	1.8	3.7	1.7
Serine	Ser	8.3	4.4	4.6	4.2	5.4	5.9	8.0	12.3	11.4	16.4	7.6	9.7	13.6	2.3
Glutamic acid	Glu	12.4	9.2	6.3	10.8	7.2	5.2	11.2	7.2	9.5	4.6	7.7	3.5	15.1	4.2
Proline	Pro	5.8	10.7	12.7	9.7	15.3	16.3	5.6	7.5	7.6	6.0	4.5	8.7	2.5	10.1
Glycine	Gly	7.3	4.8	5.0	4.3	4.4	4.0	8.9	6.7	7.0	3.1	9.6	1.1	8.9	12.6
Alanine	Ala	9.4	8.7	6.0	9.2	5.6	5.8	8.7	7.0	4.0	1.7	9.6	2.3	3.7	3.1
Half Cystine	½Cys	0.5	1.0	0.5	0.7	0.8	0.7	0.3	tr	tr	tr	tr	0.0	0.0	0.0
Valine	Val	6.6	8.6	11.7	9.7	11.7	11.8	4.7	3.8	5.1	2.9	6.1	4.6	3.6	5.1
Methionine	Met	1.0	1.2	0.7	2.0	1.0	0.8	0.7	tr	0.4	tr	tr	0.2	1.1	0.3
Isoleucine	Ile	4.5	4.8	5.9	5.7	4.9	5.4	3.2	1.7	1.7	0.5	5.5	1.4	3.1	4.6
Leucine	Leu	8.5	8.2	5.4	9.2	5.7	4.7	9.1	5.7	3.5	0.6	11.1	2.4	5.4	5.0
Tyrosine	Tyr	4.1	3.2	4.5	0.5	1.0	2.1	2.7	2.9	0.6	0.5	0.3	6.5	4.4	2.0
Phenylalanine	Phe	3.9	3.7	2.9	4.1	2.6	2.4	3.7	3.0	0.1	0.3	5.9	0.9	2.8	6.5
Lysine	Lys	1.6	2.8	3.9	7.1	5.8	6.5	7.5	8.2	10.2	10.1	6.3	9.9	6.4	5.5
Histidine	His	2.0	2.1	3.5	2.4	2.9	2.6	1.6	1.9	0.0	1.2	0.5	6.6	1.6	1.6
Arginine	Arg	3.1	4.3	4.0	3.1	1.9	1.9	4.3	2.0	1.2	0.5	3.9	0.5	3.6	0.8
*Hydroxyproline	Hyp	5.0	9.5	11.8	5.7	14.5	14.5	3.2	17.8	25.8	43.5	7.2	36.3	9.5	29.6

tr = trace

residue with chlorite/acetic acid removed the bulk of the
protein and hydroxyproline (Hyp-rich protein) and the wall
residue protein was low in proline and hydroxyproline.

Treatment of the walls with chlorite/acetic acid prior to
KOH extraction also removed hydroxyproline-rich protein, and
phenol/water partitioning of this protein gave a purified
fraction 43% in proline and hydroxyproline comparable to the
purified Hyp-rich protein from chlorite treatment subsequent
to 4M KOH.

The amino acid compositions of <u>Pinus</u> fractions, indicate that
loosely bound proteins (extracted by $100^{o}C$ H_2O through to
Na_2CO_3 treatment) are low in hydroxyproline, but relatively
high in glutamate, glycine and other amino acids (Tables
5.11, 5.12).

The amino acid composition of the loosely bound proteins
varies considerably (particularly arginine and serine)
according to the extraction procedure. Glycine is higher
in Batch 1 than Batch 2 walls. The composition resembles
that of cytoplasmic proteins ($4^{o}C$ H_2O extracts, Batch 2) and
compares with the buffer-soluble proteins from Selvendran's
wall preparation (32, 33). The variation in composition is
probably due to the existence of several different proteins.
It is also possible that an arabinogalactan protein is
present in the water-extracted fractions (197), perhaps
accounting for the hydroxproline content (see Table 5.16).

The wall proteins of <u>Pinus</u> appear to fall into two categories.
The tightly bound, low hydroxyproline proteins, removed by
alkali have amino acid compositions similar to those extracted
by aqueous extractions, but a lower level of glycine is
extracted by alkali. This compares with Selvendrans
hydroxyproline poor protein except for the relatively high
valine and low lysine. Monro found that alkali removed both
hydroxyproline-rich and Hyp-poor proteins, but this hydroxy-
proline poor protein seems to be rather different to the
one found for Pinus (see Table 5.15). The alkali extracted

Table 5.16: Mole % Composition of Amino Acids in the Arabino-
galactan-Protein Isolated from Pinus taeda callus
(197).

Aspartic acid	8.9	Isoleucine	2.4
Threonine	5.2	Leucine	6.5
Serine	15.1	Tyrosine	1.5
Glutamate	10.3	Phenylalanine	2.2
Proline	2.6	Lysine	4.1
Glycine	17.4	Histidine	1.2
Alanine	9.8	Arginine	3.6
½ Cystine	0.0	Hydroxyproline	2.7
Valine	3.8	Undetermined	2.3
Methionine	0.4		

proteins of both Batch 1 and Batch 2 walls are closely comparable, though Proline and Hydroxyproline levels are slightly higher for Batch 2 walls over Batch 1.

The second tightly bound protein is hydroxyproline-rich. It is not extracted by alkali, an observation comparable to Selvendran's results. The alkali residue (for both Batch 1 and 2 walls) has a similar composition to that of Selvendran except for high valine, low serine and lysine in walls of Pinus radiata. This could be due to plant type differences. It is noteworthy that the hydroxyproline-rich protein of Pinus radiata (Tables 5.11, 5.12, 5.15) has high Pro and low Hyp compared with the hydroxyproline-rich protein of Selvendran. The lower degree of hydroxylation of proline in the Pinus radiata hydroxyproline-rich protein could be due to growth differences since hydroxyproline and proline totals are the same for both proteins from Pinus and Phaseolus. The removal of hydroxyproline-protein is enabled by chlorite extraction, but in Phaseolus it is removed by chlorite, whereas in Pinus it stays behind and is subsequently extracted by alkali (Tables 5.12, 5.14, 5.15, Figures 5.3, 5.4).

Treatment with chlorite liberates predominantly protein and arabinose (Table 5.10). Thus the components are possibly lignin bound. But Selvendran's chlorite treatment is required to liberate hydroxyproline-rich protein from relatively non lignified walls. In Pinus, the arabinose/hydroxyproline ratio is approximately 10:1 therefore indicating an arabinan rather than just arabinose-hydroxyproline arrangements as in extensin. In the following alkali extract, the ratio is lower, approximately 6:1 and the arabinose may be associated with arabinose-hydroxyproline linkages, since gymnosperms have predominantly $(arabinose)_{3-4}$ hydroxyproline in their wall protein (31, 165). Thus this post chlorite alkali extract contains the bulk of the high hydroxyproline-containing protein, which may possess tri or tetra-arabinosides on the hydroxyproline residues.

In the final extraction residue the arabinose/hydroxyproline ratio is approx. 20:1. This final residue may contain residual pectic material (GalA, Gal, Ara) possibly bound by residual lignin.

Thus protein in the "pectic" and alkali extracts of the Pinus though containing hydroxyproline is not hydroxyproline-rich. It is possible that an arabinogalactan type protein could be present in these fractions (197), but it is only speculative that this could provide linkage between pectic polymers and xyloglucan as in the Albersheim model (8). The hydroxyproline-rich protein is strongly bound in the alkali residues, and its labilisation after chlorite treatment would appear to indicate that it is cross linked with lignin.

5.3.5 Lignin Analysis to Batch 1 Walls: Results and Discussion

Pine callus walls were shown to possess U.V. flourescence, and to stain with phloroglucinol (Section 3.7.1), which may be indicative of lignification. In view of the fact that many of the sugars and much of the uronic acid is retained in the residues to Batch 1 walls after all extractions, lignin assays were performed on these fractions. The assay was not fully quantitative due to problems of incomplete solubilisation of lignin in the acetyl bromide solution. Such colourimetric assays for lignin content were not repeated on walls of Batch 2, however in Batch 2 walls, interaction of lignin with wall polymers is implicated by the results of chlorite extraction. For Batch 1 walls the presence of lignin was demonstrated by positive assay results for several fractions. Though not detected in oxalate or EDTA fractions to either path and only slightly in Na_2CO_3 extracts, it is higher in the alkali extracts, the hot water extracts and in the residues. The level in the residues may well be a gross underestimate because of the difficulty observed in the ability of the acetyl bromide to solubilise all of the lignin present on heating as in Section 2.2.5. Data from Batch 1 walls is given in Table 5.17.

TABLE 5.17: Lignin Levels in Each Fraction. Batch 1 Walls
a) mg Lignin per fraction
b) Lignin in fraction as % total wall weight

		a	b
	$4^{\circ}C\ H_2O$	0.86	0.30
	$70^{\circ}C\ H_2O$	1.92	0.64
Path A	Oxalate	$\delta+$	$\delta+$
	EDTA	$\delta+$	$\delta+$
	Na_2CO_3	0.12	0.07
	0.5% KOH	1.00	0.60
	10% KOH	1.24	0.73
	24% KOH	1.40	0.82
	25% $NaOH-BO_3^{3-}$	0.80	0.50
	Residue	0.59	0.40
Path B	$100^{\circ}C\ H_2O$	1.07	0.82
	Oxalate	$\delta+$	$\delta+$
	EDTA	$\delta+$	$\delta+$
	6M Urea	0.05	0.04
	Na_2CO_3	$\delta+$	$\delta+$
	25% $NaOH-BO_3^{3-}$	0.72	0.60
	Residue	2.35	1.80

The retention of sugars, uronic acid, protein and lignin in the wall residues has been demonstrated by two methods, for these wall preparations. It is likely that the high residual content of pectic substances, and hydroxyproline protein in the callus walls, after alkali extraction is due to cross linking of these polymers with lignin.

Most studies on base-catalysed cleavage of ether linkages in lignin have been performed at elevated temperatures (210). Arylglycerol β-ethers have been shown to be labile (162). It is not expected however that much hydrolysis would occur at room temperature under the conditions used in this study except perhaps through aryl α-ether bonds (162) to carbohydrate which are labile in alkali at lower temperature. The lability of a wide range of carbohydrate-lignin bonds to alkali has not been investigated in detail. It is possible that some polysaccharide may be released by hydrolysis of ether linkages even though lignin breakdown is insignificant. Lignin assay on the alkali extract suggested some solubilisation at lignin by alkali, but the identity of the U.V. absorbing components in the acetyl-bromide extract was not confirmed, although the U.V. spectrum resembled that expected for lignin (see Figure 2.4, with maxima at 280 and 320 nm) more closely than that of protein.

5.4   DISCUSSION

5.4.1 Comparison between Batch 1 and Batch 2 Walls

The following points of comparison between Batch 1 and Batch 2 walls emerge from the results presented in this Chapter.

Total levels of neutral and acidic sugar are both lower for Batch 2 walls than for Batch 1 walls. However considerable uronic acid is removed in the aqueous purification step in Batch 2, and if this is also included in the total, the levels of uronic acid in the two batches are similar.

The total protein in the two wall Batches are comparable, about 50 mg/g 70°C water residue. The high protein level in the aqueous (4°C H_2O) fraction in preparation of Batch 2 walls would be cytoplasmic proteins. This does not occur in the cold water extraction of Batch 1 walls, which had been purified by wet sieving. Similarly a proportion of the 100°C water-extracted protein of Batch 2 walls may be of cytoplasmic origin. The amount of protein removed for the combined "pectic" extracts (oxalate and EDTA) of Batch 1 walls (both paths) is comparable with that of Batch 2.

There appears to be a difference in the alkali extractability of protein between Batch 1 and Batch 2 walls. Much less is removed by alkali in Batch 2 compared to Batch 1. Protein is much more tenaciously bound in Batch 2, and the bulk of it is not liberated until the chlorite treatment. This could reflect a greater lignification of Batch 2 walls and cross-linking of protein to lignin.

The apparent differences between the total carbohydrate levels in the two wall preparations may be a result of the stage of growth, or growth conditions of both batches. There should be no structural or physiological difference between batches caused by different genetic features of the callus, since both batches were derived from the same stock culture.

For the 100°C water fractions, Batch 2 has lower arabinose, slightly lower galactose, and slightly lower pectin than Batch 1. The rhamnose/galacturonic acid ratio is generally higher in Batch 2 than in Batch 1 walls, which appear to have higher levels of neutral arabinan pectic polymer. An increase in the rhamnose of pectin is borne out to some degree by methylation analysis of Chapter 6, but the high rhamnose/GalA ratio could be partly an artifact of premature extraction of pectin (with low rhamnose/GalA) by 70°C H_2O in Batch 2. Alternatively some of the polysaccharide (and uronic acid) in the 70°C water extract of Batch 2 may be of cytoplasmic nature. The higher mannose in 70°C water of Batch 2 (Table 5.8), may denote some soluble glucogalacto-

mannan.

5.4.2 Comparison Between Different Extraction Procedures; Overall Trends

The series of fractionations reported in this chapter were
designed to provide an interpretation of the different modes
of linkages of polymers within the callus walls. The
rationale for each extraction step is discussed in the
introduction to the chapter. Briefly the types of linkage
that might be disrupted by each extraction step are as listed
in Table 5.18, and the constitution of wall material removed
by these disruptions was as described (Section 5.2 - 5.3) and
is summarised here.

The carbohydrate portion of the wall extractable by the
schemes outlined in Figures 5.1 and 5.2 showed several
features. Pectic substances (uronic acid, and component
galactan (galactose) and arabinan (arabinose)) were
distributed throughout the fractions of Batch 1 and Batch 2
walls. In Batch 1 walls extraction with $100^{O}C$ water removed
some pectin which was not otherwise extracted by hot EDTA
(at lower temperature). This was perhaps covalently linked
(191) as would have been the pectic arabinan, galactan and
rhamnogalacturonan in the $100^{O}C$ water fraction of Batch 2.
The low rhamnose/GalA ratio in fractions suggests that wall
rhamnogalacturonan is not highly branched through rhamnose.
Methylation data is presented in Chapter 6 on the nature of
bonding with the rhamnogalacturonan released by $100^{O}C$ water
from walls of Batch 2.

The possibility that some of the arabinose and galactose of
cold and hot water extracts was due to a 3,6-linked
arabinogalactan (also indicated by a varying Ara/Gal ratio)
was confirmed by methylation data (Chapter 6).

In both batches pectic components were removed by alkali
(which will further β-eliminate and degrade pectin), though
a substantial amount was firmly wall bound, being resistant

Table 5.18:   Summary of Bond Disruptions Effected by Reagents

(1) 100^OC Water — β-elimination of pectin (also enables solubilisation of a linear galactan); removal of pectic polymers.

(2) Hot EDTA — Chelation of Ca^{2+} (Ca^{2+} is thought to produce ionic bridges between carboxyl groups of polyuronide) enabling solubilisation of already partly degraded pectin (132-136).

(3) GTC/Urea — Hydrogen bond disruption. Effects release of polymers held in the wall by the H-bonds only.

(4) Na_2CO_3 — Hydrolysis of ester linkages.

(5) KOH — as in (3) and (4) and the possible hydrolysis of alkali labile ethers between saccharides and lignin.

(6) Alkali & borate — Solubilisation of mannans by complex formation of borate with cis vicinal hydroxyls on mannose rings.

(7) ClO_2^-/HOAc — Oxidative degradation of lignin (and possibly protein), or possible destruction of lignin-saccharide, protein-saccharide linkages.

(8) Subsequent alkali — Solubilisation of polymers which required ClO_2^- treatment for removal but are also held by alkali labile bonds as in (3), (4), (5), (6).

(9) Residue — Contains residual lignin-bound, or cellulose-bound polymers, held in the wall by extremely resistant bonds or by physical entanglement, with cellulose and remaining lignin.

to strong alkali (for Batch 1 walls, 30% of total arabinose, 15% total galactose, and 20% of the total uronic acid). Pectic components (uronic acid, arabinose and galactose) were also found in chaotropic extracts of Batch 2 walls.

In Batch 2 walls hemicellulosic xyloglucan, xylan and small levels of mannan are found as for Batch 1 walls. In Batch 1, 6M urea removed little carbohydrate and hemicelluloses (xylan and xyloglucan) required alkali for extraction. In Batch 2, significant xyloglucan levels (from Xyl/Glc ratio) were extracted with 8M urea or 6M GTC, after some pectin cleavage by 100^OC water. This suggested that at least a portion of the xyloglucan is held in the wall by hydrogen bonds. Since xyloglucan (in Batch 2) and possibly xylan (as shown in Chapter 6) are subsequently removed by alkali, it seems other alkali-labile bonds (apart from GTC-labile hydrogen bonds) are responsible for binding some hemicellulose in the wall.

Inspection of the protein data (Tables 5.11 - 4.15, 5.10) between fractionations revealed that it is distributed amongst all fractions, but much is removed by hot water, some by chaotropic reagents, and more by alkali. Protein remains in the residues of both batches of wall studied. In Batch 1 Path B, 6M urea extracts a level approaching that of 8M urea (Batch 2). Removal of protein is less efficient with 6M GTC than 8M urea. This protein may be associated with hydrogen bonded polymers. In Batch 2 total protein labilised by Na_2CO_3 compares with protein extracted by Na_2CO_3 in Batch 1, and this protein is possibly released either by hydrolysis of ester links or by association with polymers released by hydrolysis of ester links.

Protein of the aqueous extracted fractions to both batches is hydroxyproline-poor as is the more tightly bound alkali extracted protein. Only protein of the residues of Batch 1 has elevated hydroxyproline and the protein labilised after chlorite pretreatment in Batch 2 is hydroxyproline-rich (Table 5.15).

Hydroxyproline parallels pectic substances in fractions
subsequent to Na_2CO_3 extraction, in both batches with about
40% firmly wall bound (alkali-resistant) though it is also
fairly high in some fractions which remove loosely bound
protein (Section 5.3.2), but any postulated linkage of
hydroxyproline-containing protein with pectin is tenuous.

Monro et al (2, 134) studied the distribution of polysaccharides
and protein from lupin hypocotyls. The results of this work
are in some contrast to those of Monro. In _Pinus_ callus,
pectin is not quantitatively extracted by $100^{o}C$ EDTA, or
oxalate as for Monro's results but some is removed with
chaotropic reagents and subsequent alkali. Some pectin is
therefore held in the wall by links to other polymers, such
as hemicelluloses and protein.

Monro observed that GTC treatment of lupin hypocotyl walls,
before and after dilute acid treatment did not extract
hydroxyproline, indicating that it was firmly bound, but 1M
acid ($100^{o}C$, 1 hr) completely removed hydroxyproline and
hemicelluloses. From a study of the alkaline extraction of
hydroxyproline and hemicellulose, and the effect of alkali
strength, temperature, and duration of extraction, Monro et
al concluded that the wall glycoprotein is linked to alkali
insoluble wall portions by links only slowly broken by
alkali. Since the bulk of the hemicelluloses was removed
before hydroxyproline, they concluded hydroxyproline was
either involved in alkali-labile links to hemicellulose
or unlinked (134, 135). They proposed that several linkage
types of protein-polysaccharide were possible (even amino-
acid - cellulose) (2, 134), as galactosyl-serine links were
unlikely as extraction of hydroxyproline by $0^{o}C$ alkali (which
causes β-elimination of such links) did not occur.

Only hydroxyproline-poor proteins are removed by alkali for
Pine callus. Labilisation of hydroxyproline-rich protein
after chlorite pretreatment compares more with results of
Selvendran and suggests that lignin may be cross linking
protein in the wall. Selvendran (32, 33, 67, 147) found

that a minor portion of the hydroxyproline-rich protein which
was released by acid chlorite, associated with pectic
substances on DEAE cellulose, perhaps providing support for
the Albersheim model (8).

Much of the hydroxyproline is removed with neutral poly-
saccharides and uronic acid in alkali fractions (Hyp-poor
protein) and it is possible that protein is associated with
pectin.   Uronic acid, arabinose and galactose are probably
held in the residue by lignin cross-linking.

Thus a complex pattern is already beginning to emerge, for
polymer composition and arrangement.   Pectic polymers which
are further removed with chaotropic reagents and alkali,
could be associated with hemicelluloses, some of which are
hydrogen bonded in the wall, and some which are less labile,
requiring strong alkali extraction.   Protein possibly
interacts with pectic polymers, and the low levels of lignin
complicate the picture by presumably bonding with many of
the wall components.

CHAPTER 6

METHYLATION OF SELECTED FRACTIONS OF CALLUS BATCH 1 AND 2 WALLS

6.1   INTRODUCTION

A general description of the types of polysaccharides that
are involved in the structure of the primary cell wall can be
inferred from the sugar data discussed in previous chapters.
Methylation analysis of extracted fractions provides
confirmation of the presence of these polysaccharides by
enabling linkages between sugar residues (characteristic of
distinctive classes of polysaccharides) in the polysaccharide
to be analysed. Methylation analysis has been used in the
literature on a host of plant whole cell walls, extracted
fractions, and purified and semi purified polysaccharides.
To quote but a few, for example, on whole cell wall material
of parenchyma from Phaseolus (68), on extracted wall
fragments (8, 46, 47), on semi purified (20, 43, 156) and
purified (138) polysaccharides. The technique provides
quantitative information on mixtures of monomeric methylated
sugars, after exhaustive methylation and hydrolysis of the
polysaccharide. The position of glycosidic linkages within
the polysaccharide corresponds to the positions of
unsubstituted hydroxyl groups in the methylated monosaccharides,
analysed as permethylated alditol acetates. The method gives
no information on the relative order of sugar residues, or
their anomeric configuration, which is removed by mutarotation
in hydrolysis.

Determination of the complete structure of a polysaccharide
requires complementary analysis such as graded hydrolysis by
acids or enzymes followed by isolation and identification of
the oligosaccharides formed (93). But the nature and
proportions of the O-methyglycoses provide information on
relative proportions of non-reducing end groups, the degree
of branching, the nature of the main linkage types, and the

types of interchain linkages of branch points (150) (Section 2.4).

The validity of the method requires that there be no chemical degradation of polysaccharide during methylation, that the polysaccharide is fully etherified, and that demethylation does not occur during hydrolysis (150). Conditions which achieve complete methylation and minimum degradation on hydrolysis are described in Section 2.4.3. Chemical modification of methyl esterified uronic acid residues by β-elimination under basic conditions was eliminated where it was desired to analyse uronic acid residues. The polysaccharide (as outlined in Section 2.4.4) was de-esterified before methylation, and methyl esters produced in this first methylation immediately reduced to primary alcohol groups. The neutral residues could then be safely remethylated, without degradation.

The details of the g.l.c. phases, used for the separation of the methylated alditol acetates, and details of their identification and quantitation are given in Sections 2.4.6 and Appendices.

In brief this chapter outlines the results obtained for the linkage analysis of important polysaccharide – containing wall fractions. All samples for neutral sugar linkage analysis were methylated to completion, either 3-4 times by a repeated standard Hakomori or by the multiple Hakomori procedure (methylating 4 - 5 times and using excess MeI in final methylations). The multiple Hakomori was used in analysis of methyl derivatives of the NaOH-borate fraction of Batch 1, and in analysis of derivatives of Batch 2 (as outlined on Table 6.2 Captions).

Reduction with Sodium Borodeuteride was incorporated in the methylation procedure or work-up to: -

a)   reduce methyl esters of uronic acids of the $100^{O}C$ water fraction Batch 2 walls (created in the first methylation), to primary alcohol groups. Thus the

C-6 position was then isotopically labelled in the neutral sugars so derived from uronic acid. Reduction of uronic acids with $LiAlD_4$ or reduction ($NaBD_4$) of carbodiimide products was not used due to difficulties in recovery and handling of reaction products.

b) reduce the neutral methylated sugars to alditol acetates for the 6M GTC and 10% KOH fractions of Batch 2 walls. This isotopically labelled the reducing ends of permethylated sugar derivatives, and permitted the identification by mass spectrometry of symmetric xylose derivatives. These would have otherwise been indistinguishable as they co-chromatograph on Columns 1 and 2.

Some of the terminal derivatives may have incurred small losses due to their volatility. These were difficult to measure accurately but were observed to be relatively minor between repeated evaporations in dichloromethane, the solvent used for injection onto g.c. columns.

6.2 BATCH 1 WALLS

This section outlines the principal results of methylation analyses on Batch 1 walls and the polysaccharides that are believed to be responsible for the data obtained.

The most abundant arabinose derivative is $2,3-Me_2$-arabinose corresponding to 1,5-linked arabinose. This is distributed between all fractions and represents a 5-linked arabinan, characteristic of pectic type arabinans (see Chapter 1).

The presence of $2,3,6-Me_3$-galactose in all fractions signifies the presence of a 4-linked galactan (see Chapter 1, 1.3.10). Both these derivatives are highest in uronic acid and rhamnose-rich fractions, and thus the arabinan and galactan are believed to be polymers associated with the pectic complex. The relatively high level of 3-Me-rhamnose implies

that rhamnose residues are highly branched. These would be
part of the pectin. The high levels of 2-monomethyl
arabinose, suggest the arabinan may have the typical
branching at the 3-position found in Pine arabinans (137).
The 3-monomethyl arabinose indicates a lesser degree of
branching at the 2-position.

The $2,4,6-Me_3$-galactose derivatives are relatively high in
water fractions and EDTA extractions, representing the presence
of 3-linked galactan. The existence of some branching at
position C-6 is indicated by the $2,4-Me_2Gal$. The degree of
branching in water extracts is highest in the cold water
extract and lowest in the 100^OC water extract. Thus the cold
water extracts may contain the more conventional type of
3,6-linked arabinogalactan (see Chapter 1 and 144) while the
hot water removes a less branched polymer. Small amounts of
$2,4,6-Me_3$-galactose, $2,3,4-Me_3$-galactose and $2,4-Me_2$-galactose
are present in later fractions (Table 6.1). The branched
3,6-galactan although present at low levels is evidently
generally distributed through the wall fractions.

The $2,3,4,6-Me_4$-galactose is high in some fractions and this
is discussed in Section 6.4.

The $2,5-Me_2$-arabinose, could be associated with arabino-3,6-
galactan according to the literature, but it could be equally
associated with the arabinan. In several fractions, such as
the 10% KOH fraction, I, (Table 6.1), for example, the level
of $2,5-Me_2$-arabinose is much higher than could be expected
from a classical arabino-3,6-galactan.

The $2,3,6-Me_2Glc$ represents:

1) Starch with very low branching, and
2) Xyloglucan with very high branching.

