CARBOHYDRATE FRACTIONATION AND ELONGATION OF LUPIN HYPOCOTYL CELL WALLS

A thesis presented in partial fulfilment

of the requirements for the degree

of Doctor of Philosophy

in Botany at

Massey University

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1974

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ABSTRACT

The relationship between extensibility, growth rate and carbohydrate composition in different sections of lupin hypocotyl has been investigated. Although significant differences in extensibility were found, the carbohydrate composition of elongating and non-elongating regions were similar when delignified tissue was examined. However, it was subsequently found that the delignification removed all of the wall hydroxyproline, most of the arabinose, and much galactose and that all of these were higher in non-elongating than in elongating hypocotyl. The acid conditions of delignification caused about half of the loss of the sugars but did not cause the loss of hydroxyproline.

Extraction of the hypocotyl cell walls with guanidinium thiocyanate and other denaturants, both before and after treatment with dilute acid or sodium methoxide in methanol did not dissolve the hydroxyproline, indicating that compounds containing this amino acid are probably covalently linked to insoluble wall constituents other than through acid labile arabinofuranose-hydroxyproline or ester links alone. 10% KOH extracted most of the wall hydroxyproline and hemicellulose largely as non-dialysable material. The hemicellulose thus extracted may be fractionated into hemicelluloses A and B and the latter into linear 1-4 linked polysaccharides and branched polysaccharides. Most of the hydroxyproline containing polymer is co-precipitated with the linear 1-4 linked hemicellulose-B arabinoxylan.

When cell walls from elongating and non-elongating hypocotyl sections were compared the hemicellulose-B arabinoxylan fraction from the non-elongating wall had a much higher proportion of arabinose, galactose and hydroxyproline than the same polymer from elongating wall.

Extraction of cell walls with 10% KOH at 0°C removed about two thirds of the hemicellulose-B but little hydroxyproline. Subsequent treatment with 10% KOH at room temperature removed most of the hydroxyproline and remaining hemicellulose-B. The hemicellulose-B removed at room temperature showed the greatest increases in arabinose and galactose accompanying cessation of elongation. The polysaccharide extracted at 0°C is mainly xylan while that removed at room temperature contains large amounts of galactose and arabinose. The release of galactose at room temperature was accompanied by destruction of serine and appeared to parallel β-elimination of galactosylserine The kinetics of release of arabinose and galactose at room temperature differed.

The above and other results are discussed particularly in relation to wall structure and a tentative model for the extensin-polysaccharide complex of lupin hypocotyl cell walls is proposed.

ACKNOWLEDGEMENTS

My thanks are due particularly to Drs.R.W.Bailey and E.D.Penny for their supervision encouragement and patience in directing me through this work.

I also wish to thank Mrs Sue Brennan for help in preparation of the manuscript and for some help in the practical work, and Mrs Olive Brigham for spending many sunny afternoons behind the typewriter.

To those who have contributed only through discussion I am also indebted, particularly to Vasil Sarafis who did much to give some idea of a botanical perspective.

For maintaining a broader perspective I am particularly thankful to Liz who reinforced my sense of priorities so that I was able to love life despite the thesis.

TABLE OF ABBREVIATIONS

DP	Plastic compliance (mm^2/Newton)
DE	Elastic compliance (mm^2/Newton)
DT	Total compliance (mm^2/Newton)
GTC	Guanidinium thiocyanate
HO	Hemicellulose extracted from neutral detergent treated walls by 10% KOH at 0^oC
HRT	Hemicellulose extracted from cell walls by 10% KOH at 18-22^oC after prior neutral detergent depectination and removal of HO
H24	Hemicellulose not extracted from the cell walls by 10% KOH at 18-22^oC but removed by 24% KOH
RND	Cell walls extracted with neutral detergent
RO	RND after extraction with 10% KOH at 0^oC
RRT	RO after extraction with 10% KOH at 18-22^oC
R24	RRT after extraction with 24% KOH at room temperature
IAA	Indole-3-acetic acid
ATP	Adenosine triphosphate
ATPase	ATP phosphohydrolase
Glu.	Glucose
Gal.	Galactose
Man.	Mannose
Ara.	Arabinose
Xyl.	Xylose
EtOH	Ethanol
EDTA	Ethylenediamine tetra-acetic acid

INTRODUCTION

The most stable configuration of a protoplast is spherical (Plateau, 1873) and it will assume this shape unless otherwise constrained (Errera, 1888). In plants such a constraint is provided externally by the cell wall, and where the constraint is unequally distributed growth is similarly unequal (Roelofsen, 1965). The wall present during elongation growth is defined as the primary cell wall. The primary cell wall determines the shape of the protoplast and this is fixed by the deposition of secondary wall after the period of cell enlargement. The latter component is regarded as the structural component of the plant, i.e. the plant skeleton. As the shape of the plant skeleton is determined by the primary cell wall the importance of the latter to plant morphogenesis is considerable.

Cell Wall Development and Organisation

Growth of the higher plant stem involves the processes of cell division and cell elongation. The former culminates in the formation of cross walls which later undergo the bulk of their primary synthesis during the elongation phase, prior to secondary thickening. There is no region of cell division exclusive of elongation and although the maximal rates of elongation and division may coincide they need not (Sachs, 1965). Buis (1967) has shown that in etiolated *Lupinus albus* hypocotyl maximum elongation occurs after cell division has ceased. Cell division is usually confined to the upper region and its rate generally decreases more rapidly than that of elongation.

The ontogeny of the cell wall can be traced to the telophase period of division (Northcote, 1969) when the spindle is present and chromosomal separation has taken place. At this stage vesicles appear to concentrate and coalesce at the equator of the spindle (Hepler and Newcomb, 1967) possibly guided there by the microtubular spindle fibres (Ledbetter and Porter, 1965; Newcomb, 1969). (It now seems that the microtubular element of cells is an important part of the intracellular transport system). However, while many of these electron microscopic observations strongly suggest certain roles for and relationships of the various cell organelles much of the evidence is indirect and little has actually been proved (O'Brien, 1972).

Electron microscopy has shown that in root epidermal cells (Pickett-Heaps, 1967; Northcote, 1968) these vesicles are derived from the Golgi apparatus, an organelle which has become synonymous with non-cellulosic polysaccharide synthesis. Consistent with the early radioautographic work of Northcote and Pickett-Heaps (1966) the vesicles have been shown cytochemically to be rich in pectic materials.

The zone of pectin deposited from the fusing vesicles forms a disc between the walls of the parent cell and constitutes what has become known as the cell plate (Whaley and Mollenahuer, 1963), which will contribute significantly to the middle lamella of the wall deposited by each of the daughter cells. The cell wall grows from the cell plate by the incorporation of cellulose, more pectic substances and other polysaccharides (Wilson, 1964; Northcote, 1972).

Our ability to distinguish the middle lamella from the

remainder of the cell wall depends upon the different nature of the polysaccharide deposited by the daughter cells after cell plate formation.

The reason for the qualitative change in wall synthesis is that these cells will ultimately provide directional resistance to turgor pressure (the in vivo driving force for cell expansion) and thus contribute to the elongation of the stem, and also so that they may in time provide support by resisting external forces. The rigidity is conferred upon the wall by the deposition of cellulose microfibrils and the associated annealing matrix polymers.

In higher plants the primary wall consists basically of a framework of essentially crystalline cellulose microfibrils embedded in a relatively amorphous and continuous matrix. Because of the high degree of molecular order within the cellulose microfibril it would require the longitudinal extension of either valency bonds or the deformation of valency angles to stretch the microfibrils (Preston, 1959). They can therefore be considered as having a longitudinal elastic modulus sufficiently large to render them virtually inextensible, and to be a rigid component embedded in a relatively extensible matrix of pectin, hemicellulose and protein. The microfibrils plus matrix together form the extensible framework which contains the protoplast.

It has been recognised for many years that there is a close correlation between the direction of cell extension and the orientation of the microfibrils, i.e. that anisotropic growth of a wall parallels an anisotropy of structure. In an extensive survey of the walls from cells of various shapes

Roelofsen (1965) showed that an isotropic wall has extended multidirectionally whereas an anisotropic one will have extended normal to the predominant orientation of structure.

Probine and Preston (1962) have related birefringence, ultra-structure and cell wall mechanics using the internodal cells of Nitella. They found, averaged over the whole thickness of the wall, a preferred orientation in the transverse direction (major extinction position). Extension experiments on longitudinal and transverse strips showed clearly that the cell wall is most plastic and has the lowest tensile strength in the direction which has the least cellulose microfibril reinforcement. Moreover, from later experiments Probine (1965) was able to conclude that not only do non-isodiametric cells have a mean microfibril orientation perpendicular to their axis of elongation but also that with any change in gross arrangement of the microfibrils there will be a concomitant change in the direction of growth. This was illustrated by the effects of benzimidazole (B.I.A.) in altering the preferred deposition of microfibrils from a transverse to a longitudinal orientation in pea epicotyl sections. Segments which underwent elongation in the absence of B.I.A. were found to have a transverse extinction (low birefringence) showing a transverse orientation of microfibrils. Cells which increased diameter due to the presence of B.I.A. showed extinction along the cell axis. Furthermore, placing the sections in C^{14}-sucrose in the presence of B.I.A. resulted in the longitudinal deposition of cellulose, as seen by autoradiography. B.I.A. therefore seems to stop elongation by preferentially causing longitudinal fibres to be laid down.

As early as 1935 Bonner showed that when an Avena coleoptile was stretched the change in birefringence of its walls indicated that the microfibrils had changed their major orientation from transferse to longitudinal. Also, a higher plant cell wall generally elongates over its whole surface. Furthermore Ray (1967) has shown that cellulose microfibrils are laid down by apposition, i.e. on the innermost surface of the wall adjacent to the cytoplasm. These three observations indicate that microfibrils of the outermost cell wall must have undergone considerable reorientation between the time when they were synthesised and the cell stops elongation. The microfibrils must also be free to move relative to one another to give the observed change in the orientation from transverse on the inner wall to longitudinal on the outer. Because the microfibrils are deposited essentially as lamallae, a major constraint on their movement will not be microfibril entanglement but the degree of adhesion between microfibrils and between microfibrils and the matrix.

Interaction of the microfibrils must be mediated via the matrix of the cell wall as the microfibrils are embedded in this. The tenacity with which the matrix is bound to them is such that most α-cellulose preparations from cell walls contain some sugars other than glucose. The crystalline core of the microfibril appears to be surrounded by an amorphous sheath and to be interrupted at regular intervals by regions which are paracrystalline (Muhlethaler, 1967). These regions and particularly the sheath are probably the source of non-glucose residues and the regions of interaction with the matrix polysaccharides which could be very important polymers involved

in the transfer of force from matrix to microfibril.

Both the crystallinity within the fibre and the non-crystallinity of its surface are considered to be important in determining the strength of the cell wall, the former makes possible a network of inextensible units and the latter helps the units to cohere either directly or through linkage with matrix polymers into a mechanical whole. It is the cohesion between microfibrils which is considered in this thesis, as until the microfibrils of the network are allowed to move in relation to one another cell elongation will not occur.

The Cell Wall Matrix

The primary cell wall matrix has been divided into three major structural components: pectic substances, hemicelluloses and protein. The present discussion concerns the walls of Angiosperms in which the polysaccharides show a remarkable uniformity compared with the diversity in e.g. algal groups (Northcote, 1969).

It is important to realise that the division into the pectic substances and hemicelluloses and into their subfractions is fairly imprecise since polysaccharides even with the same qualitative composition vary considerably in their degree of polymerisation and will therefore show a spectrum of solubilities. Thus in each polysaccharide fraction there coexist molecules of different structural type, because the range in molecular weight of otherwise structurally similar molecules precludes their separation into discrete fractions by the methods used.

A. The Pectic Substances

The pectic substances are by definition dissolved from the cell wall by aqueous solvents with calcium chelating agents such as ethylenediaminetetraacetic acid (E.D.T.A.) or ammonium oxalate. They have been divided by Northcote (1969) into

(i) neutral arabinogalactans.

These are basically chains of β-(1-3) linked D-galactopyranose units to which side chains are attached by β-(1-6) links. The side chains are D-galactose oligosaccharides which may carry L-arabinofuranose units, or the side chains may be simply L-arabinofuranose units.

(ii) acidic polygalacturonide or galacturonorhamnans.

These are polymers of galacturonic acid with rhamnose insertions and a variable number of side chains containing D-xylose, D-galactose, and D-galacturonic acid. In nature a large proportion of the galacturonic acid residues are methyl esterified.

The pectic materials have the ability to form gels (Rees, 1969) a property which may be important in vivo where an increase in gelation may lead to a stiffening of the cell walls. The gelation depends upon the formation of junction zones (Rees, 1969) where the polyuronide chains become aligned and form tightly ordered microcrystallites, some of which have been studied by x-ray crystallography (Roelofsen and Kreger, 1951). It seems that the alignment necessary for the formation of junction zones can be sterically prevented by the incorporation of rhamnose units which put a link in the main chain or by the presence of side chains (Gould et al., 1965; Rees and Wight, 1969; Barrett and Northcote, 1965; Rubery and Northcote, 1970). Similarly desterification can result in a

mutual coulombic repulsion of adjacent galacturonic acid containing polymers. This is why Ca^{++} chelating agents are effective in dissolving pectins from cell walls. The presence of Ca^{++} although not leading to the formation of strong calcium bridges between uronic acids, as was once thought (Bennet-Clark, 1956), does neutralize the negative charges and allow association of acidic polymer chains.

In elongating cell walls where a fluid matrix is required it seems that pectic polysaccharides are high in rhamnose and side chains and that these decrease in the pectic substances deposited as the cell ages (Bouveng, 1965; Aspinall et al., 1967; Gould et al., 1965; Rees and Wight, 1969). It is also interesting to note that cell walls which have a strong supporting funtion e.g. celery collenchyma, and would therefore require a rigid gel, are high in microcrystallites.

B. The Hemicelluloses

The hemicelluloses are those polysaccharides dissolved by alkali from the pectin extracted cell walls. Northcote (1969) has divided them into two main types

(i) the xylans and

(ii) the gluco- and galactoglucomannans.

i) The xylans are β-(1-4) linked polymers of D-xylopyranose and are generally quite large molecules with a degree of polymerization of 150-200 and glucuronic acid side chains (about 1 per 10 xylose units). Also most of the xylose is acetylated, mostly in the C3 position. Although these acetyl groups probably prevent direct H-bonding between xylose chains crystalline structures are still formed (Neiduszynski and Marchessault, 1971). Each crystallite probably involves

several polymers surrounding a stabilizing column of water which has polar interactions with the acidic side chains.

ii) Gluco- and galactoglucomannans although constituting the bulk of the Gymnosperm hemicellulose are a minor component in Angiosperms. Basically they are chains of randomly arranged D-glucose and D-mannose units in β-(1-4) linkage. The chains have a similar conformation to that of β-(1-4) glucan although molecular packing is not as light as in the case of cellulose. Terminal α-(1-6) linked galactose units are flexible and may provide non-covalent bridges with water, and other matrix polysaccharides.

Both the xylans and the gluco- and galactoglucomannans are arranged in paracrystalline array between and in the same direction as the cellulose microfibrils and are strongly adsorbed onto their surface. They are therefore very important in any discussion of interactions of microfibrils and cell wall matrix. The relevant consideration is the linearity of these polymers as it is this which allows associations (as in the formation of microcrystallites) which have a uniting effect on wall polymers.

Bauer et al. have recently claimed that the hemicellulose of Acer pseudoplatanus and Phaseolus vulgaris suspension culturesis a xyloglucan based on a repeating heptasaccharide unit which consists of four residues of β-(1-4) linked glucose and three residues of terminal xylose linked to the 6 position of three of the glycosyl residues.

Carbohydrate fractionations exist which depend partly upon the ability of iodine-potassium iodide to precipitate linear polysaccharides from aqueous calcium chloride solution

(Gaillard and Bailey, 1968). Such a fractionation has been used in the experiments reported in this thesis.

When alkaline hemicellulose extract is adjusted to pH 4.5 long chain linear xylans will precipitate, presumably because protonation of the galacturonic acid side groups allows intra- and inter-chain alignment. The polymers can be obtained by centrifugation and are referred to as hemicellulose-A.

Hemicellulose-B is the non-precipitated fraction which is then obtained from the supernatant after dialysis and freeze drying. When it is dissolved in aqueous calcium-chloride and iodine-potassium iodide solution added a precipitate is obtained which contains all those linear species (Gaillard, 1961) including arabinoxylans which did not enter the hemicellulose-A fraction. The precipitate is the linear-B fraction. The supernatant contains all the short chain or branched polysaccharide and because of its polydiversity is termed the heteroglycan-B fraction.

The fractionation of hemicellulose polysaccharides may be enhanced by varying the concentration of alkali used for extraction of the cell wall. 10% KOH will remove the xylans and arabinoxylans as well as some other polysaccharide whereas the galactoglucomannan requires 24% KOH-4% H_3BO_3 for its extraction. (fig.1, Chapter I).

The wall remaining after the above hemicellulose extraction, although termed cellulose usually contains traces of mannose and xylose probably from residual strongly adsorbed glucomannan and xylan.

C. Protein

Protein is a quantitatively important component of the

matrix (Lamport, 1965) although its role in cell wall growth is very uncertain.

The cell wall protein is unusual in that about 30% of its residues are hydroxyproline (Lamport, 1970), an amino acid confined almost entirely to the cell wall in plants. L-arabinose oligosaccharides are attached O-glycosidically to most of the hydroxyproline (Lamport, 1967) and galactose (probably of galactan) by the same type of linkage to much of the serine (Lamport et al., 1973; Keegstra et al., 1973). Lamport (1965), anticipating a role for the protein in determining wall properties has gone as far as naming it extensin, suggesting that it forms part of a glycoprotein which crosslinks the microfibrils. The fact that fragments of extensin have associated galactose and arabinose (Lamport, 1962, 1969, 1970) was used as evidence for bonding to the major wall polysaccharides. A study of the relationship of the wall protein to the various polysaccharide fractions had not been made and forms part of this thesis.

The Cell Wall and Cell Elongation

Some allusion has already been made to possible ways in which the matrix of the cell wall might influence growth. Evidence has been sought from the study of the mechanical properties of the wall, and will now be discussed.

Williams, Landel and Ferry in 1955 discovered the relationship

$$\log \frac{n}{n_s} \cdot \frac{T_s \rho_2}{T \rho} = - \frac{C_1 (T-T_s)}{C_2 + T-T_s}$$

where n = steady flow viscosity

T = absolute temperature

ρ = density

and C_1 and C_2 are constants (8.86 and 101.6) when at the characteristic temperature T_s (at which viscosity and density are n_s and ρ_s).

This equation describes the viscoelastic behaviour of a wide variety of materials, and predicts a low or rapidly increasing temperature coefficient as the temperature is lowered. In Avena coleoptile the coefficient for elongation rates between 2-11°C and 11-20°C were found to be fairly similar (Ray and Ruesink, 1962) and hence the immediate response of elongation rate to temperature has more the characteristics of a chemical process (Arrhenius equation). This suggests that the temperature effect on growth rate is mediated mainly via metabolic processes rather than a temperature dependent change in viscosity. Although these results did not rule out viscoelastic flow they do directly involve chemical reactions within the polymer system of the cell wall, and in such a system a likely reaction which can be accommodated in a plausible model is the breakage of polymer chains, which constitute most of the cell wall.

Such a model was proposed by Preston and Hepton (1960) from a knowledge of the composition of the wall. In their model it was envisaged that plasticization of the wall, as induced by indole acetic acid (IAA) resulted from an induced metabolic cleavage of matrix polysaccharides or of cross-links between them.

So far most of the evidence that the cell wall was a cross-linked polymer system had been indirect. However, using etiolated mung bean hypocotyl and the Instron stress-strain analyser Lockhart (1967) set out to confirm that

the polymer system within the cell-wall was indeed cross-linked. This confirmation could be obtained by establishing whether the irreversible deformation occurred by viscous flow or plastic deformation, for the former is characteristic of linear polymers and the latter a property of cross-linked systems. The following criteria were used to establish which of the deformation processes were occurring. The alternatives are different in that (1) the energy required for plastic deformation is independent of deformation rate while that for viscous flow is directly proportional to deformation rate. (2) Energy required for viscous flow varies with the temperature. This is essentially not true for plastic deformation.

Energy required for irreversible deformation was found to be virtually independent of rate measured over a five fold range of deformation rates and independent of temperature between 8 and 25°C. Because a polymer system is, in principle either cross-linked or linear, the results lead to the conclusion that the irreversible deformation occurred as a result of breaking cross-links. No assumption had been made regarding the chemical nature of the cross-links and the conclusions are true for whatever polymers give the mechanical properties to the tissue.

The early work of A.N.J.Heyn (1940) in measuring the bending of Avena coleoptiles from horizontal by weights with and without the application of auxin marked the beginning of research relating auxins and wall extensibility. From his experiments Heyn was able to theorise

(1) The primary factor in cell elongation is plasticity of the cell wall. The first phase in elongation consists of an

increase in wall plasticity, followed by plastic extension of the wall during which particles of the wall slide along each other. It is this plasticity (plastic extensibility) which is regulated by the growth hormone. (2) Elastic extensibility of the wall is not a factor in elongation; changes in elastic extensibility are results of actual elongation. (3) Surface enlargement of the wall does not directly depend upon production of cell-wall material (active growth of the wall), nor on the degree of elastic extension in the wall. (4) Energy necessary for surface enlargement of the wall is derived from turgor pressure.

The work of Heyn has been refined and modified by Olson et al. (1965) and further by Cleland (1967) who both used the Instron stress-strain analyser. With this, quantitative measurements of the extensibility of isolated killed cell walls became possible in a rapid and reproducible manner. The technique was originally limited in providing only a measure of total extensibility, in not accounting for variations in wall thickness and in not providing information on the type of extensibility being measured.

A modification of the original Instron technique by Cleland (1967) overcame the first two difficulties and provided information on the type of extension being achieved. This relatively quantitative technique now made possible a better understanding of the relationships between auxin treatment, extensibility and cell elongation to be gained.

With the technique Cleland (1967) was able to show for Avena coleoptiles that plastic extensibility increased following the addition of auxin to a maximum at 90-120 mins and then remained constant for up to 24 hours. Changes in

elastic extensibility were smaller but similar. When the IAA concentration was made supra optimal for elongation so that growth was arrested, the increase in plasticity was still obtained. This suggests that growth rate is influenced by other factors. Later evidence in favour of this was provided by Ruesink (1969) who found that although cellulase could markedly increase the plasticity of the wall this alone would not increase elongation.

The extension which is nearly proportional to log time has been termed the creep. Because it involves extension under a constant force creep measurement is closer to the in vivo situation where there is a nearly constant stress due to turgor. It has been shown that auxin increases not only the initial extension but also the rate of creep and stress relaxation. This lends support to the idea that the instantaneous plastic extension is merely the high speed initial creep. Creep is uaully plotted as extension vs log time, but if plotted on a linear time scale rapidly reaches a plateau.

The walls of both Nitella and Avena have been subjected to extensive creep tests (Cleland 1971) and both have the following comparable mechanical properties (1) both undergo instantaneous extension and viscoelastic creep (2) temperature has little effect on the extension of either (3) the mechanical properties are related to the in vivo elongation rate at harvest.

The fact that elongation is both inhibited by such antimetabolites as cyanide (Bonner, 1933; Ray and Ruesink, 1962) and is strongly temperature dependent (Rayle et al., 1970; Ray and Ruesink, 1962) is evidence against

viscoelastic flow alone determining the rate of elongation and suggests a biochemical alteration of the cell wall. This and the fact that cell elongation continues for far longer than mechanical extension of isolated walls leads Cleland (1967) to the more reasonable proposition that cell elongation consists of a series of independent strain-hardened plastic deformations. Each would consist of a biochemical lowering of the yield stress followed by turgor extension until the yield stress again equalled the stress from turgor. The elongation rate might therefore be determined by (i) the degree to which yield stress is lowered (ii) the turgor pressure and (iii) the number of deformation events per unit time.

Although the plastic extensibility measured with the Instron is affected by the physiological state of the tissue extended and is a definite measure of the mechanical response of walls to an applied force, its exact relevance to the in vivo situation is not certain. Differences which do exist, and which will be discussed later, may depend upon the type of stress applied to the cell; one dimensional for the Instron and three dimensional in the case of turgor pressure.

The biochemically dynamic nature of the cell wall has been recognised for some time (Lamport, 1970), both synthesis and breakdown of polysaccharides occurring together and leading to wall turnover. These processes are known to be strikingly increased by IAA (Lamport, 1970). Indeed because the cell wall polymers are mainly carbohydrate a reasonable working hypothesis has been that auxin induced elongation is the result of an increased rate of direct enzymatic cleavage of polysaccharide chains and/or of the insertion of new polymers

into the wall. However auxin can promote elongation with a time lag of only a few minutes (Nissl and Zenk, 1969) although it is generally nearly an hour before any published increase in wall synthesis (Baker and Ray, 1965; Ray and Abdul-Baki, 1968) or of polysaccharidase level (Cleland, 1971) is detectable. Moreover, when wall loosening is caused by an exogenous potent cellulase (Ruesink, 1969) elongation is not promoted.

Recently it has been discovered that wall loosening can be induced by low pH (3-4) with a lag time of less than a minute and with striking similarities to that elicited by IAA (Rayle and Cleland, 1970). The response of frozen-thawed Avena coleoptiles (in vitro system) (Rayle et al., 1970) under tension and of intact coleoptiles to a pH of 3-4.5 had in common a similar Q_{10}, a minimum yield stress, and a similar pH threshold and optimum. In both cases an increase in plasticity is obtained reaching a maximum 60-90 mins after adding the promoter. Treatment with pronase (a protease), sodium dodecyl sulphate and a temperature of 42°C altered the response to one of greater rapidity and shorter duration.

If pH induced and auxin induced elongation are identical these results rule out any necessity for wall synthesis in the loosening process as there is no synthesis in the frozen thawed system. However prior wall synthesis is possibly related to the response in maintaining the necessary wall structure.

Pretreatment with cycloheximide did not reduce the acid effect, hence it is not a response based on acid induced synthesis of a protein involved in bond breakage. Possibly auxin causes the expulsion of H^+ ions and this lowering of pH

in vivo causes chemical changes which result in wall loosening.

This is supported by the action of C.C.C.P. (n-chlorocarbonylcyanidphenylhydrazon) which is known to make membranes permeable to protons and which rapidly reduces auxin induced elongation when applied at a concentration which does not reduce respiration (Hager *et al.*, 1971). Thus it can be argued that compartmentation of H^+ ions must take place before auxin induced elongation can occur. The notion that a membrane bound ATPase accomplishes the transfer of H^+ ions is supported by the observation that ATP will under anaerobic conditions cause an immediate stimulation of elongation (Hager *et al.*, 1971).

It therefore seems possible that while auxin is necessary for continuing cell elongation and stimulates the production of enzyme systems involved in the process, its initial action in inducing elongation is due to a direct chemical effect of low pH. In the real situation this pH effect may act in concert with other auxin influenced processes, including synthesis and degradation. These processes are intimately involved in maintaining the integrity of the wall and the potential for initial and continued response to auxins. Indeed, the sudden application of auxin is unlikely to occur normally in a plant so the rapid responses due to exogenous auxin are possibly not closely related to the natural situation.

In this thesis an analysis of the cell walls of lupin hypocotyl of various growth rates has been carried out in an attempt to identify some of the changes which accompany and therefore might contribute to a cessation of growth. Because the wall is a composite structure some attention was paid to

the interrelationships of various wall components, mainly from the standpoint of the particular carbohydrate fractionation method used in this and other laboratories. During the course of the thesis it was found that the carbohydrate fractionation method used was not altogether suitable for study of the primary cell wall. The results have been considered with this limitation in mind. Once some idea of change in composition was obtained an investigation of the physiology of the process was made in the hope of gaining some understanding of the way in which growth might be related to an alteration in the degree of association of various wall constituents.

I. POLYSACCHARIDE COMPOSITION, EXTENSIBILITY AND ELONGATION IN LUPIN HYPOCOTYL

INTRODUCTION: It is known that the mechanical properties of the cell wall are related to growth rate (Heyn, 1931; Cleland, 1967) which itself is dependent on other factors such as cell turgor, the driving force of cell elongation (Ray et al., 1972). A meaningful picture of the importance of wall compostion to cell growth can be obtained by relating composition to that parameter of cell growth which is most directly dependent upon it, namely wall extensibility. In this way one is confining oneself to the cell wall without the complication of intracellular factcrs.

There have been several studies made of changes in carbohydrates with maturation of tissues, but in none of these have the mechanical properties been correlated with composition. In fact most studies are not relevent to our understanding of the reason for a diminishing growth rate of primary cell walls as they age, because they have involved a comparison of primary tissue and tissue with considerable secondary deposition. Moreover many publications report monosaccharide analysis of total walls, or the composition of fractions in terms of total pentose and/or total hexose and are therefore of limited value.

In the following work the lupin hypocotyl has been divided into various regions which have been analysed in terms of growth rate, extensibility, and wall composition, the last involving conventional polysaccharide fractionation and

measurement of the monosaccharides comprising each of the fractions.

RESULTS AND DISCUSSION

Hypocotyl Elongation and the Mechanical Properties of the Cell Wall

The elongation of hypocotyls (about 40) was measured from the photographically recorded separation of markings on them as they were elongating through 6 cm. The upper 2 cm of hypocotyl was found to contribute about 75% to growth while the lower 2 cm had ceased elongation. Once the growth pattern of the hypocotyl was clearly established it was possible to relate other parameters to the elongation.

In relating the mechanical properties and growth rate of lupin hypocotyls creep and the elastic and plastic compliance were measured for the lower middle and upper 2 cm regions of the hypocotyl. The results in Table I and II show that all parameters decrease towards the base of the hypocotyl. However, growth rate does not seem to be closely paralleled by marked changes in the physical properties of the wall except for the case of creep. This raises the question of whether DE and DP are directly concerned with cell extension.

Which of the two values, creep and DP, is most relevent to the *in vivo* determination of growth is uncertain, but the change in creep shows a closer relationship to change in growth rate than DP.

There is a well documented lack of correlation between DE and growth, although in one case DE has been suggested as a better indication of growth potential than DP (Masuda, 1969).

TABLE I PROPERTIES OF THREE 2 cm REGIONS OF 6 cm LUPIN HYPOCOTYL

Properties	Hypocotyl Region		
	Lower	Middle	Upper
Length (cm)	2	2	2
Elongation Rate (% of total for hypocotyl)	0	24	76
Total Compliance (DT) (mm^2/Newton)	1.17(±0.06)	1.43(±0.08)	1.68(±0.07)
Plastic Compliance (DP) (mm^2/Newton)	0.36(±0.024)	0.53(±0.03)	0.66(±0.03)
Elastic Compliance (DE) (mm^2/Newton)	0.81(±0.04)	0.90(±0.05)	1.0(±0.05)
Ratio DE/DP	2.27	1.71	1.55
Creep (% increase in length) *	1.21(-0.15)	1.91(-0.30)	4.35(-0.39)
Molarity of Isotonic Mannitol	0.23	0.24	0.28

* Measured between 1 and 100 min after adding 100 g wgt.

TABLE II PROPERTIES OF LOWER MIDDLE AND UPPER REGIONS OF LUPIN HYPOCOTYL EXPRESSED AS PERCENTAGE OF VALUE FOR UPPER (MOST RAPIDLY ELONGATING) REGION

Property	Region		
	Lower	Middle	Upper
Elongation	0	31.5	100
DT	70	85	100
DP	55	80	100
DE	81	90	100
$DE/_{DP}$	146.5	110	100
Creep	28	44	100
$Creep/_{DP}$	50.8	54.5	100
Isotonic Molarity	82	86	100
DPxMolarity	45	68	100
CreepxMolarity	18	29.5	100

It is noteworthy that the change in the product of creep and isotonic molarity is far more similar to that of growth rate than is the decrease in product of DP and isotonic molarity.

It should also be remembered that the creep test involves a constant force over a period of time and is probably more closely related to the situation in the elongating intact stem where a steady turgor pressure is in force.

It has been argued that DP measured in the Instron represents creep which has occurred early in log time (Cleland, 1971). The results reported in Tables I and II are not in agreement with this as there is a marked change in the ratio creep/D.P., the upper most rapidly elongating region being especially different from the other two. From the results reported here it seems that to regard D.P. as high speed creep may be an oversimplification.

The results reported here tend to be in conflict with those of Cleland and Haughton (1971) and also Yammamato et al (1970) who found that both initial extension and rate of creep of elongating segments were increased a comparable amount with auxin treatment, and their ratio remains approximately constant from experiment to experiment. (A similar parallel increase in creep and D.P. due to IAA treatment has been found in the upper section of Lupin hypocotyl (Penny, Penny and Marshall, 1974)). However their results were obtained on similar regions of the coleoptile or stem, and the period of auxin treatment may have been too short for any gross quantitative changes in carbohydrate levels to have occurred. Baker and Ray (1965) showed clearly with C^{14}-glucose that an increase in incorporation due to IAA was not detectable until about 30-60

minutes after auxin application. A similar time elapses before significant changes in D.P. and D.E. are detectable (Cleland, 1967) even though growth rate is stimulated in about 10 minutes. This similarity in kinetics of incorporation and change in properties could be used to support the concept that the change in mechanical behaviour is due to incorporated molecules. While this may be true a contribution from the bulk of the cell wall is to be expected.

That elongating and non-elongating regions of the hypocotyl are more similar in D.P. and D.E. than in creep suggests that in the hypocotyl sections D.P. and creep may be somewhat independent variables, with creep being more age dependent. If both are affected by IAA one might not therefore notice any change in the ratio of D.P. to creep in sections of the same age under different treatments.

One cannot assume that a stem which has just passed into the non-elongating phase of growth will have a similar composition to the sections from which IAA has been withdrawn even although the physical properties are not markedly different. It is probably true to a large extent, but there is likely to be an important difference between aged sections and young sections from which IAA has been withdrawn in the type and degree of wall deposition which has occurred. In the former case cessation of elongation is possibly due largely to the exhaustion of the growth limiting protein pool whose replenishment requires the continued presence of auxin (Cleland, 1971; Penny, 1971). The wall would retain juvenile structure appropriate to its rate of growth before removing auxin. In a section of non-elongating stem synthesis of

polymers which rigidify the wall will probably have started. A polymer involved in the stiffening of the wall could be the protein extensin for which hydroxyproline is a marker. Hydroxyproline has been shown to increase dramatically in pea epicotyl as it ceases elongation (Cleland and Karlsnes, 1969).

Thus while both creep and DP may be similarly affected by IAA and depend on wall structure, a change in this structure might result in a new ratio, although one which is not altered much by relatively short term IAA treatments.

A difference between the treatments of lupin hypocotyl and those of Avena coleoptiles that have been extensively used in mechanical studies is that Cleland (1967) deproteinized his sections with Pronase prior to extension analysis. It would be interesting to know how the creep/DP ratios varied in the three regions of hypocotyl with and without deproteination, especially in view of the current interest in the possibility of a protein-carbohydrate bond being cleaved by dilute acid to increase creep (Rayle, Haughton and Cleland, 1970), and in view of the inverse correlation of hydroxyproline levels and growth rate. If deproteination has decreased creep by altering the component which contributes partly to the gradual deformation then one might notice the following (1) an increase in DP due to the contribution of what would normally be protein dependent creep (pronase treatment does increase DP) (2) creep dependent only on the carbohydrate fraction of the cell wall (3) a lack of correlation of DP with growth rate. Thus it is quite possible that the change in the ratio of DP to creep in the three regions of hypocotyl is due to the increase in

extensin levels. Bearing in mind the difference in treatment of the tissues the data presented is not necessarily in conflict with Cleland and Rayles results. Whether pronase treatment of lupin hypocotyl would result in a constant ratio in the three regions, as in coleoptiles of different growth rate, is an interesting question.

The data of Rayle and Cleland (1972) shows that pronase treatment which removes 90% of the cell wall proline (Cleland, 1967) does produce a response somewhat akin to low pH. Thus it seems that partial proteolysis is giving results which hint at those obtainable from a study of the effects of complete proteolysis of wall protein, an approach which may lead to a greater understanding of the role of extensin in determining growth. Evidence so far is that enzymic proteolysis removes only part of the hydroxyproline, and that the carbohydrate attachments, which are extensive (Lamport, 1970) hinder the degradation around cross-linkage points (Brysk and Chrispeels, 1972). Certainly the fact that pronase releases a variety of extensin fragments shows that the polymer is extensively split by the enzyme, but as long as the protein and polysaccharide chains have several points of linkage with one another on these fragments (there is evidence of this (Keegstra et al., 1973)) there will be a contribution to the mechanical properties of the wall.

The lack of correlation between the growth rate, DP, DE and creep raises the question of the relevence of these quantities to the study of stem growth. Some results now suggest that DP is not a good measure of the potential of the wall to extend at a particular moment. DP is the same

in auxin treated tissue allowed to extend and in similarly treated tissue in isotonic mannitol (Cleland, 1967). Changes in DP with auxin treatment or KCN inhibition have different time courses (Cleland, 1971). Cleland is now of the opinion that DP is a measure of the average extensibility of the wall in the 60 - 90 minutes before measurement, although it is a measure of a real and physiologically dependent property of the wall (Cleland, 1971).

The lack of correlation between extensibility and growth rate reported here and the well known sensitivity of elongation to temperature and to metabolic inhibitors attests to the importance of the whole cell in determining growth.

Thus because the mechanical studies mentioned have employed killed tissue one must be mindful of the role of metabolism and biochemical changes of the wall properties.

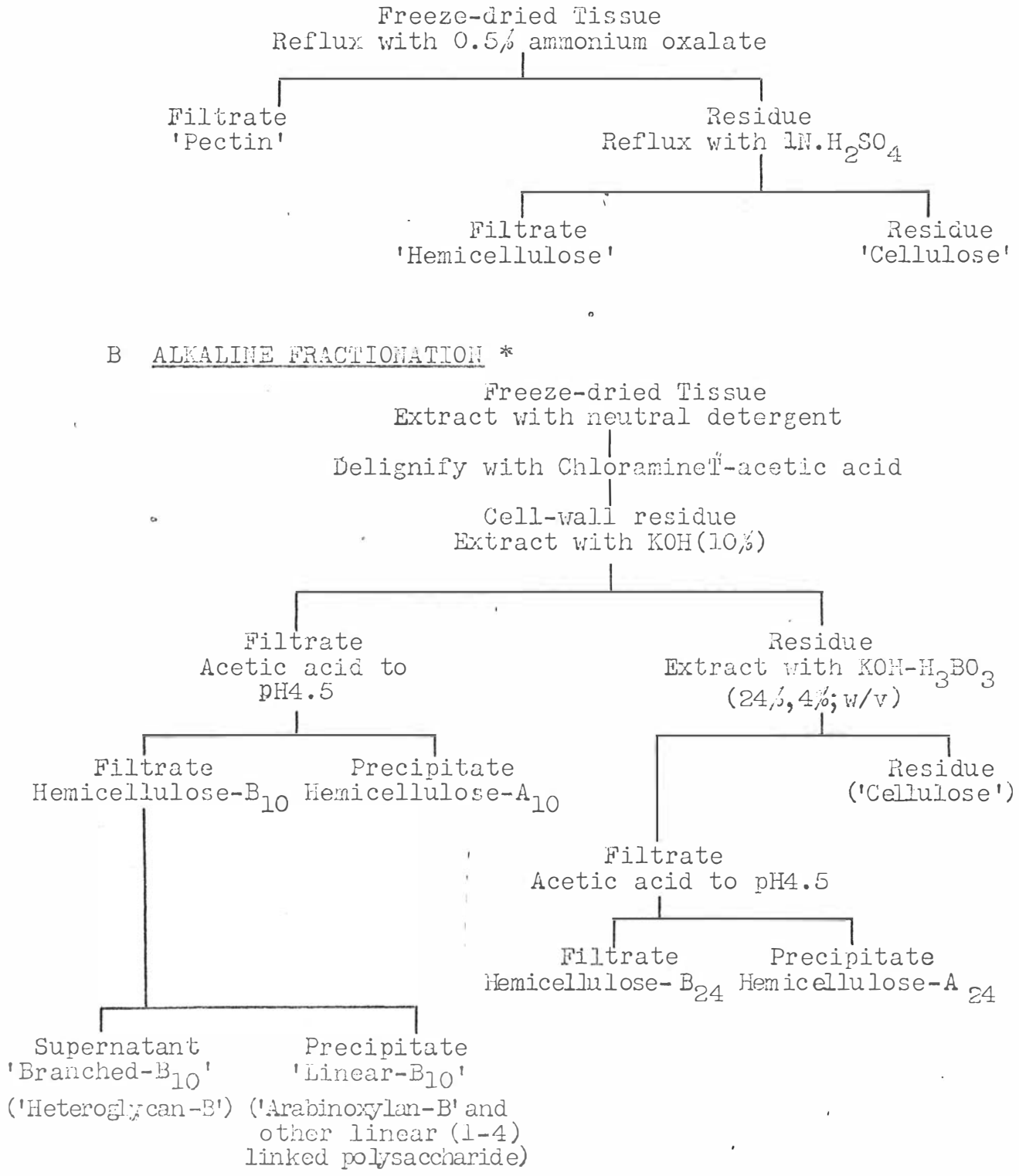
Cell Wall Composition

Hypocotyl elongation and wall composition were related by analysing the three 2 cm regions of a 6 cm hypocotyl in which relative growth rates had been established. Two analytical sequences were used and are referred to as the acid fractionation and alkaline fractionation respectively. They are shown in the flow-charts of Fig.I.

I. Acidic Fractionation

The results of the acid fractionation shown in Table III do not reveal any very marked changes in quantity of the fractions from upper middle and lower regions respectively.

FIG.I. CARBOHYDRATE FRACTIONATIONS USED IN ANALYSIS OF LUPIN HYPOCOTYL CELL WALLS



* Extracts and residues neutralized where alkaline. Extracts dialysed and freeze-dried.

TABLE III. CARBOHYDRATE COMPOSITION BY ACID FRACTIONATION OF THREE REGIONS OF HYPOCOTYL

Fraction	Hypocotyl region					
	Lower		Middle		Upper	
(a) Polysaccharide Fraction (% of hypocotyl tissue dry weight)						
Pectin	5.6		5.1		6.2	
Hemicellulose	4.3		4.7		3.6	
Cellulose	9.5		8.2		8.0	
* Lignin	1.0		0.6		0.6	
(b) Polysaccharide Fraction (% of sum of fractions of each region)						
Pectin	27.5		21		30	
Hemicellulose	21.0		25.2		19.6	
Cellulose	46.5		44.0		43.5	
Lignin	4.9		3.2		3.2	
(c) Monosaccharide content of fraction (% of fraction)	Hc**	C**	Hc	C	Hc	C
Glucose	33.9	85.9	33.4	79.8	36.4	80.8
Galactose	10.1	-	11.8	-	12.9	-
Mannose	2.6	10.2	3.1	15.2	4.1	13.5
Xylose	23.2	3.8	25.9	5.0	23.9	5.7
Arabinose	30.1	-	25.8	-	22.7	-

* Weight of solid material left after cellulose hydrolysis.

** Hc and C stand for hemicellulose and cellulose respectively.

What changes there are appear to be especially between the upper four and lower two centimetres.

There is a decrease in pectin in the middle region accompanied by an increase in the proportion of hemicellulose. It is noticeable that in the present study the total contribution of pectin plus hemicellulose in the three regions of lupin hypocotyl is similar, suggesting that in the region of deceleration of growth the pectin may become less water soluble and less hydrated thereby contributing to the rigidity of the wall. Although this idea supports the concept of Rees and Wight (1969) concerning the role of pectins in cell elongation it is not supported by the extensibility measurements of Table I. As discussed, there is the possibility that the extensibility measured on the Instron is not a true measure of the growth limiting mechanical properties of the wall, and therefore may not be related to the walls composition.

As in earlier results, there does not seem to be a change in any particular fraction associated with growth (Table III). It is difficult to extrapolate from these results to those of others. Compositional studies so far indicate a considerable variation in the cell walls from different sources. The sugar composition of cell walls has been found to vary even between species of the same genus (Nevins, English and Albersheim, 1967). The walls of various parts of bean plants were each found to have a characteristic sugar composition, and the changes in composition accompanying maturation to be different for different regions of the plant.

Thornber and Northcote (1961a, 1961b, 1962) examined the changing composition of tissue during maturation from

interfascicular cambium by analysing cambium, sapwood and heartwood from a variety of trees in terms of whole pectin, hemicellulose and cellulose. Pectin showed a large decrease while the other two fractions increased in quantity. An increase in lignin levels from zero in cambium to about the same level as cellulose indicates the degree to which secondary deposition had occurred. These results are not necessarily in conflict with the present results as the time periods over which the observed changes occurred were much greater than in the present experiment so that any short term variations in composition which might be relevant to the cessation of cell expansion are likely to have been obscured.

Nevins et al (1968) in looking at total neutral sugar composition of various bean hypocotyls found increases in xylose with maturation accompanied by a marked decrease in galactose (after an initial increase), and decreased in glucose and arabinose. They noted temporary changes taking two or three days to complete involving mainly transient increases in the proportion of galactose and to a lesser extent arabinose, followed by a decrease as elongation ceased. After this time there was little change in the relative proportions of sugars in the hypocotyl walls. These results were essentially confirmed by Jones and Albersheim (1972). However the data of both groups were derived from the analysis of whole hypocotyls and was not applied to the various polysaccharide fractions of the walls.

A study similar to this thesis is that of Jensen and Ashton (1960) who showed that Allium root cell walls change little in composition when passing into the elongating phase.

While this is consistent with the results reported here it is unfortunate that their analyses were not applied to the root as it ceased elongation.

The results of Table IIIb show that the monosaccharide composition of the hemicellulose and cellulose fractions does not change markedly between the three regions. The levels remain constant except for the case of arabinose which shows a 28% increase in the hemicellulose of the lower compared with upper region.

Although previous workers have found a decrease in arabinose with wall maturation (Nevins et al., 1968; Reid and Wilkie, 1969) and it is well established that glucose and xylose become dominant in the secondary wall (Northcote, 1969) little work appears to have been done on changes occurring during the cessation of elongation. However, the work of Nevins et al. (1968) and Dever et al. (1968) is relevant.

The earliest stage at which Nevins et al. analysed their tissues was after they had been growing for four days and thereafter they analysed at approximately daily intervals. Nonetheless the arabinose does appear to remain generally constant or increase slightly over the first few determinations before decreasing quite markedly.

Dever et al. divided corn root after about 5 days growth into meristematic (apical), elongating and mature zones. Consistent with the trend noted in this thesis they obtained an increase of about 400% in the arabinose of a 4% KOH soluble fraction. However there were concomitant changes in xylose which suggests increase in an arabinoxylan. Arabinose as a

percentage of total sugar in the walls did not change between the three regions analysed. In the present experiment no attempt has been made to distinguish between the meristematic and elongating zones, due to the impracticality of taking extensibility measurements of the former.

The increase in arabinose alone reported here would imply a rise in the levels of araban or else an increase in a molecule such as arabinoxylan with a concurrent loss of xylan from the wall - an unlikely occurrence. As mentioned earlier, the arabans known from higher plants are highly branched and water soluble (Northcote, 1972). In view of the uniformity of polysaccharide structure in Angiosperm cell walls so far examined (Northcote, 1969) it would seem that an increased incorporation of a hitherto unrecorded hemicellulosic araban peculiar to lupin hypocotyl is improbable. If this is not occurring then an oxalate insoluble polymer with arabinose side chains is probably present. Such a polymer could be the hydroxyproline rich protein known to occur almost exclusively in the cell wall (Lamport, 1965).

The work of Lamport (1967) on $Ba(OH)_2$ hydrolysates of various cell walls revealed that many of the hydroxyproline residues bear arabinose oligosaccharide side chains. In tomato suspension cultures it appears that 60% of the arabinose of the wall is bound O-glycosidically to the hydroxyproline (Lamport, 1970). Through its postulated role in controlling wall extensibility the protein has been termed extensin.

Consistent with the suggested function of the protein Cleland and Karlsnes (1967) showed that an increase in

in hydroxyproline levels is paralleled by a decrease in growth rate of pea stems. Presumably this is accompanied by an increase in protein bound arabinose. The results reported in this thesis are in agreement with this and indeed suggest that possibly the only specific sugar-containing wall polymer to increase as growth ceases is extensin. Other proteins with arabinose side chains have been reported from cell walls, e.g. peroxidase (Shannon, Kay and Lew, 1966), but do not occur in sufficient quantity to account for the arabinose increase reported here, moreover, unless it is covalently bound to the hemicellulose and/or cellulose of the wall it would be extracted during depectination. A covalent bonding between such proteins and wall carbohydrates cannot however be ruled out.

The usefulness of studies using only partial fractionation is limited in that it does not give a good indication of the number of polysaccharides involved in a change of overall monosaccharide composition, and because metabolic turnover takes place (Lamport, 1970) it may give an underestimation of changes occurring. The cell wall should be regarded as a dynamic system in which associations and solubilities of polymers are liable to change constantly. A change in monosaccharide composition in a gross fraction indicates only a change in net carbohydrate and does not tell which polymers are altering in level. It has been clearly shown that turnover occurs within the wall and that there is a precursor - product relationship between at least some polysaccharides therein. Transglycosylation can apparently occur within the matrix as neutral blocks of arabinogalactan

seem to be added to weakly acidic pectinic acid (Stoddart and Northcote, 1967).

II. Alkaline Fractionation

As in the case of measurement of total pectin hemicellulose and cellulose there is little apparent difference in composition between the various regions of hypocotyl (Table IV). These results are somewhat divergent from those of other workers.

The studies of Fuller (1958) are relevant to the problem and to a lesser extent those of MacLachlan and Duda (1965), and Northcote and Thornber (1961a; 1961b; 1962). Fuller examined the meristematic, elongating and mature regions of Vicia fabia roots. He found an increase in pectic substances, hemicellulose and cellulose during elongation, but expressed as a percentage of total cell wall polysaccharides pectic substances decreased from 70 to 48% and cellulose increased from 18 to 43% in the secondary wall. The hemicellulose decreased slightly throughout development. In the pectic fraction a galactan was found to increase per cell up to 43 mm while a second component increased only during elongation. The hemicellulose fraction could be resolved into five components of which one (a xylan) was detectable in cells only after elongation. The work does not appear to have been continued.

MacLachlan and Duda (1965) using excised pea epicotyl tissue found that cellulose and lignin increased at the expense of other fractions in sections on water. In upper sections a glucose and galactose containing fraction decreased whereas in tissue from the epicotyl base pectic acid was the only material

TABLE IV. COMPOSITION OF DELIGNIFIED HOLOCELLULOSE BY ALKALINE EXTRACTION. THE POLYSACCHARIDES EXTRACTED AND MONOSACCHARIDE COMPOSITION OF THE POLYSACCHARIES

	Polysaccharide composition of hypocotyl region (% of total polysaccharide)			Monosaccharide composition* (% of total anhydro-sugar in fraction)				
	Lower	Middle	Upper	Glu.	Gal.	Man.	Xyl.	Ara.
Branched-B_{10}	2.5	3.7	2.4	40	10	5	40	5
Linear-B_{10}	8.0	9.5	12.0	39	7	3	50	1
Hemicellulose-B_{24}	7.3	8.2	6.7	55	2	33	10	0
Hemicellulose-A_{10}	0	0	0	-	-	-	-	-
Cellulose	82.2	78.5	79.0	94	0	6	0	0

* Monosaccharide composition same for each polysaccharide from all three hypocotyl regions.

Subscripts 10 and 24 refer to 10% and 24% alkali solubility.

to decrease in concentration. How these results relate to the data presented here is difficult to ascertain as there was a constant supply of cell wall precursor for the lupin hypocotyl.

The work of Northcote and Thornber has already been related to the lupin hypocotyl.

Jensen and Ashton (1960) in their partial alkaline fractionation of onion root tip cells did not find any marked changes in relative proportions of hexose, hexuronic acid and pentose comprising the fractions. However, their fractionations did not go beyond total alkali extracts where alkali was used, so that a resolution into linear and branched species was not obtained. Furthermore analysis of hexose and pentose contents restricts interpretation, as different hexoses and pentoses can occur in different linkage and in different polysaccharides within an extract. A measure of individual monosaccharides comprising sub-fractionated alkali extracts will give a far clearer picture of changes in the cell wall.

When the sum of monosaccharides obtainable from hydrolysis of alkali fractions shown in Table IV is compared with that from acid hydrolysis fractionation (Table III) striking differences appear. There is a large loss of arabinose and to a lesser extent galactose during the analysis by alkaline fractionation. This loss could be due to (1) hydrolysis of carbohydrates due to the acid conditions used in the delignification, (2) removal of residual pectin during the delignification, (3) loss of arabinosylhydroxyproline and protein linked galactose due to the destruction of protein by the delignification treatment in which considerable halogenation

would occur (4) loss of protein bound sugars by alkaline hydrolysis of the protein during fractionation, (5) removal of arabinosyl side chains due to acid susceptibility of this linkage as has been suggested by Lamport (1970) and Rayle et al. (1970).

Probably all of the above contribute to the loss in various degrees. The delignification treatment involved heat (90-100°C) at a pH of approximately 3.6 which suggests the possibility that cleavage of acid-labile furanoside-linked sugar units from the hemicellulose xylan had occurred. While this possibility might apply to arabinose it is unlikely to explain the release of galactose, unless it is bound into the wall by some other relatively acid or chlorine labile linkage. The removal of cell wall protein should cause a large loss of arabinose. If one is to explain the loss of galactose in the same way it would be necessary to place an acid labile bond between galactose and cellulose microfibril in Lamport's (1970) model of the extensin-arabinogalactan-cellulose complex, where the galactan is bound to the extensin through an arabinose oligosaccharide.

In view of published data one would not expect the conditions used in alkaline extraction to result in sufficiently extensive protein degradation to extract extensin. Heath and Northcote (1971) used a much harsher treatment and found little extraction from sycamore suspension culture walls, which may however be somewhat different from those of lupin hypocotyl. Also the fact that the delignification will have removed much of the protein renders the contribution of alkaline degradation of extensin to the loss of arabinose and galactose rather irrelevant.

MATERIALS AND METHODS

Seeds of Bitter Blue Lupin (*Lupinus angustifolius*) were surface sterilized by immersion in 1% chloragen for 20 minutes. They were then planted in pots in pre-washed coarse exploded mica and placed under continuous light in a growth room at 23 ± 3°C. The light intensity was 14.06 W m^{-2} supplied by a combination of 95% fluorescent (Philips TLA 80W/55) and 5% incandescent light. The pots were irrigated with water daily until the seedlings were harvested, after about 4½ days.

Tissue was used in two ways.

(a) For measurement of growth intact plants were used.

(b) For measurement of osmotic concentration, extensibility, creep and carbohydrate composition hypocotyls were grown until 6 cm in length. At this stage they were harvested and cut into three 2 cm sections for comparison of the lower middle and upper regions. Sections were used immediately for measurement of osmotic concentration or extensibility. In other cases they were deep frozen within 30 minutes, and freeze dried within a few days.

Measurement of Growth Rate

Seeds were planted around the perimeter of plastic pots and allowed to germinate until they had reached a height of about 3 cm. At this stage the sides of the pot were cut away to the level of the base of the hypocotyl. Marks 2 mm apart were then placed on the hypocotyl using an implement which had 11 fine wires at 2 mm intervals. The markings were obtained by placing Gestetner ink on the wires which were then gently pressed against the hypocotyl. A plastic label placed in the pots was similarly marked and used as a reference. Elongation

was calculated from the positives of the photographically recorded separation of the markings as the hypocotyls elongated to over 6 cm in length.

Measurement of Osmotic Concentrations

The Schardakow dye method was used (Schardakow, 1948) as modified by Manachar(1965).

A number of mannitol solutions were prepared ranging from 0.1 M to 1.0 M in concentration. From each of these 5 ml aliquots were placed in corresponding small petri dishes, and a 20 ml aliquot from each was similarly placed in a test tube. A minute crystal of methylene blue was placed in each of the petrie dishes and was sufficient to dye the solution without significantly altering its osmolarity.

Groups of 5 freshly excised 2 cm sections from the lower, middle and upper hypocotyl regions respectively were placed in the petrie dishes which were then covered with lids to prevent any evaporation. The dishes were then left for 90 minutes with frequent gentle agitation. At the end of this time a drop of liquid was removed from each petrie dish with a clean pasteur pipette and very gently placed into the solution of corresponding original mannitol molarity. The drop, visible because of the dye it contained, was observed for some time and a record of its movement was made.

If the solution from the petrie dish rose in the test tube it was less dense than originally, indicating movement of solvent from the sections into the bathing solution, and thus a higher osmotic potential in the solution than in the sap. If the drop sank the opposite was the case. The osmolarity at

which no movement of the transferred drop in its parent solution occurred gives an indication of the osmolarity of the cell contents. When the osmotic pressure of section and bathing solution are the same no net movement of solvent will occur.

Measurement of Extensibility

An Instron Universal Testing Instrument was used to measure the plastic and elastic components of cell wall extension essentially as described by Cleland (1967) and modified by Penny et al. (1972). At least 18 sections from each region were measured.

Immediately after excision the 2 cm sections to be tested were quickly weighed and then boiled for 5 minutes in methanol, dried, reweighed and stored dry. Before testing the segments were soaked in methanol for 5 minutes and then in distilled water for at least 5 minutes. A thin layer of rubber was placed on the grips of the Instron and this prevented both slippage and fracture of the specimens. If the load was increased until the section broke the breakage occurred in mid-section rather than at the grips.

The initial distance between the clamps was 8 mm and they were separated at a rate of 2 mm min^{-1}. Extension was measured as the load increased from 30 to 40 g.

Values for compliance are calculated with the area estimated from the toal fresh weight of the sections. The cell wall represents about 8% of the cross sectional area of the segment and the values of the compliance could be expressed in terms of cell wall area, not total area. This has already given rise to some confusion in the literature and, as pointed

out by Cleland, compliances calculated on cell wall area and cell area c n differ by a factor of 100 for the same cell.

The Instron technique for measurement of cell wall properties was orignally introduced by Olsen et al. (1965) and improved by Cleland (1967). It is a standard fibre-testing technique and allows large numbers of measurements to be made conveniently and reproducibly. It has been described elsewhere but will be outlined here.

The sections, after rehydration, are extended with a known force and a resulting load extension curve automatically recorded. Such a curve from the extension of a lupin hypocotyl is tracing A in Fig.2.

It is in the linear region of the curve that measurements of compliance ($^{\text{strain}}/_{\text{stress}}$) are made.

The compliance $D = \text{strain} \cdot \text{stress}^{-1} = \dfrac{\Delta L / L}{F / A}$

where L is the initial length of the segment and

ΔL the change in length due to

F which is the load or force across the section.

A is the cross sectional area of these walls.

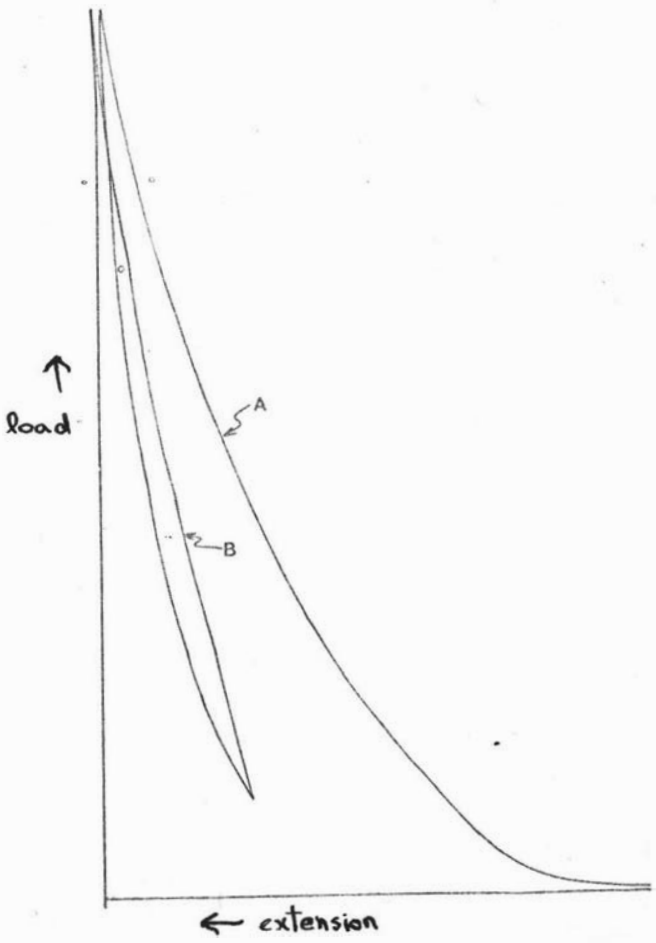
L is obtained by extending the curve back to the abscissa.

The expression $\frac{M}{l \rho w}$ can be substituted for A where M is the mass of section and ρw the density of water. If we assume the latter to equal 1 then

$$D = \frac{\Delta L / L}{F} \cdot \frac{M}{l}$$

which is a measure of total compliance, which we will now denote as DT.

FIG. 2 FIRST AND SECOND CURVES OF EXTENSION OF LUPIN HYPOCOTYL BY LOAD INCREASING FROM 30 TO 40 GRAMS



The curve obtained from the first extension gives a measure of the total compliance. If after one extension the clamps are returned to their original position and the section re-extended a second load extension curve is obtained (B of Fig.2). This represents the elastic extension (DE). The plastic extension is measured from the difference between the first and second curves.

$$DE = \frac{\Delta L/L}{F} \cdot \frac{M}{1}$$

Plastic extensibility (DP) is thus

$$DP = DT - DE$$

All extensions subsequent to the first are very similar in the case of Avena although a small increase in extension representing some sort of creep is observed (Cleland, 1967). Only the first and second extension curves were used in the experiment. Creep measurements were made separately.

Measurement of Creep

Rehydrated tissue prepared as for the Instron was employed. A modified beam balance was used for creep measurements. Sections were individually clamped at the end of one beam of a balance and surrounded by water in a plexiglass chamber. A 100 gm weight was placed in the pan held on the other beam and the extension of the section measured using an electrical transducer coupled to a digital volt meter. (Penny, Penny and Marshall, 1974). Creep was measured as percentage increase in length between 1 and 100 minutes after adding the 100 gm weight.

Carbohydrate Composition

Cell Wall Preparation: For both acid and alkaline fractionations tissue sections (60 g) were freeze dried (5 g) and ground in a Wiley Mill. In the case of acid fractionation the freeze-dried tissue was extracted with boiling 80% ETOH for 3 minutes while for alkaline fractionation it was refluxed with the neutral detergent of Van Soest for 2 hours. In 1 litre of aqueous solution this detergent contains 30 g of sodium lauryl sulphate, 18.6 g of disodium E.D.T.A., 6.8 g of $Na_2B_4O_7 \cdot 10\ H_2O$, 4.6 g of Na_2HPO_4 and 10 ml of ethylene glycol. It is therefore buffered at pH 7.

Proteins, soluble sugars and pectic materials are removed by the detergent. The cell wall preparation for alkaline fractionation was delignified essentially by the method of Gaillard (1958). Because of its mildness it was found preferable to the usual acid chlorite treatment. With mature tissue at least, the delignification can be used after extraction with ammonium oxalate without causing much loss of carbohydrates. Water was added to the detergent extracted cell wall to provide a slurry which was heated in a beaker in a simmering water bath. Chloramine T (1 gm per 75 mls water up to a maximum of 3 gm) was added followed by glacial acetic acid (1 ml to each 1 gm of Chloramine T) stirred thoroughly, covered and the mixture heated for 2 hours over a boiling water bath. The hot slurry was filtered over sintered glass and the residue washed in ethanol followed by hot 3% ethanolamine in ethanol and finally ethanol. During the ethanolamine washing the residue was kept covered with hot ethanolamine for 3 minutes before applying suction.

The entire chloramine T-ethanolamine treatment was repeated two times to give a final residue of holocellulose, which was washed with ethanol followed by acetone and dried at 40°.

Carbohydrate fractionations

(1) Acidic fractionation: After removal of pectin by refluxing the wall preparation for 1 hour with 0.5% ammonium oxalate the residue was treated with 1N.H_2SO_4 under reflux for 2 hours to remove the hemicelluloses. The residue was treated with 72% H_2SO_4 at room temperature for 4 hours then diluted to 1N.H_2SO_4 and refluxed for two hours. The hydrolysate thus obtained was used to measure cellulose, and lignin was measured by weighing the residue collected on filter paper after filtering the final hydrolysate.

(2) Alkaline fractionation: The hemicelluloses were extracted essentially as described by Gaillard and Bailey (1968), and according to the flow chart shown in Fig.1. The extractions were at room temperature with continuous stirring under nitrogen. The extracts were filtered off on sintered glass under a rubber diaphragm which excluded air. Alkaline filtrates were cooled to approximately 0°C in an ice bucket under nitrogen and then acidified with glacial acetic, also under nitrogen and with rapid magnetic stirring. The extracts were placed in a refrigerator overnight to allow hemicellulose-A to precipitate. As little turbidity developed the extracts were not centrifuged, but were dialysed directly for two days against tap water. At the end of this time they were lyophilized.

The hemicellulose-B fraction thus obtained was further fractionated into linear and branched polysaccharide species by iodine precipitation from concentrated $CaCl_2$ solution. Gaillard (1961) showed that it was possible to separate linear heteroxylans and glucans from the branched polymers in hemicellulose-B fractions by precipitating the linear polymers with an iodine-potassium iodide solution. The branched polymers are then recovered from the filtrate. There are numerous references in the literature to the reaction of iodine with hemicelluloses to give blue complexes (amyloid reaction), a reaction formerly attributed to the presence of glucose residues. However, several linear polymers devoid of glucose were found to give a heavy blue precipitate with iodine, whereas those with side chains remained in the supernatant.

In this experiment the fractionation was achieved as follows. Freeze dried hemicellulose-B (150 mg) was dissolved in $CaCl_2$ solution (25 ml Sp.G.1·3) by stirring overnight. The $CaCl_2$ solution was clarified by spinning for 15 minutes at 70,000 g and iodine solution (I_2 3%, KI 4% w/v; 4 ml) added. The blue precipitate was allowed to settle for one hour and then collected by centrifuging for one hour at 70,000 g. Iodine in both the linear-(1-4)-linked hemicellulose (linear-B_{10}) and heteroglycan-B supernatant was neutralized by treatment with conc. $Na_2S_2O_3$ solution. The solutions were finally dialysed for 24 hours against running tap water, concentrated and freeze dried.

Carbohydrate Analyses

Acid Hydrolyses: Heteroglycan-B (branched) fractions (10 mg)

were hydrolysed in N.H_2SO_4 5.0 ml by heating for 2 hours at 100°C in stoppered test tubes in a water bath. Other polysaccharide fractions (10 mg) were treated with 72% H_2SO_4 (0.16 ml) at room temperature for 2 hours, diluted with water (5 ml) and heated to 100°C as above for 2 hours.

Acid hydrolysates were neutralized with KOH when required for total reducing sugar analysis. For quantitative paper chromatography they were neutralized with $BaCO_3$, filtered or centrifuged, and freeze dried. The dried hydrolysates were then taken up in 1 or 2 ml of water. An appropriate aliquot of hydrolysate was spotted onto Whatman No.1 chromatography paper which had been washed in 0.5% oxalic acid followed by distilled water.

Paper Chromatography: Chromatograms were developed with butyl acetate-pyridine-H_2O-ethyl alcohol (8:2:1:2) and thoroughly dried before spraying. The individual monosaccharides were vizualized by the quantitative method of Wilson (1959) except that the vizualizing reagent used was aniline hydrogen phosphate rather than aniline hydrogen phthalate. The aniline hydrogen phosphate was prepared by adding 1 ml each of orthophosphoric acid and redistilled aniline to separate 100 ml volumes of water saturated butanol. The volumes were then mixed and used within one hour, although this was well within the stability range of the reagent.

The reagent was sprayed onto the chromatogram with care being taken to get an even coverage. After the first side had been sprayed the paper was dried and sprayed on the reverse side. The spray was applied almost in sufficient quantity for it to run but at no stage was it allowed to do so, and the papers were very quick to dry.

After spraying the paper was dried and placed in an oven with circulating air at 110°C for 10 minutes while the spots developed. The spots were cut out on approximately equal sized rectangles of the paper which were cut into strips and placed in test tubes. 4 mls of acidified alcohol (29 ml conc. HCl in 420 ml ETOH diluted to 500 ml with distilled water) was added to each tube which was then placed in darkness for an hour while the spot was eluted. To aid elution the tubes were shaken (gently to avoid disintegration of the paper) 3 times during the hour. The absorbance of the eluates was read on a Beckmann model DU spectrophotometer at 390 mμ in the case of hexoses and at 360 mμ for pentoses. Standard sugars (glucose, galactose, mannose, xylose and arabinose) were also spotted and run on each paper.

In a thorough investigation of this technique Wilson (1959) found the spots had a coefficient of variation of only 2%.

Uronic acids were measured by the carbazole method of Dische (1967) as described by Montreuil and Spik (1963). It is based on the fact that when sugars are treated with concentrated mineral acids they yield mixtures of products which react with various organic substances to give colours. By choosing appropriate conditions of acid concentration, time and temperature it has been possible to make some of these general reactions of carbohydrates more specific. In this case a specific reaction of hexuronic acids with H_2SO_4 and carbazole is used.

For the determination a 1 ml sample was hydrolysed after theaddition of 6 ml of concentrated H_2SO_4 by heating in a boiling water for exactly 20 minutes. The hydrolysis tubes

were then transferred to a beaker of ice and 0.2 ml of carbazole solution (0.1 gm in 100 mls ethanol) added. The colour was allowed to develop for three hours in a dark cupboard before reading at 530 m/u on a Beckman model DU spectrophotometer.

Reducing sugars were measured by the microcuprimetric method of Nelson (1944). The basis of this procedure is that reducing sugar causes the production of copper from a solution of copper sulphate in sodium potassium tartarate. The reduced copper is then measured by measuring the intensity of colour produced when it complexes with arsenomolybdate added after reduction has ceased. This widely used method is well documented and will not be detailed here.

II. THE EFFECTS OF DELIGNIFICATION ON LUPIN HYPOCOTYL CELL WALL

INTRODUCTION: In the previous section of this thesis the results of fractionation of lupin hypocotyl cell walls and of the analysis of the monosaccharide compositions of these fractions were presented. Where the monosaccharides comprising the total hemicellulose extracted in acid and alkali fractionations respectively were compared it was found that the alkali extracted hemicellulose was very low in arabinose and to a lesser extent galactose, when compared to that extracted by acid. The tissue used for the fractionations was similar except that that treated with alkali was first of all delignified.