These two polysaccharides are distinguished by the ratio of
$2,3-Me_2$-glucose/$2,3,6-Me_3$-glucose. This ratio is high in
alkali and cold water fractions, implying the presence of the
highly branched xyloglucan, and low in hot water fractions,
demonstrating the removal of residual starch from the wall

Table 6.1 Captions

Description of Fractions for Table 6.1

A $4^{o}C$ H_2O Fraction: Methylated 3 times
Purified by exhaustive dialysis against distilled water

B $70^{o}C$ H_2O Fraction: Methylated 3 times
Purified by exhaustive dialysis against distilled water.

C $100^{o}C$ H_2O Fraction, Path B: Methylated 5 times
Purified by dialysis after 1st and by Sephadex LH-20
chromatography, after the 2nd and 3rd, and subsequent
methylations.

D   0.5% Ammonium Oxalate $75^{o}C$ Fraction, Path B: Methylated
                                                    3 times
Purified by dialysis after 1st and 2nd methylations,
and by Sephadex LH-20 after the 3rd methylation.

E   0.2% $(Na)_2EDTA$, $70^{o}C$ pH 6.7 Fraction, Path B: Methylated
                                                      3 times
Purified by dialysis after 1st and 2nd, and Sephadex
LH-20 after the 3rd methylation.

F   0.2% $(Na)_2EDTA$ $70^{o}C$ pH 6.7 Fraction, Path A: Methylated
                                                      3 times
Purified by exhaustive dialysis against distilled water.

G   0.1M Na_2CO_3, $4^{o}C$ Fraction, Path A: Methylated 3 times
Purified by dialysis after 1st and 2nd methylation, and
by Sephadex LH-20 after the 3rd methylation.

H   0.5% KOH (0.5% $NaBH_4$) at $20-23^{o}C$ Fraction, Path A
Purified by dialysis after 1st and 2nd methylation, and
Sephadex LH-20 after the 3rd methylation.

I   10% KOH (0.5% $NaBH_4$) at $20-23^{o}C$ Fraction, Path A
Purified by dialysis after 1st and 2nd methylations and

Table 6.1 Captions - Continued

by Sephadex LH-20 after the 3rd methylation.

J   25% NaOH-BO$_3^{3-}$ at 20-23°C Fraction, Path B

Methylated by the multiple Hakomori procedure adopted by Albersheim (114).   Purified by dialysis.

(For the location of these fractions in the extraction pathway see the Fractionation Scheme Diagram, Section 5.2, Figure 5.1.)

Table 6.1: Methylated Polysaccharide Sugar Residues for Extracted Fractions of Batch 1 Walls: μ moles/g 70°C Water Residue

(Data was normalised as described in Section 2.4.7.3)   * Derivatives subject to some loss through volatility (low losses)
(i) Contains 2,3,6-Me$_3$Man
(ii) Contains 3,4,6-Me$_3$Gal   tr = trace amount

Sugar	O-Methyl Ether	A	B	C	D	E	F	G	H	I	J	
Arabinose	*2,3,5-	2.9	5.8	66.2	7.7	4.0	5.0	13.0	20.8	15.9	20.1	
	2,3,4-	0.0	0.0	3.4	0.0	0.0	0.0	0.0	1.0	0.0	0.0	
	3,5-	0.0	0.3	3.1	0.5	0.4	0.4	2.5	1.9	2.4	3.8	
	2,5-	2.0	2.2	36.1	4.1	2.8	3.5	6.6	8.7	8.9	11.4	
	2,3-	3.9	6.5	97.5	11.2	7.4	8.9	14.0	29.5	19.7	25.0	
	2-	2.1	3.7	40.3	5.0	4.4	4.8	8.7	18.0	14.0	16.5	
	3-	0.5	1.1	14.6	2.3	3.0	1.4	2.6	5.4	4.2	2.0	
	-	1.2	1.9	24.7	2.9	2.5	3.2	0.6	8.8	11.7	20.7	
Xylose	*2,3,4-	2.1	2.1	7.8	0.9	0.4	0.7	4.1	1.0	44.0	70.0	
	2,3-/3,4-	3.2	2.1	12.0	2.0	0.8	1.6	2.8	1.9	45.5	69.3	
	2-/3-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.1	15.8	
	-	0.3	1.3	0.0	0.3	0.8	0.3	1.9	1.2	4.3	17.9	
Fucose	*2,3,4-	0.7	0.0	3.4	0.5	0.0	0.4	0.0	0.6	13.6	25.7	
Galactose	*2,3,4,6-	3.6	6.0	60.2	7.1	4.9	5.6	20.1	9.9	29.5	39.3	
	2,4,6-	6.1	13.9	75.8	2.9	4.0	3.3	2.0	9.0	4.3	27.2	(i)
	3,4,6-	0.0	0.0	0.0	0.0	0.0	0.0	tr	tr	15.9		(ii)
	2,3,6-	3.1	6.5	49.9	3.2	7.7	5.4	5.1	17.7	12.2	30.8	(ii)
	2,3,4-	1.3	1.0	4.2	0.7	2.1	1.1	3.2	2.5	2.8	5.1	
	2,6-	0.3	0.8	2.9	0.1	1.3	0.2	0.0	0.9	0.9	0.0	
	2,3-	0.7	0.9	3.9	0.0	0.0	0.0	0.4	0.3	0.0	0.0	
	2,4-	2.9	3.4	4.9	0.0	1.1	2.4	0.7	0.0	3.3	4.1	
	2-	1.1	2.3	4.0	0.0	0.7	0.0	0.0	0.0	1.5	3.2	
Glucose	*2,3,4,6-	0.7	2.1	8.4	2.0	2.5	1.4	0.6	3.1	5.6	4.3	
	2,3,6-	7.7	21.9	69.2	8.9	4.1	11.5	7.9	7.6	90.4	107.2	
	2,3-	3.8	2.8	9.0	0.7	1.4	2.4	1.2	0.4	67.9	108.6	
	2-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	
Mannose	2,3,6-	0.0	6.0	0.0	0.0	0.0	0.0	0.8	0.0	7.5		(i)
Rhamnose	*2,3,4-	0.2	0.2	2.0	0.0	0.2	0.4	0.0	0.0	0.7	0.0	
	3,4-	0.7	0.1	1.4	0.1	0.0	0.2	0.0	0.0	1.3	0.0	
	3-	0.9	2.2	15.0	1.9	2.3	2.4	3.2	4.8	6.6	6.1	

preparation.

Terminal fucose and 2-linked galactose (3,4,6-Me$_3$-galactose) were also found in the alkali fraction, indicating that the xyloglucan is of the fucogalacto-type, as described in Chapter 1.

The presence of some highly branched xylose residues (2,4-linked xylose, 3,4-linked xylose and some completely linked xylose) in the alkali extracts suggests the presence of xylan as well as xyloglucan, and their relative quantitation is discussed subsequently (in Section 6.2) for Batch 2 walls.

The presence of mannans or glucomannans in the callus wall is suggested by the presence of 2,3,6-Me$_3$-mannose, corresponding to 1,4-linked, β-D-mannose (see examples of Chapter 1). No other mannose derivatives were detected.

6.3   BATCH 2 WALLS

The fraction of Batch 2 walls containing the highest levels of uronic acid (the 100°C water fraction) was used to study the pectic components of callus walls. The fraction was consequently methylated by the multiple methylation technique to investigate the linkage composition of neutral components. A sample (20 mg) was further α-amylase treated (5 days in 0.01M sodium phosphate buffer pH 7.0) and after de-esterification and purification by exhaustive dialysis given one methylation with excess methyl iodide to convert uronic acid carboxyl groups to methyl esters, and ring hydroxyls to ethers. Ester groups were reduced with NaBD$_4$, the sample remethylated, and reduced again (as outlined in Section 2.4.4), and finally remethylated twice.

The two other fractions for which methylation results have been obtained are (as shown in Figure 5.2) the first 6M GTC fraction containing polysaccharides removed by disruption of non-covalent bonds, and the 10% KOH fraction containing

hemicellulosic polysaccharides removed by non-covalent, and covalent bond disruption. Borodeuteride reduction of methylated sugars was employed as described in Section 6.1.b.

6.3.1 Methylation of the 100°C Water Fraction

The five times methylated whole fraction (Table 6.2, 5 x M) contains high levels of 2,3-Me$_2$-arabinose, much 2-Me-arabinose, some 3-Me-arabinose, unmethylated arabinose and some terminal (2,3,5-Me$_3$Ara) arabinose. High levels of 2,3-Me$_2$-arabinose, and 2,3,5-Me$_3$-arabinose suggest an arabinan with a 1,5-linked arabinose back bone, comparable to that of Batch 1 walls. The 2 and 3-Me-arabinose again suggest a fairly high degree of branching of the back bone.

Among the galactose derivatives, the presence of 2,3,6-Me$_3$-galactose implies (as for Batch 1 walls) relatively large amounts of 4-linked galactose from a linear pectic galactan (see Section 1.3.10). Again uronic acid and rhamnose levels are high where the pectic arabinan and galactan are found and they are likely to be part of an overall pectic complex.

The presence of 3,4-Me$_2$-rhamnose, and 3-Me-rhamnose indicates 2-linked rhamnose and 2,4-linked rhamnose as would be found in typical pectins. Rhamnose could be 2-linked to galacturonic acid in the polygalacturonic acid back bone, and the 4-linked rhamnose may provide for attachment of a pectic polymer such as the galactan.

An increase in rhamnose (as 3-MeRha) is evident after reduction and remethylation. This can be expected if non-reduced material gives aldobiouronic acids on hydrolysis. The reduced polymer would be more completely hydrolysed and therefore would give a better measure of the rhamnose content.

The 2,3,4-Me$_3$-galactose, 2,4-Me$_2$-galactose, and some 2,4,6-Me$_3$-galactose may be attributable to a 3,6-linked arabinogalactan polymer as found for Pine by Roudier and

Table Headings for Methylation of $100^{\circ}C$ Water Fraction
  of Batch 2 Walls   –   Table 6.2

(5 x M)   Methylated five times by the multiple Hakomori
           procedure; purified by exhaustive dialysis
           against distilled water.

(M)   Sample de-esterified, and given one methylation
           with excess methyl iodide, purified by dialysis
           and analysed.

(MR)   Same as for (M) then dissolved after purification
           in THF/EtOH (17/7) and reduced with sodium
           borodeuteride, then purified by dialysis.

(MRM)   Same as for (MR) then remethylated and purified
           by dialysis.

(MRMRMM)   After remethylation, a portion of the sample was
           dissolved in Dioxane/EtOH (3/1) and reduced again
           with sodium borodeuteride.   The sample was
           purified by dialysis and remethylated twice by a
           multiple Hakomori procedure and purified by
           dialysis against distilled water.

Table 6.2: Methylated Polysaccharide Sugar Residues for the $100^{\circ}C$ Water Fraction
Extracted from Batch 2 Walls: μ moles/g $70^{\circ}C$ Water Residue

Data normalised to Arabinose Totals of the 5 x M Sample (see Section 2.4.7.3).

Sugar	O-Methyl Ether	5 x M	M	MR	MRM	MRMRMM (Ave)
Arabinose						(D_2)
	*2,3,5-	18.6	14.8	12.6	18.8	21.9
	2,3,4-	0.3	0.0	0.0	0.0	0.0
	3,5-	3.6	3.5	2.7	2.9	2.4
	2,5-	12.7	8.7	9.4	12.5	12.0
	2,3-	23.4	17.1	18.0	24.8	21.2
	2-	14.5	12.9	15.3	14.5	14.6
	3-	3.8	3.1	4.6	3.7	3.7
	-	7.3	24.4	21.7	7.1	8.6
Xylose						
	*2,3,4-	2.3	2.6	3.5	4.0	10.0
	2,3-/3,4-	2.7	3.3	2.2	4.6	2.8
	2-/3-	1.0	0.0	0.0	0.0	1.9
	-	0.4	1.3	1.8	0.0	1.2
Fucose						
	*2,3,4-	1.3	0.0	0.0	0.0	3.9
Galactose						
	*2,3,4,6-	23.6	17.6	18.3	26.2	37.5 (5.4)
	2,4,6-	47.7	63.9	55.3	59.2	36.3
	2,3,6-	23.9	17.8	15.6	43.6	57.7 (38.4)
	2,3,4-	2.7	0.0	1.9	3.4	3.2
	2,6-	2.5	3.0	2.6	7.1	4.0 (1.4)
	3,6-	0.0	0.0	0.0	0.0	1.2 (0.7)
	2,3-	1.2	1.8	7.3	5.1	2.4 (0.7)
	2,4-	4.8	4.9	4.8	5.1	4.3
	2-	3.2	3.8	5.6	3.7	1.6
	-	0.0	14.5	40.0	2.9	0.0
Glucose						
	*2,3,4,6-	1.5	1.2	1.2	2.5	5.5 (0.8)
	2,3,6-	17.5	12.4	13.7	17.1	25.6 (1.0)
	3,6-	0.8	0.0	0.0	0.0	1.0
	2,3-	2.5	1.9	3.2	4.0	4.8
Rhamnose						
	*2,3,4-	0.9	0.3	0.6	0.0	0.0
	3,4-	1.6	0.7	1.5	4.8	1.8
	3-	4.1	2.2	5.1	4.6	8.0

(D_2): These data represent dideuterated derivatives (D_2 at C-6) in this column

Ave: Data is the average from duplicate hydrolyses: Maximum deviation
from the mean of ± 20%

* : Derivatives subject to some loss through volatility (small losses)

Eberhard (126). However high $2,4,6$-Me$_3$-galactose suggests
the presence of a linear galactan portion of the water
soluble molecule, or existence of a distinct polymer. It
is suggested that a water soluble arabinogalactan of the
type well characterised in larches and other coniferous
tissues (49, 50) can only be a minor component of this
fraction.

There is little evidence in this fraction of a xyloglucan,
though the low level of $2,3,4$-Me$_3$-fucose probably arises
from such a polymer.

The $2,3,6$-Me$_3$-glucose, present in high levels relative to
$2,3$-Me$_2$-glucose and $2,3,4,6$-Me$_4$-glucose, probably arise
mainly from residual starch which is solubilised by the hot
water.

The analyses of the linkages within the pectin are derived
from four sets of methylation data. In the material
methylated only once, before reduction, the proportion of
neutral derivatives to each other is similar to that for the
5 times methylated analysis, but some under methylation is
evident. (increased unmethylated Gal; Ara etc; decreased
$2,3,5$-Me$_3$Ara; $2,3$-Me$_2$Ara; $2,3,4,6$-Me$_4$Gal; $2,3,6$-Me$_3$Gal;
$2,3,6$-Me$_3$Glc). After the sample has been once reduced with
NaBD$_4$ there was an increase in the proportion of $2,3,$-Me$_2$-
galactose, 2-Me-galactose, and unmethylated galactose. These
must have resulted from reduction of galacturonic acid. Upon
a further Hakomori methylation, the relative proportions are
seen to shift to an increase in $2,3,6$-Me$_3$-galactose, and
$2,6$-Me$_2$-galactose, due to methylation of the C-6 position
(previously inaccessible in galacturonic acid). The neutral
sugar derivative ratios after this second methylation now
indicate almost complete methylation.

The results to the second reduction, and a subsequent two
Hakomori methylations, indicates a further increase in
$2,3,6$-Me$_3$-galactose, corresponding to 4-linked polygalacturonic
acid and there are increased levels of 2,4-linked rhamnose.

The 2,6-Me$_2$-galactose is reduced in proportion, so some of this may have arisen from a small amount of under methylation. Unmethylated galactose is absent now due to complete methylation of the galacturonic acid after reduction.

The increased 3-Me-rhamnose is prominent at this point indicating the high level of branching of the rhamnose in the pectin.

The analysis by combined gas chromatography - mass spectrometry of methylated and reduced samples, afforded the identification of methylated alditol acetates derived from uronic acids, by the presence in their mass spectra of ion fragments which are two mass units higher due to the incorporation at C-6 of two deuteriums (some fragments exist of 1 mass unit increase over the Do case, which have arisen from a primary fragment by loss of a molecule containing one deuterium). The uronic acid derivative was quantitated by the ratio of intensity of fragments from the dideuterated compound to the total intensity of fragments from both the deuterated and non-deuterated neutral methyl derivative. The results could be averaged for sets of fragments corresponding to the various splitting patterns for a derivative.

After methylation and a single reduction, deuterium label was found in the mass-spectrum of 2,3-Me$_2$-galactose and 2-Me-galactose confirming that those derivatives arose from uronic acid. After reduction and remethylation, deuterium label was found in 2,3,6-Me$_3$-galactose and 2,6-Me$_2$-galactose, corresponding to the increases in relative f.i.d. area. The proportions of uronic acid derivatives determined from mass spectrometry are shown in Table 6.2, and are derived as explained in Appendix 1 part 4.

In the 2,3,6-Me$_3$-galactose peak, analysis of all fragments arising from the deuterated molecule showed that approximately 67% of the peak was derived from 4-linked galacturonic acid. Thus 4-linked GalA to 4-linked galactose is 2:1.

Then allowing for this level of 4-linked GalA in the 2,3,6-Me$_3$Gal peak, the ratio of neutral galactose derivatives corresponds to the 5 times methylated analysis for neutrals. These increases determined from relative f.i.d. responses agree with calculations made from mass-spectrometry. The results suggest that the predominant linkage in the wall pectin is that of 4-linked galacturonic acid as for many wall pectins (3) with possibly some branching at C-3 of galacturonic acid.

There also appears to be some terminal galacturonic acid and a smaller amount of terminal glucuronic acid, or 4-O-methy-glucuronic acid present in this fraction. Branching appears to be provided for mainly via carbon 4 of rhamnose, 2-linked in the main chain of polygalacturonic acid.

The results of methylation and reduction analysis of the whole 100°C water fraction constitute the main evidence in this work for the nature of linkages within the pectic acid, and is further discussed in Section 6.4.

6.3.2 Methylation of 10% KOH and GTC Fractions

These fractions were methylated by the multiple Hakomori procedure, and after hydrolysis and isotopic labelling of the reducing end of sugar derivatives (Section 6.1) results were analysed by gas chromatography and combined gas chromatography-mass spectrometry. High levels of 2,3-/3,4-Me$_2$-xylose, 2,3,6-Me$_3$-glucose, 2,3-Me$_2$-glucose, 2,3,4-Me$_3$-fucose (terminal) and 3,4,6-Me$_3$-galactose (2-linked) indicated the presence of a fucogalactoxyloglucan. The high 2,3-/3,4-Me$_2$-xylose ratio suggested the presence also of some xylan. Mass spectrometry of these fractions enabled the relative quantitation of proportions of 2,3-/3,4-Me$_2$-xylose, distinguishable after reduction with borodeuteride. Any 2,3-Me$_2$-xylose should arise from a xylan, and the 3,4-Me$_2$-xylose (2-linked xylose) from xyloglucan, confirming the presence of these separate polymers. The 2,3-/3,4-Me$_2$ xylose ratio was quantitated by the m/e ratio 189/190 as

Table 6.3: Methylated Polysaccaride Sugar Residues for
             Fractions Extracted by 6M GTC, and 10% KOH,
             from Batch 2 Walls - µ moles/g 70°C Water Residue

(Data was normalised as described in Section 2.4.7.3)

Sugar	O-Methyl ether	6M GTC	10% KOH
Arabinose			
	*2,3,5-	7.2	6.5
	3,5-	0.6	1.3
	2,5-	2.6	1.9
	2,3-	5.7	6.5
	2-	3.1	2.9
	3-	1.1	1.0
	-	1.7	2.3
Xylose			
	*2,3,4-	7.8	15.8
	2,3-	6.6	4.8
	3,4-	3.3	7.2
	2-	2.1	1.5
	3-	0.8	2.2
	4-	tr	1.5
	-	0.4	1.5
Fucose			
	*2,3,4-	1.5	2.9
Galactose			
	*2,3,4,6-	7.5	9.2
	2,4,6-	0.9	1.2
	3,4,6-	3.3	4.1
	2,3,6-	3.8	4.1
	2,3,4-	5.2	0.9
	2-	0.2	1.0
Glucose			
	*2,3,4,6-	0.7	1.9
	2,3,6-	4.9	10.7
	2,3-	6.0	16.8
	6-	0.0	1.1
Mannose			
	2,3,6-	2.7	4.7
Rhamnose			
	*2,3,4-	0.9	tr
	3,4-	0.9	1.5
	3-	1.7	1.2

GTC methylated (6 times) by multiple Hakomori, purified by
                                                    Dialysis
10% KOH methylated (6 times)   "   "   "   "
* Derivatives subject to some loss through volatility
tr = trace

described in Appendix 1, Part 1, and Appendix 2.   The three
mono methyl xylose derivatives could be individually
quantitated within a peak, by mass spectrometry (ion fragment
analysis) as explained in the Appendices.   (Appendix 1, Parts
1 and 3, and Appendix 2, Part 2.)

In the GTC fraction, the ratio of 2,3- to 3,4-Me_2-xylose is
approximately 2:1 (Table 6.3) and levels of 2-, 3- and
4-Me-xylose are as indicated.   There appears to be no
4-Me-xylose in the six times methylated GTC fraction.

Similarly, the 10% KOH fraction has 2,3-Me_2-xylose/3,4-Me_2-
xylose in a ratio 2:3 from deuterium labelling of the
reducing ends.   Levels of 2-, 3- and 4-Me-xylose are as
indicated in Table 6.3.   Levels of 4-Me-xylose may be due
to a small amount of undermethylation.

The linkage analysis for xylose residues suggests that in
both fractions, both a xyloglucan and a hemicellulosic
xylan are present which is highly branched.   Thus, from the
μ moles/g 70°C water residue of above residues, it appears
that GTC removes more of the xylan, and 10% KOH, more of
the xyloglucan, about twice as much xyloglucan as in 6M GTC.
The ratio of these two polymers can be assessed by the ratio
of 2,3-Me_2-xylose (from xylan) to 3,4-Me_2-xylose (from
xyloglucan).   Thus, as deduced from the xylose/glucose
ratios in Chapter 5, the 6M GTC fraction is relatively richer
in xylan and the 10% KOH fraction in xyloglucan.   The levels
of other derivatives in Table 6.3 (2,3,4-Me_3-fucose, 3,4,6-Me_3-
galactose, 2,3,6-Me_3-glucose, 2,3-Me_2-glucose) support this
conclusion.   The actual levels of xylan and xyloglucan in
the two fractions can be assessed from the data of Table 6.3
by consideration of the structures of the two polymers.   The
xylan is highly branched (2-Me-xylose, 3-Me-xylose) and it
is possible that a 4-O-Me-glucuronoxylan is present. Terminal
arabinose derivatives in the alkali fraction (Batch 1) and
GTC and 10% KOH (Batch 2) could be attributable to xylan as
well as attributable to arabino-4-O-methyl-glucuronoxylans
of coniferous tissues (see Section 1.3.2 and 1.3.10).   The

principal types of derivatives associated with xyloglucans indicated the following types of linkages in mole ratios, compared between callus tissue and hypocotyl (145). (Table 6.4.)

Table 6.4:   Mole Ratios of Derivatives Associated with Xyloglucan, Normalised to Total Glucose (4,6-Glc plus 4-Glc).

	4,6-Glc	4-Glc	t-Xyl	2(& 4)-Xyl	t-Gal	t-Fuc	2-Gal
Callus							
alkali (Batch 1)	43	57	28	28	19	9	10
alkali (Batch 2)	61	39	57	43	32	10	14
Hypocotyl	67	33	50	17	8	8	8
* semi-purified							

In the callus alkali samples some of the xylose derivatives could be associated with xylan, such as the xylan observed in the studies described in Chapter 4, glucose derivatives with glucomannan, and some terminal galactose with galactan.

A structure for the xyloglucan cannot be properly formulated without its isolation but the data suggest a 4-linked glucan backbone, with substitution at C-6 of glucose by xylopyranose residues.   Branches at C-6 of glucose could have side chains of α-L-Fucp1→2-β-D-Galp1→2-α-D-Xylp1→C-6 and so on as in 1.3.2, Figure 1.3).   Bearing in mind the likely structures of xylan, xyloglucan and glucomannan, the derivatives corresponding to these polymers can be approximately proportioned from the data of Tables 6.3 and 5.8 for the 10% KOH fraction of Batch 2 on a quantitative basis.   This was performed assuming that for glucomannan, in the 10% KOH fraction, the glucose/mannose ratio is approximately 1:3. Once these derivatives were allotted, the derivatives for xylan and xyloglucan were proportioned for branch and terminal residues as befitted the structures expected.   The sum for each derivative equals the total level of derivative in the fraction (see Table 6.5).

Table 6.5: Quantitation of Derivatives for Some Poly-
saccharides of the 10% KOH Fraction, Batch
2 Walls. μ moles/g $70^{\circ}C$ Water Residue.

	Polysaccharides			
	Xyloglucan	Xylan	Glucomannan	$(1 \rightarrow 4) -$ Galactan
Derivatives				
Terminal Xylose	10.0	6.0		
$2,3-Me_2Xyl$		5.0		
$3,4-Me_2Xyl$	7.0			
$2-MeXyl$		1.5		
$3-MeXyl$		2.0		
Xyl		1.5		
Terminal Glucose † and Mannose	1.0		1.0	
$2,3,6-Me_3Glc$	9.0		2.0	
$2,3-Me_2Glc$	17.0			
Terminal Galactose	4.0 *			
$2,3,6-Me_3Gal$				$\sim 4.0 - 5.0$
$3,4,6-Me_3Gal$	3.0 - 4.0			
Terminal Fucose	3.0			
$2,3,6-Me_3Man$			~ 6.0	
Totals #	55	16	9	5

* residual Terminal Galactose = 5.0 μ moles: probably due to a branched galactan (Table 6.3).

Terminal Arabinose in the fraction appears to be mostly associated with an Arabinan (Table 6.3).

These are approximate levels of polysaccharides estimated.

† some Terminal Glucose associated with xyloglucan as well.

If the data is normalised to 100 mole % of total derivatives the following ratios can be compared (see Table 6.6).

Table 6.6: Derivatives as Approximate Mole % of Total for Xyloglucan and Xylan.