The alkali fractionation is a well established procedure known to lead to only minor polysaccharide degradation when oxygen is excluded from the alkaline solution. It therefore seemed that the most likely point of loss was the delignification where the high temperature, low pH, and chlorine produced under these conditions might cause some breakdown. The relatively mild method of Gaillard (1958a, b) was employed, using Chloramine T and acetic acid for the generation of chlorine in solution. This method has been widely used in studies on herbage composition. Although only small losses of carbohydrate have been reported (Gaillard, 1958b) most of the cell walls treated were predominantly secondary in contrast to those of 6 cm lupin hypocotyl which is essentially a primary tissue. It is now known that lignin can also occur in

primary and young secondary walls (Leppard et al., 1971). Whether or not one is safe in using conventional delignification on these walls without causing major losses of polysaccharide is questionable. This aspect of holocellulose preparation has been investigated and the results obtained are reported below.

RESULTS AND DISCUSSION

Effects of Chloramine T Delignification and Dilute Acid on Total Hemicellulose of Depectinated Hypocotyl

An initial comparison of the composition of tissue before and after delignification was obtained by analysing the hemicellulose from cell walls depectinated with 0.5% ammonium oxalate and from the same after the Chloramine T delignification of Gaillard. The results are presented in Table V and clearly show that Chloramine T-acetic acid treatment drastically alters the cell wall, particularly with respect to the arabinose and galactose content. Over 95% of the arabinose and about 70% of the galactose is lost.

To get some idea of the extent to which the low pH of the treatment could be responsible for the losses depectinated walls were extracted either with water, dilute acid (pH 3.6), or with Chloramine T-acetic acid (pH 3.6), all at 90-100°C for 6 hours. About 50% of the arabinose and 25% of the galactose was removed by acid alone compared to the much greater losses incurred in the presence of chlorine (Table VI). Clearly the acid conditions can be only partly responsible for the loss of these

TABLE V EFFECT OF DELIGNIFICATION ON MONOSACCHARIDE COMPOSITION OF DEPECTINATED HYPOCOTYL

Treatment	Hemicellulose monosaccharides (relative to xylose)				
	Xylose	Arabinose	Glucose	Galactose	Mannose
None	1	0.85	0.48	0.89	0.11
Chloramine T delignification	1	0.01	0.45	0.31	0.14

TABLE VI COMPOSITION OF CHLORAMINE T - ACETIC ACID, ACETIC ACID, AND WATER EXTRACTED HYPOCOTYL

Treatment*	Hemicellulose monosaccharides*			
	Xylose	Arabinose	Glucose	Galactose
Water	1	0.97	0.65	1.37
dilute acetic acid (30 ml/l)	1	0.46	0.52	1.02
Chloramine T-acetic acid	1	0.02	0.84	0.42

* 100°C for 6 hr. Tissue depectinated with neutral detergent prior to treatment.

sugars. The galactose is apparently held into the wall by less acid labile bonds than the bulk of the arabinose.

The solutions from all acid and delignification treatments were dialysed against tap water for 48 hours hydrolysed with N.H_2SO_4 at 100°C and analysed by paper chromatography. The chromatograms showed the presence of galactose and arabinose in largest amounts from the delignification treatments. Relatively small amounts of other monosaccharides were detectable. In all of the treatments the sugars were largely non-dialysable and only released by acid hydrolysis, suggesting that they were still present in polymer form.

It is known that Angiosperm arabans are branched water-soluble polymers (Northcote, 1969) which would have been removed by the depectination. It is therefore likely that the arabinose extracted in the dilute acid was largely present as furanoside-linked side chains probably of an arabinogalactan. However if all of the extracted arabinose and galactose is derived from a single arabinogalactan such a polymer has a very high proportion of arabinose side chains. It is possible that much of the arabinose lost represents side chains of a non-polysaccharide polymer as suggested in the previous section from evidence presented therein. This polymer would appear to be bound into the wall in a way that renders it insoluble in hot water or oxalate, but by links of which some are acid labile and virtually all are susceptible to delignification.

It is interesting to note that the cell wall protein extensin has been shown to bear $(arabinose)_{1-4}$ oligosaccharide side chains on the hydroxyproline residues of the peptide chain

(Lamport, 1969). Furthermore the hydroxyproline-arabinosyl glycosidic bond has been suggested as an acid-labile link involved in the control of wall elongation (Rayle et al., 1970).

Lamport (1970) has proposed a model for the involvement of glycoprotein in wall structure, in which a large portion of the arabinose side chains are directly bonded to galactan. Heath and Northcote (1971) have provided some support for this in that hydrazinolysis of the wall protein will result in release of arabinogalactan, although of short chain length. If the model of Lamport were correct and the arabinose side chains of the extensin were directly linked to galactose a large proportion of the extracted arabinose would be essentially non-dialysable. This is because of the relatively large molecular size of the arabinogalactan fragments released from covalent association with the extensin. The assumption here is that the galactan is either bound to the wall by acid or chlorine labile links, or has no further bonding to Chloramine T-acetic acid resistant portion of the wall. Arabinose oligosaccharide side chains of extensin fragments so far examined are known to be normally not more than four units in length (Lamport, 1969; Lamport and Miller, 1971) and would thus be quite readily dialysable.

In contrast to the earlier suggestion of Rayle et al. (1970) Karr and Lamport (1971) now claim that the glycosidic bond between hydroxyproline and arabinose is less sensitive to acid hydrolysis than other glycosidic bonds in the glycoprotein complex. In this case dilute acid treatment will remove largely non-glycoprotein arabinose. Thus, if the levels of hydroxyproline bound arabinose in lupin hypocotyl are anywhere near as high as in other primary tissues investigated much of

the arabinose remaining in the acid treated walls will represent extensin side chains, many of which are known to be unattached to other sugars and therefore dialysable.

As the pH of the delignification liquor was not lower than that encountered in the dilute acid treatment it would seem that the almost complete extraction of arabinose in the former case compared with the latter can be attributed to the destruction of the protein backbone of extension by the generated chlorine. Certainly such destruction will occur under the conditions used. In this case it is interesting that much galactose is lost as it suggests that some galactan of the extensin complex is bound into the wall by the protein alone.

Although it is a minor component of the walls relative to protein, lignin may be responsible for bonding some of the sugars released on delignification. A strong lignin-carbohydrate association is known (Lai and Sarkanen, 1971) and lignin fibrils are thought to occur in young walls (Leppard et al., 1971). As yet their importance in this context is unknown.

Alkaline Fractionation of Delignified and Non-Delignified Tissue

A comparison of carbohydrate fractions from tissue before and after delignification was made. Depectinated (neutral detergent and oxalate) tissue with and without delignification was extracted with 10% KOH. The alkaline solution was fractionated into hemicellulose-A (precipitated on acidification) and hemicellulose-B (remaining in the supernatant). The hemicellulose-B fraction recovered by dialysis and freeze drying of the acidified solution was dissolved in concentrated $CaCl_2$ and treated with iodine solution to give a precipitate of

hemicellulose-B arabinoxylan and soluble hemicellulose-B heteroglycan both of which were purified by dialysis and then freeze dried. The monosaccharides and hydroxyproline in each of the fractions was determined.

The protein of the cell wall was completely removed by the delignification treatment as indicated by the total absence of hydroxyproline from the delignified tissue (Table VII). The loss of hydroxyproline is accompanied in all fractions by a considerable loss of arabinose and galactose. Extraction of these sugars relative to hydroxyproline decreases from the hemicellulose-B heteroglycan to the linear hemicellulose-B to the hemicellulose-A The heteroglycan-B fraction is rich in arabinose and galactose which together with its high proportion of hydroxyproline suggests that this fraction owes its initial insolubility in oxalate and solubility in alkali to association with protein in the wall rather than to some other difference from conventional oxalate-soluble pectic arabinogalactan.

It is noteworthy that the linear hemicellulose-B (arabinoxylan-B) fraction contains a much higher proportion of arabinose to xylose than has been found in pure arabinoxylan fractions from young tissues (Northcote, 1969). Bearing in mind possible species differences one may speculate that the fraction in question consists of an association of arabinogalactan with an arabinoxylan through the protein. On the other hand a separate arabino-galactan released by delignification may co-precipitate with the hemicellulose-B arabinoxylan during alkali fractionation of non-delignified tissue. In either case the conventional name arabinoxylan as

TABLE VII COMPOSITION OF ALKALI EXTRACTED* FRACTIONS FROM DELIGNIFIED AND NON-DELIGNIFIED LUPIN HYPOCOTYL CELL WALLS

Polysaccharide fraction**	Yield	Hydroxyproline content (% of fraction)	Monosaccharide composition			
			Xyl	Ara	Gluc	Gal
Hemicellulose-A						
Delignified	20	0	1	0.04	0.23	0.04
Non-delignified	32	0.6	1	0.28	0.27	0.24
Hemicellulose-B arabinoxylan-B (linear hemicellulose)						
Delignified	69	0	1	0.12	0.62	0.29
Non-delignified	158	3.78	1	0.84	0.50	0.77
Hemicellulose-B heteroglycan-B						
Delignified	7.0	0	1	0.85	0.62	0.64
Non-delignified	17	2.15	1	17.2	0.90	5.68

* Cell walls extracted overnight at room temperature with 10% KOH

** Delignified cell wall contained no hydroxyproline

applied to this fraction may be a misnomer.

Barium Hydroxide Extraction

Treatment with hot barium hydroxide does not break glycosidic bonds, e.g. the hydroxyproline-arabinose bonds of extensin, but does break peptide links to release hydroxyproline-(arabinosyl)1-4 compounds (Lamport, 1967). Lupin hypocotyl was therefore homogenized, depectinated by extraction with neutral detergent and ammonium oxalate and subjected to barium hydroxide hydrolysis. Analysis of the walls by quantitative paper chromatography before and after showed that nearly all the arabinose and galactose had been dissolved, as shown in Tables VIII and IX. In contrast the $Ba(OH)_2$ does not appear to have solubilized the normal cell wall xylan. Only traces of xylose were present in the extract which gave intense spots for arabinose and galactose. It would seem therefore that a very high proportion of the non-pectic arabinose (i.e. not extracted by neutral detergent-oxalate) in primary walls is associated with protein, either directly or as side chains of a galactan involved in an extensin-polysaccharide complex, or with some other polymer containing alkali-labile links.

When the hydrolysate from the $Ba(OH)_2$ treatment was examined results obtained in Table X were obtained. These show that very little of the released sugar was present as monomer and that much of it (33%) was present as non-dialysable (48 hours against running tap water) polymer. The change in arabinose:galactose ratio as a result of dialysis suggests that most of the arabinose is in short chain oligomers, i.e. that if there is a spectrum of polymer sizes in the extract arabinose is

TABLE VIII BARIUM HYDROXIDE EXTRACTION OF DEPECTINATED HYPOCOTYL CELL WALL

Treatment of Wall	Hemicellulose monosaccharides* (relative to glucose)			
	Galactose	Arabinose	Xylose	Glucose
Depectinated	4.03	3.23	3.75	1
$Ba(OH)_2$ extracted	0.64	0.25	3.0	1

* Mannose present but not measured

TABLE IX BARIUM HYDROXIDE EXTRACTION OF DEPECTINATED HYPOCOTYL CELL WALL

Treatment of Wall*	Hemicellulose monosaccharides (relative to xylose)				
	Xylose	Galactose	Arabinose	Glucose	Mannose
Water	1	0.82	0.90	0.59	0.53
$Ba(OH)_2$ extracted	1	0.37	0.03	1.33	0.34

* Treatments for 10 hrs at 95°C

Data from Tables VIII and IX derived from different batches of tissue.

TABLE X CARBOHYDRATE COMPOSITION OF BARIUM HYDROXIDE EXTRACT OF DEPECTINATED LUPIN HYPOCOTYL CELL WALLS

Fraction	Reducing Sugars (glucose equivalent, mg/g of cell wall)	Arabinose:Galactose
Extract	5.14	-
Extract after acid hydrolysis	44.0	6:1
Extract after dialysis followed by acid hydrolysis	14.2	2.5:1

richer in those portions of low molecular weight. This is consistent with Lamports (1967) hydrolysis results and with the idea of an attachment of arabinose oligosaccharides to protein fragments. Measurements showed that, in agreement with Lamport (1967) the $Ba(OH)_2$ hydrolysates contained liberated hydroxyproline (5mg/gm of depectinated wall).

The almost complete removal of cell wall arabinose and partial removal of galactose by either $Ba(OH)_2$ or Chloramine T-acetic acid suggests that the same polymer is involved and that most of the cell wall arabinose and galactose not removed by depectination is present in a glycoprotein complex. Conventional carbohydrate fractionation could lose this moiety during delignification, at least in immature tissue.

Although caution is necessary in interpreting results from two such harsh treatments it is evident that a large and similar amount of the wall polysaccharide is susceptible to both Chloramine T-acetic acid and $Ba(OH)_2$ treatments, both of which cause the destruction of wall protein. It is also evident that if any changes in wall composition which accompany changes in growth rate are occurring these must be disguised by the delignification treatment. Hence in the knowledge that changes are not apparent in the delignification-resistant portion of the wall it becomes important to look at the remainder which includes the glycoprotein complex particularly as there is already evidence that the hydroxyproline levels increase in primary walls as they age (Cleland and Karlsnes, 1967; Winter *et al.*, 1971; Sadava *et al.*, 1973), and also that dilute acid is able to increase the extensibility of *Avena* coleoptile cell walls *in vivo* (Rayle and Cleland, 1970).

MATERIALS AND METHODS

Cell Wall Preparation. Cell wall material was prepared by grinding whole fresh hypocotyls in a Waring Blender with 1% 'Nonidet 90' detergent (Shell) in cold water, and extracting them with ethanol and acetone prior to refluxing for 2 hours in 0.5% ammonium oxalate to remove pectin. Where hydroxyproline determinations were to be made the tissue was refluxed successively for 1 hour with neutral detergent and 1 hour with 0.5% ammonium oxalate.

Treatment of Cell Wall. (1) Delignification and dilute acid. Cell walls were treated for 6 hours at ca.95°C with Chloramine T-acetic acid (as described in previous sections of thesis), acetic acid (30 mls glacial acetic/l) or with distilled water as a control. In each case the treated tissue was filtered and the filtrate dialysed for 48 hours against running tap water. Residues were washed with hot water, ethanol and finally acetone before drying at 40°C.

(2) Barium hydroxide. Neutral detergent-oxalate treated tissue (1 gm) was treated with 0·43 N $Ba(OH)_2$ (150 ml) for 8 hours at 90°C in a nitrogen flushed stoppered flask. It was then filtered and the residue was washed quickly with hot water. Filtrate and washings were combined, neutralized with H_2SO_4 and filtered.

Carbohydrate extractions. (1) Acidic extraction. The residues from all treatments were hydrolysed with 1 N.H_2SO_4 under reflux for 2 hours neutralized with $BaCO_3$ and filtered.

(2) Alkaline fractionation. Hemicelluloses were extracted from the delignified and non-delignified tissue (1.5 g) by stirring with 10% KOH (75 ml)

under nitrogen for 18 hours at room temperature. The extract was filtered and the filtrate acidified by stirring over ice with addition of 50% v/v acetic acid until pH 4.5 had been reached. The acidified extract was allowed to stand overnight at 0-4°C and then centrifuged at 70,000 g. The precipitate was resuspended in water, dialysed for 24 hours against tap water and lyophilized (Hemicellulose-A). The supernatant was similarly dialysed 24 hours and freeze dried (Hemicellulose-B). The hemicellulose-B (0.2 gm) was dissolved in $CaCl_2$ solution (SpG 1.3, 25 ml) with stirring overnight. Iodine solution (I_2 3%, KI 4% w/v; 4 ml) was added and the blue precipitate collected after 1 hour by centrifugation for 1 hour at 70,000 g. Iodine in the supernatant (heteroglycan-B) and precipitate (linear hemicellulose-B arabinoxylan-B) was neutralized with $Na_2S_2O_3$ solution. The fractionswere dialysed against running tap water 24 hours and freeze dried.

Hydroxyproline determination. Hydroxyproline was determined by the method of Bergman and Loxley (1963) in $Ba(OH)_2$ hydrolysates. In carbohydrate fractions it was determined by the method of Switzer and Summer (1971) after hydrolysis of the fractions with 6N.HCl under gentle reflux for 18 hours.

As it is used in all subsequent hydroxyproline determinations throughout the thesis except those of the automatic amino acid analyser the method of Switzer and Summer (1971) will be detailed here. Like most colorimetric methods for determination of hydroxyproline, including that of Bergman and Loxley, it is based on the oxidation of hydroxyproline to pyrrole which is then condensed with p-dimethylaminobenzaldehyde (Ehrlichs reagent) giving a coloured product. The method

described here is a modification of that originally devised by Prockop and Udenfriend (1960).

1 ml of hydrolysate or standard was placed in a conical 15 ml glass centrifuge tube together with a drop of 1% phenolphthalein in ethanol. Strong KOH was added dropwise until the solution had become alkaline. Using dilute HCl and KOH the pH of the solutions was readjusted until a faint pink colouration showed. The volume was adjusted to 2.5 mls with distilled water and 1 ml of 0.2M sodium borate buffer pH 8.7 added. 2 ml of 0.2 M-Chloramine T (oxidant)was added and the tubes stirred thoroughly by a vortex stirrer before being allowed to stand at room temperature for exactly 25 minutes. To stop the oxidation 1.2 ml of 3.6 M.$Na_2S_2O_3$ solution was added and the contents well mixed. A saturating quantity of KCl (1.5 g) was added followed by 2.5 ml of toluene. The tubes were tightly stoppered, shaken for 5 minutes and then centrifuged in a bench centrifuge to clearly separate the toluene and aqueous phases. The toluene which contains possible interfering compounds was carefully withdrawn with a Pasteur pipette and discarded. A glass marble was placed on the top of each tube and the rack of tubes put in a boiling water bath for 30 minutes. The tops of the tubes were subjected to a stream of cold air at the same time. When the tubes had been removed from the bath and cooled to room temperature 3 ml of Toluene was added to each. They were tightly bunged and shaken and centrifuged as before. A 2.0 ml aliquot of the Toluene phase was removed to a separate tube, 0.8 ml of Ehrlichs reagent added and the tube contents mixed on a Vortex stirrer. After standing for 30 minutes the tubes were read at 560 mμ against

a reagent blank.

The Ehrlichs reagent was prepared by slowly stirring a cold mixture of 2.74 ml of conc. H_2SO_4 and 20 ml absolute ethanol into 20 ml of absolute ethanol containing 12 g of p-dimethylaminobenzeldahyde.

Carbohydrate analyses. Fractions were hydrolysed with sulphuric acid, neutralized with $BaCO_3$ and analysed as described in the previous section of this thesis.

III. ATTEMPTS TO EXTRACT HYDROXYPROLINE FROM THE CELL WALL BY MILD METHODS

INTRODUCTION: Analyses of cell wall carbohydrates from delignified young lupin hypocotyl have revealed no major differences in wall composition between elongating and non-elongating regions. However, the delignification was found to cause the destruction of protein and the loss of nearly all of the hydroxyproline and arabinose and a substantial amount of galactose from the tissue. Thus it was not possible to reach the conclusion that measurable changes in the wall do not occur during the cessation of elongation. Rather, if there are concomitant changes in cell wall structure and tissue elongation then these changes probably involve chiefly those molecules eliminated from the wall by the delignification process. Furthermore it does not appear likely that one is able to gain a true idea of primary cell wall structure as it is related to growth unless the wall is considered as a composite structure involving both carbohydrates and proteins.

In most previous studies of the primary cell wall (Setterfield and Bayley, 1961; Wilson, 1964; Northcote, 1965; Muhlethaler, 1967) there has been a tendency to disregard the protein component on the assumption that the carbohydrates are the only important structural polymers and that any protein present was probably cytoplasmic in origin, i.e. contaminant. This assumption has been shown to be invalid since the discovery of the hydroxyproline rich protein peculiar to the cell wall.

The apparently ubiquitous occurrence of hydroxyproline in plant cell walls (Lamport 1970; Miller, Lamport and Miller, 1972) compared with the virtual absence from the cell cytoplasm (Lamport, 1965) and coupled with its extensive O-glycosidic linkage to arabinose (Lamport, 1967) has led to the postulate that the hydroxyproline rich protein extensin may control cell wall extensibility through its bonding via arabinose to polysaccharides in the wall. It would therefore act as a reinforcement by cross-linking the wall polymers to form a macromolecular complex. Some such role for the protein is supported by the finding that a decrease in growth rate is often associated with an increase in the level of cell wall hydroxyproline (Cleland and Karlsnes; 1969; Ridge and Osborne, 1970; Winter et al., 1971; Sadava et al., 1973), although under some conditions the correlation does not appear to exist (Winter et al., 1971).

Further evidence for a cross-linking role for extensin has come from the study of fragments released after enzymic (Lamport, 1969; Keegstra et al., 1973) or chemical (Lamport, 1965 and 1967; Heath and Northcote, 1971; Monro et al., 1972) degradation of the wall. They have been shown to contain various amino acids and much cell-wall galactose in addition to the hydroxyproline arabinosides (Lamport, 1969; Heath and Northcote, 1971). Lamport (1970) has therefore proposed a cell wall model, relevant to the control of extensibility, in which protein is linked via the hydroxyproline and arabinose to a galactan which in turn is linked in some way to the cellulose microfibrils (see Fig. 12a) Acid labile links are suggested between the arabinose units and alkali labile links between the

arabinose and galactose. In this regard Rayle et al. (1970) have proposed that an acid labile bond is important in the control of elongation and that its cleavage will result in a "loosening" of the cell wall.

While this thesis was being written evidence from other workers (Keegstra et al., 1973) for a galactose-serine linkage was presented. This is in agreement with the present overall findings which suggest that the hydroxyproline-arabinose links are not the only ones binding extensin into the wall. A variety of other amino-acid sugar links are possible and have already been outlined (Lamport, 1970). Nevertheless, no matter what amino-acid sugar links are likely to bind the polymers together the uniqueness of hydroxyproline in the cell wall makes it a useful indicator of the presence of extensin. Thus it is assayed in the present work to give some measure of wall glycoprotein, and not because the hydroxyproline arabinose links are thought to be necessary to the integrity of the wall glycoprotein complex.

To study the extensin-polysaccharide complex as it relates to growth one should ideally isolate it as a glycoprotein, using methods which will not result in the breaking of covalent bonds. Some attempts to remove it from the wall with simple protein extractants have been made with little success although it is readily released after partial chemical or enzymic lysis. Rees and Wight (1969) have suggested that the primary cell wall can in principle be dispersed without breaking covalent bonds. In view of this and of the possibility that significant species differences exist between the primary walls of various plants attempts have been made to extract the glycoprotein from lupin

hypocotyl by mild methods. The results are reported below.

RESULTS AND DISCUSSION

Changes in Hydroxyproline level with Growth

To confirm that there is an increase in hydroxyproline in the lupin hypocotyl with cessation of growth 6 cm hypocotyls were cut into three 2 cm regions and the hydroxyproline content of each of these determined on whole hydrolysed (6N.HCl 18 hours) sections. It was shown earlier (Chapter I) that in the 6 cm hypocotyl the top 2 cm is contributing about 75% to the increase in length of the hypocotyl whereas the bottom 2 cm has stopped elongating. The results in Table XI clearly show that there is a large increase in hydroxyproline as the tissue ages with the level in the bottom region being a twice that of the top. As there was little difference in dry weight between the three regions this represents a doubling of the absolute amount of hydroxyproline. Similar results have been reported for pea epicotyl tissue when elongation is allowed to cease normally or is induced to cease (Cleland and Karlsnes, 1969). In this former publication the hydroxyproline had increased 100% by the time a 1 cm region of pea epicotyl had elongated to its final length of about 3.8 cm.

Attempts to Extract Hydroxyproline with Non-Proteolytic Extractants.

In an attempt to remove the glycoprotein or at least its protein moiety from the wall the treatments shown in Table XII were applied to crude walls prepared by grinding whole fresh hypocotyls for a few minutes in buffer in a Waring blender and filtering. These walls were used as such or were first

TABLE XI. HYDROXYPROLINE CONTENT OF LOWER MIDDLE AND UPPER REGIONS OF 6 CM HYPOCOTYL

Region of Hypocotyl	Hydroxyproline (as a percentage of dry weight)		Dry weight of whole section as percentage of upper dry weight
	Unextracted Section	Ground Neutral detergent Extracted Sections	
Upper	0.055	0.32	100
Middle	0.085	0.46	98
Lower	0.111	0.53	105

subjected to dilute acid (pH 3.0, 100°C, 5 hours) treatment before extraction to cleave any dilute acid labile bonds which might be responsible for binding of the extensin into the walls.

The denaturing extractants tested for ability to extract hydroxyproline included 8M·Urea, 7M·Urea-$CaCl_2$ and neutral detergent, all of which disrupt the weak bonds involved in secondary and tertiary protein structure, namely interactions between polar groups (hydrogen and ionic bonds) and hydrophobic bonds. Urea is considered to be a potent hydrogen bonding agent which denatures proteins by competing with them for both protein-protein and protein-solvent hydrogen bonds, so that the molecule unfolds.

Accumulated evidence (Scheraga, 1963) suggests that the hydrophobic bonds (non-polar) also play an important role in maintaining the native conformation of protein in aqueous solution and that the powerful denaturing activity of such compounds as sodium dodecyl sulphate (SDS - principle active agent in the neutral detergent of van Soest) is due to their ability to disrupt such bonds. The effect of $CaCl_2$ which has no obvious capacity for hydrogen or hydrophobic bonding appears to be in the ability of the Ca^{++} to affect the structure of the solvent so that solvent-macromolecule interactions involved in stabilization are reduced (von Hippel and Wong, 1964). $CaCl_2$ has been shown to greatly increase the rate at which proteins are dissolved by urea (Simpson and Kauzmann, 1953). The well documented action of these agents on a variety of proteins, nucleic acids and polysaccharides indicates that under the right conditions these agents disrupt sufficient of the noncovalent bonds to allow unfolding of the molecules under the influence of

thermal forces.

The data presented in Table XII indicates that the agents tested are incapable of removing more than 5-10% of the hydroxyproline from the wall under the conditions used. Even this figure is probably an overestimate of removed wall glycoprotein as some extensin precursor would have been present either as cytoplasmic contaminant or as extensin present in, but not yet bound, to the cell wall. In agreement with the results of others (Lamport, 1970; Keegstra et al., 1973) the data presented above strongly suggests that the protein is bound into the cell wall by covalent linkages. However, Chrispeels (1969), using 0.2 M.$CaCl_2$ was able to extract over 50% of the label incorporated into carrot phloem discs after a 48 hour incubation in C^{14}-proline, but did not look for the presence of attached polysaccharide.

Cold and hot dilute acid did not solubulize the protein or increase its solubility in denaturing agents, indicating that dilute acid labile links alone are unlikely to bind the protein to other matrix polymers. Likewise the action of sodium methoxide in methanol, used to break ester linkages, did not render the protein from either buffer-ground or dilute acid-treated walls soluble in 8 M.Urea, Urea-$CaCl_2$ or in boiling neutral detergent.

Attempts at Extraction with Guanidinium thiocyanate (GTC)

Recently certain ions have been found to be highly potent in the disruption of non-covalent links and have thus been termed chaotropic ions (Dandliker et al., 1967). It has been proposed that their action depends upon the size of the negatively hydrated domain of the cation or anion so that the

TABLE XII. REMOVAL OF HYDROXYPROLINE FROM CELL WALLS BY NON-PROTEOLYTIC EXTRACTANTS

Wall* Preparation	Extractant	Extraction time (hr)	Hydroxyproline (mg/gm of original extracted wall preparation
a) Buffer Ground	PO_4 Buffer pH 7.0	16	0.25
	8M·Urea	16	0.31
	7M·Urea-$CaCl_2$	16	0.27
	Acid pH 3.0, 23°C	16	0.23
	Acid pH 3.0, 100°C	5	0.41
	5% NaOH in methanol	16	-
	followed by 8M-Urea	8	0.45
	Sodium methoxide in methanol	16	-
	followed by 8M-Urea	8	0.27**
	Neutral detergent (reflux)	3	0.36**
b) Buffer Ground-dilute Acid treated (pH2.9, 100°C 5 hrs)	8M·Urea	8	0.05
	7M·Urea-$CaCl_2$	8	0.08
	Sodium methoxide in methanol	16	-
	followed by 8M·Urea	8	0.13
	Sodium methoxide in methanol	16	-
	followed by 8M·Urea-$CaCl_2$	8	0.15
	Sodium methoxide in methanol	16	-
	followed by neutral detergent	3	0.09
	Neutral detergent (reflux)	3	0.10**

* Hydroxyproline content of buffer-ground wall 5.4 mg/gm

** Measured by difference between extracted and unextracted walls.

water molecules involved in hydrogen bonding and the hydrogen bonding groups on the macromolecule become incorporated into the domain of the ion. Where the domain is large the ion will be powerfully disruptive. One such chaotropic agent is guanidinium thiocyanate which has a great advantage over many others in that both the cation and anion have negatively hydrated domains. Thus the hydrated volume will relatively quickly approach the volume of the solution, a point where the hydrogen bond is destroyed. An attempt to remove hydroxyproline from the wall using 6M·GTC (Moldow et al., 1972) was made with little success (Table XIII). Only about 10% of the hydroxyproline was removed from the buffer ground walls. Again this is probably partly cytoplasmic contamination by the extensin precursor and partly unincorporated extensin. Brysk and Chrispeels (1972) suggest that the protein is incorporated after the addition of arabinose oligomer side chains and then is covalently linked to the wall in muro. Until this occurs the protein should be readily extracted by GTC.

Pretreatment of the walls with acetic acid (pH 2.9, 100°C, 5 hours) to break dilute acid labile bonds did not facilitate the extraction. These results (Table XIV) are consistent with the postulated role of extensin as a structural protein with covalent linkages in the wall. They are not however consistent with the idea that such linkages involve only dilute acid labile bonds, or that if a number of bond types are involved these are in series rather than in parallel with the dilute acid labile link. Such a bond could exist, as the lowering of pH to 3.6 will cause an increase in the extensibility of the cell wall in vitro (Rayle et al., 1970) and of extensibility and growth rate in coleoptiles (Rayle and

TABLE XIII. EXTRACTION OF CELL WALL BOUND HYDROXYPROLINE WITH GUANIDINIUM THIOCYANATE

Hypocotyl Batch		Hydroxyproline (mg/gm original wall preparation)		
		Cell Wall	Dialysed Extract	% remaining in cell wall
A	Initial buffer extracted walls	5.4		
	After extraction with 6M·GTC	4.7	0.49	87
B	Initial Neutral detergent extracted walls	3.7		
	After extraction with 6M·GTC	3.43	0.12	93

TABLE XIV. EFFECT OF DILUTE ACID TREATMENT ON EXTRACTION OF CELL WALL HYDROXYPROLINE

Extractions (in sequence)	Hydroxyproline (mg/gm of original buffer extracted walls)		
	Extracted	In Residue	% remaining in residue
Neutral detergent	-	3.70	
0.1 N. Acetic Acid (pH 2.9 100°C, 5 hrs	0.01	3.55	96
6 M. Guanidinium Thiocyanate	0.01	3.73	100

Cleland, 1970; Evans et al., 1971).

Even assuming that the activation energy for cleavage of this bond is biologically lowered one would expect that hot dilute acid pH 2.9, (shown to result in the loss of some cell wall carbohydrate) would cleave the bond. It therefore appears that there exist relatively stable bonds other than dilute acid labile, or methoxide labile ester links, which maintain the integrity of the extensin polysaccharide complex.

In the previous section it was shown that much wall arabinose was not extracted by hot dilute acid. This is not consistent with earlier suggestions of the acid lability of the arabinose-hydroxyproline link (Lamport, 1970; Rayle et al., 1970). However, Karr and Lamport (1972) have recently claimed that the arabinose-hydroxyproline linkage is more stable to acid than carbohydrate-arabinofuranoside bonds, and therefore cannot be considered as dilute acid labile. When these results and the data reported in this section are taken in conjunction it will be seen that they are not inconsistent with Lamport's (1970) original suggestion that the extensin is linked to other wall carbohydrates, perhaps via the arabinose. In this case hot dilute acid would render the protein extractable only if the dilute acid labile links occurred within the carbohydrate portion of the extensin complex beyond the hydroxyprolylarabinose units. However, further work (Lamport, Katona and Roerig, 1973) now suggests that the principal link from extensin to the cell wall is via galactosylserine and that the hydroxyproline has attached to it $(arabinose)_4$ oligosaccharides.

Extraction of Hydroxyproline with Alkali

In the light of failure of other mild methods of extraction

it was decided to try alkali treatment, moreover it does offer a possible direct means of studying the protein in relation to the alkali soluble carbohydrate fractions originally under investigation in this thesis. Furthermore Lamport (1970) postulated the existence of an alkali labile link between arabinose and galactose in his original model of the extensin-polysaccharide complex. Cleavage of this bond might result in the release of extensin from the wall.

When neutral detergent extracted walls were extracted by stirring at 35°C sequentially for 24 hours with dilute and strong alkali (1 gm cell wall preparation/100 ml alkali) most of the hydroxyproline was dissolved as shown in Table XV .

Similar results have been published by Cleland (1967) who was able to obtain total alkaline (1N.NaOH, 25°C, 3 hours) extraction of radioactive hydroxyproline from walls of Avena coleoptiles which had been incubated for 20 hours in C^{14}-Proline.

However the results presented here are in contrast to those obtained with sycamore (Acer pseudoplatanus) suspension cultures by Lamport (1965) who was able to obtain only partial solubilization of the hydroxyproline using 5.4% KOH. Even after extraction with 24% KOH for 3 days some remained in the "cellulose" fraction. An even greater contrast is provided in the results of Heath and Northcote (1971) who found virtually complete insolubility in strong alkali (17%) plus borate (4%).

Olson (1964) and Dougall and Shimbayashi (1960) were also unable to extract the bulk of the hydroxyproline from callus cells with cold dilute alkali. Possibly the tissue used, i.e. suspension cultured cells compared with hypocotyl, or the different species used may account for this difference in alkali

TABLE XV. EXTRACTION OF CELL WALL-BOUND HYDROXYPROLINE WITH ALKALI

	Hydroxyproline	
	mg/gm of original cell wall prepartion	% of initial cell wall hydroxyproline
In original cellwall preparation	2.14	100
Extracted by 10% KOH	1.80	84.1
Extracted by 24% KOH	0.24	11.2
In Residue	0.10	4.7

* Cell walls extracted sequentially with 10% and 24% KOH by stirring overnight at 35°C under nitrogen.

solubility, although the higher temperature employed here will have facilitated extraction to some extent.

When the 10% alkali extract of lupin hypocotyl was acidified only a slight hemicellulose-A precipitate was obtained. After dialysis and freeze-drying of the acidified solution a hemicellulose-B fraction which contained 70% of the extracted hydroxyproline was obtained. Further fractionation also involving dialysis showed that much of this hydroxyproline was non-dialysable. The hydroxyproline-rich protein has therefore been extracted as polymer and not in small degraded fragments.

When dilute (0·1M) oxalic acid (pH 2) treated walls were extracted with 10% KOH to dissolve the hydroxyproline rich polymer the results shown in Table XVI were obtained. They show that the polymer was still largely non-dialysable in spite of the cleavage of arabinofuranoside links. Thus either most of the protein fragments *per se* are non-dialysable or they are attached by bonds stable to both acid and alkali to carbohydrate of sufficient bulk to render them non-dialysable. The results suggest linkage of the extensin to the insoluble part of the cell wall complex through polysaccharides other than those involving sugar-sugar arabinofuranoside linkages.

There is little reason to doubt that the extensin is bound into the wall by covalent links. The well established alkali extraction of carbohydrates has been found to remove the protein, albeit in a partly degraded form as the partial loss during dialysis shows. It therefore appears that the investigation of alkali extracts of the cell wall may prove useful in the study of the relationship of extensin to wall carbohydrates. By using alkali at least one component, namely

TABLE XVI. EFFECT OF DILUTE ACID ON EXTRACTION OF CELL WALL HYDROXYPROLINE BY ALKALI

Extraction*	Hydroxyproline (mg/gm of original buffer extracted walls)			
	Extracted	In Residue	% Remaining in Residue*	% of extracted hydroxy-proline non-dialysable
Oxalic acid pH 2.0, 100°C, 5 hr	-	4.19		
1) 10% KOH 1 hr	1.21	2.98	71	71.8
followed by 10% KOH 5 hr	1.76	1.23	29	58.9
2) 10% KOH 9 hr	4.34	0.93	22	86.2

* Extractions with KOH at room temperature under nitrogen, after the oxalic acid treatment outlined.

the carbohydrate, may be extracted without suffering extensive degradation.

MATERIALS AND METHODS

Plant Tissue. Lupin hypocotyls were grown as described in Chapter I. Entire 6 cm hypocotyls and upper and lower 2 cm sections of 6 cm hypocotyls were excised for all wall preparations.

Cell Walls. Total cell walls were prepared by extracting the ground hypocotyls either for 2 hours with boiling neutral detergent or by grinding with buffer (0.5 M phosphate, pH 7.0) in a Wareing blender for a few minutes at room temperature.

Extractions of Cell Walls. Extractions with 8M·Urea, 7M·Urea-$CaCl_2$ guanidinium thiocyanate, and sodium methoxide or sodium hydroxide in methanol involved stirring with a magnetic stirrer in a closed flask for a specified time or overnight at room temperature (18-22°C) using 0.5 g (dry weight) of cell walls to 100 ml of solution. Alkali extractions involved similar extraction of the tissue but using 1 gm (dry weight) per 100 mls of alkali under nitrogen in a sealed flask. Dilute acid treatments involved refluxing for 5 hours in the specified acid. Flask contents were filtered through sintered glass and the residues washed quickly with distilled water. The extracts (filtrates) and washings were neutralized where necessary and were dialysed for 24 hours against running tap water, unless otherwise stated, before analysis.

Analyses. Hydroxyproline was measured by the method of Switzer and Summer outlined in Chapter II Materials and Methods, after hydrolysis for 16 hours in 6M.HCl.

IV. EXTRACTION OF CELL WALL PROTEIN AND CARBOHYDRATES WITH ALKALI

INTRODUCTION: Extraction of lupin hypocotyl cell walls with a variety of protein denaturants both before and after dilute acid and sodium methoxide treatments did not dissolve hydroxyproline, suggesting that extensin is covalently linked to insoluble wall constituents other than through dilute acid labile or ester links. The indications are that it is necessary to break covalent bonds before the hydroxyproline can be extracted, and this is consistent with the postulated role of extensin in controlling extensibility of the wall by crosslinking through such bonds to other wall polymers (Lamport, 1965).

In order to study the extensin-polysaccharide complex it is desirable to isolate it in as near to the native state as possible. How close past degradative studies have come to approaching this ideal is difficult to ascertain, but in most treatments the extensin has been broken into small fragments after enzymic (Lamport, 1969; Keegstra et al., 1973) or chemical (Lamport, 1967; 1965; Monro et al., 1972) degradation. While much about the structure of the complex has been deduced from the study of these degradation products it is felt that extra information about the extensin complex may be obtained from a study of the association of hydroxyproline with alkali soluble carbohydrate fractions, as it is known that when these are isolated under suitable conditions little polysaccharide breakdown occurs, thus essentially only one

component of the glycoprotein is degraded.

Most higher plant cell walls so far studied have a high degree of secondary deposition in which hydroxyproline is a very minor component. The cell wall has therefore usually been investigated with an understandably large bias towards carbohydrates while the protein component has been neglected, even in many investigations of primary walls.

In lupin hypocotyl polymer-bound hydroxyproline can easily be extracted with 10% KOH into non-dialysable molecules possibly associated with part of the cell wall hemicellulose. Therefore, in order to gain some more information on the relationship of extensin to the wall polysaccharides the approach has been to investigate the hydroxyproline and monosaccharide composition of carbohydrate fractions isolated from alkaline extracts of the wall.

RESULTS AND DISCUSSION

Alkali Extraction of Hydroxyproline from Cell Walls

Alkali soluble fractions for analysis of hydroxyproline and monosaccharide content were isolated from cell walls basically as described in the previous sections and shown in Fig.1 (Chapter I). After depectination the cell walls were extracted with 10% KOH under nitrogen at 35°C. The hemicellulose-B fraction, recovered by dialysing and freeze-drying the acidified extract contained 70% of the extracted hydroxyproline. This hemicellulose-B was dissolved in $CaCl_2$ and treated with iodine (Gaillard, 1961) to give a precipitate of hemicellulose-B arabinoxylan and a soluble heteroglycan-B (Gaillard, 1965), both of which were purified by

dialysis for 48 hours against running water and then freeze-dried. The residue was then extracted with 24% KOH - 4% boric acid and the extracts similarly fractionated into linear and branched species, (precipitable and non-precipitable from $CaCl_2$ with iodine respectively (Gaillard and Bailey, 1966). These and the 24% KOH - 4% boric acid extracted walls contained only traces of hydroxyproline. The 0.54% remaining in the 10% KOH treated walls appears to have been rendered dialysable by the prolonged strong alkali treatment. The results are shown in Table XVII. The monosaccharide compositions of the arabinoxylan and heteroglycan were respectively 1:0.34:0.50:0.60 and 1:5.9:4.7:0.3 for xylose, arabinose, galactose and glucose. The heteroglycan-B contained 45% and the xylan-B 25% of the hydroxyproline present in the extracted hemicellulose-B. Much of the hydroxyproline has, therefore, been extracted in a non-dialysable form and not in small degraded fragments. However the fact that 30% of the hydroxyproline in the 10% KOH extract was lost on dialysis indicates that the extensin has probably undergone a fair degree of breakdown. Based on the small amount of soluble hydroxyproline-containing glycoprotein in the cytoplasm, published results so far suggest that the native extensin molecule is far too large to dialyse and has a molecular weight of about 30,000 when incorporated into the wall (Brysk and Chrispeels, 1972).

The 24% KOH appears to have almost completely extracted the hydroxyproline remaining in the walls after 10% KOH treatment. More than 90% of this is dialysable and thus very degraded.

While association with a polysaccharide fraction does not necessarily imply linkage to that polysaccharide it is perhaps

TABLE XVII. ALKALI EXTRACTION OF POLYSACCARIDES AND HYDROXYPROLINE FROM LUPIN HYPOCOTYL CELL WALL

Fraction	Weight (mg/gm cell wall)	Hydroxyproline (mg/gm cell wall)	% of cell wall hydroxyproline in fraction	Monosaccharide composition Xyl:Ara:Gal:Glu.
Cell wall*	-	5.0	100	-
10% KOH extract	-	3.1	60	-
Heteroglycan-B	39.7	1.36	27	1:5.9:4.7:0.3
Arabinoxylan-B	79.4	0.77	15	1:0.34:0.5:0.6
Branched-B_{24}**	18.0	trace	0	-
Linear-B_{24}**	36.6	0.04	0.9	-
10% KOH extracted wall	-	0.52	7.3	-
24% KOH extracted wall	-	trace	0	-

* $CaCl_2$ wall material used had been extracted for 2 hrs with boiling neutral detergent

** Branched-B_{24} and Linear-B_{24} are those fractions extracted by 24% KOH and not precipitated and precipitated respectively from $CaCl_2$ solution with iodine.

relevant that most of the non-dialysable hydroxyproline is associated with the galactose-arabinose-rich hemicellulose heteroglycan-B; suggesting that the original cell wall glycoprotein is a source of this heteroglycan.

Rates of Extraction of Cell Wall Polymers with Alkali

Similar extractions to the above, with 10% KOH, but at 18 - 20°C for 18 - 24 hours likewise removed most of the hydroxyproline from the wall. There was still some loss (20-30%) of hyroxyproline as dialysable material. As this was probably due to alkaline degradation of the polymer, extractions at lower temperatures (0-2°C) and/or for shorter times were investigated. The results showed that only small amounts of the hydroxyproline were extracted.

The 10% KOH extraction, usually overnight at room temperature after delignification is commonly used to dissolve the bulk of the hemicellulose polysaccharides from plant cell walls. Therefore, keeping in mind the possible importance of extensin-polysaccharide associations in controlling wall extensibility, alkali extractions at various temperatures and times were further investigated in terms of both hydroxyproline and the hemicellulose polysaccharides. The results from two alkali extraction sequences are given in Tables XVIII and XIX and show that the bulk of the hemicellulose arabinoxylan-B and about half of the heteroglycan-B fraction can be removed almost independent of hydroxyproline. These results suggest that the glycoprotein is linked to the alkali insoluble portion of the cell walls by links which are only slowly broken by alkali, and is either not linked to the bulk of the hemicellulose or bound to it only by

TABLE XVIII. EFFECT OF TEMPERATURE AND TIME ON 10% KOH EXTRACTION OF CELL WALL POLYMERS

	Extractions in sequence	Time (hr)	Temperature (°C)	Hydroxyproline (mg extracted/g neutral detergent treated cell walls)	Hemicellulose
Detergent Extracted Cell Wall Batch A	1	4	2	0.172	188
	2	19	2	0.675	58
	3	13	18-20	2.55	33
Detergent Extracted Cell Wall Batch B	1	4	18-20	1.80	188
	2	16	18-20	3.13	89

TABLE XIX. POLY- AND MONOSACCHARIDE COMPOSITION OF ALKALI EXTRACTS FROM LUPIN HYPOCOTYL CELL WALLS

Sequence	Total Hemicellulose (mg/gm of initial neutral detergent extracted walls)	Hemicellulose-B Arabinoxylan-B	Heteroglycan-B	Monosaccharide composition of arabinoxylan-B (Xyl:Ara:Gluc:Gal)
A				
1*	188	135(0.162)**	7.3(0.013)**	1:0.34:0.39:0.47
3	83	46.5(2.26)	8.6(0.29)	1:0.51:0.95:2.38
B				
1*	188	155(1.60)	10.3(0.12)	1:0.46:0.29:0.52
2	89	46.5(1.70)	9.2(0.14)	1:2.64:0.51:1.17

* Extracts from batch A and B of Table XVIII

** Figures in parentheses are hydroxyproline content of hemicellulose fractions in mg.

very alkali-labile links. Such a link shown to be labile even to 0.5M.KOH at 0-4°C is the xylosylserine link (Anderson, Hoffman and Meyer, 1965). Cleavage of such a bond might release much of the bonded carbohydrate, but the existence of other alkali stable bonds within the extensin complex may account for the insolubility of the protein moiety in the 10% KOH. Also, when one considers the polymeric nature of the wall constituents it must be realized that over the length of an extensin molecule there will be a large number of linkages all of which must be cleaved before release of the protein occurs. Thus while the probability of cleavage of each of these bonds may be high the likelihood of breaking all of the alkali labile links per protein chain under the conditions used may be lower. However, alkaline cleavage of peptide links particularly through β-elimination of serine will probably have reduced the average length of the peptide chains and thus made the extraction of extensin, as fragments, more likely.

Rees and Wight (1969) have stated that the plant cell wall can in principle be completely dispersed without breaking covalent bonds by, for example, the sequential use of EDTA and cuprammonium salts. The present results suggest however that the alkaline conditions encountered in the cuprammonium solution would lead to cleavage of alkali labile bonds and partly explain its ability to disperse primary cell walls.

Relationship of Hydroxyproline and Hemicellulose Polysaccharides

The fact that extensin is not extracted by 10% KOH at 0°C indicates that its bonding to the cell wall is intact, a bonding which is probably relevant to the hypothesised wall stiffening

function of extensin. Therefore to investigate the association of extensin with wall polysaccharides more closely the fractionation of 22°C 10% KOH extracts was performed as before but on walls which had first of all been treated with 10% KOH at 0°C to remove that hemicellulose which is either attached to extensin by alkali labile bonds only, or unattached but insoluble in neutral detergent.

Matrix polysaccharides extracted from the depectinated hypocotyl cell walls with alkali at 0°C (HO) and at room temperature (HRT) were separated into the usual hemicellulose-A (xylans precipitated by acidification of the alkaline extract) and hemicellulose-B (which remains in the acidified solution). Hemicellulose-B was fractionated as before by dissolving in concentrated $CaCl_2$ solution and adding iodine to precipitate hemicellulose-B-arabinoxylan (1-4 linked polymers) and leave the arabinose-galactose-rich heteroglycan in the supernatant (Gaillard, 1961).

Total hemicellulose from several of the alkaline extracts was separated into these fractions which were analysed for hydroxyproline and monosaccharides after hydrolysis. Results from analysis of the arabinoxylan and heteroglycan are given in Table XIX. The small amounts of hemicellulose-A were discarded.

Although sequential extraction studies (Table XVIII) showed that most of the hydroxyproline was extracted at room temperature after the removal of the bulk of the hemicellulose (HO) at 0°C, it is evident that when the glycoprotein is extracted at 22°C it is largely associated with the hemicellulose-B arabinoxylan fraction (although at 35°C more

seems to be associated with the heteroglycan-B). Thus this fraction as usually isolated at room temperature contains two sub-fractions differing in the temperature required to effect their extraction and in their content of hydroxyproline. The sub-fractionation is further emphasised by the monosaccharide compositions of the arabinoxylan fractions, as the hydroxyproline-rich arabinoxylan-B is higher in galactose and arabinose than the more rapidly extracted polymer. This is consistent with the known existence of arabinosylhydroxyproline (Lamport, 1967) and galactosylserine (Lamport, Katona and Roerig, 1973) links within extensin. But in view of the β-elimination of serine which is reported to occur at 0-4°C with 2 - 15% NaOH (Anderson, Hoffman and Meyer, 1965) much of the galactose may not be in direct linkage with the protein, although it could be linked to the xylan. Protein fragments not in covalent association with the arabinoxylan fraction would possibly either be spun out of solution in $CaCl_2$ during the clarification step or, more probably because of their small size, remain in the heteroglycan-B. To test the former possibility an examination of the gelatinous mass obtained from high speed centrifugation of the solution of room temperature extracted hemicellulose-B in $CaCl_2$ was made. It revealed a large quantity of polysaccharide in the gel but an enrichment of hydroxyproline in the hemicellulose-B remaining in the supernatant. The $CaCl_2$ insoluble polysaccharide (gel) represented 50 - 60% of the polysaccharide recovered from the $CaCl_2$ solution, but it contained only about 30% of the hydroxyproline recovered. The compositions of the $CaCl_2$ insoluble polysaccharide and the supernatant were respectively

	Xylose	:	Arabinose	:	Glucose	:	Galactose
	1	:	1.45	:	0.29	:	0.66
and	1	:	3.48	:	0.28	:	4.74

Thus there is a marked enrichment of arabinose and galactose, independent of hydroxyproline, in the gel. Evidently the bulk of the hydroxyproline rich polymer is not linked to this fraction despite its enrichment in arabinose and galactose. It is noteworthy that the ratio of arabinose: galactose in the two fractions changes, suggesting that these sugars are not derived solely from an arabinogalactan present in both fractions. In fact it decreases in the fraction which increases in hydroxyproline.

The much smaller amounts of heteroglycan-B compared with arabinoxylan-B isolated from the room temperature extract contain about the same amount of hydroxyproline per unit of polysaccharide as the arabinoxylan. The amount of hydroxyproline associated with the heteroglycan increases when the temperature of extraction increases, e.g. to 35°C. It is possible therefore that this heteroglycan-B includes alkali degraded extensin complex whose yield is increased by the breakdown occurring with extraction at higher temperatures. However, because of the large proportion of heteroglycan-B extracted with 0°C, 10% KOH it does not seem likely that this fraction as usually isolated (by one room temperature alkaline extraction) owes its existence to the presence in it of extensin fragments as was suggested. But this does not rule out the possibility that it is merely conventional pectin which has become bound into the wall by establishing cold alkali labile links with wall protein containing as well as these

linkages, a more stable type which requires higher temperatures to disrupt it. However the "heteroglycan-B" (non-iodine precipitable polysaccharide) from HRT (20°C, 10% KOH soluble hemicellulose-B extracted from 0°C 10% KOH extracted cell walls) may represent a fraction derived from the extensin polysaccharide complex.

MATERIALS AND METHODS

Plant Tissue. Lupin hypocotyls were grown and prepared as described in Chapter I.

Cell Wall Material. Cell wall material for fractionation was prepared by grinding hypocotyls in iced water in a Wareing blender for 30 seconds, filtering, and extracting the residue for 2 hours in two changes of boiling neutral detergent. The walls obtained were then washed by suspending in hot distilled water and filtering two or three times.

Extraction. All alkaline extractions were carried out as before by stirring on a magnetic stirrer in a closed flask under nitrogen at the specified temperatures on melting ice in a cold room for 0°C extraction, or in the laboratory at room temperature. Where sequential extractions were made the walls were quickly filtered and rinsed after each extraction and then immediately subject to the next stage in the sequence.

Fractionations. Polysaccharide fractionations were carried out as described in Chapter I of this thesis, except that where mentioned the centrifugation of the hemicellulose-B dissolved in $CaCl_2$ solution was for 90 minutes at 70,000 g instead of for 15 minutes at 70,000 g. The hemicellulose-B was decanted off and fractionated as before, and the gelatinous mass which collected under the former centrifugation conditions was resuspended in water, dialysed 24 hours against running tap water and lyophilized.

Analyses. Details of monosaccharide and hydroxyproline analyses of the 10% KOH extracts and of the various fractions isolated therefrom have already been given.

V. HYDROXYPROLINE - POLYSACCHARIDE ASSOCIATIONS IN POLYSACCHARIDE FRACTIONS FROM ELONGATING AND NON-ELONGATING LUPIN HYPOCOTYL

INTRODUCTION: Previous studies (Chapter I) have shown that about 75% of the elongation of a 6 cm lupin hypocotyl takes place in its upper 2 cm. However little difference was found in the alkali soluble polysaccharide fractions when lower middle and upper 2 cm regions of delignified hypocotyl were compared. The delignification treatment was subsequently found to remove most of the hydroxyproline and arabinose (Chapter II) both of which are probably important components of polymers involved in the control of wall extensibility (Lamport, 1970). Hydroxyproline has been shown to increase in the wall as it ages (Lamport, 1965; Cleland and Karlsnes, 1967; Ridge and Osborne, 1970; Winter et al., 1971; Sadava et al., 1973). Also analyses of acid hydrolysates of non-delignified lupin hypocotyl cell walls suggested that there was more arabinose in the lower (least extensible) than in the upper region of the hypocotyl. It therefore became apparent that differences in wall composition between regions of differing growth rate may be determined at least partly by the hydroxyproline-rich protein (extensin) through its association with wall polysaccharides, as suggested in the extensin hypothesis of Lamport (1965). Thus it is imperative to analyse non-delignified wall where particular attention can be given to the hydroxyproline-rich component.

Most of the hemicellulose can be separated from the

hydroxyproline by extraction at 0°C leaving a relatively protein-rich, room temperature extractable hemicellulose (Chapter IV). If protein-associated polysaccharide changes during growth then it is relevant to examine the hemicellulose arabinoxylan and heteroglycan not only from a single 10% KOH room temperature extraction, but also from separate 0°C and room temperature extracts. This is because it is at room temperature that the hemicellulose fractions which appear to be most closely associated with the protein are removed. The results of such an investigation are reported below.

RESULTS AND DISCUSSION

Extraction of Cell Wall Components from Upper and Lower 2 cm Regions of 6 cm Hypocotyl

Differences in components from non-delignified cell walls of lower compared with upper regions of hypocotyl were examined by analysing monosaccharides and hydroxyproline in the fractions isolated from 10% KOH extracts. Results are presented in Table XX. They are in agreement with a higher concentration of the glycoprotein in wall constituents from the lower sections of hypocotyl, in accordance with the concept of the control of extensibility by extensin. Thus there is more hydroxyproline in the walls of the non-elongating region and higher hydroxyproline levels in its hemicellulose fractions.

A further indication of a more extensive linkage of the glycoprotein in wall polymeric material in the least extensible (non-elongating) lower region compared with the more extensible (elongating) upper region is indicated by dialysis losses of

hydroxyproline. Both the fractionation to hemicellulose-A and B and the subsequent fractionation of the hemicellulose-B involve dialysis of the fractions. The extent of loss of hydroxyproline during these dialyses may therefore be considered as an index of levels of low molecular weight material containing this amino acid and released by the alkali. This dialysis loss is very much higher for the upper hypocotyl sections suggesting either the presence in the wall of lower molecular weight glycoprotein fragments not yet extensively linked to polysaccharide, or greater susceptibility to alkaline degradation.

If the heteroglycan-B fraction contains a large number of glycoprotein fragments, and on the assumption that the peptide chain is equally susceptible to alkaline cleavage in both upper and lower regions of the hypocotyl, one might expect greater dialysis of both hydroxyproline and carbohydrate from the fraction derived from the upper section. This is because the number of polysaccharide - polysaccharide associations via protein left intact will decrease as the distance between the polysaccharides on the peptide chain increases. The results of Table XX show that only half as much heteroglycan-B is recovered from the upper region as from the lower and it contains a much lower percentage of hydroxyproline. The ratio of the absolute amount of hydroxyproline in the heteroglycan-B to that in arabinoxylan-B is 1:2 for the top region and 1:1.4 for the bottom. This is possibly a reflection not only of dialysis from the heteroglycan-B but also of an extensin molecule more frequently bonded per unit peptide length to the "arabinoxylan-B" in the lower region, so that fewer fragments are released from association with the polysaccharides by the

TABLE XX. EXTRACTION OF CELL WALL COMPONENTS FROM TOP AND BOTTOM 2 CM SECTIONS OF 6 CM LUPIN HYPOCOTYL

Component in Cell Walls*	Upper (elongating)	Lower (non-elongating)
Hydroxyproline**	3.21	5.34
Hemicellulose-A**	18.5 (0.12)***	32.5 (0.48)***
Hemicellulose-B (1-4) linked linear polysaccharide ("arabinoxylan-B")	147 (0.41)	138 (1.60)
Hemicellulose-B heteroglycan-B***	26 (1.18)	52 (3.07)
Extracted hydroxyproline lost on dialysis (%)	69.6	26.4
Monosaccharide composition of hemicellulose-B-arabinoxylan-B (Xyl:Ara:Gluc:Gal)	1:0.41:0.76:0.61	1:0.89:0.84:0.66

* Cell Walls extracted with 10% KOH at room temperature overnight.

** mg/g of neutral detergent extracted cell walls.

*** Values in parentheses are hydroxyproline as % of polysaccharide fraction.

KOH.

It is noteworthy that although there is a higher level of "arabinoxylan-B" associated galactose in the lower than in the upper region this sugar shows a much lower percentage increase between the two regions than arabinose. This is consistent with the suggestion of Brysk and Chrispeels (1970) that the extensin is incorporated with arabinose side chains only, and then becomes linked to galactose. If it were incorporated with galactan side chains one would expect a far greater increase in galactose, provided that the alkaline extraction conditions have not broken the galactan-protein linkage. If some β-elimination of galactosylserine has occurred, an increase in residual galactose might indicate that in the native state there is a marked increase in bound galactose. Much of the galactose in the arabinoxylan-B could represent extensin side chains which are precipitated by iodine or else spun out of suspension in $CaCl_2$ solution when the iodine precipitated "arabinoxylan-B" is separated from the heteroglycan-B rich supernatant.

Levels of Galactose and Arabinose in Hydroxyproline-Rich Fractions

It has been shown that both arabinose and hydroxyproline are higher as a proportion of total cell wall in the lower than upper 2 cm section of 6 cm lupin hypocotyl (Chapter III). It has also been shown that the hemicellulose in which this difference is measured can be divided into 0°C and 22°C 10% KOH extractable portions (Chapter IV). That extracted at room temperature contains nearly all of the hydroxyproline and is also higher in arabinose and galactose than the 0°C 10% KOH

soluble portion. It therefore appears that the fractions from lupin hypocotyl which are enriched in hydroxyproline have a parallel enrichment in arabinose and galactose and that this may be a reflection of the presence of extensin.

In view of the above the difference between fractions in the upper and lower sections should be made more apparent by examining the room temperature soluble hemicellulose from these two regions after removal of the portion extracted with $0^{o}C$ 10% KOH for 10 hours. A comparison of the monosaccharide composition of $20^{o}C$ 10% KOH soluble hemicellulose-B fractions isolated from upper and lower hypocotyl regions was therefore made. On the same batch of hypocotyls a recomparison of $0^{o}C$ and $22^{o}C$ 10% KOH soluble arabinoxylan was made. In both comparisons the arabinoxylan was sub-fractionated by centrifuging at high speed a solution of it in $CaCl_2$.

When the hemicellulose-B is dissolved in aqueous $CaCl_2$ SpG 1.3 prior to precipitation of the 'arabinoxylan-B' by iodine (see Fig.I B) a turbid solution is formed. This is usually clarified prior to the addition of iodine, by spinning for 15 minutes at 70,000 g. However when the solution is spun for 90 minutes at 70,000 g a gelatinous mass collects which contains as much carbohydrate as the 'arabinoxylan-B' fraction that can be precipitated from the supernatant with iodine. Furthermore, hydroxyproline analyses show that the gelatinous fraction spun out of the $CaCl_2$ solution contained one third as much hydroxyproline as the arabinoxylan subsequently precipitated from the clarified supernatant. This therefore provides a means of crudely sub-fractionating the arabinoxylan-B by separating from it a $CaCl_2$ insoluble gel which would normally

be spun down during the high-speed centrifugation after addition of iodine. A flow sheet for this sub-fractionation is shown in Fig. 3.

Heteroglycan-B was recovered from the supernatant after iodine precipitation, as usual. Although some of it may have contributed to the gel spun from the solution of Hemicellulose-B in $CaCl_2$ it cannot have contributed much as the amount of polysaccharide in the gel far exceeds the amount of heteroglycan-B usually recovered.

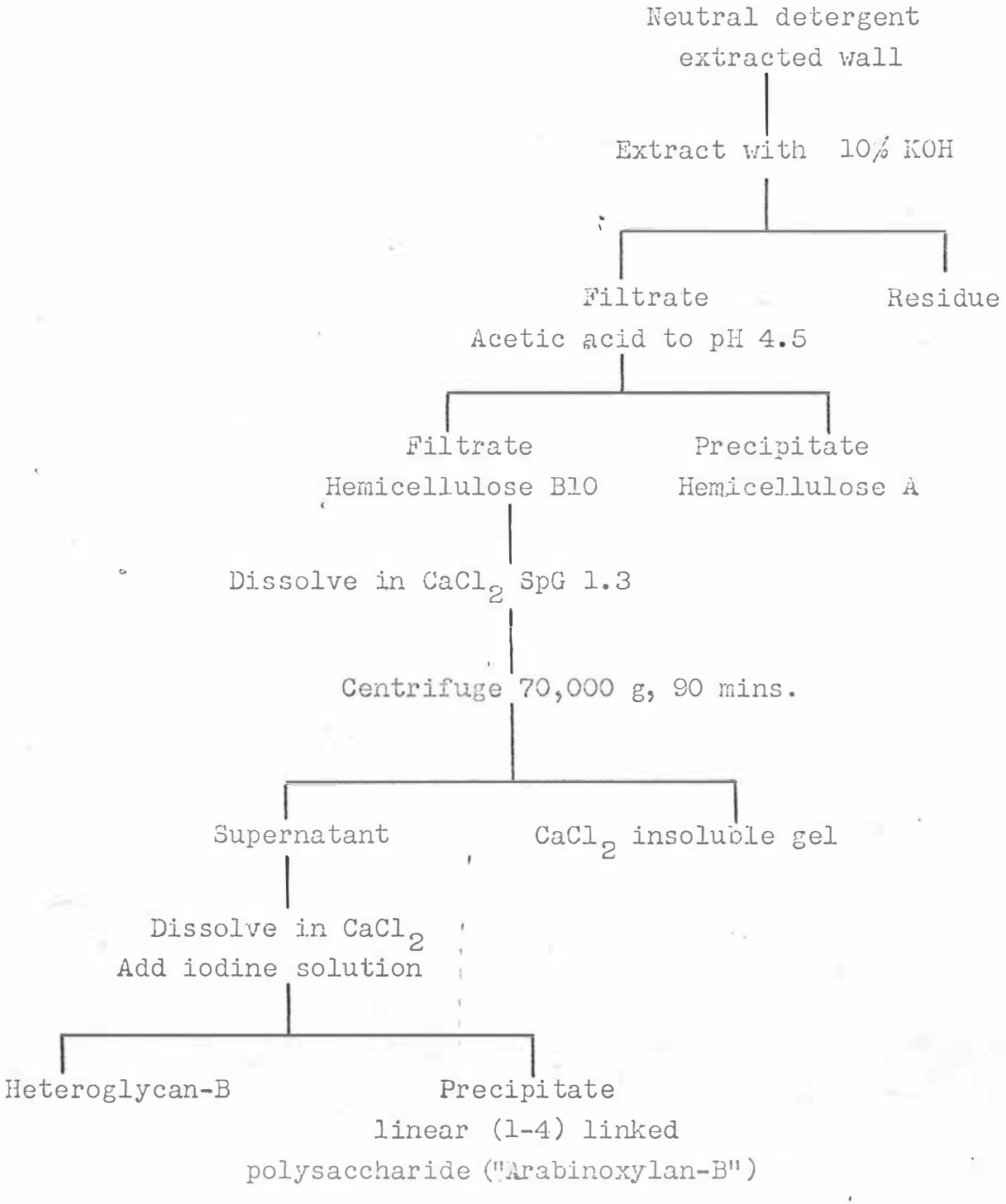
A. Total Hypocotyl

The "arabinoxylan-B" and $CaCl_2$ insoluble hemicellulose-B fractions from total hypocotyl were investigated after extraction at 0^oC and room temperature. The arabinoxylan-B was precipitated as before with iodine but only after the $CaCl_2$ solution containing hemicellulose-B had been spun at high speed to collect the gelatinous material. The results are shown in Table XXI A.

It is again clear that about 70% of the 10% KOH soluble polysaccharide can be removed by treatment at 0^oC. However the 0^oC fraction contains half as much $CaCl_2$ SpG 1.3 insoluble as it does "arabinoxylan-B" hemicellulose, whereas the room temperature extract contains rather more $CaCl_2$ insoluble than it does of arabinoxylan, even although about two thirds of the $CaCl_2$ insoluble polysaccharide is removed at 0^oC.

Compared with the "arabinoxylan-B" the material spun from the $CaCl_2$ solution has a high proportion of arabinose and galactose. Also, there is a higher proportion of these sugars in the material spun from the room temperature extracted hemicellulose-B (which contains most of the hydroxyproline) than

FIG.3. ALKALINE FRACTIONATION OF CELL WALLS MODIFIED TO INCLUDE REMOVAL OF $CaCl_2$ INSOLUBLE POLYSACCHARIDE



from that extracted at 0°C. Whether or not this reflects the presence of a glycoprotein rich in arabinose and galactose and hydroxyproline can be determined only by further work involving greater resolution of cell wall fractions. Even the separate occurrence of arabinogalactan and protein in the fraction would not rule out an in vivo association between them resulting in resistance to 0°C 10% KOH extraction, if a bond between them was cleaved by the same treatment at 20°C. This would be especially true if arabinogalactan in both fractions was bonded to long chain xylan which as well as being iodine precipitable was not very soluble in $CaCl_2$ solution.

B. Upper and Lower Hypocotyl Sections

The polymers described in Table XX were a combination of the two fractions which could have been extracted sequentially at 0°C and 20°C. The cell walls from a further batch of top and bottom hypocotyl sections were extracted sequentially with 10% KOH at 0°C and 20°C. In this experiment the hydroxyproline rich room temperature extract was examined and yielded hemicellulose-B arabinoxyland of more markedly different monosaccharide composition (Table XXIB) with

	Xylose	:	Arabinose	:	Glucose	:	Galactose ratios
of top	1	:	0.6	:	0.4	:	0.6
and bottom	1	:	1.9	:	0.4	:	1.07.

There was four times as much hydroxyproline in the bottom fraction.

The monosaccharide composition of the fractions again suggests that the polysaccharide which is being spun down from the $CaCl_2$ solution prior to addition of iodine is rich in arabinogalactan. Im this regard it is noteworthy that the

TABLE XXI. EXTRACTION OF CELL WALL POLYSACCHARIDES FROM 6 CM LUPIN HYPOCOTYL

Hypocotyl Regions	Extraction Temperature	Fraction	Weight*	Monosaccharide Composition			
				Xylose	Arabinose	Glucose	Galactose
A. Total	0	Arabinoxylan-B	86	1	0.19	0.38	0.48
Total	0	$CaCl_2$-insoluble	47	1	1.01	0.36	1.65
Total	20	Arabinoxylan-B	18	1	1.45	0.29	0.66
Total	20	$CaCl_2$-insoluble	24	1	3.48	0.28	4.74
B. Upper **	20	Arabinoxylan-B	23	1	0.59	0.41	0.61
Upper	20	$CaCl_2$-insoluble	35	1	3.19	0.24	5.31
Lower	20	Arabinoxylan-B	28	1	1.90	0.43	1.07
Lower	20	$CaCl_2$-insoluble	31	1	4.49	0.16	4.96

* mg/gm of neutral detergent extracted cell wall.

** Walls extracted for 10 hr with 10% KOH at $0^{o}C$ extraction for 24 hr.

$CaCl_2$ insoluble polysaccharide is greatest as a proportion of the total linear polysaccharide ($CaCl_2$ insoluble plus "arabinoxylan-B") in the upper region of hypocotyl. This difference is not great, but if it is real and due to an arabinogalactan then it is in keeping with results of others (Nevins, English and Albersheim, 1968; Northcote, 1972) who claim a decrease in arabinose and galactose as a tissue ages. It is possible that although such a decrease occurs it is being disguised because the fractionation has not distinguished between an arabinogalactan decreasing in quantity and an "arabinoxylan" undergoing a concomitant increase in arabinose and galactose. However, the relative changes in arabinose and galactose between the fractions from top and bottom hypocotyl regions suggests that although incorporation of an arabinogalactan may be occurring some extra arabinose is being incorporated, possibly as arabinose oligosaccharide side chains. There is evidence that these are present on protein before its secretion into the cell wall (Brysk and Chrispeels, 1972; Karr 1972).

An increase in galactose in fractions from the lower hypocotyl regions is not necessarily due to serine linked galactan. Much of it is possibly due to the presence of xylan linked galactose. In this case the increase would be independent of extensin incorporation, although it may eventually become linked to the protein.

Whether or not metabolic turnover known to occur extensively in cell walls (Lamport, 1970) takes place between the two fractions is a question for investigation. Possibly an increase in crosslinkage through extensin binds the $CaCl_2$

insoluble and $CaCl_2$-soluble fractions more extensively in the lower section of hypocotyl. This would decrease the proportion of arabinogalactan contributing to the heteroglycan-B and increase the arabinose and galactose in the so called "arabinoxylan-B" fraction.