Derivative	Xyloglucan		Xylan	
	Callus (KOH)	Hypocotyl	Callus (KOH)	Hypocotyl
Terminal - Xyl	18	26	38	34
2,3-Me$_2$Xyl			31	43
3,4-Me$_2$Xyl	13	9		
2-MeXyl			9	21
3-MeXyl			13	
Xyl			9	1
Terminal Glucose	2			
2,3,6-Me$_3$Glc	17	17		
2,3-Me$_2$Glc	31	35		
Terminal - Gal	7	4		
3,4,6-Me$_3$Gal	6	4		
Terminal - Fuc	6	4		

It must be stressed that these data are only approximations, as far as can be deduced from expected structures, and balances between terminal and branch residues for frameworks believed to be present. The xyloglucan in the 10% KOH fraction of Batch 2 callus would appear to be similar to that of hypocotyl, perhaps containing slightly longer regions of 4-linked glucose, and less terminal xylose. Thus it seems there is evidence for a xyloglucan in callus primary walls, similar to that found by J.W. Little in Pinus hypocotyl (145) as well as to that of seed amyloids (46) and to the xyloglucans observed in cambial and mature tissue of angiosperms (Section 1.3.2). The xylan of callus may be somewhat more branched than that of hypocotyl of Section 4.3.

Mannan (2,3,6-Me$_3$Man) in some fractions, appears to be only a minor hemicellulose in both Batches, agreeing with data in

Section 5.3.3). Thus the tissue is predominantly primary walled.

6.4 DISCUSSION. BATCH 1 AND 2 WALLS

As noted in Section 6.2 and 6.3, linkage residues of 5-linked arabinans and 1,4-linked galactans are present throughout wall fractions. These are highest in fractions with high uronic acid and rhamnose (e.g. 100°C water fractions Batch 1 and 2), but also present in chaotropic extracts (Batch 2), alkali extracts, and the alkali residues to both wall preparations. The methylation data (see Tables 6.2, 6.3) suggest the arabinans may be much like others for Pine (137, 140, 148) with (1 → 5)-linked arabinofuranose units with branching at C-2 and sometimes at C-3. The level of arabinan is highest in the 100°C water fraction Path B of Batch 1, much being extracted here in the place of the alkali extracts of Path A.

Lower levels of 2,3,6-Me$_3$-galactose (suggesting pectic type galactan (42, 47, 141)) are found in alkali extracts. An acidic 4-linked galactan has been found in Tamarack (139) in Pine (137) and a similar galactan has been found in compression wood of Red Spruce, in normal woods of various conifers (138), and as part of the pectin in cambial tissues of Populus tremuloides (141). In Sycamore, Albersheim et al (47) demonstrated a component bonded to pectic poly- galacturonic acid, with similar branched arabinan, and linear 4-linked galactan to those in Pine. He attributed this to pectic arabinan and galactan, directly linked to the acidic rhamnogalacturonan. Possibly in the 100°C water fractions here, the arabinan and galactan are associated covalently with the acidic wall polymers. This possibility is further explored in Chapter 7. In subsequent fractions also (Table 6.1, 6.2, 6.3) branched arabinan is probably associated with galactan in part of an overall pectic complex (198), which may be covalently linked to other polymers such as hemicellulose.

Pectin. The wall pectin (polygalacturonic acid containing rhamnose) is distributed throughout fractions as for pectic arabinan and galactan, but is high in high temperature aqueous fractions which are removed by β-elimination of uronic acids and Ca^{2+} chelation in both walls, and also high in the alkali residues. Some pectin is also extracted with hemicelluloses by chaotropic reagents and more extensively by alkali. The main component of the pectin in the $100^{O}C$ water fraction (Batch 2) was observed by methylation to be 4-linked galacturonic acid (Section 6.3). Some branching of polygalacturonic acid is possible (Table 6.2) though this appears to be minor. The relative lack of deuterium in branched galactose residues implies that GalA residues are mostly unbranched.

This would be true of the reduced, neutral residues in the chloroform/methanol soluble MRMRMM sample. However, it is not clear to what extent the sample reflects the total pectin structure. An estimate of the total uronic acid accounted for in the methylation reduction analysis can be made by comparing the yield of dideuterated residues (Table 6.2) with those of non-deuterated sugar residues (using the values for the latter given in Table 5.8). This approach yields a value for GalA of approximately 50 μ mole/g $70^{O}C$ residue, which is only 30% of the value obtained for the total $100^{O}C$ water fraction by spectrophotometric assay. It is likely therefore that a major portion of the galacturonic acid residues in the sample (∿70%) must have resisted reduction. These non-reduced acidic residues would not appear in the methylation analysis. Calculations show that in MRM, only 15% and in MRMRMM 30% of the total pectin had been reduced. Further reductions were not attempted owing to shortage of time and material. Although the galacturonan backbone in the fully reduced and methylated sample was unbranched (except for rhamnose residues), the non-reduced portion (not observed in the methylation analysis) might contain branching on GalA residues. Siddiqui and Wood (204) have proposed for Rapeseed cotyledon meal pectin, that GalA was highly branched, and they did demonstrate branched GalA residues in their methylation analysis. Their proposal was based on observing

they had not achieved complete reduction of the pectin, and a consideration of the requirement to balance terminal and branched residues in their methylation analysis. In this study also, an excess of terminal residues (e.g. high $2,3,4,6-Me_4Gal$) over branch residues was observed in the $100^{O}C$ water fraction under discussion (Table 6.3) as well as in several other fractions (Table 6.2). This too may reflect an undetected branching of GalA residues in the pectin. It is not clear however, why reduction of GalA should occur preferentially on unbranched residues.

Branching has been related to reduced cohesion for pectins in tissues undergoing rapid enlargement (43). But actively growing cells such as sycamore suspension-cultured cells (8, 46, 47) have pectin with low branching and low rhamnose. The latter situation might be a closer parallel to that of the pine callus wall.

In all fractions methylated, the presence of low levels of $3,4-Me_2$-rhamnose, and 3-Me-rhamnose, suggest that the poly-galacturonic acid is interrupted with a low level of rhamnose, 2-linked in the main chain (3). In hot water and subsequent fractions, higher levels of 3-Me-rhamnose than $3,4-Me_2$-rhamnose in both batches, imply that much of the rhamnose is branched through the 4-position, providing for the only detectable branching in the pectin. This probably represents the attachment site for the neutral arabinan or galactan to the rhamnogalacturonan backbone of the overall pectic complex. Somewhat higher levels of 3-Me-rhamnose in the alkali and residue fractions in Batch 1, might suggest that the more tightly bound pectin is more highly branched.

The distribution of pectin amongst fractionations tends to indicate that a diverse scheme of interactions might hold the pectin in the wall. It is not totally removed by conditions which degrade polygalacturonic acid by $\beta-$elimination (143, 191) or by metal ion chelators that help solubilise pectins, and more is removed by alkali after GTC, or urea extractions. After strong alkali extractions which would completely hydrolyse all ester links, pectin is still

found in the residues.   Thus, much of the pectin appears to
be held in the wall by alkali labile links, or by linkage to
polymers requiring alkali for extraction.   The residual
pectin may be interacting with lignin in the wall in some
manner preventing complete extraction with alkali.

Non Pectic Galactans.

Levels of 2,4,6-Me_3-galactose, 2,3,4-Me_3-galactose, and
2,4-Me_2-galactose, corresponding to 3-linked, 6-linked and
3,6-linked galactose are high in water soluble fractions
though present to some extent in subsequent fractions. These
have been shown to be components of 3,6-linked Type II
arabinogalactans, in coniferous tissues (49, 50) and have
been found in an arabino-galactan in Pine by Roudier and
Eberhard (126).   For Batch 1 walls, the level of 2,4,6-Me_3
Gal increases with increasing temperature in water
extractions, and in Batch 2 walls, it is again high in the
100°C water fraction.   From experiments with DEAE-cellulose
chromatography discussed in Chapter 7, it is confirmed that
most of the 2,4,6-Me_3-galactose corresponds to a 3-linked
linear galactan, requiring hot water for extraction.   In the
lower temperature aqueous extracts, a Type II arabinogalactan
structure would appear to be more dominant.   These "loosely
bound" galactans are difficult to place in a defined
category as regards bonding or function in the wall, but they
might appear to be wall components.

Hemicelluloses.

As described in Sections 6.2 and 6.3, hemicellulosic xylan
and xyloglucan are present in alkali fractions of Batch 1,
and GTC, urea, and alkali fractions of Batch 2.   Alkali
appears to remove more xyloglucan than xylan.

Methylation analyses of selected fractions of Batch 1 and
Batch 2 walls, provided further evidence for conclusions
reached from sugar analyses in Chapter 5.   These analyses
confirm the presence of pectic type arabinans, galactans,
3,6-arabinogalactan, rhamnogalacturonan and hemicelluloses,
xylan (perhaps (4-O-methylglucurono) xylan) and xyloglucan.

Table 6.7: Estimated Levels of Polysaccharides in Batch 1 Walls: mg/g 70°C Water Residue
(Estimated from methylation, and sugar data, Tables 6.1, 6.2, 6.3; 5.5, 5.6, 5.7, 5.8).

	XG	X	BG	A	G	Pectin
4° & 70°C H_2O	1.0	tr	6.0	7.0	3.0	15.0
PATH A						
Oxalate + EDTA	tr	1.5	4.5	9.0	2.5	23.0
Na_2CO_3	-	0.2	2.0	8.0	2.0	15.0
*Alkali extracts	40.0	10.0	11.0	45.0	22.0	33.0
*Alkali + BO_3^{3-}	2.5	<1.0	2.0	12.0	5.0	7.0
Residue #	7.0	1.0	tr	44.0	8.0	27.0
PATH B						
100°C H_2O	-	1.0	17.0	53.0	9.0	51.0
Oxalate + EDTA	tr	1.0	3.0	8.0	2.0	25.0
6M urea	2.0	tr	tr	2.0	2.0	4.0
Na_2CO_3	-	0.1	<1.0	7.0	1.0	12.0
*Alkali + BO_3^{3-}	36.0	10.0	5.0	18.0	16.0	14.0
Residue #	5.0	1.0	tr	25.0	13.0	31.0

XG,   Xyloglucan (glucan backbone)

X,   Xylan (4-linked xylose backbone)

BG,   Branched Galactan (includes 3-linked galactan)

A,   Arabinan (5-linked pectic arabinan)

G,   Galactan (4-linked pectic galactan)

Pectin, Rhamnogalacturonan (4-linked GalA)

tr = trace

*   contain small levels of Mannan (4-linked)

much of the glucose in the residues could have arisen from hydrolysis of cellulose, though fucose-rich xyloglucan may be present.

Table 6.8: Estimated Levels of Polysaccharides in Batch 2 Walls: mg/g 70°C Water Residue

(Estimated as in Table 6.7, from methylation and sugar data)

	XG	X	BG	A	G	Pectin	
4° + 70°C Water	2.0	tr	6.0	12.0	3.0	71.0	
H$_2$O + EDTA 100°C	1 - 2.0	0 - 1.0	14.0	25.0	6.0	34.0	XG, X, BG, A, G, Pectin; represent polymers as explained on Table 6.7
Chaotropic reagents	6.0	4.0	3.5	4.5	1.5	1.5	
Na$_2$CO$_3$ + labilised	0.2	0.1	0.8	2.1	0.6	3.0	M̲ Fraction contains low levels of Mannan
M̲ Alkali extracted	10.0	3.6	tr	10.0	6.3	9.1	
ClO$_2^-$ + labilised	1.0	2.0	tr	19.0	7.0	8.6	G̲ Much of the Glucose in this fraction could have arisen from some hydrolysis of cellulose, though a fucose-rich xyloglucan may be present.
G̲ Residue	3.4	1.0	tr	8.0	5.0	4.6	

The approximate quantitated summary of wall polysaccharides has been attempted as outlined in Tables 6.7 and 6.8. These results were obtained as in Section 6.3 for Table 6.5, by proportioning the quantities of derivatives according to the expected structures of polysaccharides. Using data of Tables 6.1 - 6.3 and 5.7, 5.8, the levels of polysaccharide were estimated and these, considered with the weights of sugars removed, enabled the approximate weight of polysaccharides in fractions to be estimated. It should be stressed that these were semi-empirical calculations and the Tables 6.7 and 6.8 show semi-quantitative rather than precise data since pure polysaccharides were not isolated.

In concluding, the following remarks seem appropriate.

In Pinus radiata callus the GalA/Rha ratio is high, as in suspension-cultured sycamore cell walls. The overall ratio in aqueous extracts is approximately 40:1 for both batches, but in subsequent extracts GalA/Rha decreases for Batch 1 and Batch 2 walls. Thus, probably the callus pectin has a low rhamnose level, and low branching of GalA, consistent with methylation data on the 100°C water fraction, for Batch 2 walls, and levels of methylated rhamnose derivatives for Batch 1. This would enable relative extraction by β-elimination of homogalacturonan regions of the pectin, leaving behind a material of high rhamnose content. The heterogeneity of the pectin as a result of selective extraction of GalA-rich polymers by β-elimination is demonstrated in the next chapter by fractionation studies of the pectin by DEAE-cellulose chromatography. Darvill et al (199) extracted pectin from sycamore cell walls with polygalacturonase instead of hot water, thus obtaining a polymeric product with much lower GalA content.

The rhamnogalacturonan would be typical of many pectins (3, 8) with some kinking of the main chain provided for by the 2-linked rhamnose. The branching of rhamnose examined by methylation analysis in Pinus callus fractions was high, usually round 75%, but branching of GalA residues was slight.

The C-4 of rhamnose may be linked to neutral polysaccharide, such as the 4-linked galactans in callus. Different pectins vary in levels of arabinose and galactose which normally appear as pectic arabinan and galactan linked to pectin through rhamnose as postulated here, into a pectic complex.

The components of a 3,6-galactan/arabinogalactan, (8, 49, 50, 126) are tentatively assigned as integral wall components, with cohesive functions. The 4-linked galactan and 5-linked arabinan are in evidence throughout wall fractions.

Much of the pectic complex requires alkali for its release, (subsequent to β-elimination of pectin) probably due to linkage to non GTC-labile hemicelluloses.

Alkali-soluble xyloglucans (after preliminary cleavage of pectin) are confirmed by methylation data in Batch 1. Methylation confirms that xyloglucan can be removed by chaotropic reagents in Batch 2 demonstrating its bonding by hydrogen-bonds in the wall. Hemicellulosic xylan and xyloglucan are confirmed in alkali fractions of Batch 2, with pectic polymers, supporting the simple sugar analyses of Chapter 5 that, more resistant bonds exist, holding much of the pectin and hemicellulose in the wall. It is expected that most ester linking would be hydrolysed by Na_2CO_3 (149, 205) and therefore alkali could be cleaving more resistant esters, or stronger bonds to lignin and/or protein, or unspecified links to cellulose (2).

CHAPTER 7

PURIFICATION OF SOME PECTIC POLYSACCHARIDES BY
CHROMATOGRAPHY ON DEAE-CELLULOSE

7.1   INTRODUCTION

The cell wall fractions studied in the previous two chapters
were highly complex, as indicated by the methylation analysis,
and evidently contained mixtures of several polysaccharides.
To investigate this heterogeneity a chromatographic fraction-
ation was attempted on the largest cell wall fraction, namely
the 100^OC water fraction, from Batch 2 walls. Analytical
data of the preceding chapters suggest that the fraction
contained pectic polymers (rhamnogalacturonan, arabinan, and
galactan) - presumably liberated by a β-elimination reaction -
as well as a Type II arabinogalactan or galactan, and some
starch and minor amounts of a xylan.

This chapter reports the results of efforts to separate some
of these components by ion exchange chromatography on DEAE-
cellulose.

7.2   DEAE-CELLULOSE CHROMATOGRAPHY OF 100^OC WATER EXTRACT
OF BATCH 2 WALLS

12ml of this fraction (100^OC water fraction of Batch 2 walls,
containing 50mg dry weight) was diluted to 44ml, with sodium
phosphate buffer pH 7.0 (.01M final concentration), this
volume being adequate for solution of much of the fraction.
The sample was brought into solution by heating briefly to
100^OC, cooling and centrifuging to remove a trace of insoluble
matter.

The sample was applied to a column of DEAE-cellulose (Whatman
DE-32, 5.8 x 2.3 cm, 24ml bed volume, pre-equilibrated with
.01M sodium phosphate buffer pH 7.0), and fractions of approx
4ml were collected by an automatic fraction collector.

Figure 7.1 : Fractionation of soluble polysaccharides of wall pectin DEAE, run 1.
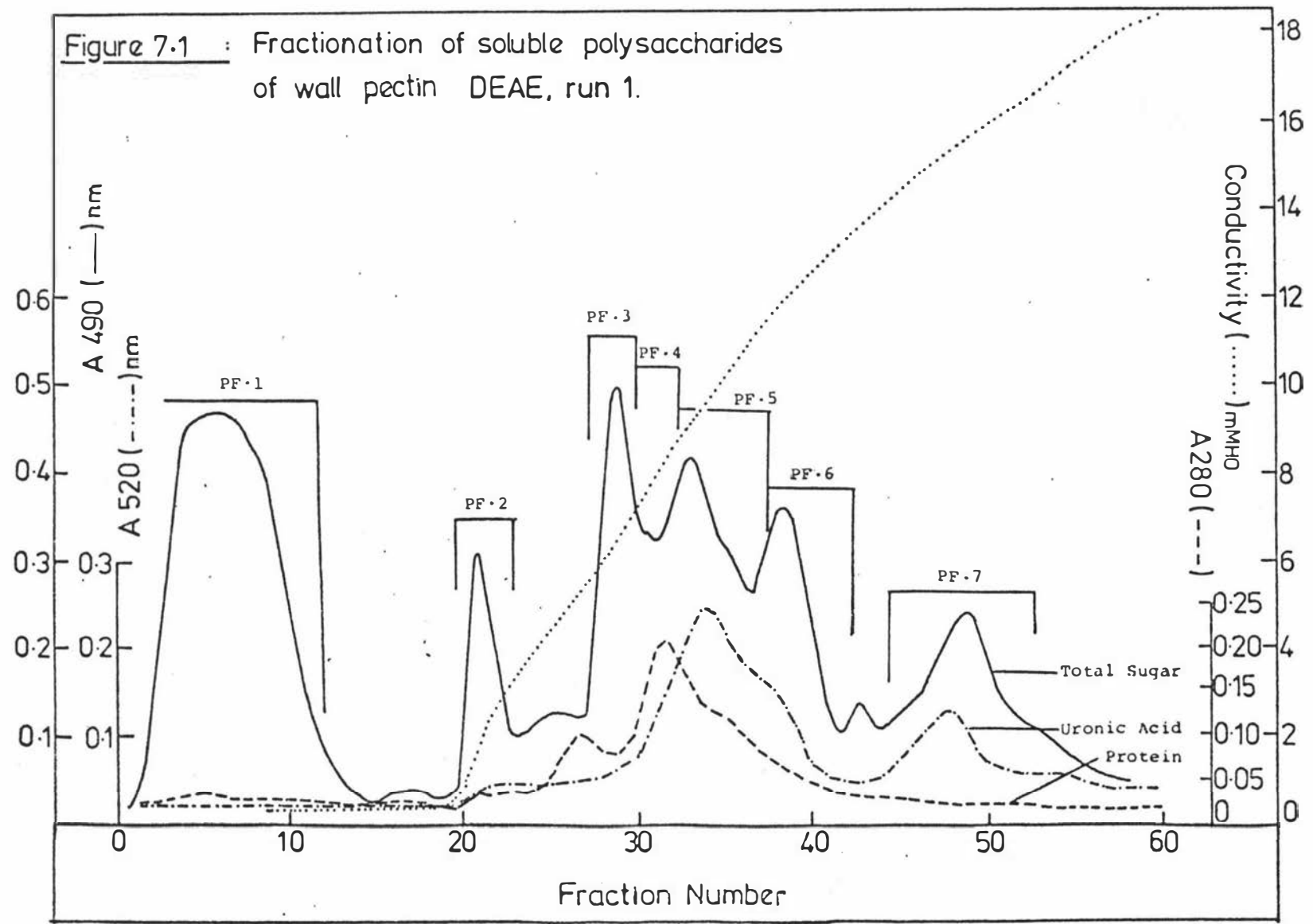
Elution was effected by 100ml of 0.01M sodium phosphate buffer pH 7.0 followed by a linear gradient of sodium chloride (0.0 - 0.5M) in .01M sodium phosphate buffer pH 7.0. Elution of material terminated in advance of residual gradient run over the column. Fractions were assayed for protein, (A_{280}), total carbohydrate (phenol-sulphuric acid assay on 200μl aliquots of each fraction) and uronic acid (m-hydroxydiphenyl assay on 100μl aliquots). The results are plotted as an elution profile in Figure 7.1. The conductivity of fractions is also indicated.

Figure 7.1 shows that a large neutral carbohydrate fraction was eluted first, followed by a heterogeneous series of acidic fractions, containing varying amounts of uronic acid and/or protein.

Fractions were pooled as shown on Figure 7.1, each fraction corresponding to a peak in the elution profile of carbohydrate or protein. The neutral fraction (PF.1) was divided in the ratio 4:1 and the larger portion set aside for methylation analysis. The smaller portion and all the other pooled fractions were analysed for monosaccharide composition as described in methods Section 2.3.1. Hydrolysis of the lyophilised fractions was performed with HNO_3/urea.

The monosaccharide analytical data are shown in Table 7.1. It is clear that separation of distinct polysaccharide components has occurred. The major components are tentatively identified on the table, with comments below. In fraction PF.1 it appears that there was a neutral galactan or arabinogalactan perhaps contaminated with a small amount of glucan (possibly residual starch). Methylation analysis (Section 7.3) confirmed this identification. Fraction PF.2 may be an arabinogalactan, or arabinogalactan-protein, while Fraction PF.3 appears to be a distinct polysaccharide, possibly an acidic arabinan.

Although fractions PF.5, 6 and 7 apparently represented three distinct pectin fractions; fraction PF.5 was the major uronic acid fraction. It is eluted at lower salt concentration than

Table 7.1:   Neutral Sugar Percentage Composition of Fractions of DEAE-Run 1

Fraction (PF)	Sugars							Polysaccharide
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	
1			6.3			86.0	7.9	Galactan
2		1.3	43.0	2.0		54.0		Arabinogalactan (-protein)
3	0.4	0.3	85.4	-		14.0		Acidic Arabinan
4	5.4	0.81	63.0		0.6	29.0	1.1	*Arabinogalactan (-protein)
5								Pectin 1 (Sample lost)
6	11.7	2.5	58.0	1.0	0.35	26.0	0.93	Pectin 2
7	2.7	2.1	71.0			24.1		Pectin 3
								* or Arabinan and Galactan

Unfortunately fraction 5 containing much of the uronic acid was lost, and neutral sugar data to this fraction is not available.

Fraction PF.6 which has a relatively higher neutral sugar content (see Figure 7.1). The order of elution of these two fractions may be governed by the degree of esterification of the uronic acid carboxyl groups (42). Alternatively covalent association with protein may have a role here.

The association of arabinose and galactose in all the acidic fractions may indicate covalent linkage to either pectin or protein. Further information on the arabinan fraction was obtained from a subsequent DEAE-cellulose fractionation, the results of which are presented in Sections 7.4 - 7.6.

The major protein peaks in Figure 7.1 do not correspond to the carbohydrate peaks and no association between protein and polysaccharide can be inferred from the data presented here. The protein analytical data discussed in Chapter 5 suggest that much of the protein in the $100^{\circ}C$ water fraction of Batch 2 walls was, in fact, cytoplasmic protein. Amino acid analyses on selected fractions eluted from DEAE-cellulose are presented in Section 7.5 and show no features that would indicate that they are wall proteins.

7.3   METHYLATION OF NEUTRAL GALACTAN

The major portion of pooled fraction 1 from the DEAE-cellulose chromatography described in Figure 7.1 was permethylated, hydrolysed and converted to partially methylated alditol acetates as described in methods Section 2.4.3.1. G.C.-M.S. of the resulting mixture (see Section 2.4.7) revealed the composition indicated in Table 7.2. The glucose derivatives were present in proportions consistent with their origin from starch. This would be a small amount of starch which had resisted α-amylase digestion, as suggested in Chapter 6. The rather high degree of branching of this residual starch (ca. 15% $2,3$-Me_2-glucose) suggests a possible reason for its resistance to α-amylase.

The remaining residues were mostly galactose and arabinose derivatives. Of these, $2,4,6$-Me_3-galactose represented about

Table 7.2: Methylation Data for DEAE - Run 1, Fraction 1
Relative Mole % Composition of Methylated
Polysaccharide Sugar Residues

Monosaccharide	O-Methyl ether	Mole %
Arabinose	*2,3,5 - tri	0.8
	2,3,4 - tri	0.06
	3,5 - di	0.1
	2,5 - di	0.9
	2,3 - di	2.5
	2 - mono	4.6
	- - nil	2.8
Xylose	*2,3,4 - tri	0.1
	2,3-/3,4 - di	0.3
Galactose	*2,3,4,6 - tetra	1.9
	2,4,6 - tri	72.4
	2,6 - di	1.0
	2,3 - di	0.6
	2,4 - di	1.8
	2 - mono	1.1
Glucose	* 2,3,4,6 - tetra	0.6
	2,3,6 - tri	6.9
	2,3 - di	1.3
Rhamnose	3,4 - di	0.1
	3 - mono	0.2

* Derivatives subject to small loss through volatility

80 mole %. This suggests that the polysaccharide material
of fraction 1 was largely a linear (1 → 3)-galactan. A low
degree of branching at position 6 of the galactose is
indicated by the $2,4\text{-Me}_2$-galactose. The arabinose residues
may or may not be an integral part of the galactan molecule.
The structure of this linear galactan is considered further
in Section 7.7.

7.4   SECOND (LARGER SCALE) DEAE-CELLULOSE CHROMATOGRAPHY
OF 100^OC WATER EXTRACT OF BATCH 2 WALLS

The sample of 100^OC water fraction (250mg) was suspended in
a total volume of 200ml 0.01M sodium phosphate buffer pH7.0.
The sample was brought into solution by heating briefly to
100^OC, cooled and centrifuged at 2900g for 5 minutes in a
Sorval centrifuge, to remove a trace of insoluble material.

The supernatant was applied to DEAE-cellulose (Whatman DE-32,
8.2 x 2.3cm, 34 ml bed volume, pre-equilibrated with 0.01M
sodium phosphate buffer pH 7.0), and fractions 7 - 8ml were
collected by a timed automatic fraction collector. After
application of the 200ml of sample, it was washed through
with 100ml sodium phosphate buffer and acidic polysaccharides
were eluted with 400ml 0.01M sodium phosphate buffer pH 7.0
containing NaCl (0 - 1.0M linear gradient). Elution was
complete before the last of the gradient was passed over the
column. The increased final salt concentration over that
used in the previous separation (Section 7.2) provided for
the elution of any more tightly bound acidic material.