The hemicellulose-B is totally soluble in $CaCl_2$ solution which also contains urea. This suggests that the usual insolubility of some of the hemicellulose polysaccharide is due to inter- and/or intramolecular associations, and possibly reflects a capacity for inter-polymer associations within the wall. Such non-covalent interactions between wall polysaccharides have been demonstrated (Rees, 1969) and for some time been considered important in determining the properties of the primary wall (Gould et al., 1965). This has usually been discussed in relation to pectic substances which are present in higher concentration in the wall than is the $CaCl_2$ insoluble polysaccharide (see Chapter I Table I).

The $CaCl_2$ insoluble polysaccharide does not increase as a proportion of the total hemicellulose-B in the bottom compared with the top hypocotyl region. This suggests that the gelation capacity of the hemicellulose-B per se is not a primary determinant of growth rate.

However, the ability of the fraction to form gels may not be directly related only to the amount of arabinose-galactose-rich $CaCl_2$ insoluble polysaccharide contained in it but also to the fine structure of this polysaccharide. There is evidence that such polymers may undergo slight internal modifications such as the insertion of rhamnose units or side chains which affect their linearity (Gould et al., 1965; Aspinall et al.,

1967; Rees and Wight, 1969; Rubery and Northcote, 1970).

Although polymers of varying gelation capacity may be spun with equal ease from the $CaCl_2$ solution, it is not necessarily a reflection on the strength of their non-covalent bonding within the wall, although it does suggest an ability to associate. One must take into account the environment within the cell wall where as well as a much lower ionic concentration there are a number of other molecules present which might influence the ability to form gels. For example, extensin, by forming glycosyl linkages to galactose or other sugars may sterically hinder the formation of non-covalent interpolymer junctions while at the same time forming a rigid network based on irreversible formation of covalent bonds. The transition from a pH dependent non-covalent to a covalent type of linkage may be the basis of the loss of elongation response to auxins in primary cell walls as they age.

The origin of the arabinose and galactose(?arabinogalactan) in the hemicellulose-B is of interest, as it is possibly derived largely from pectic arabinogalactan which is rendered insoluble in neutral detergent by covalent association, perhaps through extensin, with other wall polysaccharides. In this case it might enter the wall as pectin and later become bound into what is known coventionally as the hemicellulose fraction. Such a precursor product relationship between two wall fractions has been suggested by Stoddart and Northcote (1967) and Rubery and Northcote (1970) as a result of their work on turnover of pectic substances in sycamore suspension cultures.

Whether the arabinogalactan of the room temperature

extracted arabinoxylan-B fraction is derived metabolically from the 0°C soluble fraction, from conventional pectin, or neither, should be apparent from labelling experiments. That is, the degree of specificity involved in glycosylation of the extensin molecule during fabrication of the extensin-polysaccharide complex of the cell wall should become apparent.

The above results clearly show that application of the alkali extraction procedure to cell walls from top and bottom 2 cm regions of a 6 cm lupin hypocotyl provides evidence of a difference in polysaccharide composition between elongating and non-elongating regions. It seems significant that this difference is largely in a fraction with which most of the extracted hydroxyproline is associated. Whether or not this association involves direct covalent linkage remains to be seen.

The data contributes to the growing body of evidence which suggests that although the cell wall is predominantly carbohydrate, a relatively small fraction, the glycoprotein extensin, is of great importance in the determination of cell elongation. Further study of its chemical integration into the wall is important to an understanding of its mode of action. In this connection it seems likely that glycosylserine links are not the sole ones joining the amino acids to the cell wall as these links appear to be broken by β-elimination in 10% KOH at 0°C (Anderson, Hoffman and Meyer, 1965), to permit extraction of hydroxyproline. It seems that on the whole the role of the hydroxyproline rich glycoprotein in controlling extensibility involves the formation of more than one kind of link with the cell wall polysaccharide and may even involve amino acids linked to the "cellulose" fraction (Monro, Bailey

and Penny, in press).

MATERIALS AND METHODS

Plant Tissue. Seeds of blue lupin (Lupinus angustifolius) were germinated and grown under previously described conditions (Chapter I). Hypocotyls ($6 \pm 0{\cdot}2$ cm in length) were harvested. Either whole hypocotyls were used or they were cut into three 2 cm sections of which the top and bottom regions were analysed in this experiment.

Cell Wall Preparation. The hypocotyls and sections were ground in a Wareing blender in iced water for 30 seconds, filtered and extracted for 2 hours in 2 changes of boiling neutral detergent. The walls were washed by suspending in hot distilled water and filtering two or three times.

Extractions. All alkaline extractions were carried out basically as described in Chapter I of this thesis, except that the tissue used here was not delignified. Comparisons of upper and lower regions were made in terms of total 10% KOH soluble hemicellulose and in terms of the 18-22°C 10% KOH soluble hemicellulose removed from the walls after a prior extraction for 10 hours with 10% KOH at 0°C. For comparison of hemicelluloses derived from 10% KOH extraction at 0°C and subsequent extraction at 18-22°C respectively walls derived from whole hypocotyls were used. All extractions were done under nitrogen.

Fractionations. For comparison of top and bottom hypocotyl regions total 10% KOH soluble hemicellulose was fractionated into hemicellulose A and B and the hemicellulose-B sub-fractionated into linear and branched species by iodine precipitation of the linear species from solution in aqueous $CaCl_2$ SpG 1·3, as has been described in Chapter I.

For comparison of hemicellulose from top and bottom hypocotyl by extraction with 10% KOH at room temperature after prior 0°C 10% KOH extraction, and for comparison of both 0°C and room temperature 10% KOH soluble hemicelluloses from total hypocotyl, a modified fractionation was used. In this the hemicellulose was dissolved in $CaCl_2$, SpG 1.3 by stirring overnight and the solution was spun at 70,000 g for 90 minutes. The $CaCl_2$ insoluble material (gel) was resuspended in water, dialysed 24 hours against running tap water and freeze dried. The supernatant was dialysed and freeze-dried before redissolving in $CaCl_2$ solution and submitting to iodine precipitation to separate linear (1-4) linked polysaccharides and the heteroglycan-B, as described in Chapter I.

Analyses. Carbohydrates were analysed in acid hydrolyses of the fractions by the quantitative paper chromatographic method of Wilson (1964) as described in Methods Chapter I. Hydroxyproline was measured in 6 N·HCl hydrolysates by the method of Switzer and Summer described in Methods, Chapter II.

VI. THE AMINO ACID COMPOSITION OF POLYSACCHARIDE FRACTIONS ISOLATED FROM LUPIN HYPOCOTYL CELL WALLS

INTRODUCTION: The protein "extensin" is characteristic of the cell walls of higher and many lower plants (Lamport, 1970; Miller et al., 1972; Lamport and Miller, 1971; Gotelli and Cleland, 1968). Most of the cell hydroxyproline is located in this protein, which is confined to the cell wall (Lamport, 1965).

The isolation of arabinosylhydroxyproline and peptides containing hydroxyprolyl(arabinose)$_{1-4}$ from cell wall hydrolysates (Lamport, 1967) lead to the idea that extensin acts to crosslink wall polysaccharides and thereby control the extensibility of the wall. This was supported by the physiological evidence that hydroxyproline increased in walls as they ceased elongation (Cleland and Karlsnes, 1969).

Extensin fragments were later found to contain both arabinose and galactose (Lamport, 1969) and this lead to the postulation that the wall protein was bound via arabinose oligosaccharide-linked galactan to the wall microfibrils (Lamport, 1970). More recently however, it has been shown that isolated arabinosylhydroxyproline is not involved in any direct link to polysaccharide, at least in fragments so far isolated. Moreover galactose associated with wall peptides has now been shown to be involved in a galactosylserine linkage (Lamport et al., 1973) and although as yet only single galactose units have been found attached to the peptide, it is thought that protein linked galactan conjugates the other

polysaccharides and the protein of the wall (Keegstra et al., 1973).

The ability of pronase to release polysaccharides from polysaccharidase treated walls (Keegstra et al., 1973) adds weight to the concept of crosslinks between the protein and major wall polysaccharides. Based on these and other (Talmadge et al., 1973; Bauer et al., 1973) results Keegstra et al. (1973) have now proposed a general model for the primary cell wall of sycamore suspension cultures in which the extensin and most of the wall polysaccharides are united into a macromolecular complex. The essential feature of Lamport's original model (Lamport, 1970) is preserved and that is that the protein molecule crosslinks through polysaccharides of the wall matrix to the microfibrils and can therefore retard their movement relative to one another. Any study of the primary cell wall matrix is therefore now to be regarded as a study of the extensin-polysaccharide complex and has an important bearing on the understanding of cell elongation.

In addition to enzymic and harsh chemical degradation used by the above workers, mild chemical means may be used to fragment the wall and enable isolation of various polymers. These may then be characterized and fitted into a hypothetical model of the wall.

It was shown in Chapter IV that total 10% KOH soluble hemicellulose (HT) can be extracted in two fairly discreet stages. The first of these is at 0°C and removes about two thirds of the hemicellulose (HO) and little hydroxyproline. The resistant hemicellulose (HRT) is readily extracted along with most of the wall hydroxyproline when the temperature is

increased to room temperature (18-22°C). It was therefore suggested that HRT is bound into the wall through its involvement in the extensin polysaccharide complex and is not released until the complex is dissociated through the rupture of alkali labile bonds.

Such a bond is the galactosylserine link of extensin. It appears to be resistant to 0°C 10% KOH (Lamport et al., 1973), although this is considerably more stable than a glycosylserine link reported from collagen (Anderson, Hoffman and Meyer, 1965). The alkaline cleavage of this bond is accompanied by destruction of the serine through β-elimination (Fig.5a).

Evidence for the importance of the galactosylserine to the association between various wall polymers may therefore be obtained from a study of their amino acid compositions. Furthermore, as a variety of amino acid-carbohydrate bonds are possible (Lamport, 1970), it is relevant to consider the levels of amino acids other than serine, as an enrichment of a particular amino acid in any fraction might indicate a bond between this amino acid and a specific polysaccharide in that fraction. This approach may also provide information useful to the methodology of extensin isolation and will help clarify the relationship of extensin and the alkali-soluble plant cell wall carbohydrates. The results of such a study are reported below.

RESULTS AND DISCUSSION

Extraction of polysaccharide fractions and protein

Lupin hypocotyls were ground and extracted with neutral

detergent. They were then subjected to the alkaline fractionation shown in Fig.3 Chapter V which is basically according to Gaillard and Bailey (1967) with the 0°C and 18-22°C alkaline extractions. Tissue is extracted with 10% KOH first at 0°C for 8 hours to remove 0°C soluble hemicellulose (HO) and then at 18-22°C for 24 hours to remove the room temperature soluble fraction (HRT). The HO has been shown to contain little hydroxyproline (Chapter IV) and was not fractionated further. The HRT was dissolved in $CaCl_2$ SpG 1.3 and separated by centrifugation at 70,000 g into $CaCl_2$ SpG 1.3 insoluble material, and soluble material, and the latter fractionated further into linear and branched species (arabinoxylan-B and heteroglycan-B) by the addition of I_2:KI solution (Gaillard, 1961; Gaillard and Bailey, 1966). Hemicellulose-A was not removed prior to this fractionation as, apart from being low in quantity in this tissue (Monro, Bailey and Penny, 1972), it contains very little hydroxyproline (Monro Bailey and Penny, 1974; Chapter V). The 10% KOH extracted residue was treated with 24% KOH for 24 hours at room temperature.

The results are given in Table XII in terms of polysaccharide fractions and protein extracted. They confirm the results already presented in Chapters IV and V although the arabinoxylan-B : $CaCl_2$ insoluble polysaccharide ratio is somewhat higher than formerly obtained when approximately equal quantities of the two fractions were found. As before, the heteroglycan is a minor component.

Of the loss in weight of the residues with the 0°C and 18-22°C 10% KOH extractions about 30% is recovered in the freeze dried extracts after 48 hours dialysis. This possibly

indicates a high degree of degradation of the polymers, the presence in the wall of material of low degree of polymerization, or of some bacterial degradation.

The weights of HO and HRT after 24 hours dialysis are given in parentheses in Table XXII. The loss of these fractions is only about half that encountered over 48 hours.

The percentage and weight of protein per gram of detergent extracted starting wall is also shown in Table XXII. The figures are obtained from the amino acid analyses shown in Table XXIII. No extraction of protein was recorded with the 0°C 10% KOH extraction and most of it (77%) was removed by the 18-22°C extraction of RO. In agreement with this Olson (1964) has shown that 85% of the hydroxyproline (an index of wall protein) of tobacco callus cells is resistant to 0°C alkali extraction, and Dougall and Shimbayashi (1960) and Lamport (1965) have found the bulk of the wall-bound hydroxyproline in sycamore callus cells to be resistant to cold alkali. Of the 25.2 mg of protein in the 0°C 10% KOH extracted residue (RO) only 8.1 mg are accounted for in the sum of protein in the room temperature 10% KOH extracted residue (RRT) plus the three fractions derived from HRT. This means that in the 18-22°C 10% KOH extraction and fractionation some 67% of the protein in RO before extraction has been lost. A similar figure for the loss of total fraction can be deduced from Table XXII. Hence there is no evidence for independent dialysis of the protein and polysaccharide.

The amino acid analyses (Table XXIII) show that of the hydroxyproline not extracted at 18-22°C two thirds was removed by the 24% KOH leaving approximately 11% of the protein

TABLE XXII. ALKALINE EXTRACTION OF POLYSACCHARIDE AND PROTEIN FROM NEUTRAL DETERGENT TREATED LUPIN HYPOCOTYL CELL WALLS

* Extraction Conditions	Cell Wall After Extraction (mg)	Polysaccharide Extracted		Protein in Extracted Residue		
		Total Fraction (mg)	Fraction Isolated from HRT**	mg/gm of Extracted Residue	mg/gm of Initial Cell Wall (RND)	% Extraction of Protein
Neutral Detergent (4 hr, 100°C)	1000	-	-	24.26	24.26	-
10% KOH (0°C 8 hr)	764.8	103.3 (H0) (287.9)**		32.96	25.21	0
10% KOH (18-22°C 24 hr)	626.7	45.3 (HRT) (85.4)**	23.0 (arabinoxylan B) 10.6 ($CaCl_2$ SpG. 1.3 insoluble 4.1 (heteroglycan-B)	8.99	5.63	77
24% KOH (18-22°C 24 hr)	512.0	30.0 (H_{24})		5.02	2.57	89.4

* Extractions in sequence

** Figures in parentheses are weights recovered after 24 hr. dialysis, other figures are for total fraction after 48 hr. dialysis.

TABLE XXIII. AMINO ACID ANALYSES OF FRACTIONS AND RESIDUES OBTAINED FROM EXTRACTION SEQUENCE SHOWN IN FIG.4

Amino Acid	Sample and Amino-acid content mg/gm sample						
	RND	RO	RRT	$CaCl_2$ insoluble	Arabino-xylan-B	Hetero-glycan-B	R24
Lysine	2.28	3.16	0.56	14.61	13.05	10.39	0.36
Histidine	1.20	1.96	0.20	13.31	8.78	2.55	0.10
Ammonia	0.13	0.17	0.05	2.87	0.99	5.02	0.051
Arginine	0.24	0.32	0.12	2.64	0.71	5.86	0.053
Hydroxyproline	7.54	9.33	1.89	32.48	48.12	15.37	1.93
Aspartic Acid	0.89	1.03	0.49	6.48	3.72	13.05	0.29
Threonine	0.61	0.69	0.20	3.68	2.38	6.05	0.11
Serine	2.51	3.51	0.42	10.38	12.90	22.05	0.15
Glutamic Acid	0.83	1.05	0.42	9.58	4.62	24.60	0.27
Proline	2.11	2.82	0.86	19.51	11.52	4.03	0.66
Glycine	1.76	2.14	1.33	5.76	1.48	14.43	0.82
Alanine	0.66	0.83	0.27	4.49	3.01	6.07	0.20
½ - Cystine	-	-	-	-	-	-	-
Valine	1.16	1.58	0.48	8.57	6.11	5.76	0.33
Methionine	0.06	0.08	0.03	0.70	0.24	1.81	0.02
Isoleucine	0.54	0.73	0.37	3.52	1.90	4.99	0.30
Leucine	0.84	1.10	0.46	7.68	3.22	8.74	0.33
Tyrosine	1.24	1.58	0.29	7.04	8.61	7.17	0.13
Phenylalanine	0.66	0.88	0.55	3.14	1.15	4.53	0.42
Total (mg/gm sample)	24.26	32.96	8.99	156.4	132.5	162.5	5.02
Sample weight (mg/gm of RND)	1000	764.8	626.7	10.6	23.0	4.1	512.0

originally in the RND in the R24. Similarly, the data of Lamport (1965) indicates that some 20% of the wall-bound hydroxyproline of sycamore suspension culture cells was not removed after 3 days in 24% KOH - 4% H_3BO_3. One should however, be cautious in extrapolating from walls of intact tissue to those from suspension cultures, as some differences between the two in wall structure are apparent.

Herth et al. (1972) found an alkali stable fraction from Pleurochrysis scales to contain 32% protein of which 38% was serine. No hydroxyproline was present although Miller et al. (1972) and Lamport (1970) have shown hydroxyproline to be widespread throughout the plant kingdom. This indicates that perhaps it is not always valid to use hydroxyproline as an indicator of extensin in wall fractions.

The nature of the tenaciously bound hydroxyproline is of interest in view of the suggestion that the extensin-polysaccharide complex of the wall matrix controls wall extensibility by crosslinking between the cellulose microfibrils. One of the obvious points for control of extensibility is the microfibril-glycoprotein junction, presumably left intact and possibly concentrated in the protein of the R24. Keegstra et al. (1973) have proposed a fairly indirect linkage of the protein through serine → arabinogalactan → pectic rhamnogalacturonan → araban and galactan → xyloglucan, with the interconnections uniting most of the wall polysaccharide. The xyloglucan is thought to be attached by hydrogen bonds to the microfibril surface and to be able to creep along the microfibril at a rate dependent on wall pH and thereby rapidly influenced by auxin. While the above

model may be true for suspension culture cell walls, from which much unbound polysaccharide will have been leached into the suspending medium (Becker, Hui and Albersheim, 1964), it does not appear to adequately accommodate what is known of the primary walls of intact tissue. The incomplete solubility of hydroxyproline in alkali and the differential removal of most of the protein, xylan and pectic polymers during the sequence of neutral detergent, 0°C 10% KOH and 18-22°C 10% KOH extractions suggests that the microfibril-matrix and matrix polymer inter-relationships are somewhat different than envisaged by the above authors.

Amino Acid Composition of Wall Fractions and Residues

Those residues, extracts and derived fractions obtained from the experimental sequence shown in Fig. 4 and which were subjected to automatic amino acid analysis are shown in Table XXII.

The HO and 24% KOH extracts were not fractionated or analysed as they contain very little protein (Monro, Bailey and Penny, 1972). The R24 similarly contains little but as discussed this protein is probably a very important component of extensin due to its intimate association with the microfibrils.

The percentage amino acid composition of the protein in each fraction is given in Table XXIV. Overall, a considerable variation is apparent involving a large number of amino acids. This, coupled with uncertainty due to dialysis losses makes it difficult to obtain a basis for the comparison of the various fractions. A drawback in using percentage compositions is that variation in each amino acid of a fraction affects the figure

FIG.4. ALKALINE EXTRACTION AND FRACTIONATION OF PLANT CELL-WALL POLYSACCHARIDES AT 0°C AND 18-22°C

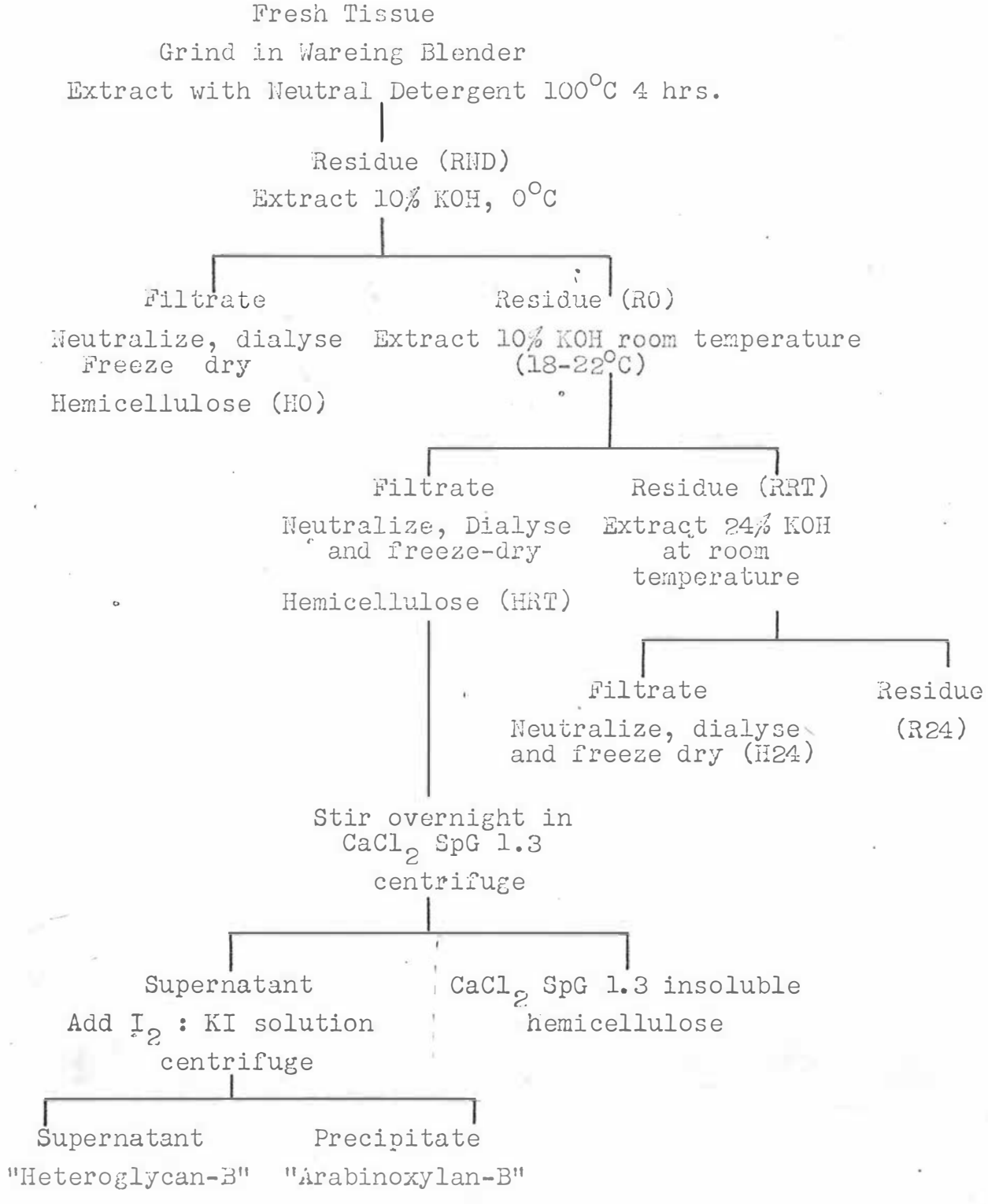


TABLE XXIV. AMINO ACIDS OF THE FRACTIONS AND RESIDUES SHOWN IN TABLE XXIII AS A PERCENTAGE OF TOTAL AMINO ACIDS FOR EACH ANALYSIS

Amino Acid	Sample and Percentage Amino Acid Content						
	RND	RO	RRT	$CaCl_2$ insoluble	Arabinoxylan-B	Heteroglycan-B	R24
Lysine	9.40	9.59	6.23	9.34	9.85	6.40	5.53
Histidine	4.95	5.95	2.22	8.51	6.63	1.57	1.58
Ammonia	0.54	0.52	0.56	1.83	0.75	3.09	0.78
Arginine	0.99	0.97	1.33	1.69	0.54	3.61	0.81
Hydroxyproline	31.08	28.31	21.02	20.76	36.31	9.46	29.58
Aspartic Acid	3.67	3.13	5.45	4.14	2.81	8.03	4.40
Threonine	2.51	2.09	2.22	2.35	1.80	3.72	1.65
Serine	10.34	10.65	4.67	6.64	9.74	13.57	2.27
Glutamic Acid	3.42	3.19	4.67	6.12	3.49	15.14	4.17
Proline	8.70	8.56	9.57	12.47	8.69	2.48	10.13
Glycine	7.25	6.49	14.79	3.68	1.12	8.88	12.61
Alanine	2.72	2.52	3.00	2.87	2.27	3.74	3.07
Half-Cystine	-	-	-	-	-	-	-
Valine	4.78	4.79	5.34	5.59	4.61	3.55	5.07
Methionine	0.25	0.24	0.33	0.45	0.18	1.11	0.26
Isoleucine	2.23	2.21	4.12	2.25	1.43	3.07	4.57
Leucine	3.46	3.348	5.12	4.91	2.43	5.38	5.04
Tyrosine	5.11	4.79	3.23	4.50	6.50	4.41	1.99
Phenylalanine	2.72	2.67	6.17	2.01	0.87	2.79	6.48

obtained for all others in the same fraction. However, despite the limitations, some meaningful points are apparent and are discussed below.

Extraction of RND with 10% KOH at 0°C for 24 hours causes very little change in amino acid composition of the extracted cell wall. This indicates that if glycosylserine bonds exist in the lupin hypocotyl they are not as susceptible to β-elimination as the xylosylserine reported from chondroitin sulphate peptides of cartilage (Anderson, Hoffman and Meyer, 1965) and which were destroyed by 2% NaOH at 0-4°C for 19 hours. It also shows that the bulk of the hemicellulose is not involved in a polysaccharide-protein complex similar to that proposed by Keegstra et al. (1973), where the galactosyl bond is the only covalent link between the protein and polysaccharide moieties and where this and interpolysaccharide covalent links result in the wall matrix being effectively a macromolecule. According to their model the HO should not have been extracted from RND unless accompanied by β-elimination of galactosylserine or simultaneous extraction of protein. The results suggest that HO must be bound into the wall by other more alkali labile links which may still involve extensin. Ester linkages between the carboxyls of aspartic and glutamic acids and sugar hydroxyls could be involved (Lamport, 1970) although sodiummethoxide was ineffective in extracting hydroxyproline from the wall (Chaper III). It is interesting to note that levels of these amino acids have often been high in published cell wall analyses (Lamport, 1970; Lamport et al., 1973; Brysk and Chrispeels, 1972) although in the present case they represent only a few per cent of the RND protein.

However, assuming crosslinks between the HO polysaccharides it would only take a few amino acid-sugar ester links to account for the insolubility of HO in pectic solvents.

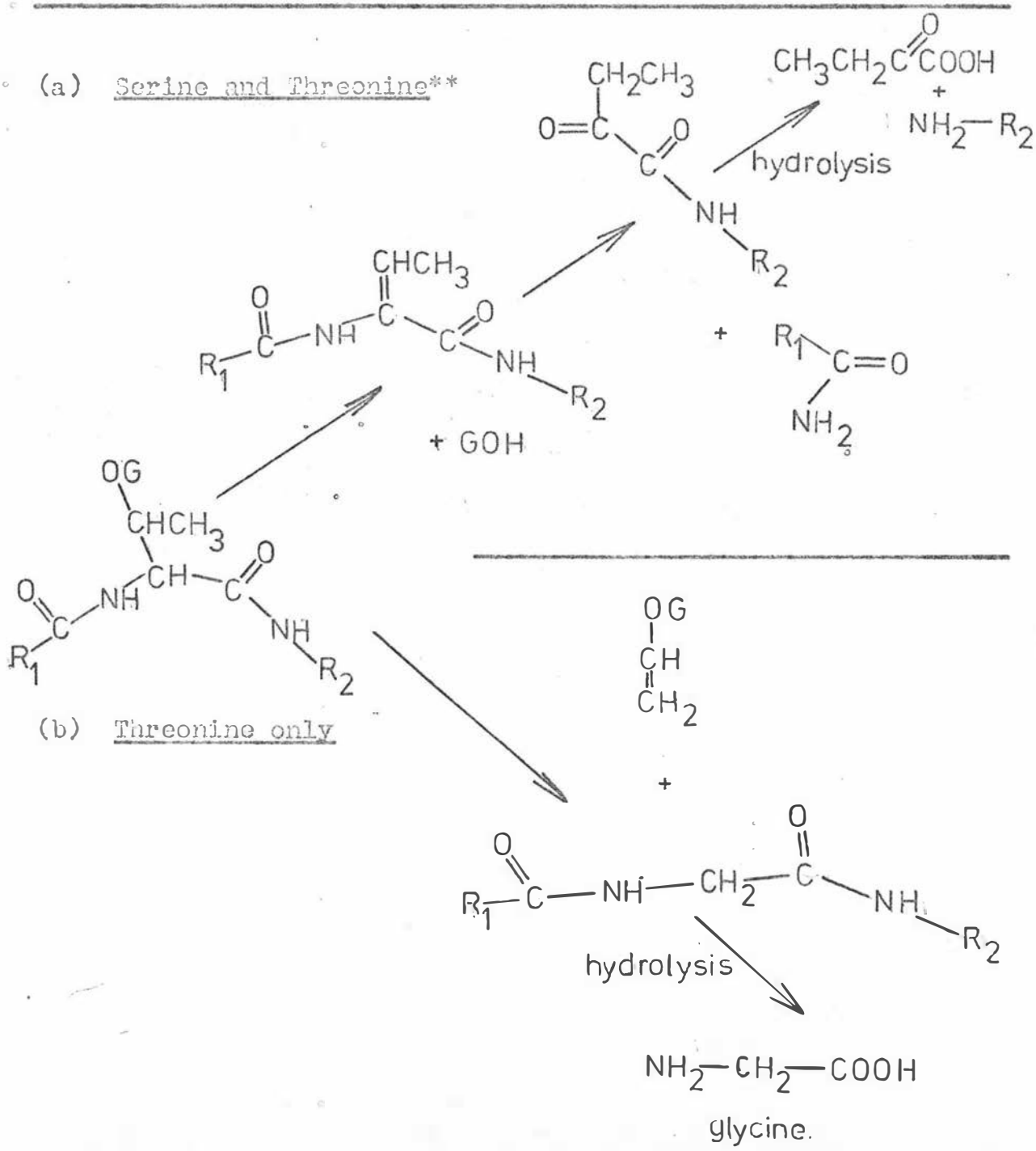
The amino acid composition of RND is fairly similar to that reported for a variety of other higher plant cell walls and has the typically high hydroxyproline (Lamport, 1970; Brysk and Chrispeels, 1972).

Extraction of RO with 10% KOH at 18-22°C for 24 hours to give RRT caused a marked change in amino acid composition. It should be remembered that the RRT protein represents only a portion of that present in RO, the remainder having been dissolved from the wall (as HRT) and recovered to some extent in the fractions isolated from the extract.

Most of the changes involve increases in the amino acid percentage in the protein of RRT compared with that in RO or RND. This no doubt reflects the decrease in the case of both hydroxyproline and serine to the extents of 7.3% and 6% respectively between RO and RRT. This means that the hydroxyproline and serine have dropped to 75% and 50% respectively of their original percentages.

That the amount of serine in the protein has halved probably indicates that β-elimination has occurred. During this reaction (Fig.5a) an unstable dehydropeptide intermediate (2-aminoacrylic acid) is formed. This easily undergoes hydrolysis under mild alkali or acid conditions resulting in the formation of a $-\overset{O}{\overset{\|}{C}}-NH_2$ terminal and α-oxopyruvic acid. Thus the peptide chain is cleaved. The amide formed will release ammonia upon acid hydrolysis.

FIG. 5. β - ELIMINATION OF O-GLYCOSYLATED SERINE AND THREONINE*



* Reference - Adams, 1965.

** in (a) the methyl is replaced by hydrogen

R_1 and R_2 are the N-terminal and C-terminal residues respectively, and G a glycosyl unit.

Lamport has reported alkaline destruction of serine in sycamore cell walls under conditions similar to those encountered here, but not at 0°C. The extraction of 0°C 10% KOH resistant hemicellulose HRT from RO can be explained in terms of glycoprotein complex similar to that proposed by Keegstra et al. (1973) if one assumes that the alkali cleaves the galactosylserine bond and at the same time dissociates the polysaccharides from the microfibrils. If on the other hand the presence of 24% KOH resistant protein indicates a more direct bonding of extensin to microfibrils, then release of the polysaccharide may depend to some extent on peptide chain cleavage as well as on β-elimination. Distinguishing between such alternatives may prove difficult due to the extent of crosslinking within the wall. However, a study of the kinetics of extraction of the various wall polymers after a variety of treatments could provide results relevant to the interpretation of wall structure.

It is probably significant that the extracted protein which has remained with the arabinoxylan-B fraction (i.e. excluding dialysed, $CaCl_2$ insoluble and heteroglycan-B protein) has relatively high serine levels accompanied by high hydroxyproline. The arabinoxylan-B serine is at a similar level to that in RND and RO and this suggests that the arabinoxylan-B protein is that which has not yet been β-eliminated from association with polysaccharide. On this basis, the heteroglycan-B serine may be part of fragments of protein containing non-glycosylated serine as well as serine glycosylated but unattached to a xylan and therefore not precipitated with iodine. However, if the region of extensin which is bonded to polysaccharides is enriched in serine then

the levels will be raised in the fraction enriched in the polysaccharide, even if extensive β-elimination has occurred.

A marked increase in glycine in the cell wall residue occurred after treatment of RO with the room temperature KOH. It is interesting that glycine can be the product of the β-elimination of O-glycosylated-L-threonine by the reaction shown in Fig.5 b in which a vinyl glycoside is formed and decomposed in alkali to free sugar and acetaldehyde (Adams, 1965). The threonine residue is converted to glycine and the peptide chain remains intact.

The constancy of threonine levels argues against this reaction being involved here although when protein losses are considered it must be regarded as possible. In this case, the high glycine observed in the residue (12.6%) compared with only a few percent in the arabinoxylan-B and $CaCl_2^{\circ}$ insoluble material would suggest protein containing glycosylated threonine associated with the microfibrils and protein with glycosylated serine associated primarily with the matrix. During the β-elimination of threonine depolymerization and release of the peptide chain would not occur with the same frequency as during serine destruction. This could partly account for the increased level of glycine in the residue.

Hydroxyproline levels vary between residues and fractions. In its glycosylated and non-glycosylated states it is stable to both acid and alkali and is therefore not likely to have been destroyed. That it is not enriched in the residue is consistent with evidence that its arabinosyl side chains are not further attached to microfibril-linked polysaccharide (Lamport et al., 1973), as was originally suggested by Lamport

(1970). It is however markedly enriched in the arabinoxylan-B fraction compared with RRT, although the average proportion of hydroxyproline in the arabinoxylan-B and $CaCl_2$ insoluble fractions is fairly similar to that in the original cell wall (RND).

That hydroxyproline can increase in proportion relative to other amino acids to the extent it has suggests (i) that its distribution along the peptide chain is uneven and concentrated in those regions where a glycosylserine bonding of extensin fragments to the arabinoxylan-B remains or (ii) that it is involved itself in direct linkage to the polysaccharides of this fraction or (iii) that the $CaCl_2$ insoluble heteroglycan-B fractions are contaminated with residual cytoplasmic protein which dilutes out the hydroxyproline. The assumption in these cases is that the solubility in $CaCl_2$ SpG 1.3 solution of any protein is enhanced by attachment to the soluble arabinoxylan-B.

Evidence in favour of (i) lies in the results of those who have analysed extensin fragments (Lamport, 1969; Lamport et al., 1973). The proportion of hydroxyproline and serine in the amino acids of such fragments is generally much higher than in intact wall protein. In the isolated fragments of extensin so far sequenced by Lamport the lowest hydroxyproline content was 60% of constituent amino acids and in the fragment in which a galactosylserine was identified (Lamport et al., 1973) the peptide had the composition $Gal_2Ser_2Hyp_5Lys_1$. In most cases hydroxyproline and serine predominate so that it appears that where galactosylserine links exist, there will be an enrichment in hydroxyproline. The arabinoxylan-B composition

is consistent with this, although galactosylserine links have not actually been demonstrated in this case. It is however relevant that a high level of hydroxyproline is present in a fraction in which high serine levels also occur and in which the protein and polysaccharide have shown parallel solubility in $CaCl_2$ SpG 1.3 and precipitability with I_2:KI.

The minor heteroglycan-B fraction, although high in serine, is relatively low in hydroxyproline and is more markedly different in amino acid composition from RND and RO than are the other fractions. It is also high in glutamic acid which is more characteristic of the cytoplasmic protein of lupin.

In an earlier experiment conducted at 35-40°C arabinoxylan-B + $CaCl_2$ insoluble and heteroglycan-B were separated. Despite the same alkaline treatment allo-hydroxyproline was found only in the latter fraction to the extent of almost 50% of its total hydroxyproline. This suggests that none of the hydroxyproline in the heteroglycan-B was glycosylated and that if chain cleavage occurred then where glycosylated hydroxyproline was concentrated the cleavage was sufficiently frequent to render the hydroxyproline-$(arabinose)_{1-4}$ fragments dialysable. Such is further evidence for the occurrence of arabinosylhydroxyproline and galactosylserine in the same region of the extensin molecule.

The composition of the $CaCl_2$ insoluble polysaccharide is in many respects intermediate between that of the arabinoxylan-B and the heteroglycan-B. It is probably a mixture of two protein types. (1) Those typical of heteroglycan-B and consisting of small residual cytoplasmic

and extensin fragments. (2) Those typical of arabinoxylan-B and representing larger protein fragments than in the heteroglycan-B, and extensin-polysaccharide with its solubility in $CaCl_2$ reduced due to greater cleavage from $CaCl_2$ soluble polysaccharide. The lower levels of hydroxyproline and serine in the $CaCl_2$ insoluble fraction support this. Also, this fraction has been found to contain a lower percentage of xylose than the arabinoxylan-B and higher levels of galactose and arabinose, particularly if derived from HRT (as in this experiment) compared with HO. The parallel drop in hydroxyproline and serine in the $CaCl_2$ insoluble fraction compared with the $CaCl_2$ soluble (1-4) linked linear fraction is further evidence for some association between the two amino acids such as sharing the same regions in the peptide chain of extensin.

The 24% KOH extracted residue (R24) has very low serine levels and compared with RRT is high in hydroxyproline. The most marked difference is in the very low serine suggesting that the protein is not bound into the R24 by galactosylserine links as the model of Keegstra et al. (1973) suggests. While this portion of the wall protein may include the very important point of crosslinkage of matrix and microfibril, it is quite possible that no covalent linkage is involved, and that the resistance to alkali treatment is due to encrusting alkali resistant polymers. Such a situation would be similar to that reported for tryptophan in tobacco mosaic virus where buried and exposed residues are respectively resistant and susceptible to attack by N-bromosuccinimide (Witkop, 1961). The low serine and threonine levels do not, however, support the idea of

hydroxy-amino acids being involved in linkage of protein in R24.

Whether or not a covalent bonding is involved, the 24% KOH resistant fraction of the extensin and polysaccharide is closely associated with the cellulose microfibrils and therefore very relevant to any consideration of the interaction of microfibrils and matrix. In work aimed at providing information necessary for the construction of a cell wall model related to what is known of polysaccharide composition, microfibril orientation, metabolic control of elongation etc., one would do well to closely examine this fraction of the cell wall.

It is clear from the above results that in the present state of knowledge, any cell wall model, although useful, must be regarded as transient.

MATERIALS AND METHODS

Growth, preparation, and extraction of tissue has been dealt with in earlier sections of the thesis as well as in some detail in the text of this section.

Amino Acid Analyses. 10 mg of total sample was hydrolysed with 6 N.HCl in an evacuated tube for 16 hours. They were subjected to analysis on a Beckmann Model 120C automatic amino acid analyser.

VII. KINETICS OF EXTRACTION OF HYDROXYPROLINE AND SUGARS FROM DEPECTINATED LUPIN HYPOCOTYL CELL WALLS WITH 10% KOH AT 0°C AND 18-22°C

INTRODUCTION: Extraction of hemicellulose from cell walls with alkali has generally employed successive treatments with dilute and concentrated alkali at room temperature. These methods have been developed as a result of work on plant tissue with a high degree of secondary wall deposition. It has become apparent however that such methods are not altogether suitable for the study of primary cell walls in which the polymers appear to be less tightly knit.

A major difference between primary and secondary walls which drastically affects the extraction of their polymers is the presence of lignin in the secondary walls.

Another major difference has now become clear, namely the presence of a hydroxyproline-rich protein in the primary wall (Lamport, 1965). This protein is thought to be involved in the control of cell elongation and has therefore been termed extensin. Because over about 95% of the cell hydroxyproline is found in the cell wall this amino acid may be used to indicate the presence and approximate amount of extensin.

Extensin fragments have been found to contain a large proportion of the primary wall arabinose as hydroxyproline linked $(arabinose)_{1-4}$ oligosaccharides (Lamport, 1970) and more recently have been shown to contain galactosylserine (Lamport, Katona and Roerig, 1973). Current thinking is that the galactosylserine is the point of attachment of extensin to

the remaining matrix polysaccharides, themselves covalently interlinked, and the whole extensin-polysaccharide complex non-covalently bound to the network of cellulose microfibrils. Keegstra et al. (1973) have proposed a cell wall model to this effect based on work with sycamore suspension cultures.

Results from this laboratory are not in complete agreement with the above model in that it has been found that the bulk of the hemicellulose can be removed at 0°C with 10% KOH. Under these conditions the galactosylserine bond is not cleaved and only a small portion of the wall hydroxyproline is extracted. 10% KOH at room temperature appears to effectively rupture the galactosylserine bond with β-elimination of serine (Lamport, Katona and Roerig, 1973; Chapter VI) and remove the remaining 10% KOH soluble hemicellulose (Chapter IV).

In previously published fractionations using 10% KOH no distinction has been made between the 0°C and room temperature soluble cell wall fractions. Insofar as these two fractions may be a reflection of involvement or non-involvement of hemicellulose in the extensin polysaccharide complex the separation is relevant to the study of primary wall structure. Whether or not there is any marked difference in the polysaccharides extracted at each temperature is not yet known and is a question which has important implications that are discussed below.

The present experiment has been aimed at defining more clearly the separation of the 0°C 10% KOH and 18-22°C 10% KOH soluble wall fractions by sequential extractions at the two temperatures.

RESULTS AND DISCUSSION

Total Fraction Extracted as a Function of Time

The time course of extraction of 0°C soluble hemicellulose (HO) and room temperature extracted hemicellulose (HRT) with 10% KOH from lupin hypocotyl was first established. This was obtained by extracting a batch of depectinated (neutral detergent 4 hours, 100°C) cell walls with 10% KOH sequentially for 1, 1, 2, 4, and 4 hours at 0°C and then for 1, 1, 2, 4, 4, 12, and 24 hours at 18-22°C. Thus the walls were filtered 1, 2, 4, 8, and 12 hours after the start of extraction at 0°C and 1, 2, 4, 8, 12, 24 and 48 hours after the temperature of extraction had been raised to 18-22°C (Table XXV). The extracts were neutralized, dialysed and freeze dried.

The total hemicellulose extracted up to each time, obtained from the sum of the weights of hemicellulose extracts up to that time is shown in Fig.6 and Table XXV column a. There is clearly a rapid extraction at 0°C which soon levels off. When the temperature is raised to 18-22°C there is a rapid increase in extraction again levelling off fairly quickly. In agreement with earlier results (Monro, Bailey and Penny, 1974) HO represents about two thirds of the total hemicellulose usually extracted over 24 hours at 18-22°C.

In the previous section it was shown that there was no loss of serine or any other amino acid with 0°C treatment of lupin hypocotyl. It therefore seems that HO is being released due to the action of alkali on some bond other than the O-glycosidic linkage between a sugar and a hydroxyamino acid, or else it is not linked to the alkali

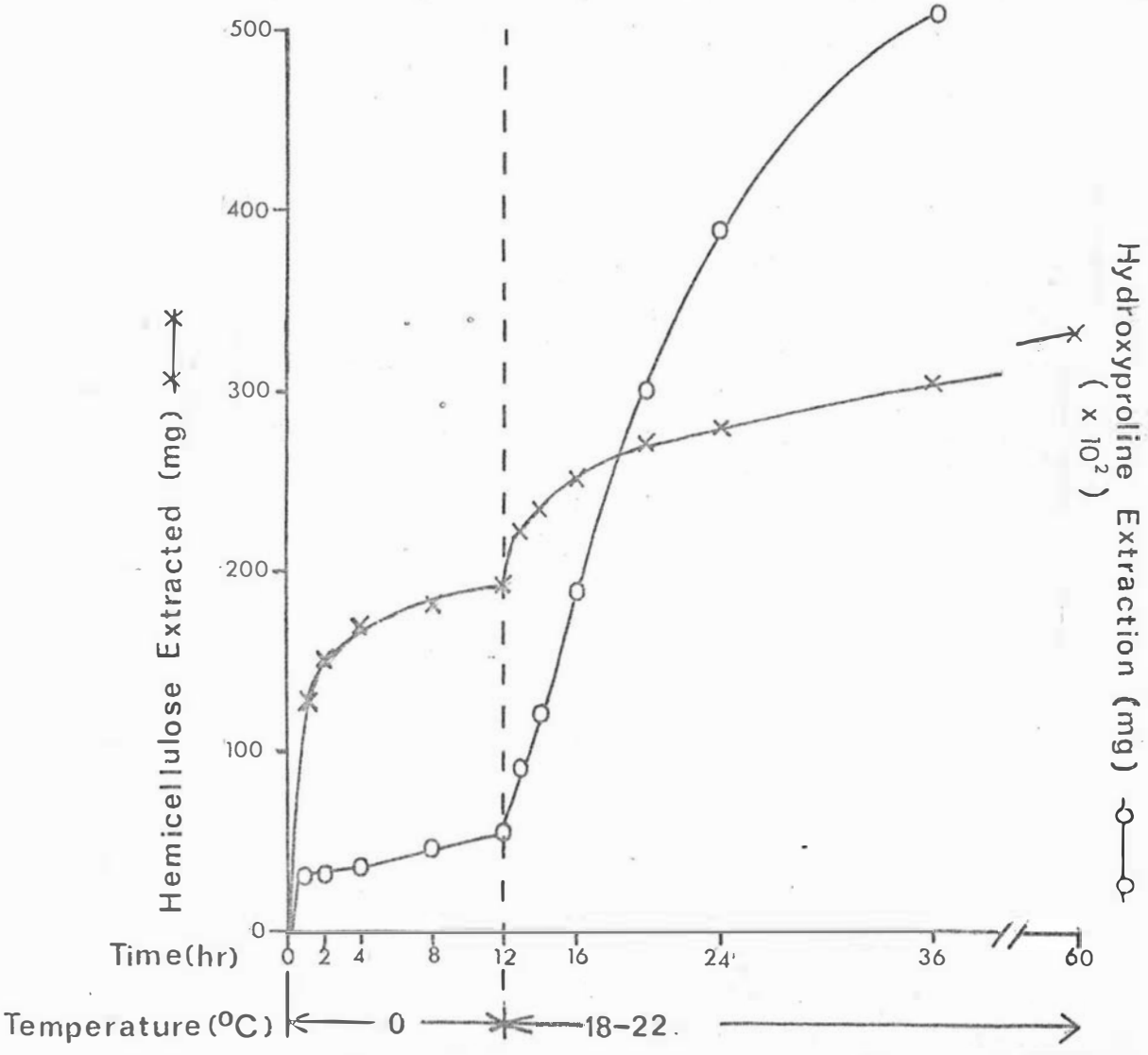
TABLE XXV. REMOVAL OF HEMICELLULOSE AND HYDROXYPROLINE FROM DEPECTINATED LUPIN HYPOCOTYL CELL WALL BY SEQUENTIAL EXTRACTIONS WITH 10% KOH AT 0°C AND 18-22°C

Temperature	Number of Stage in Sequence	Total Time in Extraction Sequence (hr)	Duration of Stage in Sequence (hr)	Hemicellulose Extracted* (mg)			Hydroxyproline Extracted* (mg)		
				Total up to time given a	Per Stage b	Rate of Extraction (mg/hr) c	Total up to time given d	Per Stage e	Rate of Extraction (mg/hr) f
0°C	1	1	1	128.9	128.9	128.9	0.280	0.280	0.28
	2	2	1	152.8	23.9	23.9	0.310	0.030	0.30
	3	4	2	167.8	15.0	7.5	0.352	0.044	0.022
	4	8	4	181.8	13.3	3.3	0.440	0.088	0.022
	5	12	4	193.9	12.8	3.2	0.570	0.130	0.033
18-22°C	6	13	1	221.1	27.2	27.2	0.88	0.31	0.31
	7	14	1	235.2	14.1	14.1	1.20	0.32	0.32
	8	16	2	250.2	15.0	7.5	1.88	0.68	0.34
	9	20	4	268.5	18.3	4.6	3.00	1.12	0.28
	10	24	4	281.6	13.1	3.3	3.88	0.88	0.22
	11	36	12	306.1	24.5	2.0	5.13	1.25	0.104
	12	60	24	332.8	26.7	1.1	-	-	-

* All figures are per g of neutral detergent depectinated starting cell wall.

FIG 6.

EXTRACTION OF TOTAL HEMICELLULOSE AND HYDROXYPROLINE FROM LUPIN HYPOCOTYL CELL WALLS BY 10% KOH AT 0° AND AT 18-20°C AS A FUNCTION OF TIME



insoluble wall.

Rate of Extraction of Hemicellulose and Hydroxyproline at 0°C and 18-22°C with 10% KOH

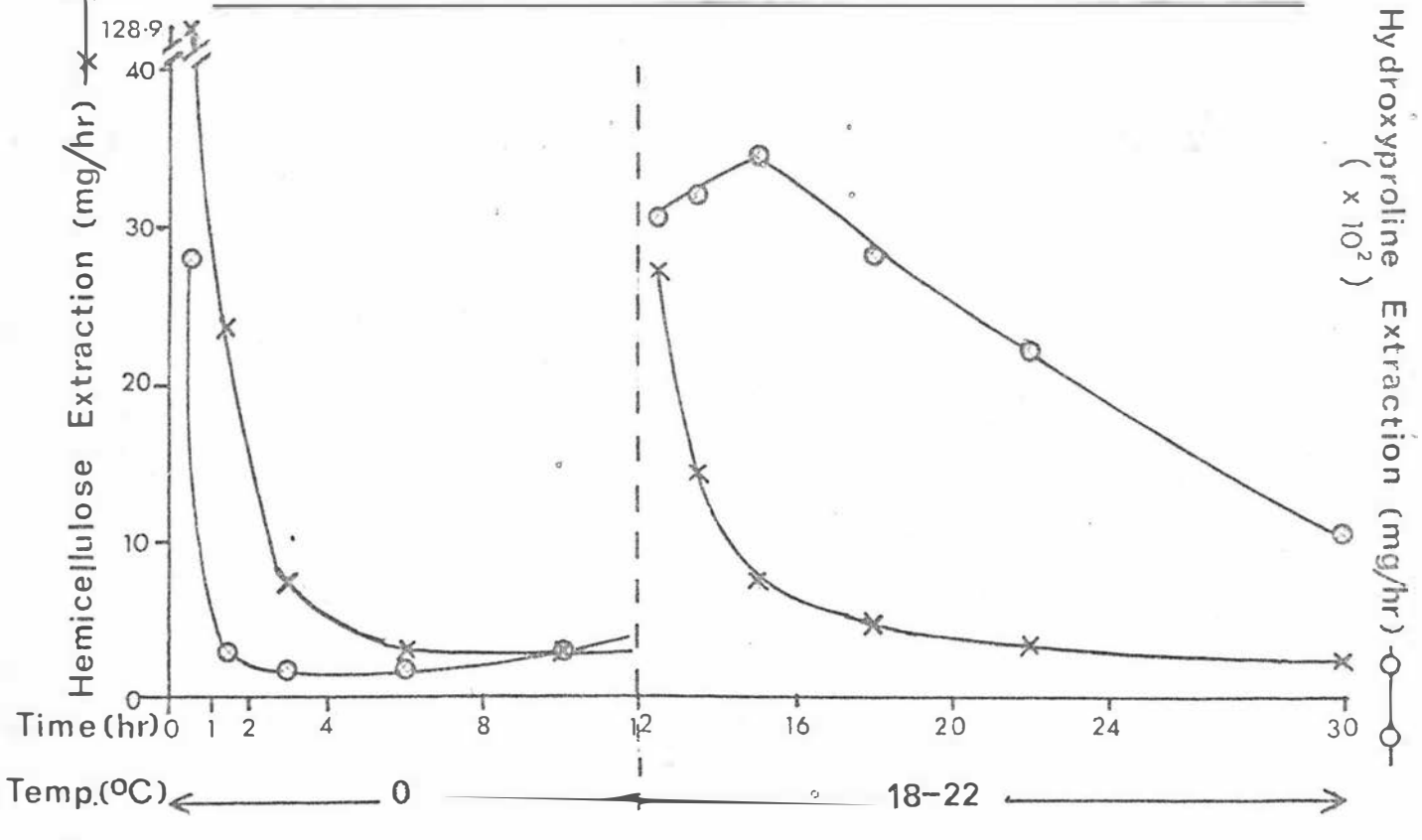
When the result of each extraction of hemicellulose shown in Table XXV column b is expressed as mg. hemicellulose released per hour the figures in Table XXV column c are obtained. Results from hydroxyproline analyses of acid hydrolysates of the dialysed freeze dried extracts from each step in the extraction sequence are shown in Table XXV column e. Expressed on a mg hydroxyproline extracted per hour basis they give the figures which are presented in Table XXV column f and graphically in Fig.7. Total hydroxyproline extracted up to each filtering time is shown in column d.

HO has clearly a very high rate of extraction compared to HRT. Over the first hour at each temperature 4.7 times as much hemicellulose is extracted at 0°C than at 18-22°C, although over 12 hours at each temperature the HO:HRT ratio is only 1:0.45. Expressed otherwise, of the HO extracted over 12 hours 66.5% is removed during the first hour, whereas for HRT the figure is 40.2%. Whatever reaction is involved in the release of HO at 0°C would seem to have a higher rate constant than that involved in the release of HRT at 18-22°C.

The HO contained only 7% of the hydroxyproline present in the starting depectinated cell wall while the HRT contained 56.5% of the starting hydroxyproline and 19% remained in the residue after 24 hours in 10% KOH at 18-22°C. Some 82% of the hydroxyproline is therefore accounted for. This is somewhat higher than in earlier experiments probably because

FIG 7

RATE OF EXTRACTION OF HEMICELLULOSE AND HYDROXYPROLINE FROM LUPIN HYPOCOTYL CELL WALLS BY 10% KOH AT 0°C AND 18–22°C

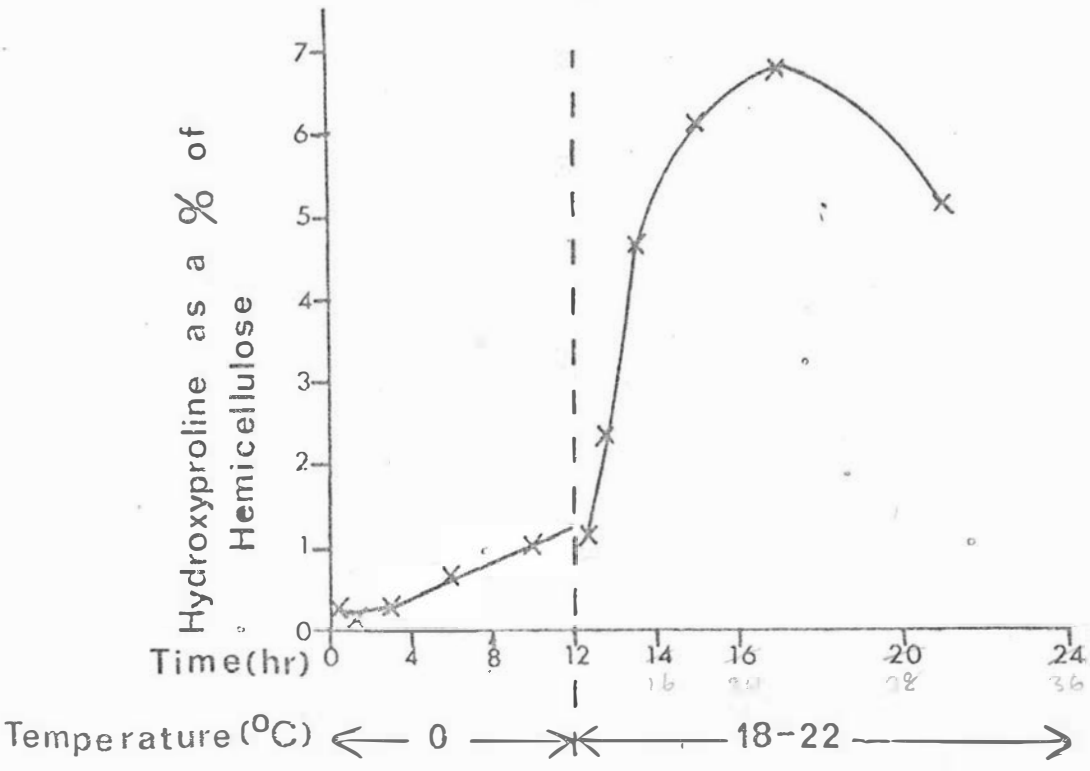


the hydroxyproline containing protein once extracted was neutralized within a few hours in most steps in the sequence of extractions, and therefore was not subjected to continuing attack by alkali. In previous experiments the degradation would have continued for the duration of the total extraction.

The data in Fig. 7 shows that although the initial rates of extraction of hydroxyproline at 0°C and 18-22°C are similar there is a sharp decline at 0°C, so that the average rate of extraction is much higher at 18-22°C. Of the HO hydroxyproline 50% is extracted during the first hour of the 12 hours in 10% KOH at 0°C. This can be explained in terms of protein-polysaccharide crosslinks when one considers the kinetics of hemicellulose extraction compared with hydroxyproline extraction at the two temperatures. At 0°C both curves are approximately hyperbolic and asymptotic but at 18-22°C only the hemicellulose extraction curve shows these characteristics while the removal of hydroxyproline decreases almost linearly after the first few hours. Thus at 0°C the amounts of hydroxyproline and polysaccharide extracted are proportional to one another. It is likely that the removal of much of the hydroxyproline from the walls in the first hour is due to removal of the bulk of the HO in this time. If there is even only a small degree of linkage between extensin and the HO polysaccharide then a large extraction of this polysaccharide will be accompanied by a proportionally large extraction of hydroxyproline. This is borne out by the data presented graphically in Fig. 8 where it can be seen that at least over the first few hours hydroxyproline does not make up

FIG 8

HYDROXYPROLINE AS A PERCENTAGE OF HEMICELLULOSE FROM EACH STAGE IN EXTRACTION SEQUENCE OF TABLE 1.



more than 0.3% of the HO and does not change appreciably as a percentage of the fraction. The initially extracted HO hydroxyproline could indicate the presence of a portion of extensin which is incompletely bound into the extensin polysaccharide complex. Pulse labelling with C^{14}-proline may give some indication of whether or not this represents a precursor of the HRT fraction.

Between 2 and 12 hours at $0^{o}C$ hydroxyproline increases linearly as a percentage of HO extracted at each stage, from about 0.3 to 1.0%.

When the temperature is raised to 18-$22^{o}C$ the rates of hemicellulose and hydroxyproline extraction jump to about 8 times the rates of extraction of the preceding hour at $0^{o}C$. The rate of HRT extraction then drops rapidly while that for hydroxyproline is more sustained. The effect of this on the composition of HRT can be seen in Fig.8 where the hydroxyproline accounts for as much as 7% of the HRT extracted between 8 and 12 hours at 18-$22^{o}C$. Assuming that hydroxyproline comprises about 30% of the amino acids present in this fraction the HRT extracted at this stage is over 20% protein. However the grounds for making this assumption are based on figures for total HRT from one 24 hour extraction (Chapter VI) where there may have been differential dialysis of amino acids after the prolonged exposure to alkali.

Fractionation of HO and HRT

The HO from steps 1 and 4 and HRT from steps 6 and 11 in the extraction sequence were fractionated into linear and branched species by precipitation of the linear molecules from

$CaCl_2$ solution by the addition of I_2:KI solution (Gaillard and Bailey, 1966). The small amount of Hemicellulose-A present in lupin hypocotyl (Chapter V) will have contributed to the arabinoxylan-B fraction. The blue precipitate was collected and both precipitate (arabinoxylan-B) and supernatant (heteroglycan-B) neutralized with sodium thiosulphate prior to dialysing for 24 hours. The fractions were recovered by freeze-drying the dialysed solutions. The results of the fractionation are shown in Table XXVI.

The heteroglycan-B is the minor component of all the total hemicelluloses fractionated, in agreement with earlier results. Because of this it is unlikely that the earlier suggestion that heteroglycan-B could represent degraded extensin complex is correct, at least in the case of the HO derived fractions.

Although the 24-36 hour extracted HRT is only about 10% xylose (see Fig.9) 75% of it is precipitated by iodine solution including the hydroxyproline-rich protein, as earlier results indicate (Monro, Bailey and Penny, 1974). This implies linkage of most of the components of HRT at this time to xylan or other 1 — 4 linked linear polymers. These may have a low degree of polymerization but because of interpolymer aggregation in the $CaCl_2$ solution react positively with iodine (Gaillard and Bailey, 1966).

It is interesting that the hemicellulose from stages 4 and 6 in the extraction sequence show similar dialysis losses and contain similar amounts of hydroxyproline (Fig.8). Hemicelluloses from stages 1 and 11 and probably also from stages 4 and 6 contain similar amounts of the various monosaccharides (Fig.9). It would seem that at $0^{\circ}C$ a small

TABLE XXVI. FRACTIONATION OF HEMICELLULOSE EXTRACTED FROM LUPIN HYPOCOTYL CELL WALL WITH 10% KOH AT 0°C AND 18-22°C

Parent Hemicellulose			Derived Fractions		
Stage in Extraction Sequence (Table XXV)	Extraction Temperature (°C)	Weight Taken (mg)	Arabinoxylan-B (linear (1-4) linked polysaccharide)	Heteroglycan-B	% recovery
1	0	100	94.6	7.5	102
4	0	100	65	18.2	83
6	18-22	100	71.0	13.0	84
11	18-22	100	74.6	19.2	94

amount of material typical of HRT as well as 0°C 10% KOH soluble xylan is being removed due to some cleavage of the 18-22°C 10% KOH labile bond.

Monosaccharide Composition of Hemicellulose Removed at Each Stage in Extraction Sequences at 0°C and 18-22°C

The monosaccharide composition of extracts is shown in the histograms of Fig. 9. The figures are derived from the G.L.C. of the alditol acetates of monosaccharides released from the hemicellulose by acid hydrolysis. They are consistent with earlier results (Monro, Bailey and Penny, 1974; Chapters IV and V) which showed a higher proportion of arabinose and galactose in the hemicellulose extracted at room temperature than in that removed in a prior 0°C 10% KOH treatment.

A large unidentified peak occurred between arabinose and xylose in the HRT chromatograms and has not been included in the data of Fig. 9. It can be seen on the gas chromatograph of stage 7 shown in Fig.10.

The monosaccharide composition of HO depends on the time in the extraction sequence at which it is removed from the wall. During the first hour when the bulk of HO is extracted, xylose and glucose in the ratio of 2:1 account for over 60% of the extracted polysaccharide. Thereafter xylose decreases while arabinose and galactose increase until between 8 and 12 hours xylose, arabinose, galactose and glucose make up about 90% of the HO in approximately equal proportions. It appears that at least two different components are being extracted, that dominating in the later phase having a high

FIG 9

MONOSACCHARIDE COMPOSITION OF HEMICELLULOSE FROM SEQUENTIAL EXTRACTIONS OF LUPIN HYPOCOTYL CELL WALLS WITH 10% KOH AT 0°C AND 18–22°C AS INDICATED IN TABLE 1

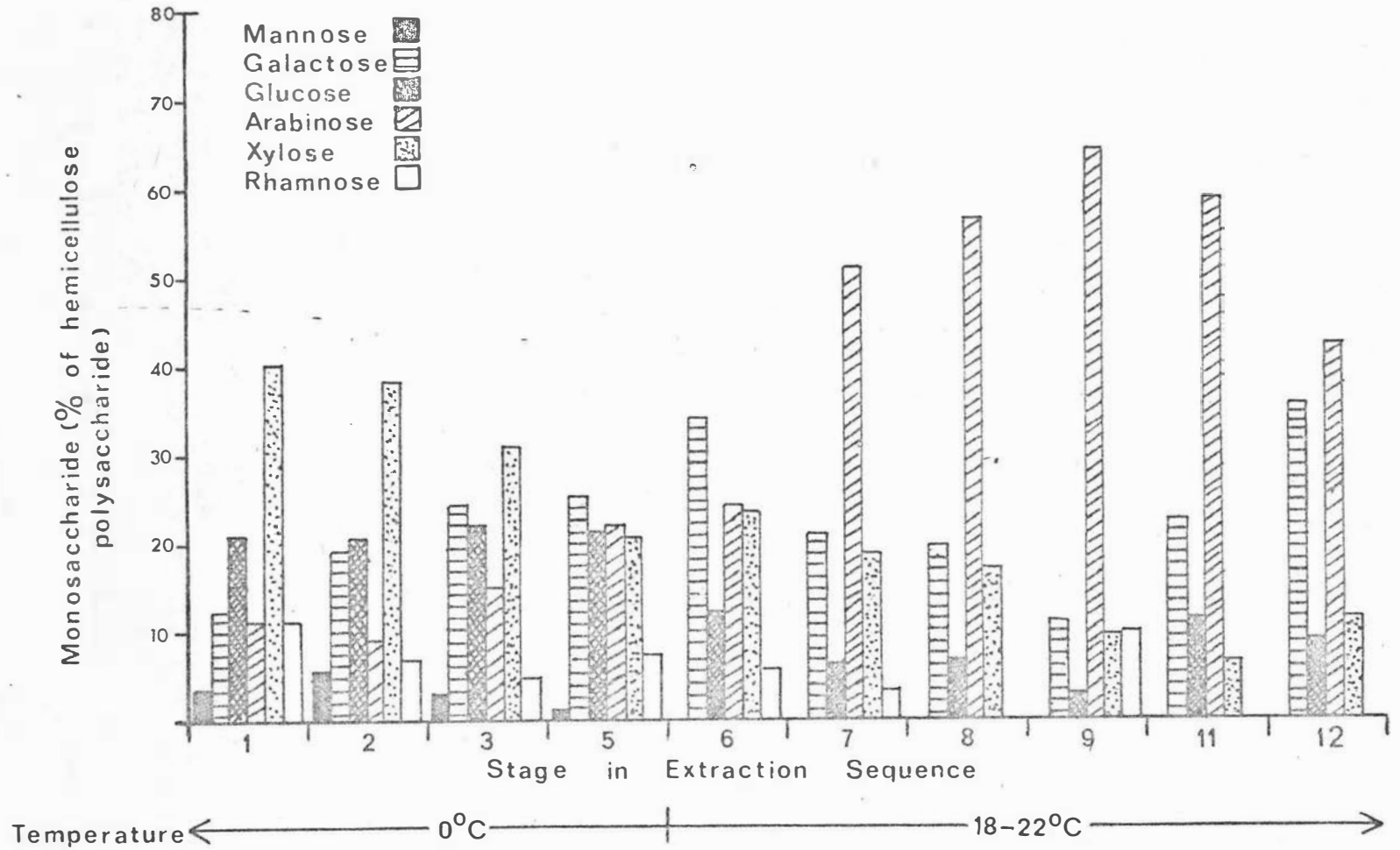
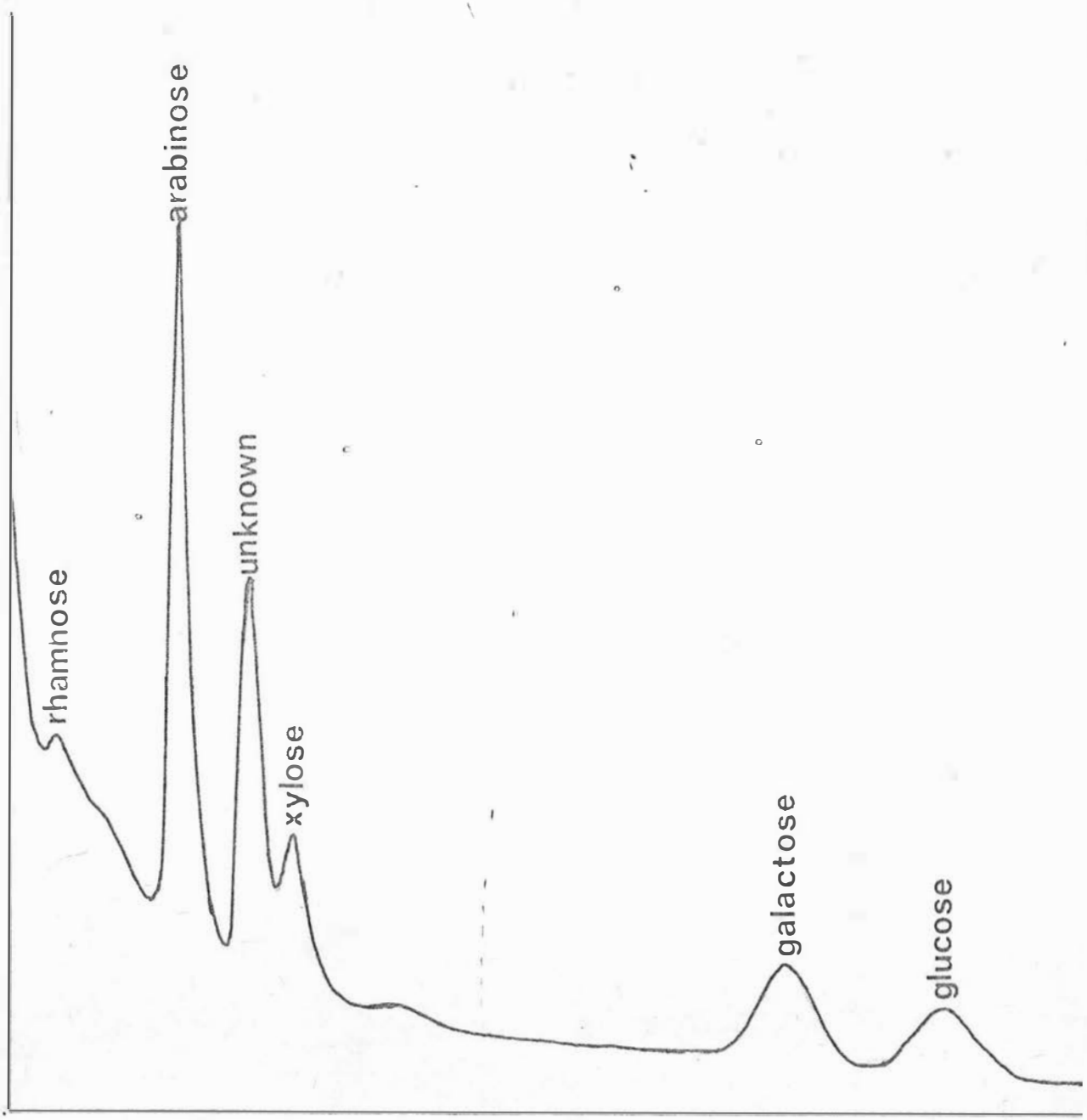


FIG 10

GAS-LIQUID CHROMATOGRAM SHOWING MONOSACCHARIDE COMPOSITION OF HRT FROM STEP 7 OF EXTRACTION SEQUENCE



arabinose-galactose content.