As shown in Figure 7.2 the rather steeper gradient used in
elution of the second run on DEAE-cellulose resulted in a
crowding of some of the fractions that had been separated
previously (Figure 7.1). In particular the fraction
identified as an arabinan, (fraction PF.3 in Figure 7.1)
now appeared as a shoulder in the succeeding fractions. In
addition, the separation of distinct uronic acid-containing
fractions was obscured on the second run. On the other hand

Figure 7.2   Fractionation of Soluble Polysaccharides
              of wall pectin DEAE run 2
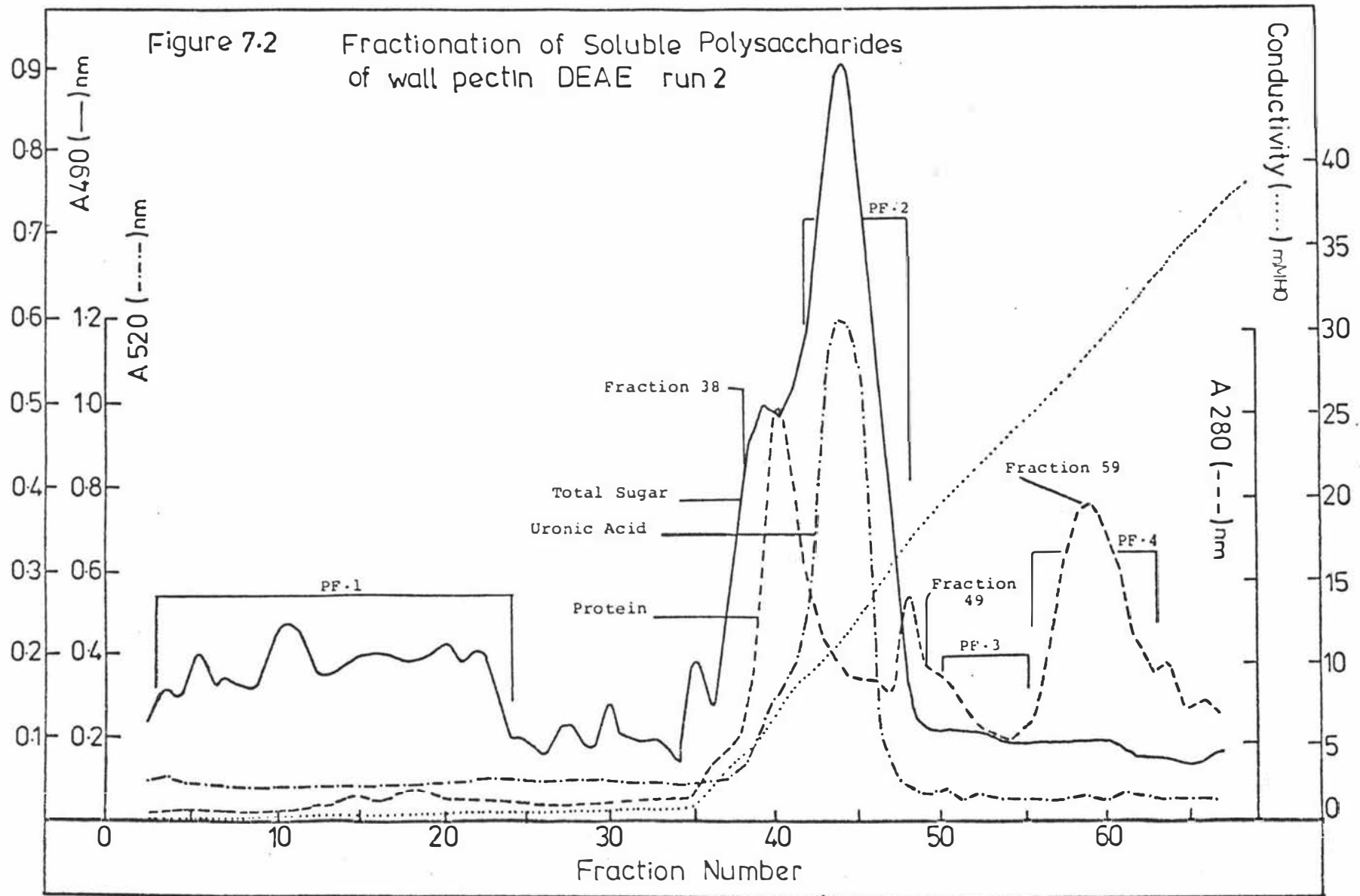
the higher salt concentration revealed a rather tightly
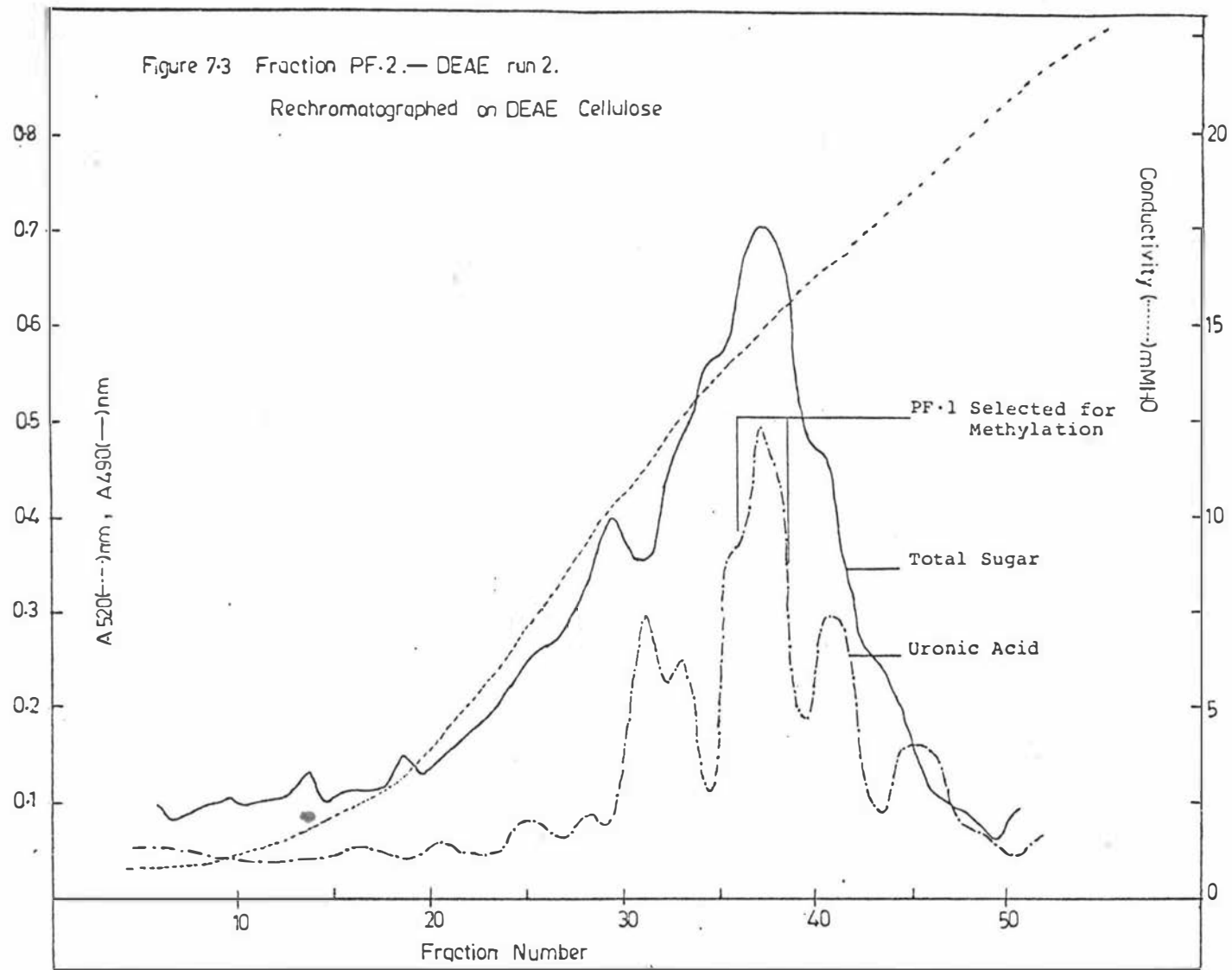bound protein (Figure 7.2).

Selected fractions from the elution profile of Figure 7.2
were pooled for monosaccharide analysis or subjected to
amino-acid analysis (Section 2.2.3). The results are
discussed in Section 7.5. A fraction was selected from the
presumed arabinan region of the profile (Fraction 38,
Figure 7.2) for methylation analysis. This is described
in Section 7.6 below.

Fractions from region PF.2 (Figure 7.2) were pooled and
applied again to a DEAE-cellulose column for gradient
elution with NaCl (0 - 0.5M) in 0.01M sodium phosphate buffer
pH 7.0 as described in Section 7.2, in an attempt to
fractionate the uronic acid-rich polysaccharides. 5ml
fractions were collected. The resulting elution profile
is indicated in Figure (7.3). As can be seen, separation
of several distinct uronic acid-rich fractions was achieved.

One fraction from this re-run was selected for methylation
analysis, by the methylation-reduction technique described
in Chapter 6, for study of uronic acid-containing polymers.
However, the results indicated that selective methylation
of the neutral components of the fraction had occurred and
only a trace of dideuterated $2,3,6-Me_3$-galactose was
obtained. The small sample size may have led to low recovery
of methylated galacturonan, and shortage of material and
time did not permit further structural investigation of these
uronic acid-rich fractions.

7.5   ANALYSIS OF FRACTIONS FROM THE SECOND DEAE-CELLULOSE RUN

The elution profile again shows a neutral fraction and several
acidic fractions as in the first run (Figure 7.1), but with
a further major protein peak at high salt concentration.

Figure 7·3   Fraction PF·2.— DEAE run 2.

Rechromatographed on DEAE Cellulose

PF·1 Selected for Methylation

Total Sugar

Uronic Acid

Conductivity (------)mMHO

A 520(---)nm , A490(—)nm

Fraction Number

The monosaccharide and amino acid analytical data for
selected fractions indicated on Figure 7.2 are presented in
Tables 7.3 and 7.4 respectively.

Table 7.3: Monosaccharide Data, DEAE - Run 2
            Neutral Sugar Mole Percentage Composition of
            Some Fractions

Fraction	Sugars						
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
PF.3	2.9	10.2	38.3	0.0	0.0	26.4	22.1
PF.4	4.6	4.0	67.7	0.0	0.0	18.3	6.1

Table 7.4: Amino Acid Composition of Fractions from DEAE-
            Cellulose Chromatography (as indicated in
            Figure 7.2 and described in the text)
            Amino acids expressed as Mole % for each fraction

Amino Acid	Column fraction		
	40	49	59
Asp	10.9	13.8	7.5
Thr	4.9	4.9	3.8
Ser	9.9	5.8	8.7
Glu	14.8	17.9	11.5
Pro	5.8	5.8	2.3
Gly	10.2	11.5	25.9
Ala	8.6	8.6	5.8
½Cys	δ+	δ+	δ+
Val	6.8	5.8	5.8
Met	1.7	1.15	1.45
Ile	3.6	2.9	2.9
Leu	5.6	5.8	5.8
Tyr	4.0	2.90	4.0
Phe	2.6	2.9	3.8
Lys	2.5	2.0	2.3
His	3.3	2.0	4.9
Arg	2.6	3.8	3.5
Hyp	2.3	2.6	1.2

Fractions 40 and 49 both had low levels of hydroxyproline but no other noteworthy features. Fraction 59 had high glycine levels. It is probable that much of the protein of this 100°C water fraction is not structural wall protein and is of no particular relevance here. Other fractions, which were not investigated from this DEAE run, may have had more hydroxyproline but the predominant fractions appear to be of hydroxyproline-poor protein.

Neutral sugar analyses were obtained for the fractions eluted by high salt concentration, and hence not examined in the first column run. These fractions contained little uronic acid and it is likely that the neutral sugar components were contained in glycoprotein molecules. No further study of soluble glycoproteins was made.

7.6   METHYLATION OF THE ARABINAN-RICH FRACTION

The results of methylation analysis of Fraction 38 (Figure 7.2) are presented in Table 7.5. The level of galactose derivatives is higher than expected when compared with the corresponding pooled fraction 3, (PF.3), of Table 7.2. This may reflect a genuine difference in composition of the fraction resulting from the different sodium chloride gradient used on the second run, or it may result from selective methylation of a galactose rich portion of Fraction 38.

The results in Table 7.5 suggest the presence of a typical pectic arabinan, with high levels of 5-linked, 3,5-linked, and terminal arabinose residues, but the level of 3-linked arabinose residues (shown as 2,5-Me$_2$-arabinose) is unusual and these residues may be associated with an arabinogalactan.

The data for galactose derivatives, suggests the presence of a pectic 4-linked galactan, but also shows a very high ratio of terminal to branched residues. It may be that some of the galactan residues were terminal on a branched arabinan or arabinogalactan. Although the level of uronic acid was

Table 7.5:   Methylation Data of Arabinan-Containing Fraction
            (DEAE run 2, Fraction 38)
            Relative Mole % Composition of Methylated
            Polysaccharide Sugar Residues

Monosaccharide	O-Methyl ether	Mole %
Arabinose	*2,3,5 - tri	11.0
	2,3,4 - tri	0.2
	3,5 - di	0.9
	2,5 - di	9.7
	2,3 - di	12.0
	2 - mono	9.1
	3 - mono	2.7
	- - nil	5.1
Xylose	*2,3,4 - tri	0.3
	2,3-/3,4 - di	0.6
Galactose	*2,3,4,6 - tetra	15.3
	2,4,6 - tri	6.3
	3,4,6 - tri	1.0
	2,3,6 - tri	14.1
	2,3,4 - tri	1.0
	2,6 - di	0.6
	2,3 - di	0.5
	2,4 - di	1.0
	2 - mono	1.4
Glucose	*2,3,4,6 - tetra	1.0
	2,3,6 - tri	1.5
	2,3 - mono	0.1
Rhamnose	3,4 - di	0.5
	3 - mono	4.0

*Derivatives subject to small loss through volatility

relatively low in this Fraction 38 (see Figure 7.2, 15% total carbohydrate of the fraction), the rhamnose level was fairly high, and some of the terminal galactose residues may have been associated with branched rhamnose residues.

7.7   DISCUSSION

The chromatographic runs, reported in this chapter have demonstrated a lack of correspondence between the uronic acid-containing fractions and protein in the 100°C water extracted material.

The protein fractions were probably mostly cytoplasmic and contained only very low levels of hydroxyproline. An association of some sort between protein and pectin in the cell wall residues after a series of mild extractions may be inferred from the data of Chapter 5, but attempts to demonstrate any such association chromatographically were confined to the 100°C water extract reported in this chapter.

The $(1 \rightarrow 3)$-galactan identified here is probably a β-D-galactan related to the 3,6-linked arabinogalactan Type II of Aspinall, (reviewed by Anderson, Clarke and Stone(5)). Such arabino-galactans have often been characterised as water soluble components of pine wood (Section 1.3.10). The methylation data of Chapter 6 suggest that such arabinogalactans were present in the 4°C, and 70°C water extracts of Batch 1 walls. As discussed in Chapter 6, the ratio of $2,4\text{-Me}_2$-galactose to $2,4,6\text{-Me}_3$-galactose in methylation analyses decreased progressively through the 4°C, 70°C and 100°C water extracts. It thus appears that we are dealing with two polysaccharides here: an arabino-3,6-galactan and a linear $(1 \rightarrow 3)$-galactan. The latter is evidently relatively insoluble in cold water but sufficiently soluble to chromatograph successfully at room temperature on DEAE-cellulose.

A linear β$(1\rightarrow3)$-D-galactan has been described in hot water extracts from Rosa glauca callus tissue by Mollard et al

(154). This galactan was insoluble in cold water and could
not be chromatographed at room temperature though the Pinus
galactan was soluble enough for chromatography at room
temperature. This galactan appears to be analogous to the
galactan found here. Mollard et al found the galactan was
always obtained in the water soluble pectin fraction, but it
was also removed by prolonged aqueous buffer washing. They
suggested that the Rosa glauca galactan may function as a
protective covering to the wall and its role as a tightly
bonded structural entity was questioned.

CHAPTER 8

CONCLUSION

The evidence discussed in this thesis has established the
presence of a range of different polysaccharide types in Pinus
radiata callus cell walls.   Though information is available
on the nature of polysaccharide components of mature walls in
gymnosperms there has been little information on the polymeric
components and their organisation in gymnosperm primary cell
walls.   This study has significantly improved the understanding
of the nature of the polysaccharides and protein in this new
area.   The systematic wall fractionations carried out enabled
the types of polysaccharides selectively extracted to be
studied by methylation, for important cell wall fractions.
The fractionation schemes were more detailed than used
previously on primary gymnosperm cell walls by Northcote (130,
131) who did not support sugar analyses of "crude" fractions
with methylation data.   This current work permitted more
accurate assignment of polysaccharides for distinctly defined
chemically removed fractions.   Also the methylation of
particular fractions gave a more detailed description of the
types of polysaccharides distributed between wall fractions,
compared to the cursory methylation study of whole wall of
Pseudotsuga menziesii (54).   Thus, likely polysaccharides are
more clearly established than in previous work.   The following
principal types may be identified: an arabinan, a pectic
galactan, an arabino-3,6-galactan, a linear (1 → 3)-galactan,
a xyloglucan, a xylan (probably (4-O-methylglucurono) xylan
similar to that found in other gymnosperms (125) and Pinus
tissue (127)), and a rhamnogalacturonan, with which much of
the arabinan and galactan is probably covalently associated
through rhamnose residues, as an overall pectic complex.
The linear 3-linked galactan, and xyloglucan are new poly-
saccharide components in gymnosperm primary walls.

The extraction sequences of this work examine the possibility
for several bonding arrangements of the polysaccharides.
Extractions with cold and warm ($70^{o}C$) water, preceding further

extractions, remove polysaccharides not firmly bound in the wall. Polymers extracted thereafter are somewhat arbitrarily considered as integral wall components. Such a conclusion is justified by the similarity in composition of walls prepared from broken cells by the two procedures of wet sieving and buffer extraction.

The role of protein in primary cell walls is not understood but, from the work of Selvendran (32, 33, 67, 147, 151) and others reported in Chapter 5, it appears that two distinct classes of integral wall proteins exist. Albersheim et al (8) in their early cell wall model, proposed a covalent link between rhamnogalacturonan and protein. The evidence was somewhat tenuous and their revised model did not include protein (9).

In the work of Monro et al (132-136), GTC at neutral pH, which does not split ester links, could solubilise appreciable proportions of the glycoprotein (133), especially from the pectin-rich upper hypocotyl segments. Addition of EDTA caused a further increase in the amount of hydroxyproline extracted by 6M GTC at 20°C. This could be evidence for a protein-pectin linkage and some pectin was extracted by EDTA at 37°C. With 6M GTC however only a minor proportion of the pectic substance is removed.

Release of wall protein with 10% KOH was temperature dependent (135). None was extracted at 0°C (132), but the bulk was removed at 22°C regardless of prior removal of pectic substances. A more direct link was envisaged between protein and cellulose by Monro et al (2, 132).

Failure of prolonged treatment with 10% KOH to remove all of the non-glucose polysaccharides and protein, suggested a more direct association of protein and polyuronide with cellulose, but the proposal of Monro et al (2, 132) involving a covalent linkage cannot be sustained without further evidence. Extraction of polyuronide from lupin and mung bean hypocotyl with neutral detergent or ammonium oxalate, while removing pectin, extracted little of the wall protein (133, 136).

Protein could possibly be envisaged to be removed with pectin in the Albersheim model (2, 8), but it is likely that pectin removal by hot neutral detergent or hot oxalate involves degradation by β-elimination, and hence cleavage of any pectin-protein complexes. The protein might therefore not be expected to be removed with the pectin. Treatment with hot neutral detergent or oxalate (2) would be analogous to the polygalacturonase treatment used by Keegstra et al (8, 47). This enzymic treatment liberated pectic polysaccharides and oligosaccharides leaving proteins and hemicellulose in the wall residue.

The association of protein with cell wall polysaccharides in Pinus radiata callus walls can be considered in the light of fractionation studies reported in this thesis. In pine callus we note that much of the polyuronide was removed along with hydroxyproline by conditions which cleave the pectin, such as 100^OC water. Extraction with 6M GTC, or 8M urea did not liberate appreciable levels of hydroxyproline here. However, the proteins of aqueous fractions resemble hydroxyproline-poor cytoplasmic proteins. The less readily extractable "wall proteins" in alkali extracts (subsequent to Na_2CO_3 extraction) and in the residue are found in fractions with elevated levels of uronic acid. If this correlation between hydroxyproline (and protein) levels and uronic acid in the alkali fractions is significant, it might yield considerable information on wall structure. On the other hand, little protein was removed by chaotropic reagents, which did remove hemicellulose after pectic cleavage. This might suggest that much protein is not linked via pectin-hemicellulose. Alternatively, it may be that a protein-pectin complex is initially present but is degraded to such an extent by prior β-elimination that some hemicellulose can now be extracted without the protein.

The role of pectin in primary cell walls is not clear but has been envisaged to link protein and hemicelluloses in the wall (3, 8), though an opposing view has been taken by others (2), and thus its precise function is controversial (211).

Much of the pectin in Pine Callus could be removed by a hot
water treatment which presumably promotes degradation and
release of pectin, through a β-elimination reaction. The
removal of a further portion of pectin by alkali extraction
might suggest that along lengths of the rhamnogalacturonan
there could be polysaccharide bridges connecting some of this
pectin in the wall by alkali labile bonds (e.g. ester links)
to other wall polymers (e.g. lignin) or that linking poly-
saccharides may be hydrogen bonded to cellulose. Probably
ester links are not the only alkali-labile bonds, since
pretreatment with Na_2CO_3 at pH 10.0, followed by 6M urea and
hot EDTA, did not remove a large amount of polysaccharide, as
was subsequently removed by alkali. Cleavage of ester links
by sodium carbonate (weakly basic) renders an appreciable
level of arabinose and galactose soluble in walls of Batch 1,
and though less in walls of Batch 2, some pectin and
xyloglucan is then recovered by 8M urea and an additional
EDTA extraction. Pectic arabinans, galactan, and polyuronide
continue to be released by alkali along with hydroxyproline,
as would occur if polyuronide was bound to protein and vice-
versa by alkali-labile links as in the Albersheim model (8,
53).

Classically, hemicelluloses have been operationally defined as
those polysaccharides which are extractable from holocellulose
by concentrated alkali. A more chemical description might be
that of β-(1 → 4)-linked non cellulosic polysaccharides which
have a tendency towards hydrogen bond formation with cellulose.
They include (in Pinus radiata) glucomannans, xylans and
xyloglucans. Albersheim et al (46) showed that xyloglucans
could be extracted from sycamore primary cell walls by 8M
urea, provided that pectin had been previously degraded.
Monro et al (170) similarly found an almost quantitative
release of xyloglucan from lupin hypocotyls by 6M GTC, whereas
arabinoxylans required mild alkali for extraction. On the
other hand Bailey and Kauss (133) observed that extraction of
hemicelluloses could be accomplished without appreciable
degradation of pectin.

With Pinus radiata callus cell walls, the hemicellulose
situation is more complicated. A certain amount of xyloglucan
and galactoglucomannan are evidently extracted by cold water;
6M GTC then extracts a large part of the xylan and some of
the xyloglucan. Subsequent treatment with alkali removes
most of the remaining hemicelluloses, with more severe
conditions required for the glucomannan, but even after this,
a portion remains in the residue and requires chlorite
treatment followed by alkali for its extraction.

Removal of xyloglucan along with pectic components in alkali
fractions,after pectin has already been partly removed by
β-elimination, supports the hypothesis (2, 8) that linking
polysaccharides (xyloglucan and possibly xylan) between
protein, pectin and cellulose may be hydrogen bonded to the
cellulose. The simultaneous removal of hydroxyproline would
not be inconsistent with an alkali-labile link between protein
and pectic polymers (perhaps via galactosyl-serine) (8, 28,
29, 53). A galactosyl-serine link is questioned by Monro
et al (134, 135, 136) on the grounds that alkali-treatment
does not cause the expected decrease in serine level.

Xyloglucan and xylan, the predominant hemicelluloses can also
be partially solubilised from the wall by the chaotropic
agents, 6M GTC, and 8M urea, after preliminary β-eliminative
cleavage of pectin. These solubilised fractions also contain
arabinan and galactan and rhamnogalacturonan, an observation
which would be consistant with, but not proof of, some form
of bonding between polyuronide of the wall and hemicellulose.
This would support the proposal of links between polyuronide
and cellulose as in the Albersheim model (8). Bailey and
Kauss (133) showed that pectin polyuronide of mung bean
hypocotyls was completely resistant to extraction by 10%
alkali at $22 - 24^{\circ}C$, a treatment which might be expected to
cleave polyuronide by the β-eliminative mechanism, although
most of the hemicellulose and glycoprotein was extracted by
this treatment. This result appears to be in conflict with
the Albersheim model.

It is noted in contrast to Monro et al (2, 134, 135) that considerable levels of hemicelluloses are left after strong alkali extraction in Pinus. Most of this material was liberated by oxidative degradation of lignin with acid chlorite and subsequent extraction with 10% KOH. It is proposed that firmly bound material in the pine callus wall occurs by association with lignin. In the present studies the partial resistance to alkali extraction of pectic substances, hemicelluloses and hydroxyproline-rich protein may be due to lignin cross-linking and rather high levels of lignin appear to be present. A mild treatment with acid chlorite, sufficient to cause marked delignification (as evidenced by the bleaching of the sample) but causing very little degradation of protein or carbohydrate, led to nearly all the remaining protein and non-cellulosic polysaccharide becoming alkali-extractable.

The resistance of about 40% of all the pectin and protein to alkali extraction is attributed to some residual bonding and meshing of the polymers with lignin, found at low levels in these walls. With the onset of lignin synthesis the possibility exists for coupling between phenolic residues of lignin and polysaccharides and protein during polymerisation of lignin, as observed by Whitmore (6, 7). This coupling could be via ether linkages formed through reactive free radical intermediates and ether links could be formed to sugar ring hydroxyls or carbonyl groups of protein. The introduction of the lignin polymer with the capacity for ester, ether, and C-C bond formation increases the possibility of cross-linking between wall polymers enormously. Treatment of the walls after all other extractions with chlorite provides strong evidence for lignin-polysaccharide (6) and lignin-protein interactions (7). The subsequent release of the non-cellulosic polysaccharide by alkali, suggests that these (mostly pectic polysaccharides indicated by the release of arabinose and galactose; some polyuronide, xyloglucan and protein) are involved in the lignin cross-linking.