The increase in arabinose and galactose paralleled by the increase in hydroxyproline probably indicates fragmentation of the extensin-polysaccharide complex. Extensin fragments have been shown to bear arabinose oligosaccharide side chains (Lamport,1967) and in many cases galactose (Lamport, 1969). This and the similar dialysis losses during fractionation of steps 4 and 6 of the extraction sequence suggest that some removal of the extensin complex is occurring.

A small and decreasing amount of mannose and rhamnose is extracted. Mannose is typical of the room temperature 24% KOH-4% H_3BO_3 soluble wall fraction (Chapter I). It is interesting that there should be cold (0^oC) 10% KOH extractable and 24% KOH extractable mannose and none removed by an intermediate 18-22^oC 10% KOH. It is apparent that the action of alkali on cell walls has some specificity and that there is a considerable difference between the wall bonds labile to cold 10% and room temperature 24% alkali. If the HO mannose is a precursor to the 24% KOH soluble mannose, it does not seem to pass through a fraction of intermediate solubility. This would suggest that at least some of the HO and HRT polysaccharides are metabolically independent of one another once incorporated into the wall.

The monosaccharide composition of HRT shows several significant changes with time of room temperature extraction which is between 12 and 60 hours at 18-22^oC. They are (1) a decrease in the level of xylose from 23% in the thirteenth hour (stage 6) to 7% between 24 and 36 hours (Stage 11);

(2) a drop in galactose to about one third of its initial value by 16-20 hours (stage 9) followed by an increase to its original value between 36 and 60 hours (stage 12); (3) a threefold increase in the HRT arabinose extracted between 12-13 hours (stage 6) and that extracted between 16-20 hours (stage 9) followed by a decrease.

The arabinose level is much higher in HRT than in the bulk of the HO, in agreement with earlier results. Reference to Fig.8 shows that the increase in arabinose is paralleled by an increase in hydroxyproline as a percentage of HRT from 1 to 6.7%. Being expressed on a % basis these figures do not represent absolute amounts. However, when the rate of arabinose extraction is plotted in mg/hr for the 18-22°C portion of the extraction sequence (Fig.11) a curve similar to that for hydroxyproline extraction (Fig.7) is obtained. It is rather more hyperbolic than that for rate of hydroxyproline extraction probably because not all of the arabinose is present as hydroxyproline linked arabinose side chains.

From the data of Fig. 7 and Fig. 9 it is clear that hydroxyproline and arabinose are extracted independently of most of the other HRT sugars. Furthermore the shape of the curve for rate of extraction of arabinose against time (Fig. 11) is more similar to that of hydroxyproline extraction than to that of HRT extraction. While arabinose shows a percentage increase in parallel with that of hydroxyproline the galactose decreases until it is at its lowest when hydroxyproline and arabinose have reached peak extraction rate (Fig. 9.). Arabinose oligosaccharides have been shown to be in alkali stable linkage with hydroxyproline (Lamport, 1967).

In higher plants most of the hydroxyproline appears to be glycosylated, and the arabinose oligosaccharides 4 units in length. The present data indicates that at the stage of the extraction sequence where the HRT is 6.2% hydroxyproline and 6.4% arabinose the molar ratio of hydroxyproline to arabinose is 1:8.7. About half of this arabinose is therefore not present as side chains of extensin.

It is clear from the above results and those presented previously that different alkali labile bonds are involved in the binding of HO and HRT respectively to the 10% KOH insoluble wall. No change in amino acid occurs in walls treated for 10 hours at 0°C with 10% KOH (Chapter VI). The release of HO is therefore not due to β-elimination of galactosylserine but due to cleavage of some more alkali labile bond. If the HO polysaccharide is involved in the extensin-polysaccharide complex some of the wall xylan or xyloglucan must be linked in a different way to that suggested by Keegstra et al. (1973). The two step release of xylose at 0°C and 18-22°C suggests two types of bonding or two types of polymer. In their model of sycamore suspension culture cell walls all the xylose is present in xyloglucan and is similarly linked to the pectic and cellulose fractions.

Most of the hypocotyl wall pectin is extracted by the 4 hour neutral detergent treatment. In this respect the results are at variance with those of Keegstra et al. who include nearly all the polyuronide and other wall matrix polysaccharides in their model of the extensin polysaccharide complex of sycamore suspension culture cell walls. However this may simply be a reflection on the loss

of unbound pectin to the suspension culture medium and of the absence, perhaps due to pectin solubility, of the pectin-rich middle lamella typical of the cell walls of intact higher plant tissue. In other words the extensin-polysaccharide complex basically as proposed by Keegstra et al. may be present in intact tissue cell walls but in addition to other polysaccharide which is unbound and lost from suspension culture cell walls. The cold alkali may be responsible for breaking ester linkages both in the acidic amino acids of extensin (Lamport 1970) as well as in the polyuronides. Deesterification of pectin is likely to cause a marked lowering of hydrogen bonding capacity between polyuronide chains if some remain after the detergent extraction. Polyuronides have not been measured in fractions in the present experiment. It seems that they may be important to consider in future analyses. Dilute base has also been shown to partially extract xyloglucan non-covalently bound to cellulose (Bauer et al., 1973; Aspinall et al.,1969). Thus the extraction of HO may be due to disruption of covalent and non-covalent bonds.

From extraction step 6 onwards there is a lack of coincidence between release of arabinose and galactose suggesting that a bond between the major galactose and arabinose portions of HRT is cleaved by 10% KOH at room temperature. In this regard it is relevant that between extraction steps 2 and 5 at $0^{o}C$ there is a parallel increase in arabinose and galactose. This suggests the presence of a $0^{o}C$ 10% KOH stable linkage between the two, and that a bond between arabinose - galactose containing polymers and the $0^{o}C$ 10% KOH insoluble wall is being cleaved.

In the previous chapter it was seen that significant β-elimination of serine does not occur after 10 hours in 10% KOH at 0°C. It is unlikely that linkage to arabinogalactan side chains would be sufficient to render the extensin inextractable unless it was otherwise bonded in some way to the remainder of the cell wall. A further linkage is not indicated in the model of Keegstra et al. Unfortunately a timecourse of β-elimination of serine has not been determined in the present work. However Lamport et al. (1973) have examined β-elimination of galactosylserine under a variety of conditions. They found that 4.4 M.NaOH for 10 hours at 23°C could cause β-elimination of just under 50% of the serine residues of tomato suspension cultures whereas N_2H_4 for 10 hours at 105°C caused about 75% destruction of serine. Assuming the same lability of galactosylserine in lupin hypocotyl cell walls it seems that release of galactose from the walls approximately parallels β-elimination of serine.

The β-elimination reaction when carried out under the conditions employed here results in peptide chain cleavage. Assuming that the serine linked galactan is bound to the insoluble portion of the wall, and hydroxyproline to arabinose oligosaccharides, release of segments of peptide chain will occur when adjacent galactosylserine units are β-eliminated. Therefore there will be a lack of coincidence in galactose and arabinose-hydroxyproline extraction as is observed here, with galactose being freed before most of the arabinose.

Earlier work in this laboratory has shown that the iodine precipitable hemicellulose-B can be divided into $CaCl_2$ soluble and $CaCl_2$ insoluble fractions. The former has an amino acid

composition similar to that for the whole wall and therefore probably contains segments of extensin which contain non-β-eliminated galactose-linked serine. Hence it appears that elimination of adjacent galactosylserines is probably not absolutely necessary for the release of extensin segments. The serine linked galactose present in the iodine precipitable fraction may be further bonded to xylan or xyloglucan and if not may represent portions of polysaccharide which was linked according to the model of Keegstra et al. and which has become separated due to action of alkali on bridging molecules. Further chromatographic analysis of these fractions is important to determine how much of the iodine precipitated hydroxyproline containing polymer is covalently linked to xylose-rich polymer.

During the stage of HRT extraction when most of the hydroxyproline is removed the glucose-xylose ratio remains approximately constant at about 1:2.5. This is somewhat different to the 3:4 (1:1.3) ratio for the xyloglucan of sycamore suspension culture cell walls claimed by Bauer et al. (1973). In the model of Keegstra et al. (1973) based partly on the results of Bauer et al. it is proposed that all of the wall glucan is present as xyloglucan, which constitutes the hemicellulose of the wall. Wilder and Albersheim (1972) report the same structure for the hemicellulose of Phaseolus vulgaris suspension culture walls. The results here suggest that in lupin hypocotyl there is either a larger number of xylose side chains on the glucan than in sycamore suspension cultures, or more likely, that some xylan is present in the HRT.

The increasing proportion of galactose in stages 11 and 12 of the extraction sequence (when most of the wall protein has been removed) suggests that there is some galactan released by cleavage of other than galactosylserine bonds. Such a bond is possibly responsible for the bonding of some of the serine-linked galactan to the remainder of the cell wall.

The parallel release of xylose and galactose in HRT does suggest that these two are liberated by cleavage of the same bond or bonds which differ from those involved in release of HO. If this cleavage occurred in the polyuronide section of the extensin-polysaccharide complex of Keegstra et al. the galactose would be liberated instead of xylose unless the xylose was also dissociated from the cellulose microfibrils. It does in fact appear that the xylose is fairly readily separated from its hydrogen bonding with the microfibrils (Bauer et al., 1973; Aspinall et al., 1969). Keegstra et al. suggest that hydrogen bonding is the only link between the xylose and the microfibrils. In this case it is interesting that such solvents as guanidinium thiocyanate will not remove extensin from the wall. Also, if hydrogen bonding is important and is disrupted by 10% KOH one might expect a more rapid release of extensin instead of separate extraction of the bulk of the HRT polysaccharide and hydroxyproline. It seems that even though the polysaccharide of the extensin complex as envisaged by Keegstra et al. may separate from the microfibrils further bonds must be broken before it can be released from the wall. The pattern of monosaccharide extraction suggests that this further cleavage must be between the galactan and hydroxyprolylarabinose, and therefore probably

at the site of β-elimination of serine, which breaks the peptide chain.

The results indicate that the β-elimination must occur either to reduce the size of the complex so that it becomes sterically feasible to extract it or so that some highly resistant bond between extensin and the microfibrils is cleaved. In relation to this question the earlier results of Lamport (1969) are of interest. Where pure polysaccharidases were used in an attempt to extract extensin from suspension culture cell walls there was little success. This indicates that disruption of the polysaccharides is not sufficient to render extensin extractable. However, where some protease was present considerable extraction was achieved. Wall fractions isolated from sycamore suspension cultures by the action of endopolygalacturonase contained little protein although this was readily removed from the walls with attached polysaccharide when the walls were treated with pronase (Talmadge et al., 1973; Bauer et al., 1973; Keegstra et al., 1973). It therefore seems that cleavage of the peptide chain is a prerequisite to removal of extensin. The results here suggest that it is not until β-elimination has caused breaks in the peptide chain that the protein is extractable, although it has not been shown that cleavage of other alkali labile bonds is not also required. Other bonds will probably have been broken by the relatively non-specific action of aqueous alkali on wall polymers, which makes it difficult to ascribe polysaccharide release to cleavage of any particular bond. However, the fact that differences in extraction rate do occur indicates the presence of bonds differing in susceptibility to alkali.

In the previous chapter and in the work of Lamport (1965), Olson (1964) Dougall and Shimbayashi (1960) an alkali resistant fraction of the wall protein was found. If this represents portions of many of the peptide chains of the extensin-polysaccharide complex then proteolysis will be necessary to extract the extensin by non-cellulolytic means. This very important point of attachment to the microfibrils has not been included in either the model of Lamport (1970) or that of Keegstra et al. (1973) as being distinct from the bonding via serine-linked galactose, although it does appear to be very significant. It is interesting that it survives extraction even where the serine is reduced to very low levels as a percentage of the 24% KOH insoluble wall protein, as results of the previous section showed. Galactosylserine therefore does not appear to be important to association of the alkali resistant protein and the microfibrils. Whether this association of protein and wall cellulose is non-covalent due to the trapping of protein amongst alkali insoluble polysaccharides or involves a protein-carbohydrate bond is a question worthy of consideration.

One way in which further information on the differences between the HO and HRT fractions can probably be obtained is from a study of the metabolic relationship of the two fractions. It is of interest to find out whether or not HRT polysaccharide is derived from the HO fraction due to the formation of $0^{o}C$ 10% KOH resistant cross-links within the wall as the work of Brysk and Chrispeels (1972) suggests. Pulse labelling experiments should show whether there is a precursor-product relationship between the two fractions.

Although the construction of cell wall models has involved a limited number of bondings and relationships of wall polymers the true situation appears to be very complex and probably involves a far larger number of combinations of wall polymers than has been used in the wall models so far proposed.

MATERIALS AND METHODS

Plant Tissue

Seeds of bitter blue lupin (*Lupinus angustifolius*) were germinated and grown under previously described conditions (Penny, 1969). Hypocotyls about 5 cm in length were harvested and deep frozen for no longer than two days. They were ground for 30 seconds in a Wareing blender in distilled water, filtered over sintered glass and immediately extracted under reflux for a total of 4 hours in two changes of the neutral detergent of Van Soest (1963). The walls were filtered, washed twice by resuspending in hot distilled water for 5 minutes and filtering, and finally washed with ethanol and acetone and dried at 40°C overnight.

Extractions

Tissue (5.52 g) was subjected to a series of extractions by stirring with 200 ml volumes of 10% KOH at 0°C in a flask thoroughly flushed with nitrogen. The temperature was maintained at 0°C by standing the flask in a container of ice on a magnetic stirrer in a cold room at 4°C. At the end of each extraction the walls were filtered quickly on a porosity 1 sintered glass funnel and then immediately submitted to the next extraction in the sequence.

The times of filtering to 0°C extractions were 1, 2, 4, 8 and 12 hours from the start of the experiment. After the final 0°C extraction the walls were neutralized with dilute acetic acid, washed with distilled water, ethanol and acetone and dried overnight before weighing.

The room temperature (18-22°C) extractions were carried

out as for the 0°C 10% KOH extractions but in the laboratory, and were filtered 1, 2, 4, 8, 12, 24 and 48 hours after the start of the room temperature extraction.

All extracts were neutralized with acetic acid immediately after filtering, refiltered through a porosity 4 sintered glass funnel and dialysed for 24 hours against running tap water. At the end of this time they were concentrated on a rotary evaporator at 40°C to about 80 ml and then freeze dried and weighed.

Polysaccharide fractionation

Freeze dried hemicellulose extracts (50 or 100 mg) were dissolved in $CaCl_2$ solution SpG 1.3 (15 or 30 ml) clarified by spinning at 70,000 g for 15 minutes, and iodine solution (I_2 3%, KI 4% w/v; 2.5 or 5.0 ml) added. After standing for an hour the solutions were centrifuged and the heavy blue precipitate and supernatant separated by decantation and neutralized with $Na_2S_2O_3$ solution. The solutions were dialysed 24 hours, and freeze dried.

Hydroxyproline analyses

Wall residues and freeze dried cell wall extracts (20 mg) were hydrolysed under gentle reflux with 6N·HCl (8 ml) for 18 hours. The hydrolysates were analysed for hydroxyproline by the method of Switzer and Summer.

Carbohydrate analyses

(1) Hydrolysis of fractions. Wall residues and freeze dried cell wall extracts (20 mg) were placed in 50 ml boiling tubes. 72% H_2SO_4 (0.7 ml) was added and after 3 hours distilled water (20 ml). Each tube was then covered with a watchglass and placed in a water bath at 100°C for 3 hours. The

hydrolysates were neutralized with $BaCO_3$, filtered and freeze dried.

(2) Preparation of alditol acetates for gas-liquid chromatography. The freeze dried hydrolysates were dissolved in 2 ml distilled water and 3 ml $NaBH_4$ (0.3M) added and allowed to react for 3 hours in a cold room (0-4°C). The reaction was stopped by the addition of acetic acid and the solution passed through a column of amberlite resin IR-120 (H+) and eluted with 5 bed volumes of distilled water. The solutions were evaporated to dryness in small round-bottomed flasks and taken up in dry methanol, which was evaporated to dryness in reaction vials on a hot plate at 135°C under a stream of air. When the samples were completely dry the vials were cooled and pyridine:acetic anhydride 1:1 (0.4 ml) was added. The vials were tightly stoppered and placed in an oven at 110°C for 1 hour with periodical shaking. They were then cooled and chloroform (0.4 ml) added.

(3) Separation of Alditol Acetates. The alditol acetates were separated with a Varian Aerograph series 1400 gas chromatograph using a 2 m stainless steel column of outside diameter 1/8th inch. The gas chromatographic packing was 3% ECNSS-M by weight on GAS-CHROM Q, 100/120 mesh (Applied Science Laboratories, Inc., P.O.Box 440, State College, P.A.). Gas flow rates were N_2, 20 ml/min; H_2, 20 ml/min; air, 300 ml/min. 1 μl of sample was injected. Temperatures used were column, 165°C (isothermal); injection, 210°C; and detector 220°C.

CONCLUSION

The data presented has resulted from an investigation of the composition of primary cell walls of lupin hypocotyl in relation to hypocotyl elongation. Because of the predominantly carbohydrate nature of the wall the initial approach was to study the monosaccharide composition of fractions isolated therefrom by procedures used in this laboratory mainly for the study of hemicellulose. To this end the alkaline fractionation method developed by Gaillard (1961) and basically as described by Gaillard and Bailey (1968) was used (Fig.1). During the course of the work it became apparent that one of the wall components probably most relevant to the control of elongation is the hydroxyproline-rich cell wall protein extensin. Because hydroxyproline is confined mainly to the cell wall protein it was used as an indicator of extensin, and a study of the relationship of hydroxyproline to wall fractions and to stem elongation made. In the light of the results obtained some preliminary incorporation experiments were done.

Although the approach has been to study the cell wall with techniques used widely for carbohydrate studies these have proved inadequate in several respects. It is clear that while significant points have arisen from the work on cell wall fractions more meaningful results will emerge when these fractions are further analysed with the high resolution offered by chromatography, electrophoresis etc.

The methods used here distinguish between classes of one

type of polymer, namely the carbohydrates. The polysaccharide fractions are classed according to their structure only in so far as this is reflected in the capability of a reagent to precipitate or extract the polysaccharide. Thus the terms hemicellulose-A, arabinoxylan-B refer to fractions enriched in certain carbohydrates but certainly not pure in the sense that they each contain molecules of only one structure. The hemicellulose-B-linear 1-4 linked polysaccharide for example is a fraction extracted from the wall by 10% KOH and precipitated from $CaCl_2$ solution with iodine, and the uniformity between cell wall polysaccharides leads us to extrapolate to lupin hypocotyl and assume the presence of arabinoxylan or xyloglucan, although galactose also occurs in the fraction. The names traditionally given to wall fractions isolated by the methods used here are not entirely accurate even where the name refers to a specific structure. The polydispersity of wall polysaccharides or of fragments of the cell wall complex make it difficult to achieve clear cut results by such methods. It is characteristic of the human mind to reduce any complex to units. It seems that in the case of the cell wall one must recognize that in a sense the complex <u>is</u> the unit.

Clearly the cell wall must be regarded as a complex of several types of polymer - carbohydrate, protein, lignin, lipid and possibly others. Use of methods designed for the study of one of these types will provide data, but it will be difficult to interpret in the context of the whole cell wall which from the point of view of experimental design should

perhaps be regarded more as a macromolecule.

As far as relating the hydroxyproline-rich protein to the cell wall fractions is concerned the results are of interest, but because these fractions probably do not contain discreet species it is not possible to say with certainty to which polysaccharide the protein is bonded, if it is at all, but only that it is associated in certain proportions with fractions enriched in polysaccharides of certain composition. However the results are indicators upon which a model of the cell wall, and working hypotheses can be founded.

It is now necessary for this work to be extended by more complete resolution of wall components so that individual fragments containing both extensin and wall polysaccharide or other polymer can be isolated prior to identifying the bonds linking the different moieties. While it would provide more information on the nature of wall fractions from the fractionation of Gaillard and Bailey if they were resolved further, as much information on the molecular structure of the cell wall could be obtained by resolution of the total alkaline extracts by chromatography, electrophoresis etc., in other words by considering the extract to contain a spectrum of polymers derived from the degradation of a macromolecule. A difficulty inherent in the method used here is that the relatively non-specific action of alkali probably makes the spectrum of derived polymers far broader than it would be if agents (such as enzymes) which acted on specific bonds had been used.

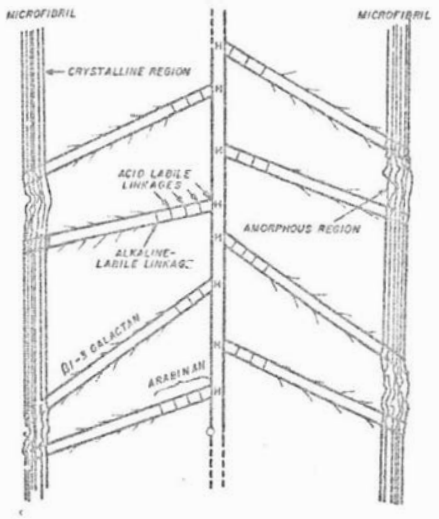
The alkaline extraction provides a means of obtaining fairly large fragments which once separated could be

subjected to the action of proteases or carbohydrases as an approach to identifying any alkali stable linkage points. Although a similar approach has been taken by Bauer et al. (1973) their work could also be extended by use of alkali extraction which seems to remove a greater proportion of hydroxyproline at least from lupin hypocotyl and Avena coleoptile walls.

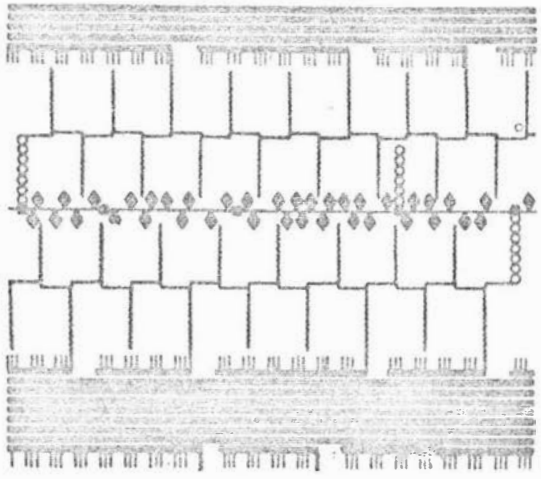
Two models of the primary cell wall extensin-polysaccharide complex have been proposed in recent years; those of Lamport (1970)(Fig.12a) and of Keegstra et al. (1973)(Fig.12b). In the former the protein backbone is linked to the microfibrils via a galactan which is bound to the hydroxyprolyl(arabinose)$_{1-4}$ of extensin. The galactan-arabinose oligosaccharide linkage was suggested due to the co-occurrence of arabinose and galactose in fragments of extensin (Lamport, 1969) and was thought to be anomalously alkali labile as only arabinose oligosaccharide had been found attached to hydroxyproline in alkaline hydrolysates of cell walls (Lamport, 1967). With the discovery of the galactosylserine bond the galactan-hydroxyprolylarabinose linkage was no longer necessary for a wall model. This and other evidence lead to the second model (Keegstra et al., 1973) in which the arabinose oligosaccharide side chains of hydroxyproline are free and the main linkage of the protein to the wall is through galactan → rhamnogalacturonan → arabinan or galactan → xyloglucan, the last being attached by hydrogen bonds to the microfibrils. The hydrogen bonds are the point of movement of the matrix and microfibrils and it is suggested that these bonds are influenced by pH, itself controlled by an auxin sensitive hydrogen ion pump in the plasmalemma.

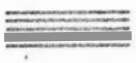
FIG.12 MODELS FOR EXTENSIN-POLYSACCHARIDE COMPLEX OF CELL WALLS

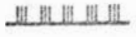
(a) Lamport (1970)

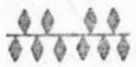


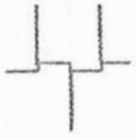
(b) Keegstra, Talmadge, Bauer and Albersheim (1973)




cellulose elementary fibril


xyloglucan


wall protein with arabinosyl tetrasaccharides glycosidically attached to the hydroxyproline residues


total pectic polysaccharide

rhamnogalacturonan main chain of the pectic polysaccharide

arabinan and 4-linked galactan side chains of the pectic polymer


3,6-linked arabinogalactan attached to serine of the wall protein


unsubstituted seryl residues of the wall protein

On the basis of results presented in this thesis some modifications to the above models may be made, although support for many of their features has also been obtained. The results pertinent to the construction of a modified wall model are summarized below.

(1) The average composition of the 6 cm lupin hypocotyl cell wall is as follows: pectin 27%, hemicellulose 19.8%, cellulose 42.3%, lignin 3.3% and protein 7.9%.

(2) Changes in cell wall composition related to wall elongation occur in a protein and arabinose-rich fraction of the wall, and involve percentage increases in both hydroxyproline and arabinose.

(3) Most of the arabinose and much galactose is removed from depectinated cell wall by the delignification treatment of Gaillard (1958a). Other sugars comprising the hemicellulose are not greatly affected.

(4) Hydroxyproline is completely removed from the cell wall by the delignification treatment which therefore disrupts the wall protein.

(5) Dilute acid at the temperature and pH of the delignification will remove much of the wall arabinose and galactose, but less than is extracted by the delignification treatment, whose effect on the monosaccharide composition of the wall is therefore not due solely to the cleavage of dilute-acid labile bonds.

(6) Dilute acid did not remove the wall hydroxyproline, indicating that the extensin is not bound into the wall by dilute acid labile bonds as has been implied. An acid labile linkage has been proposed (Lamport, 1970) between the

arabinose-oligosaccharides and the hydroxyproline of extensin, and there was the suggestion that such a link could be the site of bond cleavage by acid during wall loosening induced by low pH (Rayle et al., 1970). The results presented here do not provide evidence for such an acid labile bond. Hot dilute acid did not release extensin or facilitate its extraction from the wall with other solvents.

(7) Urea and 6M guanidinium thiocyanate are ineffective in removing hydroxyproline from the wall, indicating that covalent bonds are probably involved in the bonding of extensin to the wall polysaccharides. Dilute acid pretreatment did not facilitate hydroxyproline extraction by the above agents showing that the bonds responsible for binding extensin into the wall are probably not dilute acid labile alone.

(8) Sodium methoxide does not facilitate extraction of extensin from acid (pH2.9, 100°C, 5 hr) or non-acid treated walls, indicating that ester links alone do not bind extensin to the wall.

(9) Barium hydroxide hydrolysis which cleaves peptide links removes most of the cell wall arabinose and galactose and hydroxyproline, but not the xylose. If these portions of the wall are linked their connection is therefore through an alkali-labile bond.

(10) When the barium hydroxide hydrolysate is dialysed the loss of arabinose is much greater than the loss of galactose indicating that the arabinose is present in smaller wall fragments than the galactose. The more rapidly dialysed arabinose probably represents the arabinose oligosaccharide side chains of extensin linked glycosidically to

hydroxyproline. This suggests that the linkage of hydroxyprolyl(arabinose)1-4 and galactose is either alkali labile as suggested by Lamport in his model, or non-existent, as in the model of Keegstra et al. The nature of a non-alkali-labile bond between hydroxyprolylarabinose oligosaccharide and galactan is hard to vizualize when one considers that the primary reducing group in both the galactose and arabinose is already involved in bonding.

(11) Most of the hydroxyproline can be extracted from the wall, with most of the hemicellulose, by 10% KOH. Alkali-labile bonds are therefore probably involved in binding hemicellulose and extensin to the alkali-insoluble wall fraction.

(12) Delignification of the hypocotyl cell walls prior to alkali extraction leaves a 10% KOH soluble hemicellulose-B fraction which is rich in xylose. This xylose is therefore present in a polymer which does not depend on the hydroxyprolylarabinose molecule for its retention in the wall.

(13) Alkaline fractionation reveals that the hemicellulose-B can be divided into a major iodine precipitable and minor non-iodine-precipitable wall fraction. Hence most of the wall hemicellulose is associated with linear (1-4) linked polysaccharide. Most of the hydroxyproline is associated with the linear fraction.

(14) Delignification causes a great loss of arabinose from both of these fractions.

(15) If the 10% KOH extraction is done at 0°C two thirds of the 18-22°C 10% KOH soluble hemicellulose is removed from the wall. Little hydroxyproline is extracted at 0°C. The

remaining hemicellulose and the hydroxyproline is removed when the temperature is raised. Thus bonds of two different alkali labilities are involved in attachment of the room temperature 10% KOH-soluble hemicellulose to the alkali insoluble portion of the wall.

That most of the hemicellulose can be extracted separately from the extensin by 10% KOH at 0°C indicates that either the bulk of it is not bound to extensin or that it is in extremely alkali-labile bonding to it. It is possible that there is a linkage between some of the hemicellulose and the remaining wall which is susceptible to 10% KOH at 20°C but not at 0°C. Although there is no direct evidence for a 0°C 10%KOH-labile bond between the hemicellulose and extensin it is a possibility, and if it exists at least two types of alkali-labile bonds to extensin should be envisaged.

(16) Destruction of serine by β-elimination does not occur in 10% KOH at 0°C. When the temperature is raised to 18-22°C there is a marked loss of serine. Thus cleavage of galactosylserine is probably necessary for the release of HRT but not of HO. HRT is therefore probably linked to extensin via galactan or galactose.

(17) The release of HO takes place in two overlapping stages. During the first there is release of xylose and during the second a fraction more enriched in arabinose galactose and hydroxyproline occurs. This is consistent with an initial release of xyloglucan or xylan and a slower but increasing release of material derived from extensin more slowly, perhaps due to cleavage of another 0°C 10% KOH labile bond.

(18) The release of HRT also varies with time. As a percentage

of HRT all sugars except arabinose decrease and then galactose and to a lesser extent xylose again increase. There appears to be an initial release of xylan (or xyloglucan) and galactan and then a continued but diminishing release of arabinose. This can be explained by the cleavage of a bond between the galactose and arabinose-rich portions of the wall in such a way that the break allows immediate release of galactan, while the release of arabinose depends on other factors such as proximity of the β-eliminative cleavage of the peptide chain.

(19) The release of galactose approximately parallels the β-elimination of galactosylserine. The fact that arabinose release does not parallel this can be explained by the necessity for β-eliminations in the same region of the extensin so that the resulting peptide chain cleavage can release an arabinose-rich portion of extensin.

(20) Pronase treatment of the hypocotyl walls (Appendix D) causes a loss of arabinose but not of galactose from the arabinoxylan-B suggesting that galactan but not araban is involved in linkage of extensin to the fraction. The independence of arabinose and galactose is further emphasised by the greater dialysability of arabinose compared with galactose in $Ba(OH)_2$ hydrolysates of depectinated cell walls (see 9 and 10).

(21) Much of the linear 1-4 linked polymer (i.e. the $CaCl_2$ soluble iodine precipitated portion) released appears to contain a non-β-eliminated material, suggesting that β-elimination of adjacent serines is not absolutely necessary for release of extensin. Therefore some of the serine-linked side chains are probably cleaved from attachment to

microfibrils, but not from the extensin chain. Thus there may be two alkali labile bonds in the series of polymer connections between the microfibrils and the serine of extensin.

(22) A 24% KOH resistant protein fraction exists within the wall. It is rich in hydroxyproline but contains very little serine, which does not therefore appear to be involved in bonding of this fraction to the 24% KOH insoluble polysaccharides.

Although the 24% KOH treatment will remove nearly all of the hemicellulose and not all of the protein this does not provide grounds for suggesting a direct link between the protein and the cellulose microfibril (β-1,4-linked glucan), as traces of sugars other than glucose remain in the extracted cell walls. Also, when one considers the highly ordered nature of the cellulose microfibril it will be realized that most of the cellulosic glucose is probably unavailable for linkage, although in Lamport's model galactan is bound to paracrystalline regions of the microfibril. At the surface of the microfibril, where glucose may be available for bonding, other molecules of hemicellulose may exist in quite high concentration relative to the superficial glucose. It is with these sugars that a 24% KOH resistant protein may link.

The wall models of Lamport and Keegstra et al. predict the behaviour of wall components under a variety of treatments. Some of these predictions are not borne out by the results presented here. For instance, according to Lamport acid or alkaline treatment should release the glycoprotein. Acid will not readily release extensin and alkali is not totally effective in doing so.

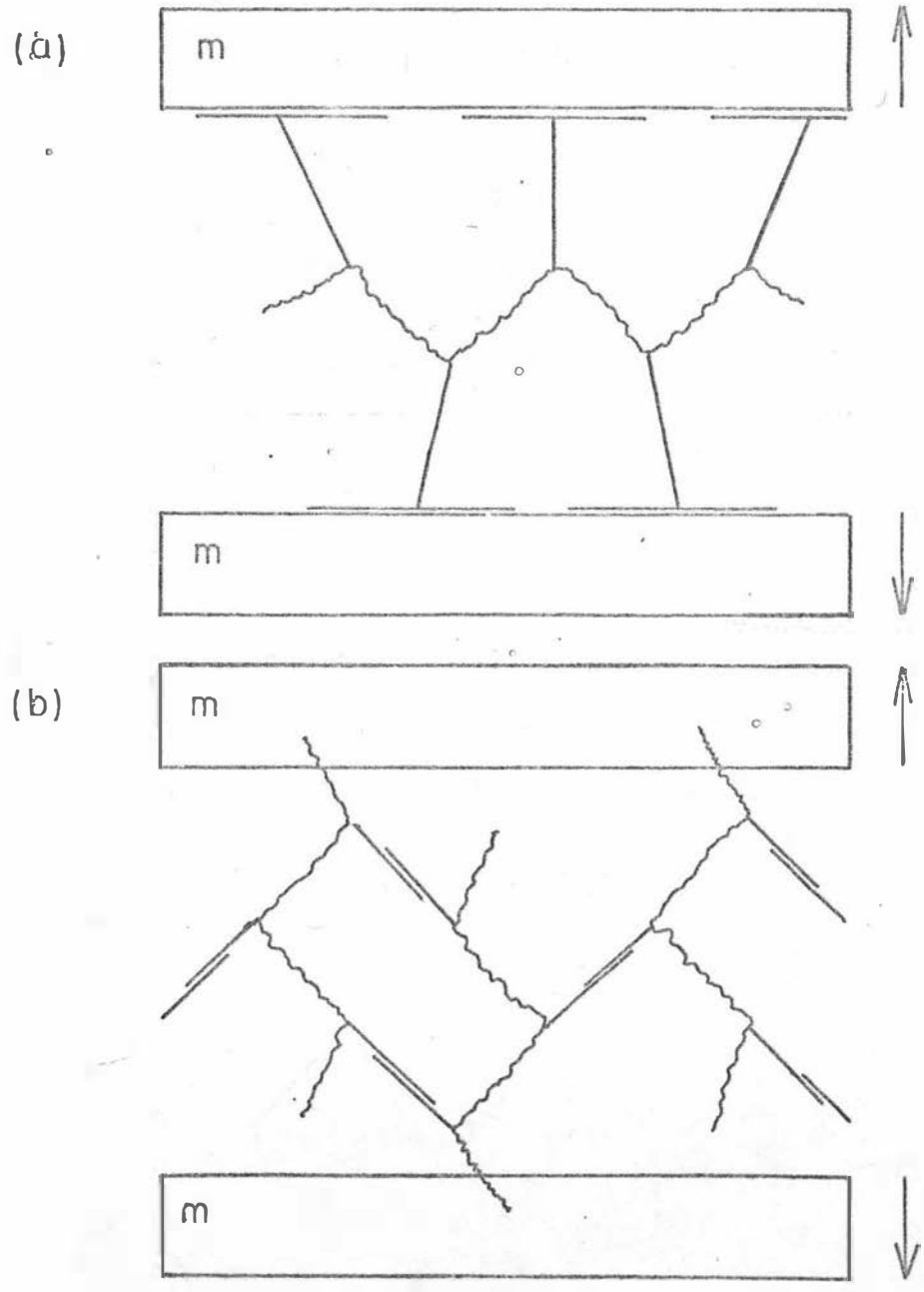
Similarly, in the model of Keegstra et al. There are several points of conflict with the results obtained here. (1) It should not be possible to remove pectin independently of extensin according to their model, as all of the extensin-microfibril links are through a polyuronide molecule and all of the wall pectin is envisaged to be involved in the extensin-polysaccharide complex. (2) Removal of xylose polymer with alkali should be accompanied by the release of extensin. This does not occur with the 0°C 10% KOH extraction. (3) Extensin should require the presence in it of galactosylserine to be retained in the wall, yet the 24% KOH resistant fraction is very low in serine.