A linear, 3-linked galactan was shown to be present in the 100^OC water extract of both batches of pine callus cell wall. A similar polysaccharide was isolated by Mollard et al (154, 155) from <u>Rosa glauca</u> cell walls. Although β-elimination of pectin would have occurred during isolation of the fraction containing the linear galactan, it does not follow that the galactan was originally covalently linked to pectin. The uronic acid content of the galactan after DEAE-cellulose chromatography was in fact very low (not detectable), and it is likely that it existed as a free neutral polymer, either in the wall, or as suggested by Barnoud et al (154), for the rose callus galactan perhaps as a peripheral protective agent.

The model of Keegstra et al, (1973) (8) envisages polymers joined to one another with a sequence of glycoprotein-pectin-xyloglucan-hydrogen bonds to cellulose. In the Monro model for lupin (2, 132, 134, 135, 136) and mung beans (2, 132) the complete resistance of the pectin polyuronide to alkali extraction which liberated most of the hemicellulose and glycoprotein (133), suggests that the compounds are either not extensively linked to the pectin or only through alkali-labile ester-links. The alkali treatment of Bailey and Kauss, although not removing pectin, did render it more readily extractable by mild reagents, such as 0.5% ammonium oxalate at 37^OC. This suggests that some degradation of pectin by β-elimination may have occurred with alkali, thus partially obviating the necessity for hot detergent treatment to bring about the same degradation. Extraction of the degraded pectin would require removal of Ca^{2+} by oxalate or EDTA. By contrast hot water alone was fairly effective on <u>Pinus radiata</u>, but "pectin" is nevertheless subsequently removed with hemicelluloses. Pectin removed by Bailey and coworkers (132-136) apparently seems to have required both:

1) β-elimination (hot water or cold alkali) and
2) Ca^{2+} removal (oxalate/or EDTA).
Hemicellulose removed by Bailey and Kauss (free of pectic material) required only alkali.

It is proposed that Pinus radiata callus walls conform to a similar model to that of Albersheim's for sycamore, but with reservations as to the details of polymer interconnections as recently admitted by Albersheim (211), for the overall wall structure.

The different polysaccharides do however exhibit differences in modes of bonding in the wall. Thus;

1) cold water removes xyloglucan, arabinogalactan and galactoglucomannan

2) hot water removes pectin, $(1 \rightarrow 3)$-galactan, pectic galactan and arabinan

3) treatment with alkali removes a xylan which is extracted more readily than the xyloglucan, and the glucomannan (with strong alkali), and pectic substances are further removed

4) and pectic substances and hydroxyproline-rich protein remain in the residue apparently cross-linked by lignin.

Hydrogen bonding holds much of the hemicellulose, which may also be bonded to pectin in the wall. Pectin in turn could cross-link much of the protein in the wall though a protein-polysaccharide (arabinogalactan) complex has not been isolated. Since weak alkali (Na_2CO_3) removes some poly-saccharides or renders them extractable subsequently, there may also be ester linkages of pectins and hemicelluloses (involving uronic acid residues) to other wall components, but cleavage of the pectin is probably a necessary prerequisite for removal after Na_2CO_3 treatment. It is proposed that residual polysaccharides and protein are held in the wall by lignin and not by alkali resistant bonds to cellulose as proposed by Monro et al.

This work has laid a framework for further studies on the intermolecular bonding within Pinus radiata cell walls, but much further work is needed in this area generally to elucidate the covalent and non-covalent bonding between wall polymers (211).

The nature of the polysaccharide components is now fairly clear; some understanding of the proteins present has been gained and ideas have been formulated concerning the linkage between polymers on the basis of sequential extraction studies. It has also been possible to test some aspects of cell wall models, such as the confirmation of hydrogen bonded xyloglucan, but some hemicellulose is also linked in another manner. There is also evidence that ester links hold a small amount of polysaccharide in the wall which can be subsequently released by chelating and chaotropic reagents.

The _Pinus_ _radiata_ callus wall differs from previously studied primary walls in its higher lignin content and in the rather tight binding of much of the pectic material within the wall, perhaps by covalent linkage through lignin and/or protein.

Further elucidation of the structure within the _Pinus_ _radiata_ cell wall, will be facilitated by using a lignin-free tissue such as cambium.

The structures reported in this thesis provide an essential ground-work for future investigation of the covalent association between polymers in _Pinus_ _radiata_ important for elucidation of the structure, growth and elongation processes. Development of the column chromatographic approach explored in Chapter 7 should prove useful in separating fragments and elucidating associations between neutral, acidic (pectic), and protein, polymeric fragments released in fractions, and hence polysaccharide-polysaccharide, and protein-poly- saccharide linkages in the wall.

CHAPTER 9

APPENDICES

Appendix 1:   Identification of Running Parameters for Partially
              Methylated Alditol Acetates on Columns 1, 2, and
              Methods of Quantitation.

Part 1:   Procedure

Capillary columns were not available for the work of this thesis
and conventional columns as described in Section 2.3.1.4 were
used.   Methylated alditol acetates of preliminary samples were
identified by their running positions relative to 1,5-di-O-
acetyl-2,3,4,6-tetra-O-methyl-D-glucitol,(T. values) the
standard which is usually used in the literature.   The
reproducibility of relative retention times was generally
within 1% provided that two internal standards, with consider-
ably different retention times were used, and that the values
were determined by interpolation.   However more use was made
here of programmed runs, where T values could not be directly
inferred.   Instead the absolute retention times were compared
for samples with large numbers of known compounds as on the
charts in this appendix.   As a beginning to the solution of
locating running positions on our columns some standard poly-
saccharides were methylated, hydrolysed and derivatised, and
used as markers (see Methods Section).

An arabinoxylan (wheat flour), galactomannan (Carob bean) and
a mixture of these two methylated polysaccharides, were used to
produce standard samples of partially methylated alditol
acetates.   These were;

a) run over all columns along with $2,3,4,6-Me_4Glc$ marker to
   begin the delineation of positions,
b) run before samples and
c) coinjected with some samples where necessary to confirm
   the presence by g.c. of a peak.

Some undermethylated soluble starch, delineated several
permethylated glucose positions.

As early samples were analysed more peaks were verified by mass spectrometry and the list of running positions, and fine distinctions between some of the derivatives increased. The sets of running parameters to the results of standards and mass spectrometry of early samples were built up as shown in this appendix.

Along with gas-chromatographic identification, all derivatives were verified by g.c.-mass spectrometry (Table 9.1), and spectra were correlated with spectra in the literature (95). The chromatographing positions of derivatives are shown in Figures 9.1 - 9.3.

FIGURE 9.1 RUNNING PARAMETERS FOR PERMETHYLATED ALDITOL ACETATES ON OV225, COLUMN 5(2.3.1.4.). PROGRAMMED 150°-210°C at 1°C/min.
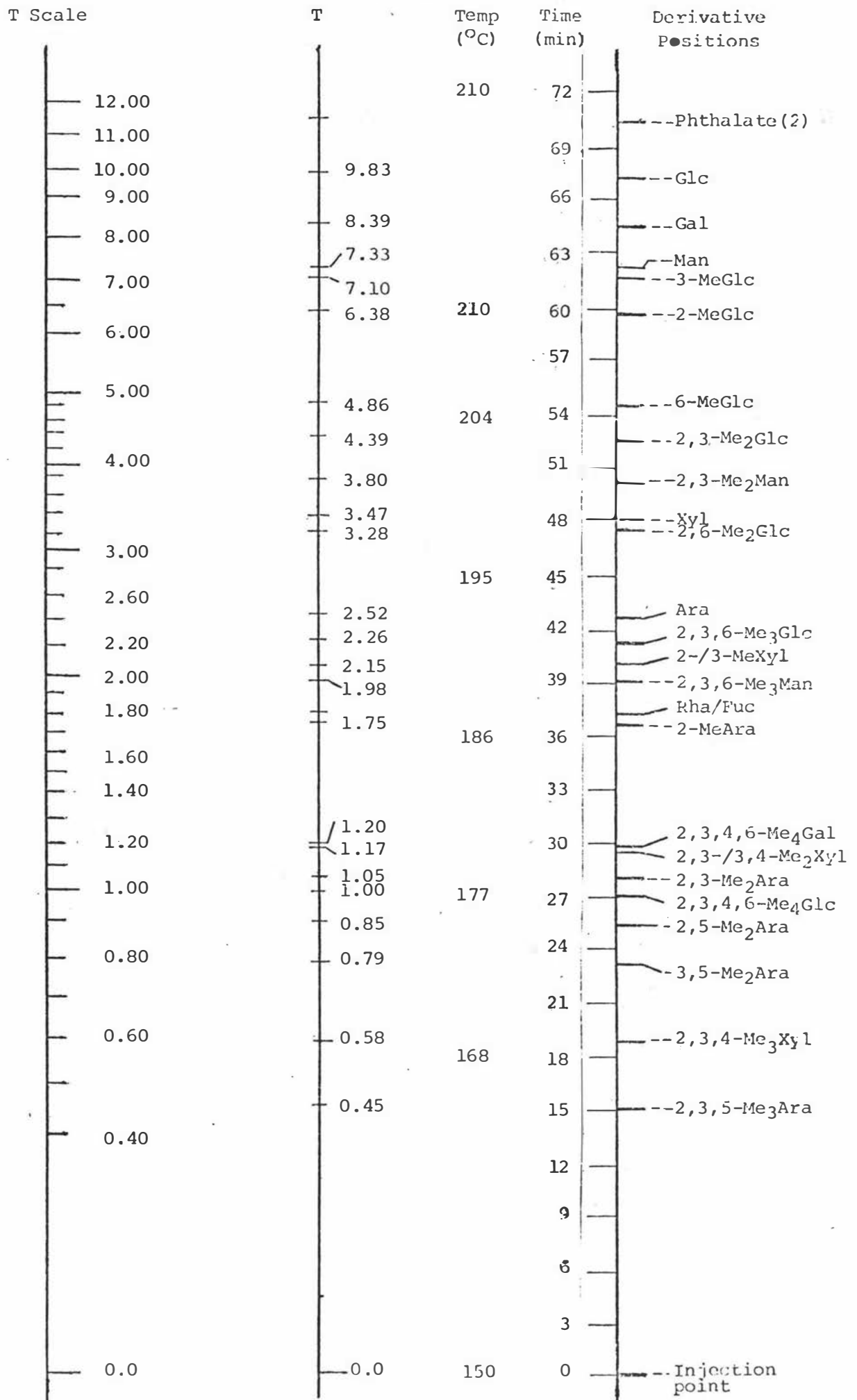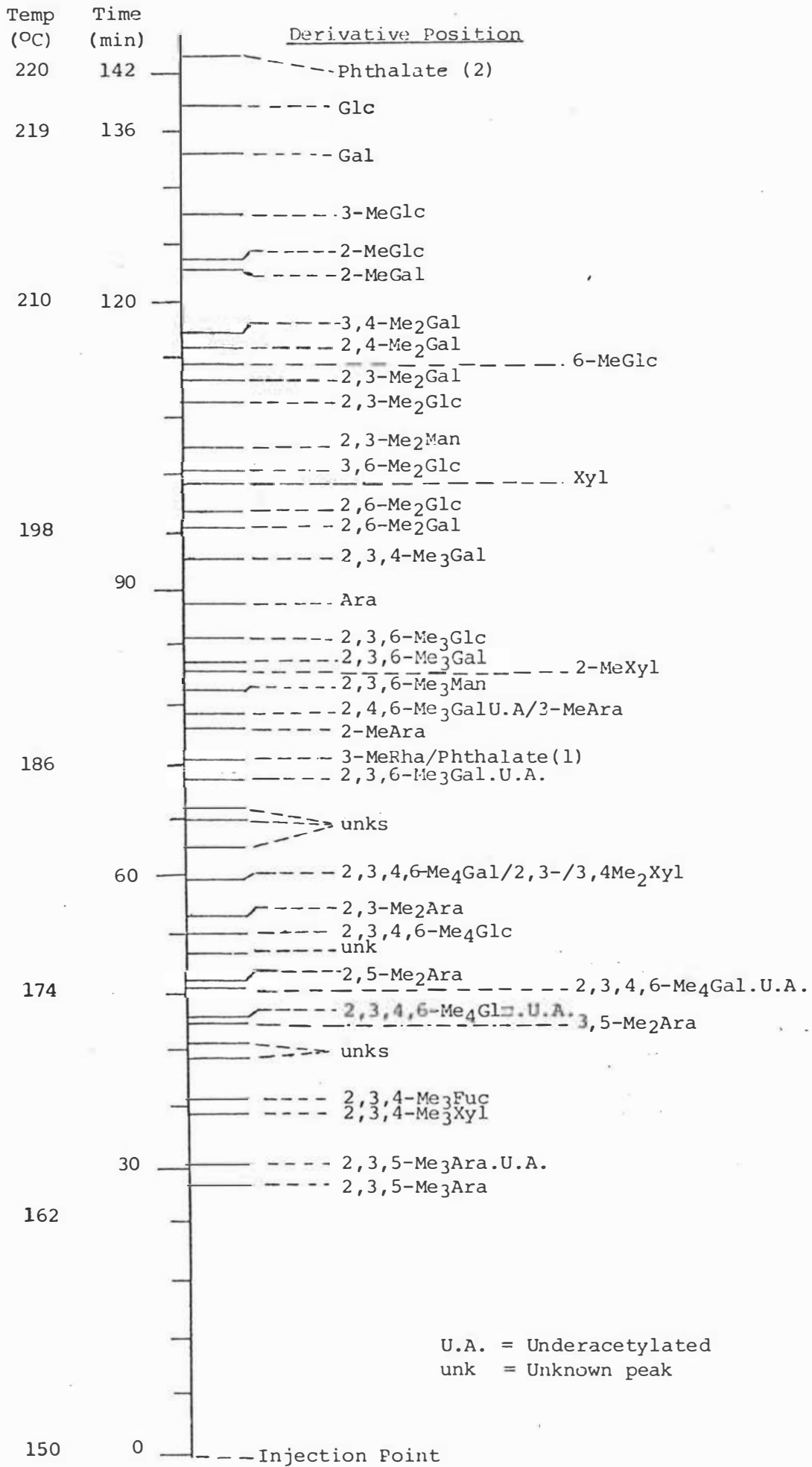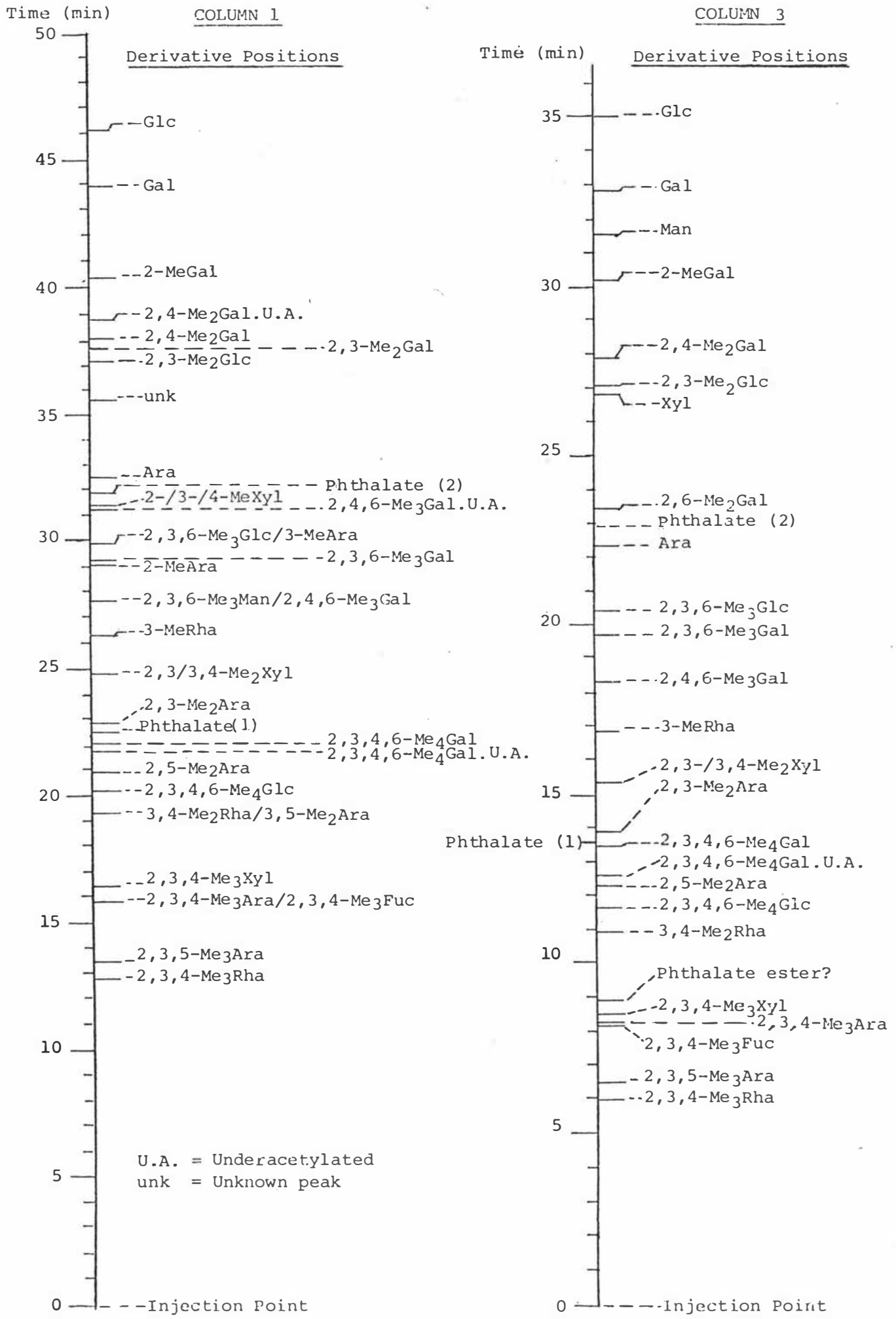
FIGURE 9.3   RUNNING PARAMETERS FOR PERMETHYLATED ALDITOL ACETATES
ON SP2340, COLUMNS 1 AND 3 (2.3.1.4.).   PROGRAMMED
150°-230°C at 2°C/min.

APPENDIX 1

Table 9.1:   Identification of Methylated Sugar Derivatives

Every peak was identified by running the sample on two columns (some were run on OV225 isothermally at $160^{\circ}C$), OV225 and SP2340. All peaks were identified by mass-spectrometry,* but in some cases on some chromatograms, the identity of pure peaks could be verified by relative retention times on OV225, and SP2340. (* Ionising Potential 70eV, accelerating voltage 4K.V., emission current 100μ amp, ion source temp. $250^{\circ}C$).

Derivative	(T-OV225)	Identifying Features
$2,3,4\text{-Me}_3\text{Rha}$	0.35	Runs before $2,3,5\text{-Me}_3\text{Ara}$ on SP2340. Confirmed by mass-spec. on SP2340 by m/e 89, 115, 117, 131, 161, 175.
$2,3,5\text{-Me}_3\text{Ara}$	0.41	Clearly distinguished on OV225 and SP2340. Confirmed by mass-spec. by m/e 45, 117, 161.
$2,3,4\text{-Me}_3\text{Ara}$	0.53	$2,3,4\text{-Me}_3\text{Xyl}$ and $2,3,4\text{-Me}_3\text{Fuc}$ separated on both columns. $2,3,4\text{-Me}_3\text{Ara}$ runs with $2,3,4\text{-Me}_3\text{Fuc}$ on SP2340, as a shoulder on $2,3,4\text{-Me}_3\text{Xyl}$ on OV225 (separated by isothermal run). Figures 9.2, 9.3.
$2,3,4\text{-Me}_3\text{Xyl}$	0.54	
$2,3,4\text{-Me}_3\text{Fuc}$	0.58	Quantitation of $2,3,4\text{-Me}_3\text{Ara}$, Xyl, Fuc. was usually from a combination of programmed runs on two columns. $2,3,4\text{-Me}_3\text{Ara/Xyl}$ confirmed by mass-spec. by m/e 101, 117, 161. $2,3,4\text{-Me}_3\text{Fuc}$, confirmed by mass-spec. by m/e 117, 131, 161, 175($\rightarrow$115).
$3,5\text{-Me}_2\text{Ara}$	0.80	Clearly distinguished on OV225 and SP2340. Confirmed by mass-spec. by m/e 45, 161, 189.
$3,4\text{-Me}_2\text{Rha}$	0.87	Runs with $3,5\text{-Me}_2\text{Ara}$ on SP2340. Confirmed by mass-spec. by m/e 131, 189.

Appendix 1:   Table 9.1 Continued

Derivative	(T-OV225)	Identifying Features
$2,5\text{-}Me_2Ara$	0.89	Clearly distinguished on OV225, SP2340. Confirmed by mass-spec. by m/e 45, 117, 233($\rightarrow$113).
$2,3,4,6\text{-}Me_4Glc$	1.00	Clearly distinguished on OV225 and SP2340. Confirmed by mass-spec. by m/e 45, 117, 161, 205($\rightarrow$145) and <u>101</u>.
$2,3\text{-}Me_2Ara$	1.07	Clearly distinguished on OV225 and SP2340. Confirmed by mass-spec. by m/e 117, 189($\rightarrow$129).
$2,3\text{-}/3,4\text{-}Me_2Xyl$) $2,3,4,6\text{-}Me_4Gal$)	1.19	Run together on OV225. Separate isothermally at $160^{O}C$, and well separated on SP2340. $2,3,4,6\text{-}Me_4Gal$ confirmed by mass-spec. by m/e <u>101</u>, 205($\rightarrow$145). $2,3\text{-};3,4\text{-}Me_2Xyl$ distinguishable by m/e 117, 129, 189. Approx. quantitation by 101/129, or ratio 189/205, peak intensities. The ratio of $2,3\text{-}/3,4,\text{-}Me_2Xyl$ within the peak, was by labelling the reducing end of the alditol with Deuterium (Appendix 2, Part 2) and quantitation was by the 117/118, 129/130 peak intensities.
3-MeRha	1.67	Runs with phthalate ester (No. 1) on OV225. Clearly separated on SP2340. The contribution from phthalate can be subtracted. 3-MeRha confirmed by mass-spec. by m/e 143.
2-MeAra	1.82	Clearly distinguished on OV225. Confirmed by mass-spec. by m/e 117, 261.

Appendix 1:   Table 9.1 Continued

Derivative	(T-OV225)	Identifying Features
3-MeAra	1.98	Clearly distinguished on OV225 (runs before $2,4,6-Me_3Gal$, but well separated on OV225). Confirmed by mass-spec. by m/e 87, 129, 189.
$2,4,6-Me_3Gal$) $2,3,6-Me_3Man$)	2.03	Run together on OV225 and SP2340. Mass-spec of $2,4,6-Me_3Gal$ confirmed by m/e 117, 129 (high). $2,3,6-Me_3$ Man confirmed by mass-spec. by m/e 117, 233→113 129(low). Approximate quantitation by one of m/e 113/117/129 ratios. Better quantitation by use of ion fragments/ f.i.d. response parameters (see Appendix 1, Part 3).
2-MeXyl 3-MeXyl 4-MeXyl	2.15	Run together as shoulder on $2,3,6-Me_3$ Gal and $3,4,6-Me_3Gal$ on OV225. Clearly distinguished on SP2340, but all run together. 2- and 4-MeXyl confirmed by mass-spec. by m/e 117, 261, 3-MeXyl by m/e 87, 129, 189.

On OV225 determination from mass-spec. ratios is difficult, better to check quantitation from SP2340. Relative proportions of 2-,3-,4-MeXyl calculated by mass spec. ion fragment/f.i.d. response parameters (Appendix 1, Part 3). (ions m/e 129, 189 for 3-MeXyl, and 117, 127, 261, for 2- and 4-MeXyl). Separate quantitation of 2- and 4-Me Xyl was only on deuterated samples and this was done by 117/118, 261/262 ratios.

Appendix 1:   Table 9.1 Continued

<u>Derivatives</u>   (T-OV225)   <u>Identifying Features</u>

3,4,6-Me$_3$Gal ⎤   2.22   Run together on OV225 and SP2340.
2,3,6-Me$_3$Gal ⎦   3,4,6-Me$_3$Gal confirmed by mass-spec.
                            by m/e 189, 161→101.   2,3,6-Me$_3$Gal;
                            mass spec. by <u>117</u>, 233→113;   can be
                            quantitated by 189, 117, 233, 113 ion
                            fragments (Appendix 1, Part 3).
                            (2,3,6-Me$_3$Gal-d$_2$ quantitation, see
                            Appendix 1, Part 4).

2,3,6-Me$_3$Glc   2.32   Clearly separated on OV225 programme.
                            Confirmed by mass-spec. by 117, 233→
                            113.   Compare with 2,3,6-Me$_3$Gal.

Ara   2.60   Clearly separated on OV225 and SP2340.
               Confirmed by mass-spec. by m/e 103/115.

2,3,4-Me$_3$Gal   2.89   Clearly identified by retention time
                            on OV225.   Confirmed by mass-spec. by
                            m/e 117, 161→101, 189→129, 233 (113
                            not prominent as in 2,3,6-Me$_3$Gal etc).

Xyl   3.60   Cleary distinguishable on OV225.
               Mass spec. as for Ara.

2,3-Me$_2$Man   3.92   Clearly distinguishable on OV225.
                          Mass-spec. m/e 117, 261.

2,3-Me$_2$Glc   4.50   Clearly distinguishable on OV225.
                          Mass-spec. m/e 117, 261.

2,3-Me$_2$Gal   Clearly distinguishable on OV225.
                      Mass-spec. m/e 117, 261.

2,4-Me$_2$Gal   5.1   Clearly distinguishable on OV225.
                        Mass-spec. m/e 117, 189.

Appendix 1:   Table 9.1 Continued

Derivative   (T-OV225)   Identifying Features

2,6-Me$_2$Gal   also distinguishable on OV225 and
3,6-Me$_2$Gal, Glc;   SP2340 by retention time and mass
2-MeGlc, 2-MeGal;   spec. as given in reference (95).

Part 2:   Gas Chromatograms of some Programmed Runs on OV225

Figures 9.4 and 9.5, are chromatograms of fully methylated,
fully acetylated samples. The diagrams indicate fairly clean
traces, with all of the peaks identified.

The 2,3-/3,4-Me$_2$-xylose, and 2-/3-/4-Me-xylose, could be
separated from the galactose derivatives on SP2340, column 1,
under programme.

To make sure of peak identity in complex samples, regions,
such as from 3-Me-rhamnose to 2,3,6-Me$_3$-glucose on these
chromatograms were scanned by mass spectrometry, using an
m/e range at 43-300, 1.0 sec scans, every 20 secs of
programmed run on OV225. Runs were repeated on OV225 and
scans again carried out across crowded derivative positions.

The mass spectrometry operating conditions were as listed at
the beginning of Table 9.1.

Figure 9.6 shows the better resolution of data using glass
columns and ensured acetylation conditions (with pyridine as
catalyst), as used for all reported methylation data in this
thesis.

Figure 9.4: Gas Chromatogram of Permethylated Alditol Acetates of Methylated Sugars of the 10% KOH Fraction Batch 2 Walls, 6 Times Methylated. Conditions: injection onto OV225 (Column 2) in CH_2Cl_2, temperature programmed 150-225°C at 0.5°C/min, N_2 carrier gas flow rate 30ml/min.

Key to Derivatives

(1) $2,3,5-Me_3Ara$ (half actual peak height)
(2) $2,3,4-Me_3Xyl$
(3) $2,3,4-Me_3Fuc$
(4) $3,5-Me_2Ara$
(5) $2,5-Me_2Ara$
(6) $2,3,4,6-Me_4Glc$
(7) $2,3-Me_2Ara$
(8) $2,3,4,6-Me_4Gal/2,3-/3,4-Me_2Xyl$
(9) 3-MeRha, + Phthalate Ester No. 1
(10) 2-MeAra
(11) $2,3,6-Me_3Man/2,4,6-Me_3Gal$
(12) 2-/3-/4-MeXyl (from mass spec)
(13) $2,3,6-/3,4,6-Me_3Gal$
(14) $2,3,6,-Me_3Glc$
(15) Arabinose
(16) $2,3,4-Me_3Gal$
(17) Xylose
(18) $2,3-Me_2Glc$
(19) $2,4-Me_2Gal?$
(20) 2-MeGal
(21) 3-MeGlc?
(22) Phthalate Ester No. 2
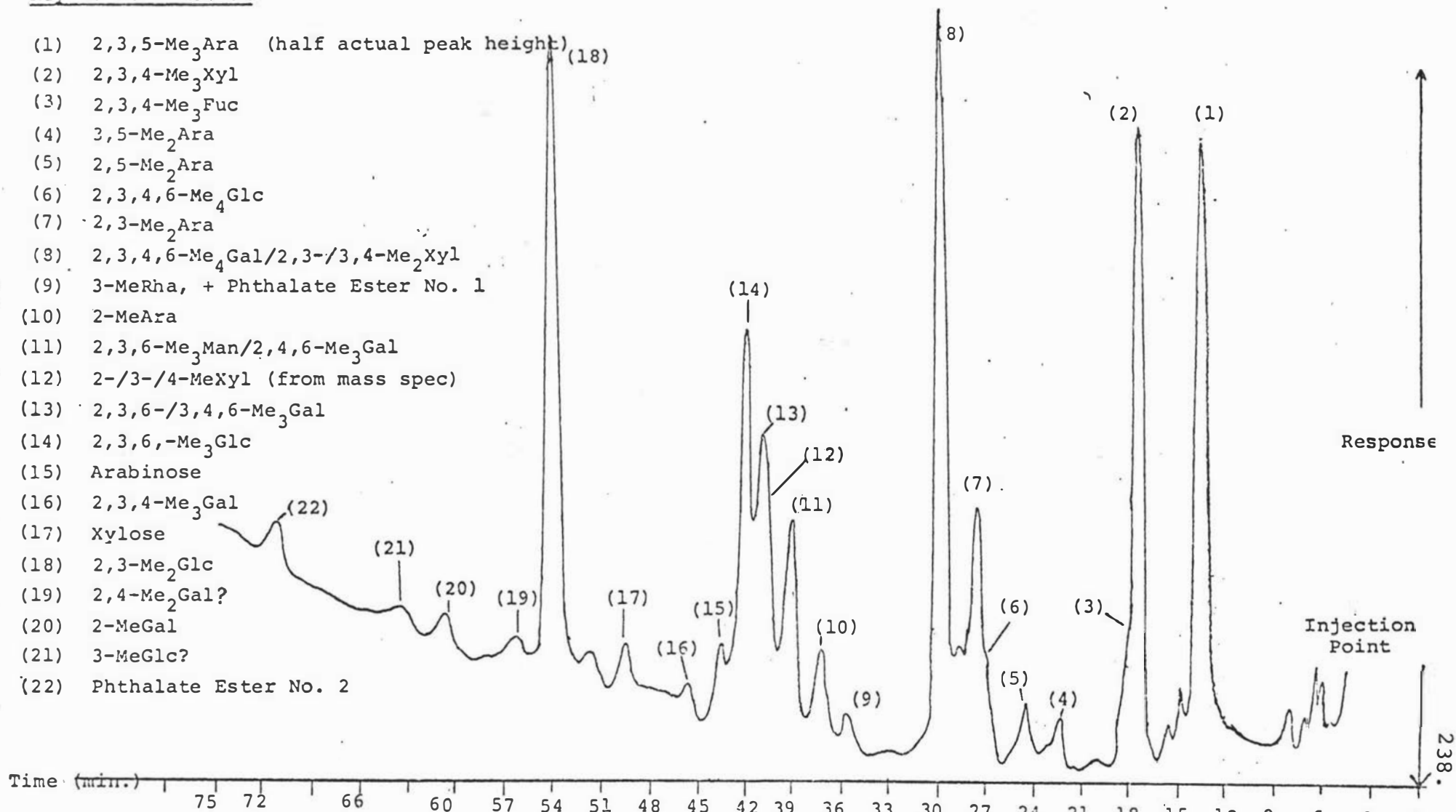
Figure 9.5: Gas Chromatogram of Permethylated Alditol Acetate Derivatives of Methylated Sugars of the EDTA Fraction, Path A Batch 1 Walls, 3 Times Methylated. Conditions: injected onto OV225 (Column $\underline{2}$) in CH_2Cl_2, temperature programmed 150-225°C at 0.5°C/min, N_2 carrier gas flow rate 40ml/min.

Key to Derivatives

(1) $2,3,5-Me_3Ara$
(2) $2,3,4-Me_3Ara/2,3,4-Me_3Xyl$
(3) $2,3,4-Me_3Fuc$
(4) $3,5-Me_2Ara$
(5) $2,5-Me_2Ara$
(6) $2,3,4,6-Me_4Glc$
(7) $2,3-Me_2Ara$
(8) $2,3,4,6-Me_4Gal/2,3-/3,4-Me_2Xyl$
(9) $3-MeRha$
(10) $2-MeAra$
(11) $2,4,6-Me_3Gal$
(12) $2,3,6-Me_3Gal$
(13) $2,3,6-Me_3Glc$
(14) Arabinose
(15) $2,3,4-Me_3Gal$
(16) $2,6-Me_2Gal$
(17) Xylose
(18) $2,3-Me_2Glc$
(19) $2,4-Me_2Gal$

Response

Injection Point

Time (min)

239.
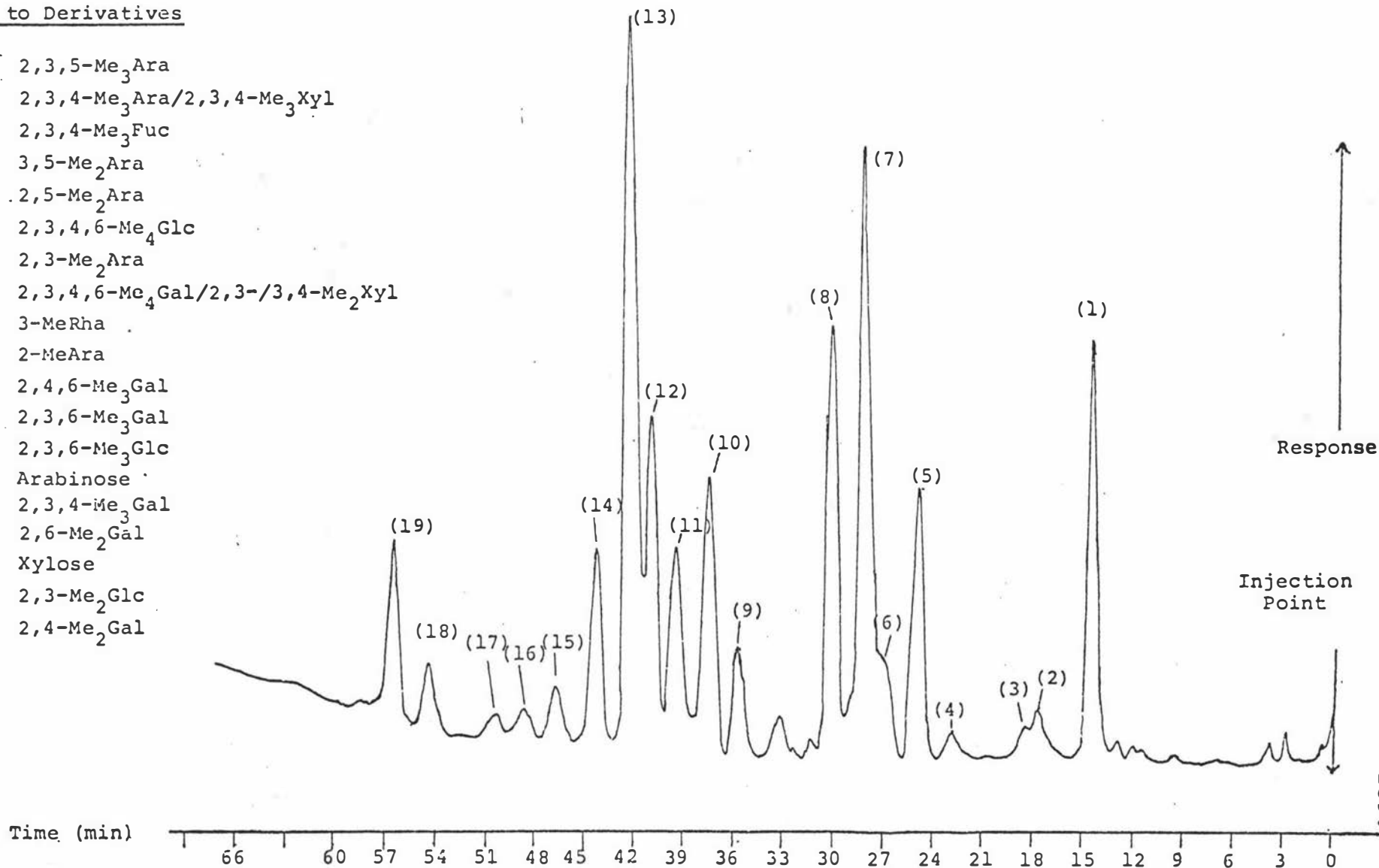
Figure 9.6: Appendix 1

Effect of Glass Columns and Complete
Acetylation on Gas Chromatographic Traces.

Chromatograms of Partially Methylated
Alditol Acetates of Batch 1, 100°C Water
Fraction, Methylated 3 Times.

Details of the Figure are given in the Footnotes.

Trace C — Full acetylation and glass column – conditions used for reporting all thesis results.

Trace B — Under acetylation and a glass column.

Trace A — Under acetylation and a steel column.
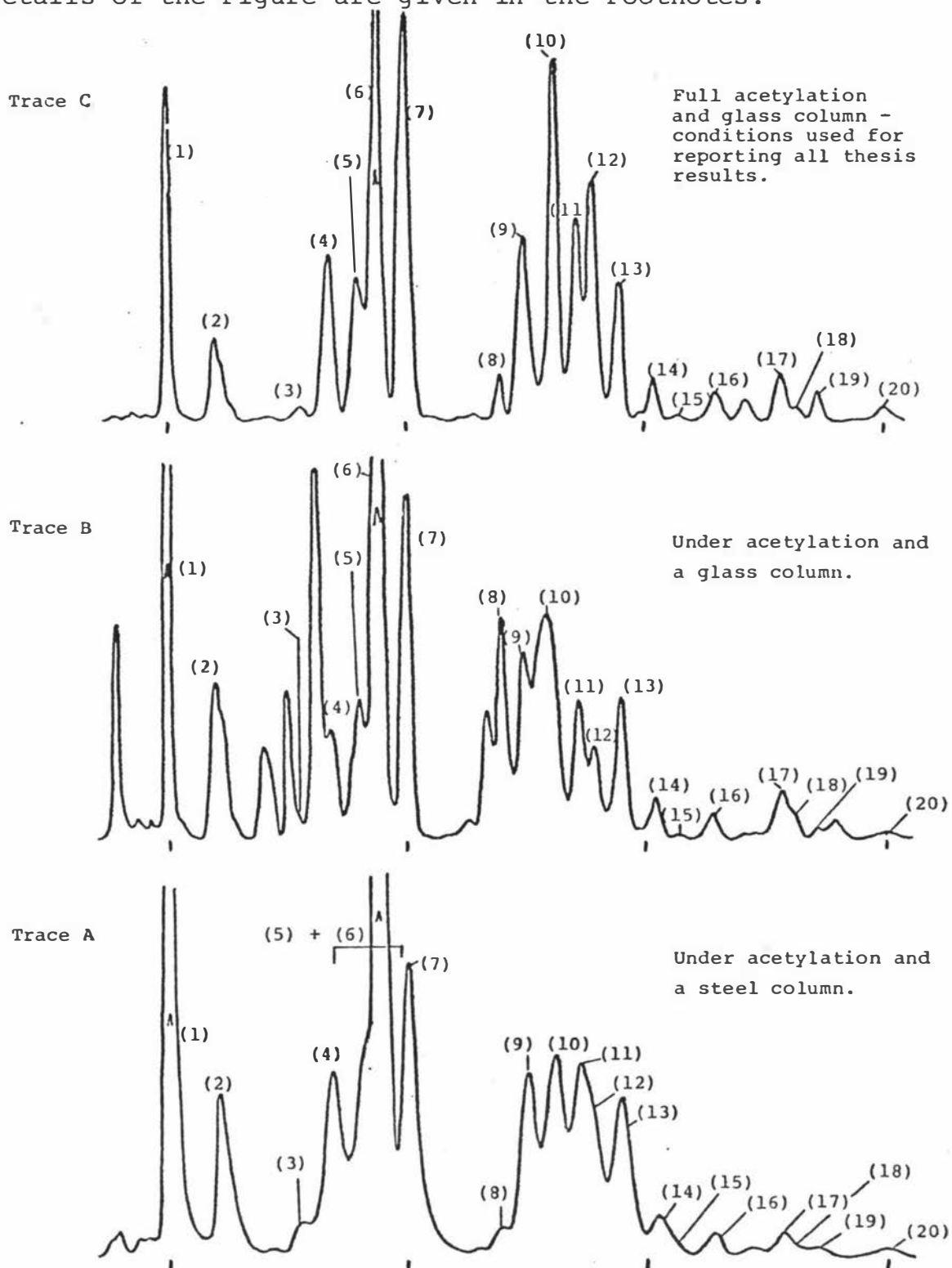
Footnotes to Figure (9.6) Appendix 1.

Chromatograms of Partially Methylated Alditol Acetates of Batch 1, 100°C Water Fraction, Methylated 3 Times.

Trace A: Acetylated with acetic anhydride at 100°C, 1 hr, NaOAc present. Sample injected in CH_2Cl_2 onto steel column. (Column 5). Run programmed 150-210°C at 1°/min. N_2 flow 30 ml/min.

Trace B: Acetylated with acetic anhydride at 100°C, 1 hr, NaOAc present. Sample injected in CH_2Cl_2 onto glass column. (Column 2). Run programmed 150-225°C at 0.5°/min. N_2 flow 40 ml/min.

Trace C: Acetylated with acetic anhydride/pyridine 100°C, 1 hr, NaOAc present. Sample injected in CH_2Cl_2 onto glass column. (Column 2). Run programmed 150-225°C at 0.5°/min. N_2 flow 40 ml/min.

Peak Identities:

(1) $2,3,5-Me_3Ara$
(2) $(2,3,4-Me_3Ara/2,3,4-Me_3Xyl),2,3,4-Me_3Fuc$
(3) $3,5-Me_2Ara$
(4) $2,5-Me_2Ara$
(5) $2,3,4,6-Me_4Glc$
(6) $2,3-Me_2Ara$ (peak drawn at ½ height)
(7) $2,3,4,6-Me_4Gal/2,3-/3,4-Me_2Xyl$
(8) 3-MeRha + Phthalate ester 1
(9) 2-MeAra
(10) $2,4,6-Me_3Gal$
(11) $2,3,6-Me_3Gal$
(12) $2,3,6-Me_3Glc$
(13) Ara
(14) $2,3,4-Me_3Gal$
(15) $2,6-Me_2Gal$
(16) Xylose
(17) $2,3-Me_2Glc$
(18) $2,3-Me_2Gal$
(19) $2,4-Me_2Gal$
(20) 2-MeGal

Part 3: Quantitation of Permethylated alditol acetates by
         Gas chromatography-mass spectrometry.

Description of Technique

Derivatives which were difficult to quantitate from their
f.i.d.responses alone on either OV225 or SP2340 could be
quantitated to about 10% accuracy by combined g.c.-mass
spectrometry. If the quantitation was to be made in a broad
peak region (containing 2-3 derivatives, running close
together), the mass spectra were scanned across the region at
20 sec. intervals (1 sec. scans) as in Appendix 1, Part 2.

The quantitation was made by use of Ion Fragment/F.I.D.
Response Parameters between the derivative to be quantitated
and an identified pure derivative on the same chromatogram.
An appropriate m/e range was selected so that diagnostic
ions for derivatives could be monitored (usually m/e 43 - 300).

The Ion Fragment Responses for equal areas of pure derivatives
on f.i.d., were calculated and are shown in Table 9.2.
Combinations of ions were used for a derivative which were
diagnostic under the chromatographic separation conditions
used.

Details of the mass spectra of derivatives are given in
Appendix 2.

Appendix 1:   Table 9.2:   Response Factors Between Ion Fragments and F.I.D. Response for Partially Methylated Sugars

Compound	m/e	A (m/e)(Int)	A Area	B (m/e)(Int)	B Area	C (m/e)(Int)	C Area	D (m/e)(Int)	D Area	E (m/e)(Int)	E Area	F (m/e)(Int)	F Area	A	B	C	D	E	F	Average
Phthalate Ester	149	63.0	21.2	16.0	16.1	6.0	4.3	19.0	7.6					100	100	100	100			100
3-MeRha	143	18.0		40.0		10.0		26.0						100	100	100	100			100
2,5-Me₂Ara	113	12.5	3.2	50.0	12.0	12.0	5.6	33.0	10.3	17.0	10.8	67.0	67.0	100	100	100	100	100	100	100
2-MeAra	117/118*	94.0	5.5	350.0	26.4	70.0	9.6		14.4			355.0*	82.0	440	300	340			430	380
	127	12.5		50.0		12.0						49.0		60	45	60			60	55
2-MeXyl	261	4.5		13.0		3.0								21	12	12				15
3-MeAra	129	63.0	3.8	82.0	7.9	86.0		61.0	4.3					430	240	450	440			390
3-MeXyl	189	16.0		19.0		22.0	8.9	15.0						100	60	110	100			90
3,5-Me₂Ara	101									9.0	4.0							150		150
	161									8.0								130		130
3,4,6-Me₃Gal	145											41.0	76.0						50	50
	189/190*					17.0		4.0				42.0*				40	40		50	45
2,3,6-Me₃Gal I	117/118*	150.0		400.0		80.0		90.0				350.0*		380	340				300	350
	233	35.0	10.3	80.0	28.0	20.0	20.0	19.0	9.6			66.0	126.0	90	70	93	93		60	80
	113	90.0		240.0		44.0		47.0				260.0		230	200	240	240		200	200
2,4,6-Me₃Gal	117	40.0	5.3	100.0	14.0									200	170					185
2,3,6-Me₃Man I	129	52.0		155.0										250	260					255
	201	1.5		4.0										7	7					7
	161#																			

Area = Relative Peak Areas on OV225
(m/e) = Relative Intensity for ion fragments
(Int) for derivatives on each chromatogram

Relative Intensity for ions for equal f.i.d. Area Responses of peaks on chromatograms

More prominent in 2,4,6-Me₃Gal

2,3,6-Me₃Man ——Use responses for 2,3,6-Me₃Gal above

* Fragment arising from incorporation of single Deuterium at reducing end of sugar

Table Captions

A, Batch 1 EDTA Fraction, Path B
B, Batch 1 0.5% KOH Fraction, Path A
C, Batch 1 10% KOH Fraction, Path A
D, Batch 1 Na₂CO₃ Fraction Path A
E, Data from Methylated Arabinoxylan
F, Batch 2. GTC Fraction (NaBD₄ reduced)

2,- and 3-MeAra can be measured in area, hence a fragment/area response may be calculated. Though 2- and 3-MeXyl cannot be easily calculated in area, on OV225, response parameters can be determined, since they have identical mass-spectra to 2-, and 3-MeAra.

*, I and——, are given in Footnotes to Table 9.2 on next page.

Footnotes to Table 9.2

*   For the GTC fraction of Batch 2 walls, ion intensities
as for 117, 189, in the undeuterated molecule are
replaced by intensities corresponding to the next
highest mass number, since the reducing end is now
labelled with deuterium.

I $3,4,6\text{-Me}_3\text{Gal}$ and $2,3,6\text{-Me}_3\text{Gal}$
$2,4,6\text{-Me}_3\text{Gal}$ and $2,3,6\text{-Me}_3\text{Man}$
These pairs are not completely separable on OV225 & SP2340.
Ion fragment intensity/area response is difficult to
establish. For cases where mass-spec. implies only one
derivative present on the chromatogram, ion fragment/
area responses were calculated.

___   An underlined figure, stands for the area of the
peak of the pure derivative on that line, under compound.

Part 4: Quantitation of the Proportion of Dideuterated
         Derivatives arising from Uronic Acids

The structure of 2,3,6-Me$_3$-hexitol-tri-acetate is as shown
(Figure 9.7) and prominent peaks observed in the mass
spectrum arise by splittings as shown in Figure 9.7 and as
outlined in Appendix 2.

Figure 9.7:   Fragmentations of 2,3,6-Me$_3$-galactitol-tri
              acetate
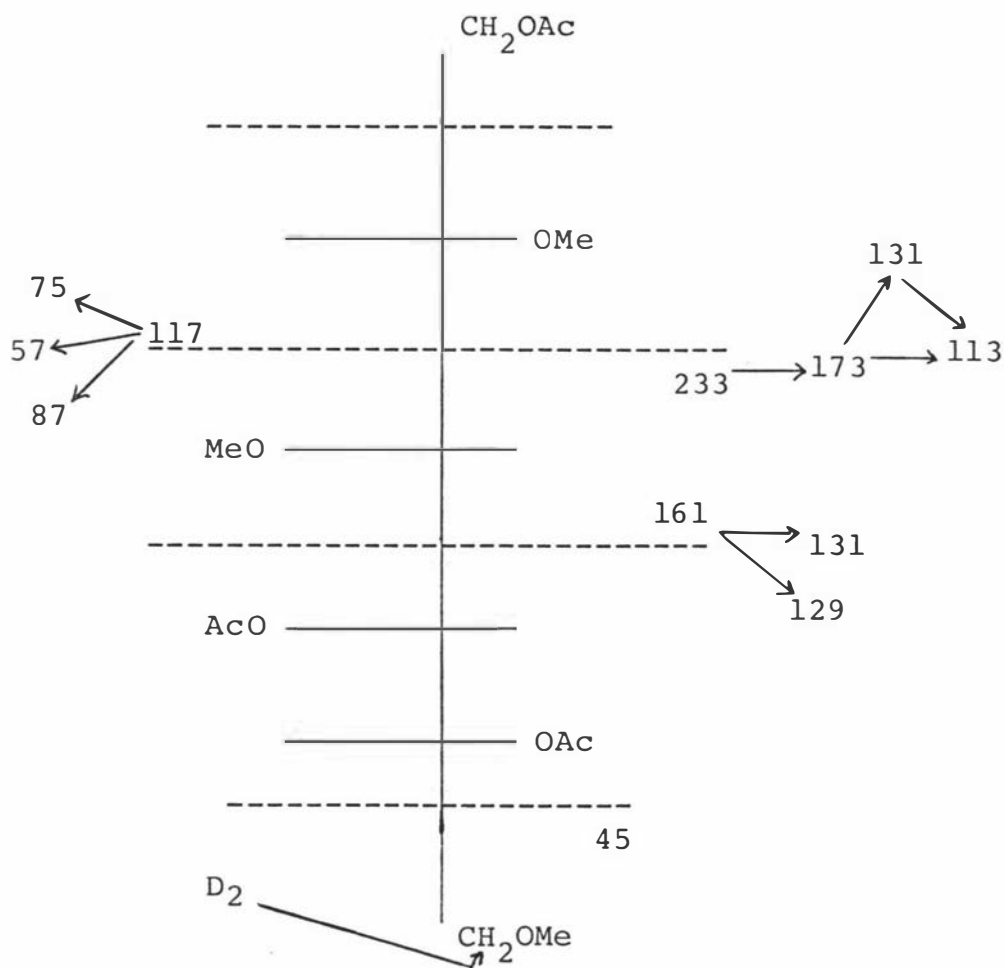
For the 2,3,6-Me$_3$-galactose derivative, to the doubly reduced
and completely methylated sample (sample MRMRMM) the follow-
ing fragment intensities were observed.

	Dideuterated (D_2)	Undeuterated (D_0)
173	11.0	11.0
174	5.0	1.3
175	27.0	0.7
233	20.5	12.3
235	38.0	0.3

The contribution to the 174, 175 and 235 m/e fragments, (prominent peaks in the deuterium labelled derivative) in the mass spectrum from undeuterated $2,3,6$-Me_3-galactose were subtracted. (This was applied for the quantitation of the other deuterated derivatives).

The proportion of dideuterated derivative was then calculated from the ratio of the fragment intensities arising from the dideuterated molecule to the fragment intensities summed from both molecules present. i.e. from the above data subtracting contributions to 174, 175 and 235 fragments that arise naturally from D_0, then for the combination of peaks in D_2, the ratio $\dfrac{174 + 175}{173 + 174 + 175}$ for m/e intensities, implies that 69% of the $2,3,6$-Me_3-galactose is from 4-linked galacturonic acid in the MRMRMM sample. Similarly the 233 and 235 m/e fragments imply approx 64% 4-linked galacturonic acid. An examination of all fragments gives 67%. Thus 4-linked GalA to 4-linked galactose is approx. 2:1.

The other peaks quantitated with dideuterium label (as in Table 6.2) were estimated by the same procedure as above for $2,3,6$-Me_3-galactose. For $2,3,4,6$-Me_4-glucose and $2,3,4,6$-Me_4-galactose contributions to m/e 205, 207 and 145, 146, 147 were used. For $2,3,6$-Me_3-glucose, m/e 173, 174, 175 as for $2,3,6$-Me_3-galactose were used. $2,3$-Me_2-galactose from uronic acid was quantitated by m/e 261, 262, 263, $2,6$-Me_2-galactose was quantitated using m/e 45, 47 and m/e 189, 190, 191 and $3,6$-Me_2-galactose by m/e 233, 235 and m/e 189, 190 and 191.

Appendix 2:   Mass Spectral Fragmentation Patterns Arising
From Partially Methylated Alditol Acetates.

Part 1:   Origin of Fragments

Partially methylated sugars identified tentatively on the
basis of g.l.c. alone, can be confirmed by their mass spectra,
if a combined g.l.c.-m.s. system is available. Mass
spectrometric analysis of compounds are performed as
described in Appendix 1.

Each methylated sugar is transformed on reduction and
acetylation into a single derivative.

Stereoisomeric partially methylated alditol acetates give
very similar mass spectra and it is not possible to determine
from the mass spectrum if a compound derives from e.g.
glucose, galactose, mannose. But methylated alditol acetates
with different substitution patterns can be distinguished by
their different mass spectra.

A detailed account of the theory of fragmentation pathways
is given by Lindberg et al (95, 96, 209). Use is made of
deuterium labelling to understand fragmentation pathways
(163, 209). Briefly the mass fragments arise in the following
manner.

Molecular ions are rarely observed. Primary fragments are
generated by fission between carbon atoms in the chain.
The fragment with the methoxyl group carries the positive
charge. Fission between two methylated carbons (I) is more
abundant than fission between one methoxylated and one
acetoxylated carbon (II), which in turn is more abundant than
fission between two acetoxylated carbons (III). In case (I)
both fragments appear as strong ions. In case (II) only the
methoxylated fragments appear as strong ions. In (III) both
ions are weak except for alditol acetates and mono-O-methyl
alditol acetates in which the methoxyl is terminal.

Secondary fragments are formed from the primary fragments, by single or consecutive loss, of acetic acid (m/e 60), ketene (m/e 42), methanol(m/e 32) or formaldehyde (m/e 30). Methanol and acetic acid are lost by β-elimination and acetic acid by α-elimination. For example m/e 161, for the primary fragment gives the following secondary fragments via the indicated losses.

$$
\begin{array}{c}
\text{CHOMe} \\
| \\
-\text{C}\!-\!\text{OMe} \\
| \\
\text{CH}_2\text{OAc}
\end{array}
\qquad
\begin{array}{c}
\text{m/e} \\
\underline{161}
\end{array}
\;
\xrightarrow{\hspace{2cm}}
\;
\begin{array}{c}
\underline{101} \\[4pt]
\underline{129} \xrightarrow{\;-\text{CH}_2\text{C=O}\;} \underline{87} \\[4pt]
\underline{131}
\end{array}
$$
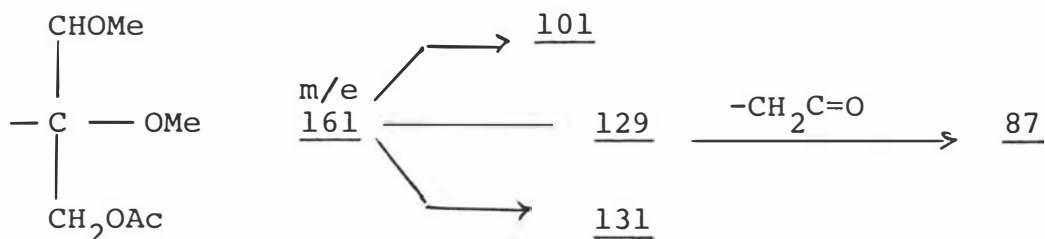
Thus it is possible to determine the substitution pattern in a partially methylated alditol acetate by comparing the spectrum with previously determined spectra and by analysing it according to the principles of cleavage to give primary fragments, and losses of acetic acid, ketene, methanol, and formaldehyde to give the secondary fragments.

On reduction some methylated sugars e.g., 2-O-methyl, and 4-O-methyl-pentose give alditols with the same substitution pattern. The information lost on reduction to alditols can be compensated if the reduction is performed with sodium borodeuteride. This enables the fragment which has arisen from the reducing end of the molecule to be distinguished and the substitution pattern is elucidated. Similarly 2,3- and 3,4-Me$_2$-xylose inseparable by g.l.c. can be distinguished by different mass spectra on reduction to alditols with borodeuteride.

$$
\begin{array}{ll}
\begin{array}{c}
\text{CHDOAc} \\
| \\
\text{H}-\text{C}-\text{OCH}_3 \\
\underline{118}\;\text{-}\;\text{-}\;\text{-}\;\text{-}\;| \\
\text{CH}_3\text{O}=\text{C}-\text{H}\;\;189 \\
| \\
\text{H}-\text{C}-\text{OAc} \\
| \\
\text{CH}_2\text{OAc}
\end{array}
&
\begin{array}{c}
\text{CHDOAc} \\
| \\
\text{H}-\text{C}-\text{OAc} \\
| \\
\text{CH}_3\text{O}-\text{C}-\text{H}\quad \underline{190} \\
\text{-}\;\text{-}\;\text{-}\;|\;\text{-}\;\text{-} \\
117\;\text{H}-\text{C}-\text{OCH}_3 \\
| \\
\text{CH}_2\text{OAc}
\end{array}
\end{array}
$$
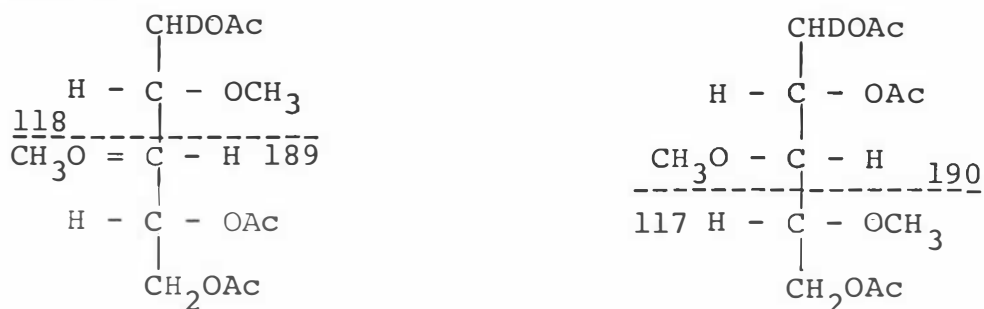
The primary fragmentation patterns for alditols of importance are listed diagrammatically in the following sections of this appendix.

Detailed spectra are recorded in Part 3.

Part 2:   Fischer Projections of Permethylated Alditol
          Acetates showing Fragmentation Positions Producing
          Primary Fragments

Key to Formulae

Pentose Derivatives; - arabinose and xylose derivatives are shown.

Hexose Derivatives; - glucose derivatives are shown.

6-Deoxyhexose Derivatives; - rhamnose derivatives are shown.

The m/e ratio for a primary fragment is shown on the side of the line designating fragmentation, corresponding to that fragment.

The substitution pattern of methyl groups is designated under each derivative.

The primary m/e fragments arise as described in Appendix 2 Part 1.

Pentose Derivatives

(1)

2,3,5 —   2,5 —   3,5 —   5 —

(2)

2,3,4 —   2,3 —   2,4 —   3,4 —

(3)

2 —   3 —   4 —   nil
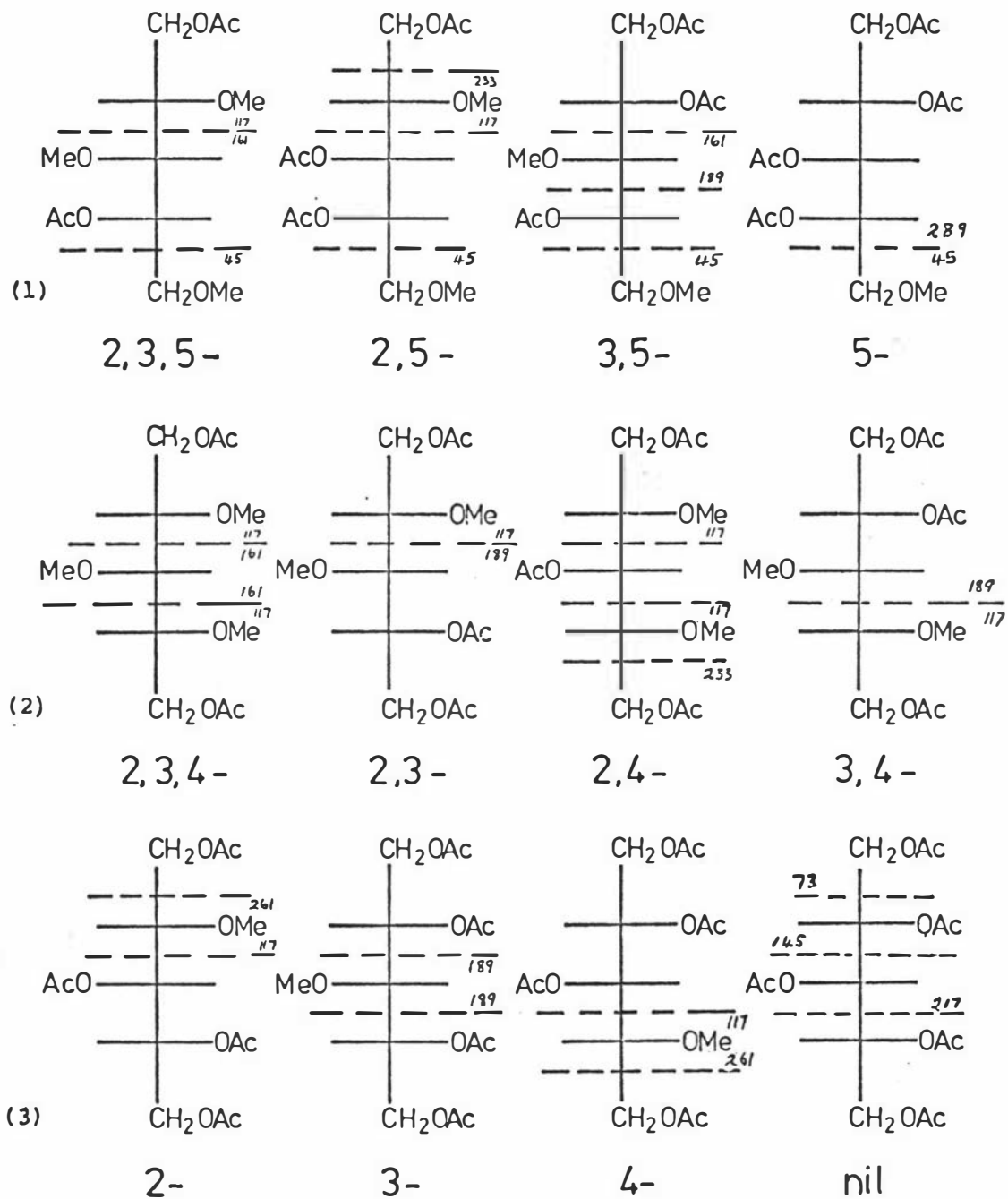
Fragmentation patterns for corresponding derivatives of
different pentoses are closely similar.
Primary fragmentations for L-arabinose derivatives, Line (1)
and D-xylose derivatives, Lines (2) and (3), are given here.

Hexose Derivatives

Fragmentation patterns for corresponding hexose derivatives of different sugars are all similar.

D-glucose derivatives are shown here.

6-Deoxyhexose Derivatives

CH₂OAc —— OMe *117* —— OMe *175* ; *161* MeO —— *131* ; AcO —— CH₃

2,3,4-

CH₂OAc —— OMe *117* ; *203* —— OMe ; AcO —— ; AcO —— CH₃

2,3-

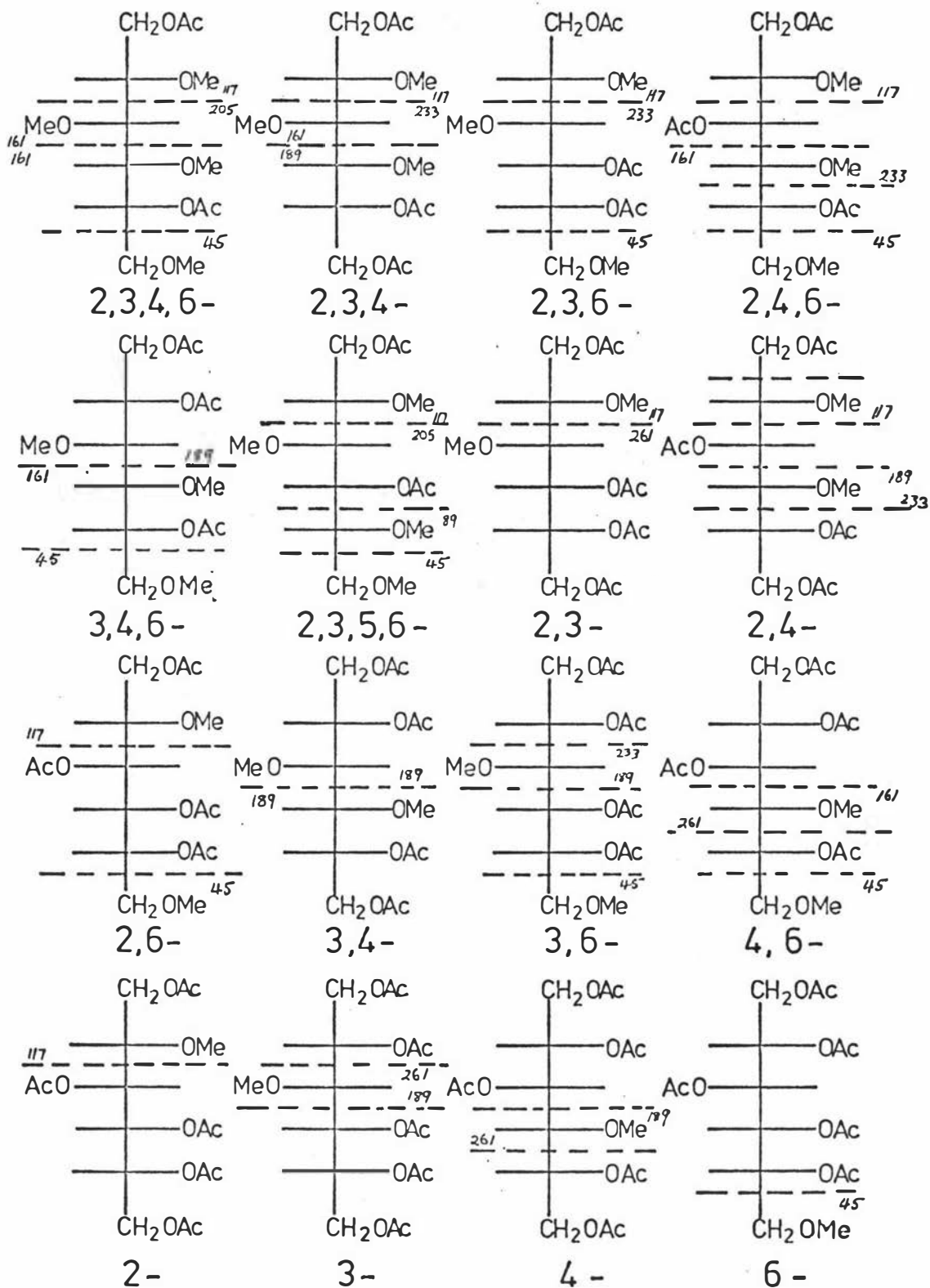CH₂OAc ; *117* —— OMe —— OAc ; MeO —— *131* ; *233* ; AcO —— CH₃

2,4-

CH₂OAc —— OAc —— OMe ; *189* MeO —— *131* ; AcO —— CH₃

3,4-

CH₂OAc ; *117* —— OMe —— OAc ; AcO —— ; AcO —— CH₃

2-

CH₂OAc —— OAc ; *203* —— OMe *189* ; AcO —— ; AcO —— CH₃

3-

CH₂OAc —— OAc —— OAc ; MeO —— *131* ; *261* ; AcO —— CH₃

4-

CH₂OAc ; *73* ; *145* —— OAc —— OAc ; AcO —— *159* ; AcO —— *89* ; CH₃

nil

Fragmentation patterns for L-rhamose derivatives are the same as for corresponding L-fucose. L-rhamnose (6-deoxy-L-mannose) derivatives are shown here.

Part 3:   <u>Detailed Mass Spectra have been recorded for pure</u>
          <u>samples, of partially methylated derivatives</u>
          <u>observed in this work</u>

Mass-spectrometry conditions used were;
                ionising potential, 70eV.
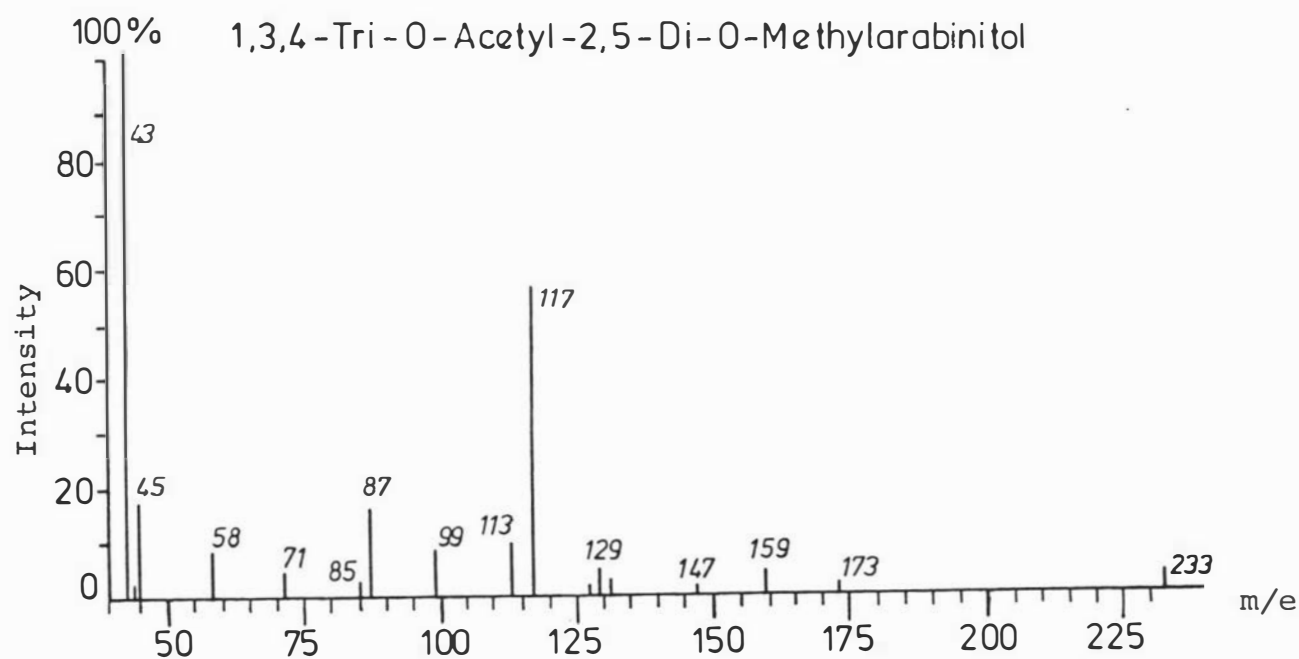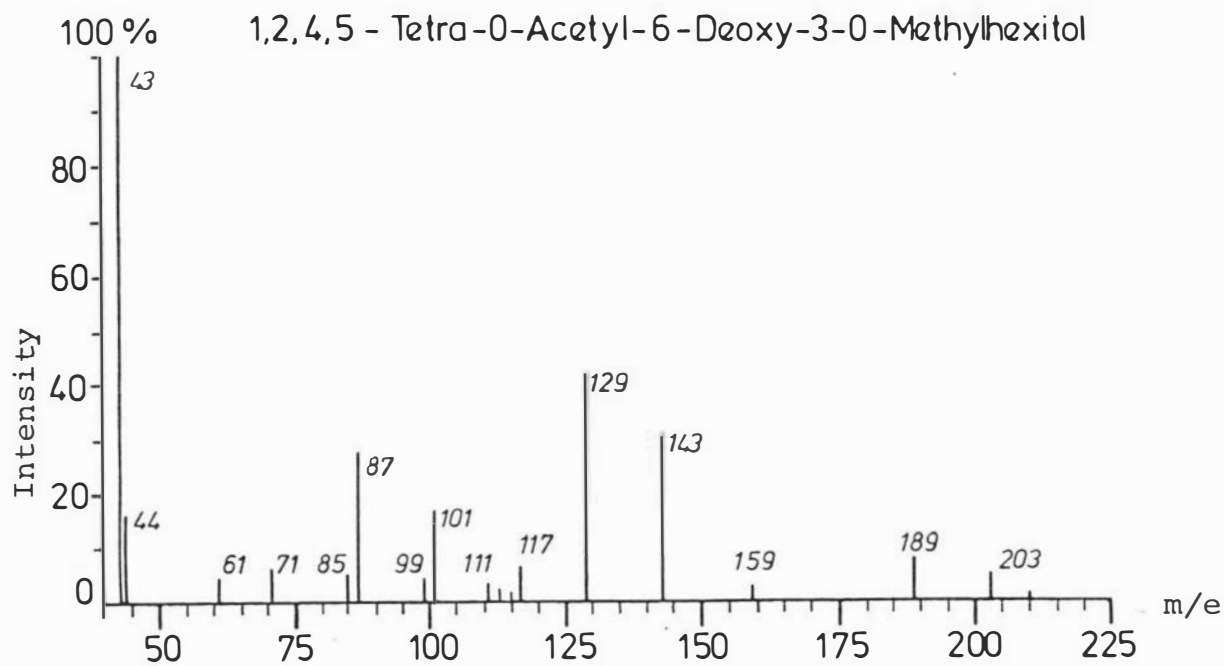   accelerating voltage, 4 K.V.
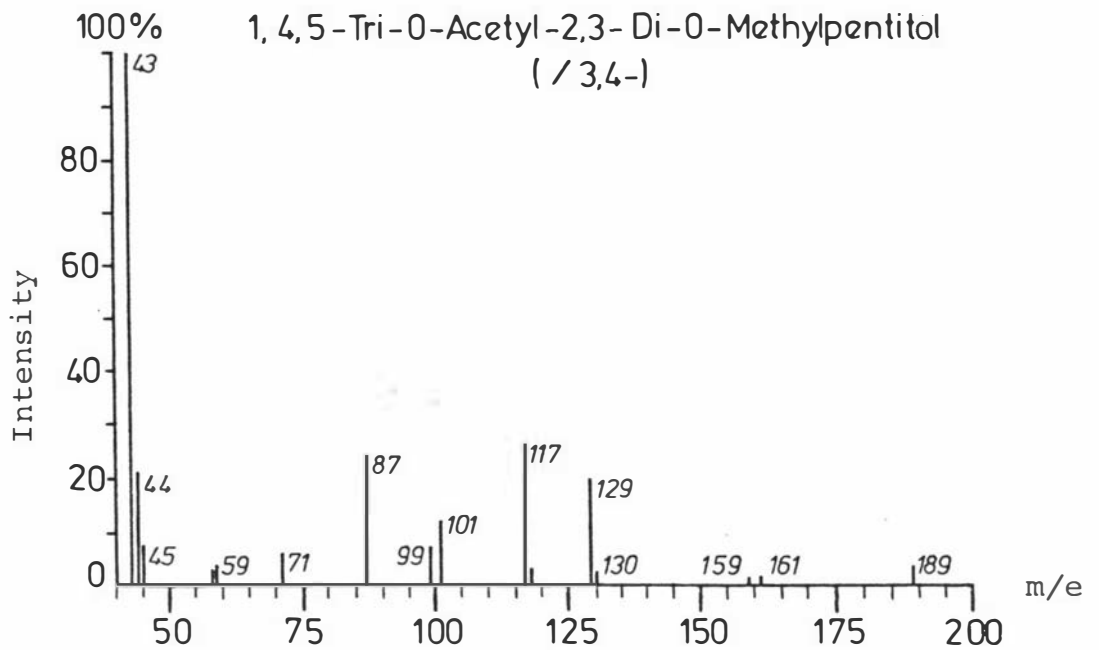   ion source temperature, $250^{O}C$
                emission current 100 μ amp

Compounds for which detailed spectra are shown: -

1.   1,2,4,5-Tetra-O-Acetyl-6-Deoxy-3-O-Methyhexitol
2.   1,3,4-Tri-O-Acetyl-2,5-Di-O-Methylarabinitol
3.   1,2,4-Tri-O-Acetyl-3,5-Di-O-Methylpentitol
4.   1,4,5-Tri-O-Acetyl-2,3/3,4-Di-O-Methylpentitol
5.   1,2,4,5-Tetra-O-Acetyl-3-O-Methylpentitol
6.   1,3,4,5-Tetra-O-Acetyl-2-O-Methypentitol
7.   1,4,5-Tri-O-Acetyl-2,3,6-Tri-O-Methylhexitol
8.   1,3,5-Tri-O-Acetyl-2,4,6-Tri-O-Methylhexitol
9.   1,2,5-Tri-O-Acetyl-3,4,6-Tri-O-Methylhexitol

For each spectrum the vertical axis represents percent of
base peak (in most cases the base peak is m/e 43) which is
taken at 100%. The m/e ratio is given on the horizontal
axis and mass numbers are listed against important mass
spectral fragments.

1,2,4,5 - Tetra-0-Acetyl-6-Deoxy-3-0-Methylhexitol

1,3,4-Tri-0-Acetyl-2,5-Di-0-Methylarabinitol

1,2,4-Tri-0-Acetyl-3,5-Di-0-Methylpentitol

1,4,5-Tri-0-Acetyl-2,3-Di-0-Methylpentitol
(/ 3,4-)

1,2,4,5-Tetra-O-Acetyl-3-O-Methylpentitol

1,3,4,5-Tetra-O-Acetyl-2-O-Methylpentitol

100% 1,4,5-Tri-O-Acetyl-2,3,6-Tri-O-Methylhexitol

100% 1,3,5-Tri-O-Acetyl-2,4,6-Tri-O-Methylhexitol

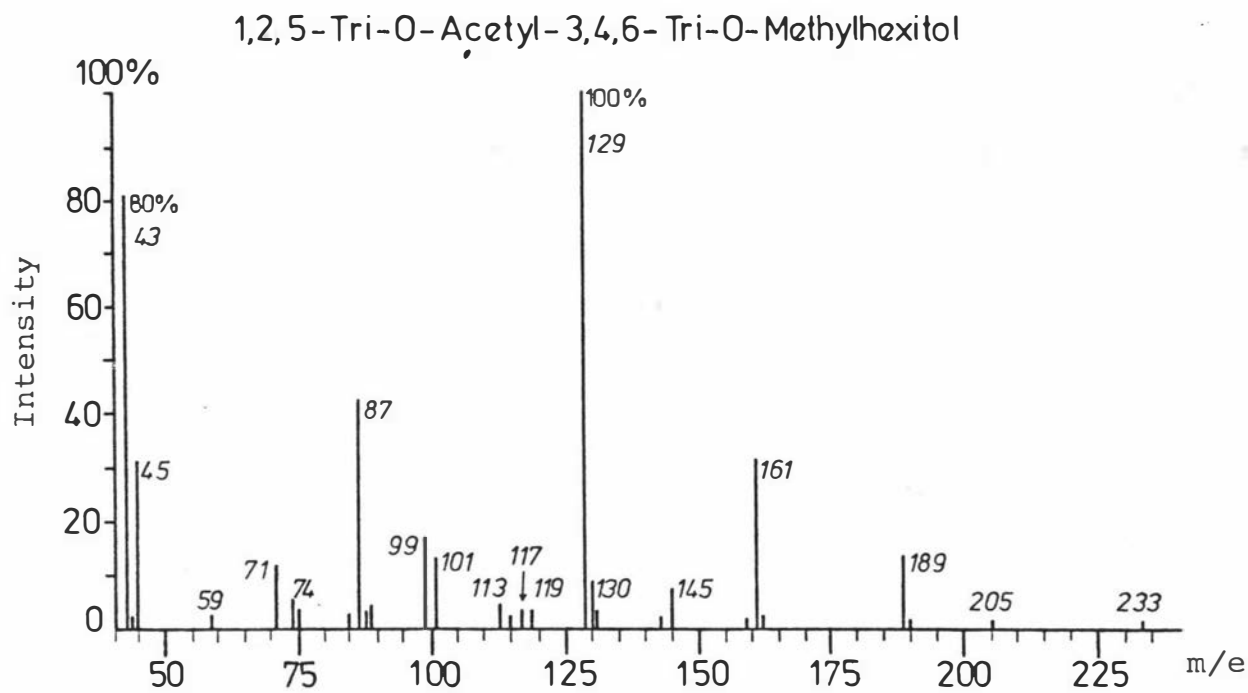1,2,5-Tri-O-Acetyl-3,4,6-Tri-O-Methylhexitol

REFERENCES

1. J.C. Roland, International Review of Cytology, 36 (1973) 45-92.

2. J.A. Monro, D. Penny and R.W. Bailey, Phytochemistry, 15 (1976) 1193-1198.

3. R.D. Preston, Annu. Rev. Plant Physiol., 30 (1979) 55-78.

4. A.E. Ahmed and J.M. Labavitch, Plant Physiol., 65 (1980) 1009-1013.

5. A.E. Clarke, R.L. Anderson and B.A. Stone, Phytochemistry, 18 (1979) 521-540.

6. F.W. Whitmore, Phytochemistry, 17 (1978) 421-425.

7. F.W. Whitmore, Plant Science Letters, 13 (1978) 241-245.

8. K. Keegstra, K.W. Talmadge, W.D. Bauer and P. Albersheim, Plant Physiol., 51 (1973) 188-197.

9. P. Albersheim, Scientific American, 232 Part 4, (1975) 80-95.

10. M. McNeil, P. Albersheim, L. Taiz and R.L. Jones, Plant Physiol., 55 (1975) 64-68.

11. F.W. Whitmore, Plant Physiol., 53 (1974) 728-731.

12. D. Sadava and M.J. Chrispeels, Develop. Biol., 30, (1973) 49-55.

13. G. Fellenberg, Progress in Botany, 38 (1976) 175-179.

14. R. Cleland, Proc .Natl. Acad. Sci. U.S.A., 70 (1973) 3092-3093.

15.   D.L. Rayle, and R. Cleland, Planta (Berl), 104 (1972)
      282-296.

16.   M. Jacobs and P.M. Ray, Plant Physiol., 56 (1975) 373-376.

17.   J.D. Blake and G.N. Richards, Carbohydr. Res., 8 (1968)
      275-281.

18.   D. Sadava and M.J. Chrispeels, Science, 165 (1969) 299-300.

19.   S. Wada and P.M. Ray, Phytochemistry, 17 (1978) 923-931.

20.   J.M. Labavitch and P.M. Ray, Phytochemistry, 17 (1978)
      933-937.

21.   J.A. Monro. Ph. D. Thesis 1974 Massey University, N.Z.

22.   D. Sadava, F. Walker and M.J. Chrispeels, Develop. Biol.,
      30 (1973) 42-48.

23.   D.T.A. Lamport ., Advan. Bot. Res., 2 (1965) 151-218.

24.   A. Hager, H. Menzel and A. Krauss, Planta 100 (1971)
      47-75.

25.   J.W. Hardin, J.H. Cherry, D.J. Morré and C.A. Lembi,
      Proc. Natl. Acad. Sci. U.S.A., 69 (1972) 3146-3150.

26.   D.H. Northcote, International Review of Cytology, 14
      (1963) 223-265.

27.   D.H. Northcote, Annu. Rev. Plant Physiol., 23 (1972)
      113-132.

28.   D.T.A. Lamport, Nature, 216 (1967) 1322-1324.

29.   D.T.A. Lamport, in "Biogenisis of Plant Cell Wall
      Polysaccharides",   F. Loewus Ed. (1973) p. 149.
      Academic Press, New York.

30. R.M. Haslemore. Ph. D. Thesis (1973) Otago University, N.Z.

31. D.T.A. Lamport and D.H. Miller, Plant Physiol., 48 (1971) 454-456.

32. R.R. Selvendran, Phytochemistry, 14 (1975) 2175-2180.

33. R.R. Selvendran, A.M.C. Davies and E. Tidder, Phytochemistry 14 (1975), 2169-2174.

34. D.H. Miller, D.T.A. Lamport and M. Miller, Science, 176 (1972) 918-920.

35. D.H. Miller, I.S. Mellman, D.T.A. Lamport and M. Miller, J. Cell Biol., 63 (1974) 420-429.

36. Y.L. Lau, H.W. Scheld and J.R. Cowles, Plant Physiol., 59 (1977) Abs. 85.

37. F.W. Whitmore, Phytochemistry, 15 (1976) 375-378.

38. R.D. Hartley and E.C. Jones, Phytochemistry, 16 (1977) 1531-1534.

39. S.C. Fry, Planta, 146 (1979) 343-351.

40. W.T. Jones and J.L. Mangan, J. Sci. Fd. Agric., 28 (1977) 126-136.

41. D.H. Northcote, S.E.B. Symposia, 17 (1963) 157-174.

42. G.O. Aspinall, in "Biogenisis of Plant Cell Wall Polysaccharides".   F. Loewus Ed. (1973) pp 95-115. Academic Press. New York.

43. D.A. Rees, and N.J. Wight, Biochem. J., 115 (1969) 431-439.

44. R.W. Stoddart and D.H. Northcote, Biochem. J., 105 (1967) 45-49.

45.   M. Knee, Phytochemistry, 17 (1978) 1257-1260.

46.   W.D. Bauer, K.W. Talmadge, K. Keegstra and P. Albersheim,
      Plant Physiol., 51 (1973) 174-187.

47.   K.W. Talmadge, K. Keegstra, W.D. Bauer and P. Albersheim,
      Plant Physiol., 51 (1973) 158-173.

48.   I.R. Siddiqui and P.J. Wood, J. Sci. Fd. Agric., 28 (1977)
      530-538.

49.   T.E. Timell, Adv. Carbohydr. Chem., 19 (1964)
      247-302.

50.   T.E. Timell, Adv. Carbohydr. Chem., 20 (1965)
      409-483.

51.   R.W. Bailey, in "Chemistry and Biochemistry of Herbage."
      G.W. Butler and R.W. Bailey Ed. (1973) pp 157-211.
      London Academic Press.

52.   G.O. Aspinall, Adv. Carbohydr. Chem., 14 (1959)
      429-468.

53.   P. Albersheim, W.D. Bauer, K. Keegstra and K.W. Talmadge,
      in "Biogenisis of Plant Cell Wall Polysaccharides,"
      F.A. Loewus Ed. (1973) pp 117-147. Academic Press. New
      York.

54.   D. Burke, P. Kaufman, M. McNeil and P. Albersheim.
      Plant Physiol., 54 (1974) 109-115.

55.   G.O. Aspinall, J.A. Molloy and J.W.T. Craig, Can. J.
      Biochem., 47 (1969) 1063-1070.

56.   A.T. Mankarios, M.A. Hall, M.C. Jarvis, D.R. Threlfall
      and J. Friend, Phytochemistry, 19 (1980) 1731-1733.

57.   K.C.B. Wilkie and Woo Swee Leng, Carbohydr. Res., 57
      (1977) 145-162.

58. A.J. Buchala, C.G. Fraser and K.C.B. Wilkie, Phytochemistry 11 (1972) 2803-2814.

59. R.L. Anderson and B.A. Stone, Aust. J. Biol. Sci., 31 (1978) 573-586.

60. D.J. Mares and B.A. Stone, Aust. J. Biol. Sci., 26 (1973) 793-812.

61. G.B. Fincher, J. Inst. Brew., 81 (1975) 116-122.

62. I.S. Forrest and T. Wainwright, J. Inst. Brew., 83 (1977) 279-286.

63. D.J. Mares and B.A. Stone, Aust. J. Biol. Sci., 26 (1973) 1005-1007.

64. P. Albersheim, in "Plant Biochemistry", Bonner and Varner 3rd Edn. (1976) pp 225-274, Academic Press, New York.

65. R.D. Preston, in "The Physical Biology of Plant Cell Walls", Chapman and Hall (1974) pp 40-42, p. 49.

66. J.S.G. Reid, and K.C.B. Wilkie, Phytochemistry, 8 (1969) 2045-2051.

67. M.A. O'Neill and R.R. Selvendran Biochem. J., 187 (1980) 53-63.

68. M.A. O'Neill and R.R. Selvendran, Carbohydr. Res. 79 (1980) 115-124.

69. D.L. Rayle, Planta (Berl) 114 (1973) 63-73.

70. G.A. Gilbert and S.P. Spragg, Methods in Carbohydrate Chemistry Vol. 4 (1964) p. 168.

71. J. Okuda and I. Miwa. Methods of Biochemical Analysis Vol. 21 (1973) 169-173.

72. M.J. Karnovsky, J. Cell. Biol., 27 (1965) 137A.

73. J.H. Venable and R. Coggeshall, J. Cell Biol., 25 (1965) p. 407.

74. T. Murashige and F. Skoog, Physiol.Plant., 15 (1962) 473-497.

75. E.M. Linsmaier and F. Skoog, Physiol. Plant., 18 (1965) 100-127.

76. H. Geissbühler and F. Skoog, Tappi, 40 (1957) 257-262.

77. F.A.L. Clowes and B.E. Juniper, Botanical Monographs J.H. Burnett ed. 8 (1968) p. 228, Blackwell Scientific Publications.

78. M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Robers and F. Smith, Analytical Chemistry 28 (1956) 350-356.

79. J. Immers, Journal of Chromatography, 15 (1964) 252-256.

80. M. Messer and A. Dahlqvist, Analytical Biochemistry, 14 (1966) 376-392.

81. N. Blumenkrantz and G. Asboe-Hansen, Analytical Biochemistry 54 (1973) 484-489.

82. B.R. Switzer and G.K. Summer, Analytical Biochemistry, 39 (1971) 487-491.

83. D.J. Prockop and S. Udenfriend, Analytical Biochemistry, 1 (1960) pp. 228-239.

84. D.B. Johnson, W.E. Moore and L.C. Zank, Tappi, 44 (1961) pp 793-798.

85. P. Albersheim, D.J. Nevins, P.D. English and A. Karr, Carbohydr. Res., 5 (1967) 340-345.

86. T.M. Jones and P. Albersheim, Plant Physiol., 49 (1972) 926-936.

87. M.A. Jermyn and E.A. Isherwood, Biochem. J., 64 (1956) 123-132.

88. P.M. Ray and D.A. Rottenberg, Biochem. J., 90 (1964) 646-655.

89. J. Martin, Tappi 50 (1967) 335-337.

90. I.M. Morrison, J. Sci. Fd. Agric., 23 (1972) 455-463.

91. M.O. Bagby, R.L. Cunningham and R.L. Maloney, Tappi, 56 (1973) 162-163.

92. R.W. Bailey, N.Z. Journal of Agricultural Research, 7 (1964) 496-507.

93. H. Björndal, C.G. Hellerqvist, B. Lindberg and S. Svensson Angew. Chem. (International Edn), 9 (1970) 610-619.

94. B. Lindberg, J. Lonngren, J.L. Thompson and W. Nimmich, Carbohydr. Res., 25 (1972) 49-57.

95. P.E. Jansson, L. Kenne, H. Liedgren, B. Lindberg and J. Lonngren, Chem. Comm.,(Stockholm) 8 (1976).

96. B. Lindberg, Methods in Enzymology, Vol 28 (1972) Part B pp 178-195.

97. G.G.S. Dutton, Adv. Carbohydr. Chem. Biochem., 28 (1973) 11-160.

98. N.K. Matheson and H.S. Sainai, Phytochemistry, 16 (1977) 59-66.

99. D.J. Mares, Ph. D. Thesis 1973 La Trobe University, Australia.

100. H. Björndal, B. Lindberg and S. Svensson, Acta. Chem. Scand., $\underline{21}$ (1967) 1801-1804.

101. T.E. Timell, Sven. Papperstidn., $\underline{63}$ (1960) 668-671.

102. A.T. Mankarios, C.F.G. Jones, M.C. Jarvis, D.R. Threlfall and J. Friend, Phytochemistry, $\underline{18}$ (1979) 419-422.

103. B.J.H. Stevens and R.R. Selvendran, Phytochemistry, $\underline{19}$ (1980) 559-561.

104. M.C. Jarvis and H.J. Duncan, Phytochemistry, $\underline{15}$ (1976) 171-174.

105. R.W. Bailey in "Data for Biochemical Research", R.M.C. Dawson, D.C. Elliott, W.H. Elliott and K.M. Jones Ed. 1968 p.541, Oxford University Press.

106. R.W. Bailey, ibid., p. 544.

107. J. Hickman and G. Ashwell. J. Biol. Chem., $\underline{241}$ (1966) 1424-1428.

108. D.J. Brasch and L.E. Wise, Tappi, $\underline{39}$ (1956) 581-588.

109. D.J. Brasch and L.E. Wise, ibid., $\underline{39}$ (1956) 768-774.

110. G.G.S. Dutton, Adv. Carbohydr. Chem. Biochem., $\underline{30}$ (1974) 9-110.

111. S. Hakomori, J. Biochem., $\underline{55}$ (1964) 205-208.

112. H.E. Conrad, Methods Carbohydr. Chem., $\underline{6}$ (1972) 361-364.

113. D.P. Sweet, R.H. Shapiro and P. Albersheim, Carbohydr. Res., $\underline{40}$ (1975) 217-225.

114. B.S. Valent, A.G. Darvill, M. McNeil, B.K. Robertson and P. Albersheim, Carbohydr. Res., $\underline{79}$ (1980) 165-192.

115. J. Lonngren and A. Pilloti, Acta. Chem. Scand., $\underline{25}$ (1971) 1144-1145.

116. A.G. Darvill, M. McNeil and P. Albersheim, Plant Physiol., $\underline{62}$ (1978) 418-422.

117. G.O. Aspinall, I.W. Cottrell, J.A. Molloy and M. Uddin, Can. J. Chem., $\underline{48}$ (1970) 1290-1295.

118. B. Lindberg and J. Lonngren, Methods in Enzymology, Vol. 50 Part C (1978) pp 20-24.

119. R.L. Taylor and H.E. Conrad, Biochemistry, $\underline{11}$ (1972) 1383-1388.

120. H.D. Van Etten and D.F. Bateman, Phytopathology, $\underline{59}$ (1969) 968-972.

121. F.L. Humoller, Methods Carbohdr. Chem., $\underline{1}$ (1962) 102-104.

122. R.W. Bailey, N.Z. Journal of Agricultural Research, $\underline{10}$ (1967) 15-32.

123. W.E. Trevelyan, D.P. Procter, and J.S. Harrison, Nature, $\underline{166}$ (1950) 444-445.

124. K.S. Jiang and T.E. Timell, Cellulose Chem. Technol., $\underline{6}$ (1972) 493-498.

125. T.E. Timell, Sven. Papperstidn., $\underline{64}$ (1961) 748-750.

126. A.J. Roudier and L. Eberhard, Bull. Soc. Chim. (France), (1963) 844-850.

127. A.J. Roudier and J. Galzin, Bull. Soc. Chim. (France), (1966) 2480-2485.

128. T.E. Timell, Tappi, $\underline{44}$ (1961) 88-96.

129. T.E. Timell, Sven. Papperstidn., 64 (1961) 651-661.

130. J.P. Thornber and D.H. Northcote, Biochem. J., 81 (1961) 449-455.

131. J.P. Thornber and D.H. Northcote, ibid, 81 (1961) 455-464.

132. J.A. Monro, R.W. Bailey and D. Penny, Phytochemistry, 15 (1975) 175-181.

133. R.W. Bailey and H. Kauss, Planta (Berl), 119 (1974) 233-245.

134. J.A. Monro, R.W. Bailey and D. Penny, Phytochemistry, 13 (1974) 375-382.

135. J.A. Monro, R.W. Bailey and D. Penny, Carbohydr. Res., 41 (1975) 153-161.

136. J.A. Monro, R.W. Bailey and D. Penny, Phytochemistry, 11 (1972) 1597-1602.

137. Y.U. Fu, and T.E. Timell, Cellulose Chem. Technol., 6 (1972) 513-515.

138. Hans. R. Schreuder, W.A. Coté and T.E. Timell, Sven. Papperstidn., 69 (1966) 641-657.

139. K.S. Jiang and T.E. Timell, Sven. Papperstidn., 75 (1972) 592-594.

140. A.J. Roudier and L. Eberhard, Bull. Soc. Chim. (France), (1965) 460-464.

141. B.W. Simson and T.E. Timell, Cellulose Chem. Technol., 12 (1978) 79-84.

142. J. Aitken and K. Horgan, Production Forestry Division, Tree Physiology Report No. 33 (1978) ODC, 168: 174-7 Pinus radiata. Forestry Research Institute. Rotorua N.Z.

143.   J.J. Doesburg, in "Phytochemistry", L.P. Miller Ed.
        Vol 1 (1973) p.278. Van Nostrand-Reinhold, New York.

144.   B.W. Simson and T.E. Timell, Cellulose Chem. Technol.,
        12, (1978) 63-77.

145.   J.W.L.Little, M. Sc. Thesis (1980) Massey University.

146.   D.T.A. Lamport, Annu. Rev. Plant Physiol., 21 (1970)
        235-270.

147.   R.R. Selvendran, Phytochemistry, 14 (1975) 1011-1017.

148.   H. Meier and K.C.B. Wilkie, Holzforschung, 13 (1959)
        177-182.

149.   M.C. Jarvis, D.R. Threlfall and J. Friend, Biochem. Soc.
        Transactions, 6 (1978) pp 391-392.

150.   G.O. Aspinall, in "Elucidation of Organic Structures",
        Bently and Kirby 2nd Edn., pp 379-450. Wiley International.

151.   S.G. Ring and R.R. Selvendran, Phytochemistry, 19 (1980)
        1723-1730.

152.   W.A. Coté, P.A. Pickard, T.E. Timell, Tappi, 50 (1967)
        350-356.

153.   B.W. Simson and T.E. Timell, Cellulose Chem. Technol.,
        12 (1978) 51-62.

154.   A. Mollard and F. Barnoud, Carbohydr. Res., 39 (1975)
        C16-C17.

155.   A. Mollard, F. Barnoud and G.G.S. Dutton, Physiol. Veg.,
        14 (1976) 101-108.

156.   G. Chambat, J.P. Joseleau and F. Barnoud, Phytochemistry,
        20 (1981) 241-246.

157. Y. Takeuchi and A. Komamine, Physiol. Plant., 48 (1980) 271-277.

158. R.W. Stoddart, A.J. Barrett and D.H. Northcote, Biochem. J., 102 (1967) 194-204.

159. G.C. Hoffmann and T.E. Timell, Tappi, 53 (1970) 1896-1899.

160. G.A. Towle and R.L. Whistler, in "Phytochemistry" L.P. Miller Ed. Vol. 1., (1973) p. 208. Van Nostrand Reinhold, New York.

161. J.A. Smelstorius, Holzforschung, 28 (1974) 99-106.

162. B. Kosikova, D. Joniak and L. Kosakova, Holzforschung, 33 (1979) 11-14.

163. J. Lonngren and S. Svenson, Adv. Carbohydr. Chem. Biochem., 29 (1974) 41-106.

164. R. Cleland, Annu. Rev. Plant Physiol, 22 (1971) 197-222.

165. D.T.A. Lamport, Recent Adv. Phytochem., 11 (1977) 79-115.

166. B.W. Simson and T.E. Timell, Cellulose Chem. Technol., 12 (1978) 39-50.

167. H. Meier, Journal of Polymer Science, 51 (1961) 11-18.

168. R.P. Kibblewhite and D. Brookes, Wood Science and Technology, 10 (1976) 39-46.

169. R.P. Kibblewhite, N.S. Thompson and D.G. Williams, Wood Science and Technology, 5 (1971) 101-120.

170. J.A. Monro, R.W. Bailey and D. Penny, Phytochemistry, 15 (1976) 175-181.

171. J.E. Darvill, M. McNeil, A.G. Darvill and P. Albersheim, Plant Physiol., 66 (1980) 1135-1139.

172. R.G. Fulcher, M.E. McCully, G. Setterfield and
     J. Sutherland, Can. J. Botany, 54 (1976) 539-542.

173. J.P. Joseleau and F. Barnoud, Phytochemistry, 13 (1974)
     1155-1188.

174. B.S. Valent and P. Albersheim, Plant Physiol., 54 (1974)
     105-108.

175. G.C. Hoffman and T.E. Timell, Sven. Papperstidn., 75
     (1972)   135-142.

176. Y. Fu, P.J. Gutmann and T.E. Timell, Cellulose Chem.
     Technol., 6 (1972) 507-512.

177. J.E. Darvill, M. McNeil, A.G. Darvill and P. Albersheim,
     Plant Physiol., 66 (1980) 1135-1139.

178. P.S. Baur and C.H. Walkinshaw, Can. J. Botany, 52 (1974)
     615-619.

179. J.E. Hodge and B.T. Hofreiter, Methods Carbohydr. Chem.,
     1 (1962) 389-390.

180. E. Marré, Annu. Rev. Plant Physiol., 30 (1979) 273-288.

181. P.M. Ray, Plant Physiol., 59 (1977) 594-599.

182. D.G. Pope, Planta, 140 (1978) 137-142.

183. K.V. Ramalingam and T.E. Timell, Sven. Papperstidn.,
     67 (1964) 512-521.

184. W.H. Loescher and D.J. Nevins, Plant Physiol., 52 (1973)
     248-251.

185. J. Washer, K.J. Reilly and J.R. Barnett, N.Z. Journal of
     Forestry Science, 7 (1977) 321-328.

186. H. Rauvala, Carbohydr. Res., 72 (1979) 257-260.

187. L. Kenne, B. Lindberg, K. Petersson and P. Unger, Carbohydr. Res., 84 (1980) 184-186.

188. R.P. Kibblewhite, Cellulose Chem. Technol., 7 (1973) 659-668.

189. P. Albersheim, Biochem. Biophys. Res. Commun., 1 (1959) 253-257.

190. R. Waite and A.R.N. Gorrod, J. Sci. Fd. Agric., 10 (1959) 317-326.

191. P. Albersheim, H. Neukom and H. Deuel, Arch, Biochem. Biophys., 90 (1960) 46-51.

192. M. Knee, Phytochemistry, 12 (1973) 637-653.

193. G.O. Aspinall and D. McGrath, J. Chem. Soc., C (1966) 2133-2139.

194. C. Tanford, Adv. in Protein Chemistry, 24 (1970) 1-95.

195. G.T. Grant, C. McNab, D.A. Rees and R.J. Skerret, Chem. Commun., Part 14 (1969) 805-806.

196. P.H. Von Hippel and K.Y. Wong, Science, 145 (1964) 577-580.

197. J.F. Bobalek and M.A. Johnson, A.G.P. News, 3 (1980) 18-25.

198. G.O. Aspinall and K.S. Jiang, Carbohydr. Res., 38 (1974), 247-255.

199. A.G. Darvill, M. McNeil and P. Albersheim, Plant Physiol., 62 (1978) 418-422.

200. O. Perila and C.T. Bishop, Can. J. Chem., 39 (1961) 815.

201. M. Mori, S. Eda and K. Kato, Carbohydr. Res., 84 (1980) 125-135.

202. M. Marx-Figini and G.V. Shultz, Biochem. Biophys. Acta, 112 (1966) 81-101.

203. D.A. Rees and I.W. Steele. Biochemistry, 5 (1966) 3108-3110.

204. I.R. Siddiqui and P.J. Wood, Carbohydr. Res., 50 (1976) 97-107.

205. M.C. Jarvis, M.A. Hall, D.R. Threlfall and J. Friend, Planta, 152 (1981) 93-100.

206. S. Ishii, Plant Physiol., 62 (1978) 586-589.

207. Bruce Christie, Personal Communication, Dept. Plant Science and Horticulture, Massey University.

208. P. Halmer and T.A. Thorpe, Phytochemistry, 15 (1976) 1585-1588.

209. H. Bjorndal, B. Lindberg, A. Pilotti and S. Svennson, Carbohydr. Res., 15 (1970) 339-349.

210. A.F.A. Wallis, in "Lignins", K.V. Sarkanen and C.H. Ludwig Ed. (1971) p. 361. Wiley International.

211. A. Darvill, M. McNeil, P. Albersheim and D.A. Delmer in "The Biochemistry of Plants" P.K. Stumpf and E.E. Conn. Edn., Vol. 1, (1980), pp 137-143, Academic Press, New York.

212. R.G. Brown and W.C. Kimmins, Plant Phsiol., 63 (1979) 557-561.

213. D.A. Rees and N.G. Richardson, Biochemistry, 5 (1966) 3099-3107.