The model proposed here and that of Keegstra et al. are basically in agreement in that the extensin is linked to the remainder of the wall through a glycosylserine bond, while the model of Lamport contains no such link.

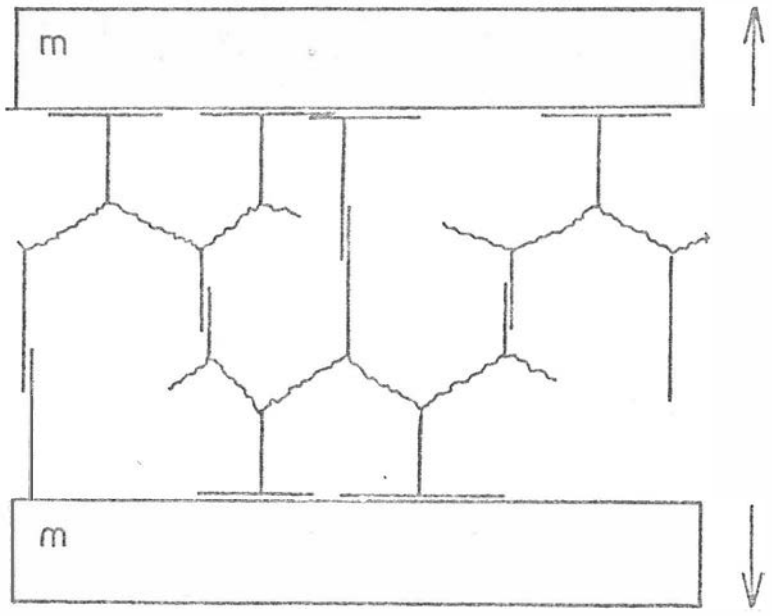
In both the models of Keegstra et al. and Lamport the extensin chain is oriented parallel to the cellulose microfibrils (Fig.13a).The results presented here suggest a strong direct link of a portion of the extensin to the microfibrils, and if this represents attachment of the protein running between microfibrils the orientation must be more at right angles to them as shown in Fig.13b. It has been suggested that wall creep is due to the cleavage and reformation of hydrogen bonds under tension, implying the movement of the bonding polymers parallel to one another i.e. chain slippage. If the resistance to elongation is resistance to separation of microfibrils, and if creep involves their slow separation it would seem more logical that

FIG 13

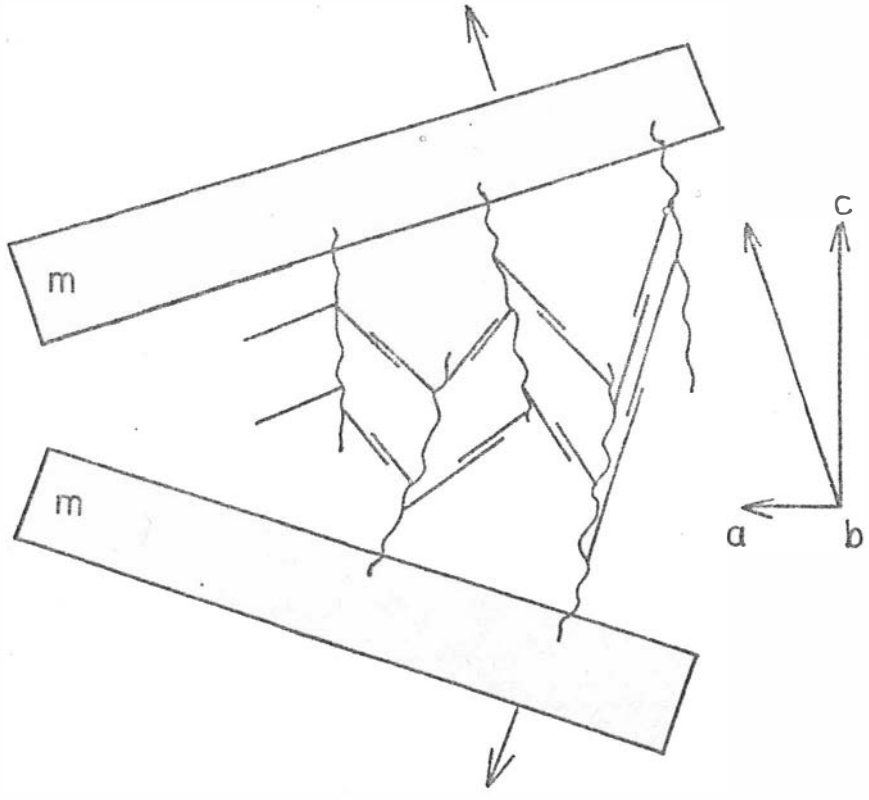
RESISTANCE TO CELLULOSE MICROFIBRIL MOVEMENT BY NON-COVALENT BONDS BETWEEN POLYSACCHARIDES LINKED TO EXTENSIN



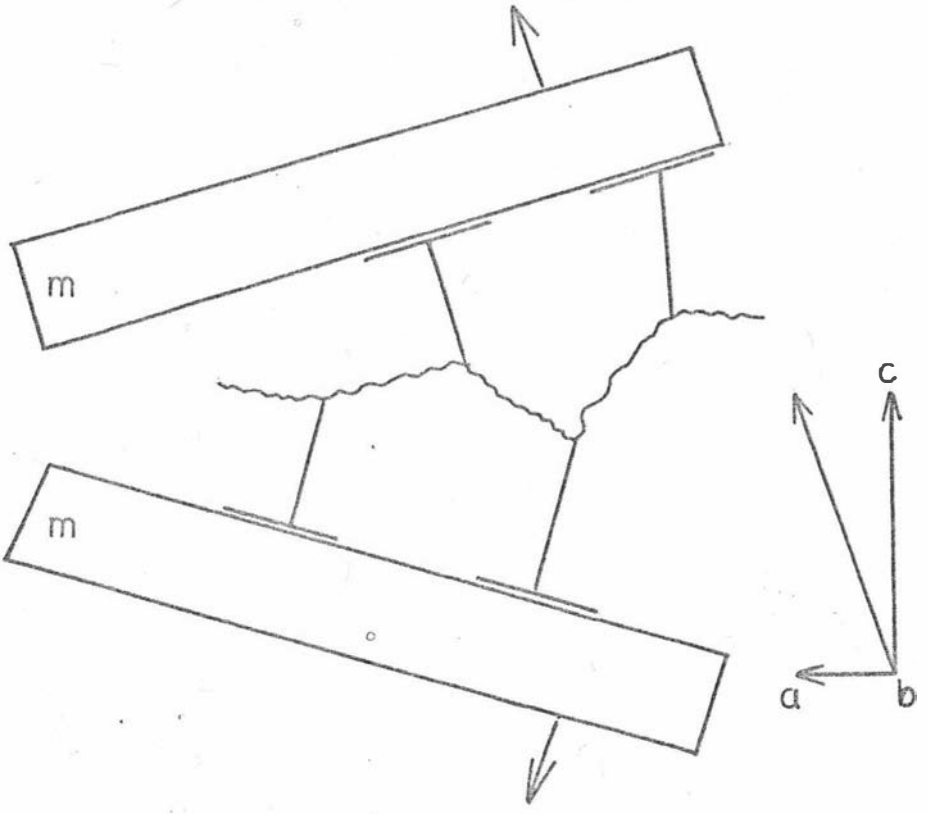
(c)



(d)



(e)



m - Cellulose microfibril

⇅ - direction of microfibril movement

═══ - regions of non-covalent bonding and potential ch slippage

(a) - microfibrils parallel, little stress in directio of chain slippage

(b) and (c) - microfibrils parallel, shearing of non-covalent bonds

(d) and (e) - microfibrils not parallel, shear component parallel to direction of potential chain slippage

the hydrogen bonding polymers should be at an angle to the microfibrils. The situation where extensin is parallel to the microfibrils and chain slippage at right angles to them is shown in Fig.13c . However if the microfibrils are at an angle to one another considerable stress will be placed on the bonds between polymers stretched to their maximum (Fig.13 d and e) and even for bonds which lie parallel to the microfibril there will be a shearing component (ab of Fig.13d and 13e).

A modified model of the primary cell wall based on the above results and on the models of Lamport and Keegstra et al. is shown in Fig.14. It has the following features.

(1) Extensin is bound to the wall covalently.

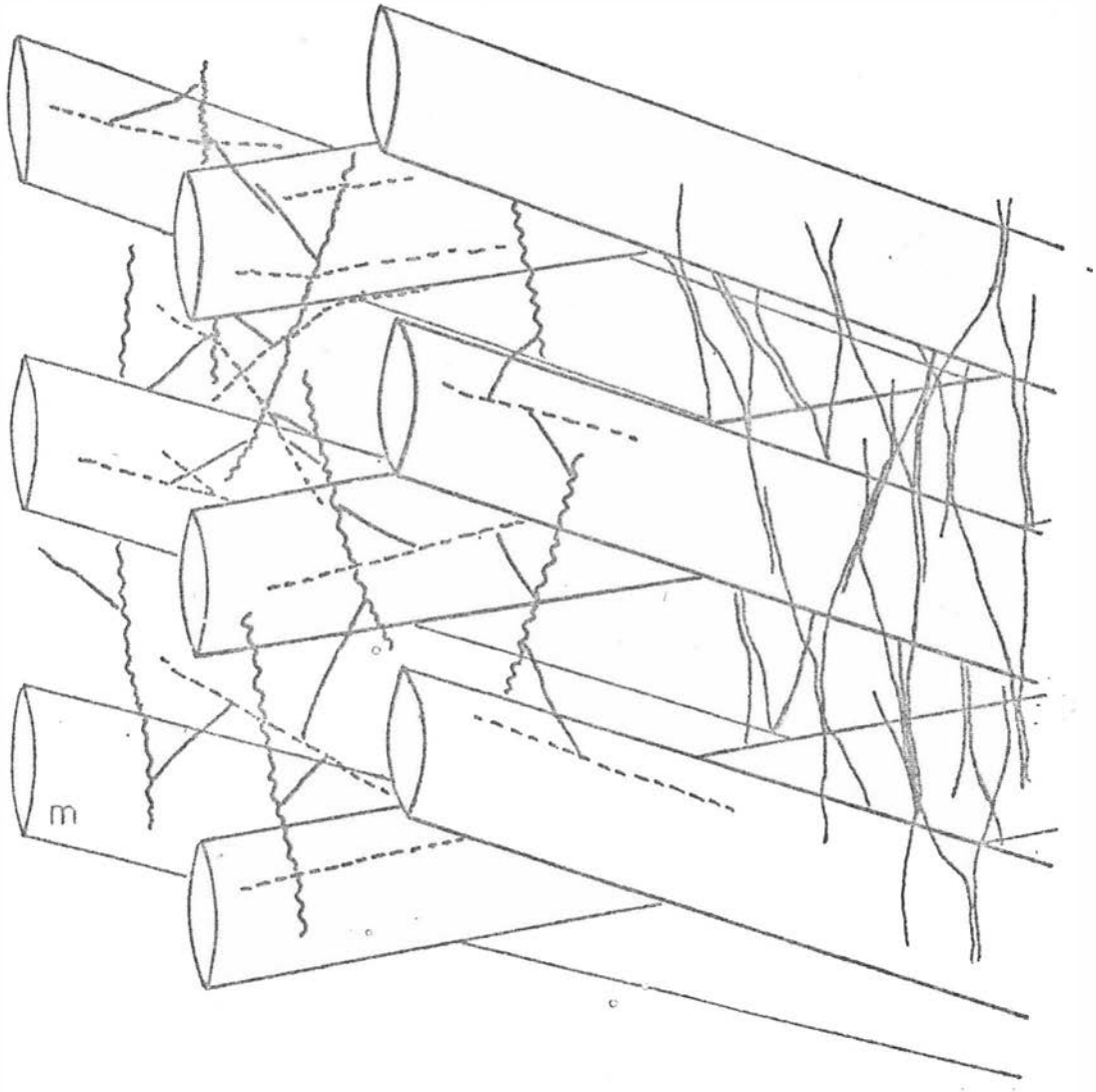
(2) Most of the pectin is not involved in linkage of the protein and microfibrils, in contrast to the model of Keegstra et al.

(3) Much of the wall xylan is not necessary for the bonding of protein to cellulose microfibrils, and is attached to the wall by $0^{o}C$ 10% KOH labile bonds. This also contrasts with the model of Keegstra et al. where all of the xylan is involved, as xyloglucan.

(4) There is a close association between a portion of the extensin and the microfibrils. This fraction of extensin is high in hydroxyproline and low in serine. Such an association is not emphasised in either of the above models.

(5) An alkali labile bond exists between the wall arabinogalactan-extensin and the wall xylan or xyloglucan. Dialysis of alkaline hydrolysate suggest that the alkali separates much of the wall arabinose and galactose. In the model of Lamport an anomalously alkali-labile bond was postulated between hydroxyprolylarabinose and galactan but

FIG.14. TENTATIVE MODEL FOR EXTENSIN-POLYSACCHARIDE COMPLEX OF LUPIN HYPOCOTYL



Extensin-polysaccharide complex.

Pectin network.

For convenience the extensin-polysaccharide complex and pectin network have been drawn separately.

Extensin with hydroxyproline-linked $(arabinose)_{1-4}$

Galactan linked to extensin

Xylan (HO and HRT)

Junction zone in pectic gel

m Cellulose microfibril

this idea has now been discarded by Lamport.

(6) Dilute acid labile links bind much of the wall arabinose and galactose to the remainder of the cell wall.

(7) Each extensin chain has several linkages through galactosylserine and polysaccharides to the microfibrillar portion of the wall. Thus several cleavages of the peptide chain due to β-elimination must occur before sections of the chain are released. This high degree of linkage is consistant with both models but in that of Lamport the linkage is not through galactosylserine.

A model is made more relevant to the <u>in vivo</u> situation if scale is introduced by consideration of microfibril size and separation and the length of some of the molecular species in the wall.

The assumption is made that the wall is 60% water and 40% polymers by weight (Roelofsen, 1959). As the average density of wall polymers is about 1.5 (Mark, 1967) the ratio of polymer volume to water volume in the wall becomes $\frac{40}{1.5}$: 60 = 26 : 60.

Thus 30.7% of the wall volume is polymer.

42.3% of the polymer volume is cellulose which therefore occupies $\frac{42.3}{100} \times 30.7 = 13\%$ of the wall volume.

But as cellulose in contact with water is 16% water the actual volume occupied by cellulose microfibrils will be $(1.5 \times 13 \times \frac{16}{100}) + 13 = 16\%$ of the total volume of the wall.

The distance between cellulose microfibrils can be calculated appoximately by reference to Fig.15. The area of interfibrillar space enclosed by the four microfibrils is the area of the parallelogram A B C D minus the area of one

microfibril. If the area ABCD is 100 square units the area of the microfibril will be 16 square units.

Where r is the radius of a microfibril (say 100 Å) and ℓ is the length of the sides DC and BC

the area of ABCD = ℓ x BE

$= \ell \times 0.5\ell \tan 60$

The cross sectional area of a microfibril (πr^2) is 16% of the area of ABCD.

$$\pi r^2 = \frac{16 \times 0.5 \ell^2 \tan 60}{100}$$

$$\ell^2 = 2.271 \times 10^5 \text{ Å}^2$$

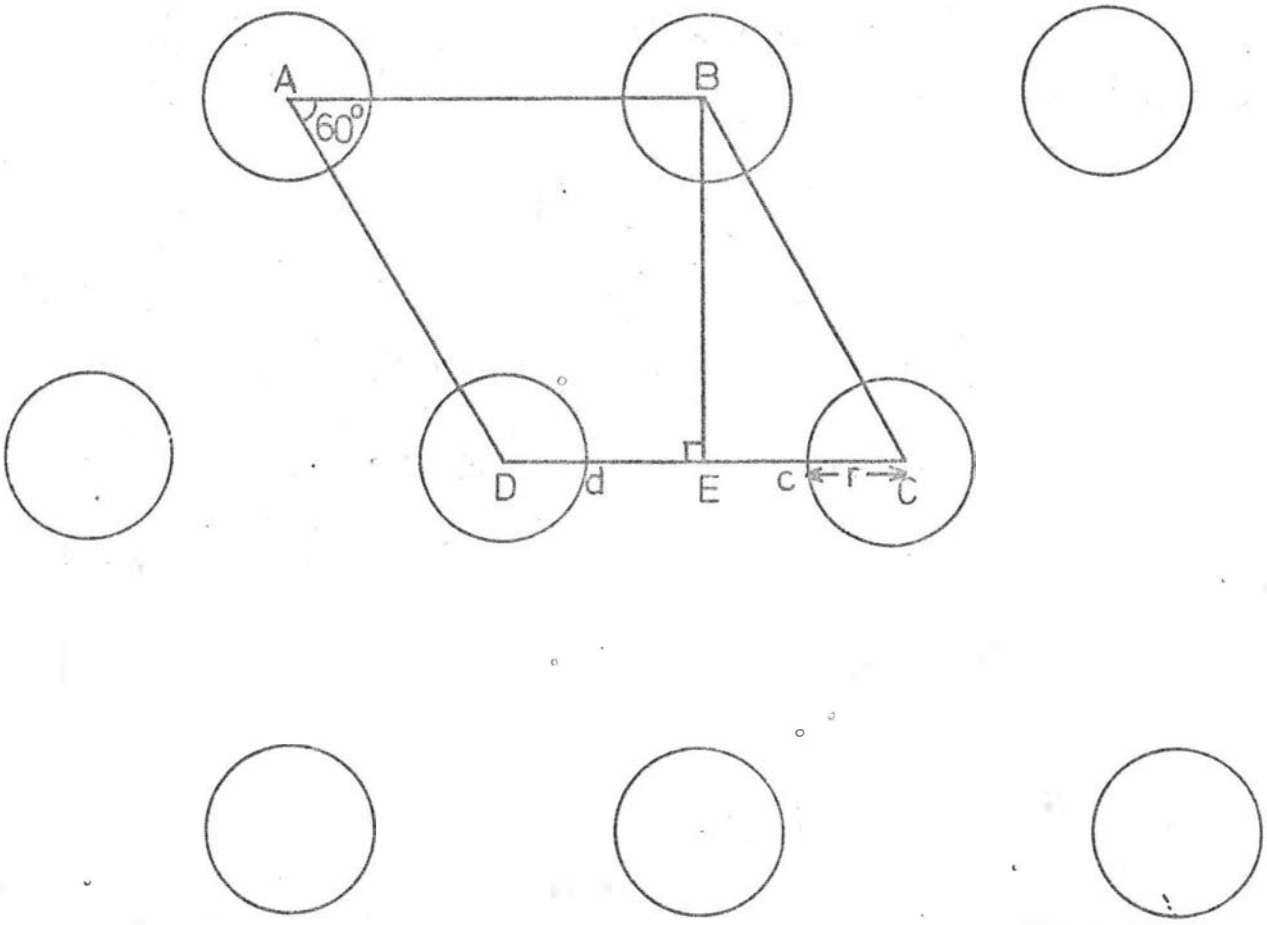
$$\ell = 469 \text{ Å}$$

As Cc and Dd are each 100 Å in length the closest distance between microfibrils is (469-200) Å, that is, 269Å. This value for intermicrofibrillar distance is probably an overestimation because the pectic substances have been included in the wall matrix in the above calculation. The pectins are known to be enriched in the middle lamella rather than distributed evenly throughout the wall.

The cohesive capacity of the wall matrix will probably depend partly on the lengths of its constituent polymers. Some figures for molecular lengths have been published. Pectins are generally over 500Å in length and would therefore easily stretch between two microfibrils. Moreover several different pectin chains are capable of joining through non-covalent bonds to form regions of association known as junction zones. The result is that a network of pectin molecules in the solvent is formed and a gel results. The junction zones may be sufficiently ordered to form microcystallites. When the formation of junction zones between

FIG 15

DISTANCE BETWEEN CELLULOSE MICROFIBRILS IN LUPIN HYPOCOTYLS



The microfibrils have been equidistantly spaced so that they are at their greatest minimum separation.

Fig.15 applies to pp.143 and 144 of text.

different pectic polymers is considered it is clear that many microfibrils will fall within the sphere of a gel involving connected pectic polymers.

Xylans generally have a degree of polymerization of about 150-200. The cellobiose unit of cellulose is estimated to be 10.3 Å. Assuming a xylobiose unit to be of similar dimensions and rembering that side chains do not contribute to molecular length, a conservative figure of about 500 Å would be the length of a xylan molecule. Xylans have been shown to be capable of forming junction zones (Neiduszynski and Marchessault, 1971) and microcrystallites as are found in pectins *in vivo* and in pure pectin gels, so that it would seem that networks of both xylans and pectins are probably present in the wall. As well as binding to one another, xylan chains have been shown capable of noncovalent binding to cellulose (Clayton and Phelps, 1965; Luce and Robertson, 1961) and to be closely applied to the microfibril surface. 10% KOH at 0°C may, due to disruption of non-covalent bonds (Aspinall *et al.*, 1969) cause release of xylose polymer from the cellulose microfibrils and from association in junction zones.

Brysk and Chrispeels (1972) have estimated 35,000 as the molecular weight of a hydroxyproline rich cytoplasmic protein thought to be extensin precursor. A lower figure of about 11,000 can be obtained from the figures of Lamport (1970). The average molecular weight of the amino acids of extensin is about 114 giving a degree of polymerization of 307 in the case of Brysk and Chrispeels and 97 for Lamport's protein. If one assumes that the extensin has a polyproline configuration, as is found in collagen, a distance of 3.12 Å

will be travelled per residue, parallel to the helix axis. Thus the lengths of the polypeptide chains in the case of Brysk and Chrispeels, and Lamport, will be 950 Å and 303 Å respectively.

If the xylan or xyloglucan, pectin, galactan and protein are covalently linked in series a molecule of at least 2000 Å in length, capable of stretching across five microfibrils as they are spaced within the wall, will be formed. Although the evidence is against linkage in series the dimensions of the individual polymers make it clear that wherever the points of covalent linkage of the polymers are, a macromolecule capable of spanning the intermicrofibrillar space will be formed.

The cell wall model drawn to scale is shown in Fig.14.

It is important to remember that the cellulose microfibril is essentially inextensible and deformation of the cell wall depends primarily upon bonding between rather than within the microfibrils. The modulus of elasticity in the polymer chain direction for crystalline native cellulose has been calculated as 1.37×10^4 kg/mm^2 (Sakurada *et al.*, 1962).

The figure given for microfibril separation is an average throughout the thickness of the wall. However it is known that the microfibrils are not evenly distributed through the wall, but are arranged in lamellae which are separated by the matrix polysaccharides (Roelofsen, 1965; Probine and Preston, 1961). In this case one would expect most of that intermicrofibrillar interaction mediated through covalent bonds to be within the lamellae, while that due to pectin-pectin links, in gel formation to be between the

lamellae. In Nitella at least, there appears to be little radial reinforcement i.e. there is little reinforcement between the inside and outside of the wall (Probine and Preston, 1961). If this is the case most of the covalent resistance to cell elongation will be due to longitudinally oriented bonds.

It appears that the microfibrils in elongating cells are deposited by apposition with a mean transverse orientation which becomes predominantly longitudinal as elongation proceeds. Thus the microfibrils of the inner lamellae are crossed and subtend a small angle with the horizontal. This angle increases greatly as the cell elongates (and other microfibrils are added by apposition) so that the length of microfibril capable of linking to a microfibril which crosses it is greatly reduced. Consider Fig.16

If the maximum distance over which the covalent crosslinks can form is AC, the length of microfibrils not separated by more than AC is AB and the angle between the microfibrils is θ it can be seen that

$$\text{Cosec } 0{\cdot}5\,\theta = \frac{AB}{0.5\ AC}$$

As AC is constant $L \propto \text{Cosec } \theta$

A graph of θ vs Cosec θ is shown in Fig.17 and shows that a change from almost parallel microfibril orientation at deposition on the inner wall surface to an angle of $\theta = 10^{\circ}$ would be accompanied by a 10 fold decrease in longitudinal bonding. In other words the longitudinal covalent bonding would have been reduced 90%.

The degree of resistance of the wall to cell turgor pressure may also be controlled by the ease with which

FIG 16

DECREASE IN LENGTH OF MICROFIBRIL BONDED FROM AB TO A'B WITH INCREASE IN ANGLE BETWEEN MICROFIBRILS FROM 6° TO 20°

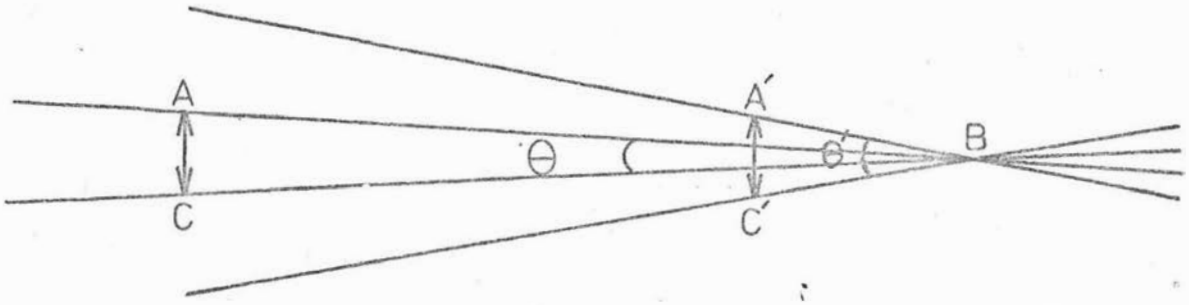
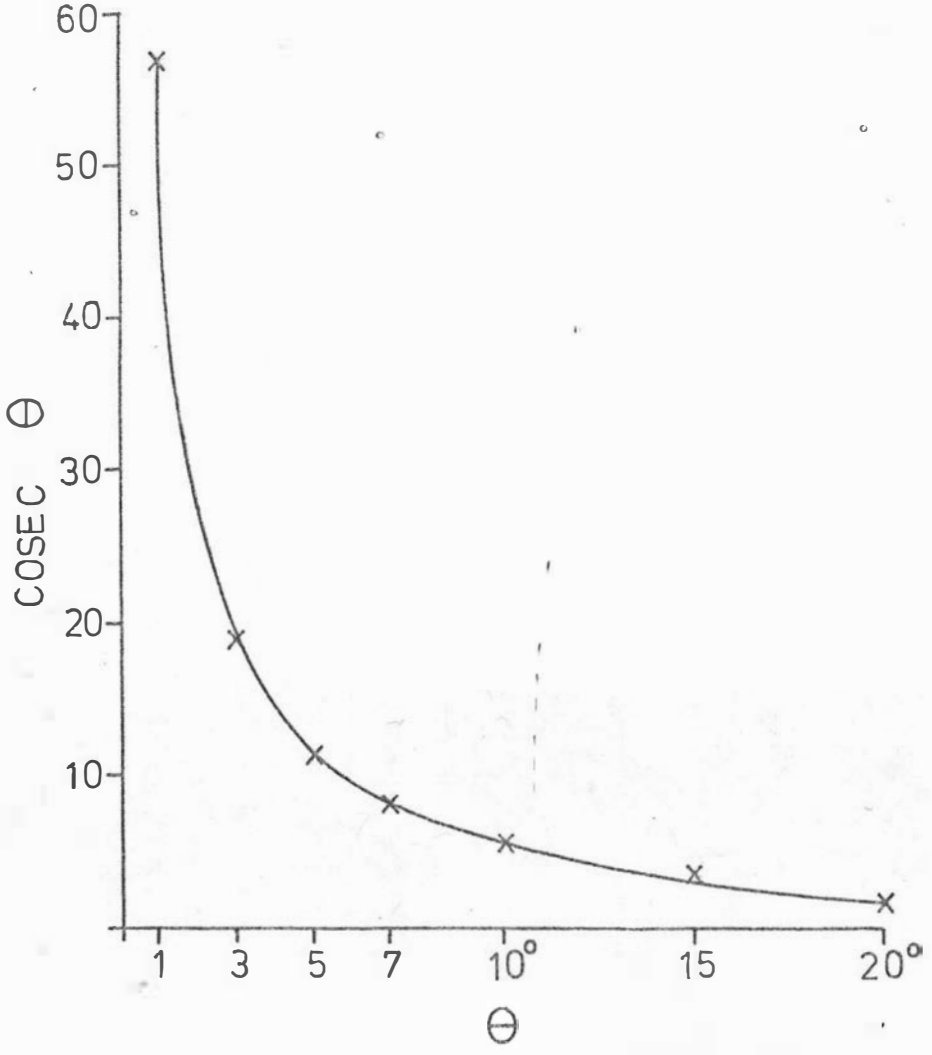


FIG 17 GRAPH OF ANGLE θ vs COSEC θ BETWEEN 0 AND 20 DEGREES.



microfibrils can move across one another, as elongation is not accompanied by a marked decrease in cell diameter. This is presumably also a function of intermicrofibril angle. If bond reformation occurs readily and the resistance is dependent on the number of bonds to be broken, then the most resistance will occur at the microfibril orientation, where the greatest microfibril movement occurs for any change in angle.

Consider simplified case in Fig. 18 where two microfibrils aa′ and bb′ with fixed points A and C have a moving point of intersection at B. BD is parallel to the longitudinal axis of the cell and increases with increasing cell length to DE. AD is constant.

Thus $\text{Tan } \theta = \frac{BD}{AD}$

Therefore increments in θ decrease for every unit increase in BD.

Also $AB = \frac{BD}{\text{Sin } \theta}$

Thus, as $\text{Sin } \theta$ decreases as θ increases AB increases with increase in BD. Thus the degree of movement of the microfibrils across one another increases with every unit increase in cell length. If the microfibril reorientation approaches 90° increase in AB $\rightarrow$ increase in BD (i.e. AE approximately equals DE). As this stage is approached constraint on elongation would also come from within the microfibril due to valency bonds resisting its extension, unless the microfibril could move along its entire length, through the matrix. The least bonding about point B will occur when the microfibrils have each moved 45° from their original orientation at right angles to the longitudinal axis

FIG 18

MOVEMENT OF MICROFIBRILS aa′ AND bb′ WITH CELL ELONGATION ALONG THE AXIS yy′

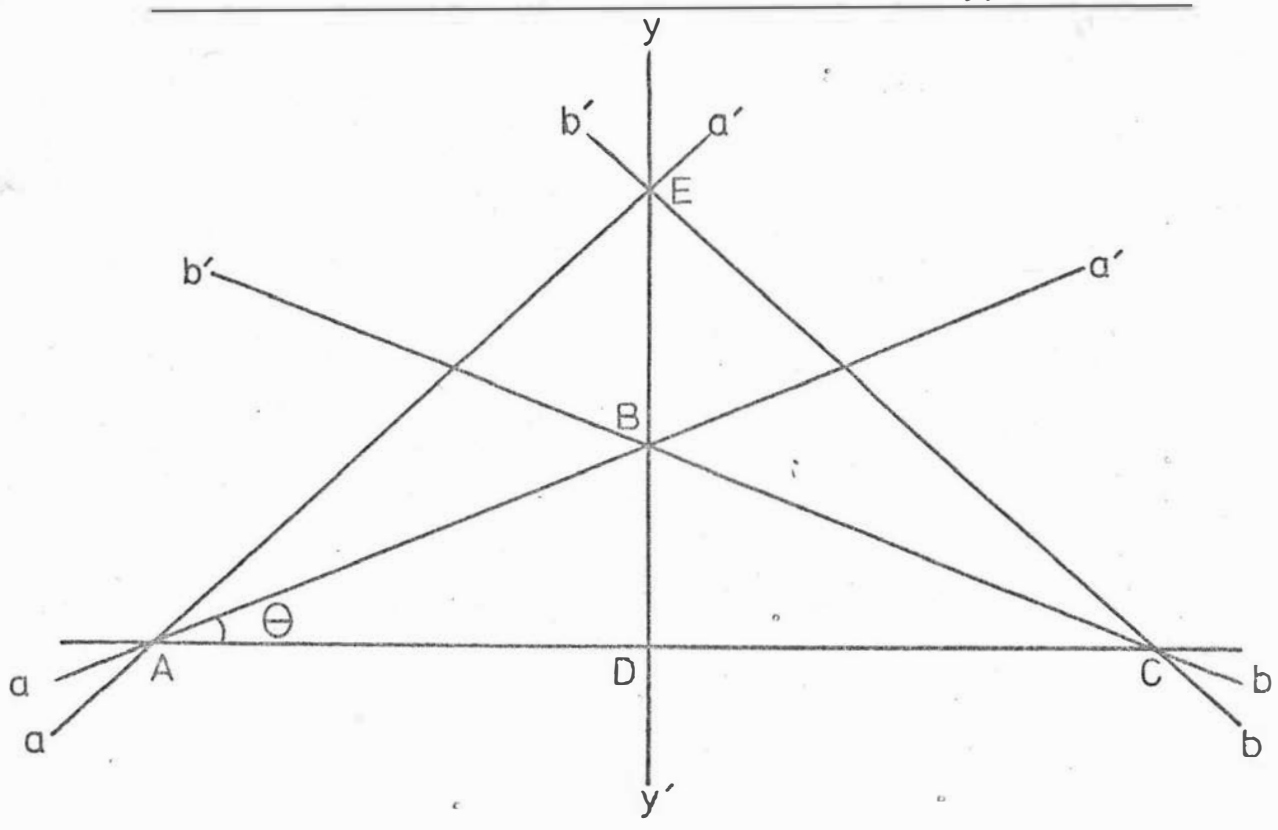


Fig.18 applies to page 148 of the text.

of the cell. Beyond this stage there will be a rapid increase in bonding as θ increases.

Therefore there are at least two interactions of microfibrils which contribute to wall strength at different stages in elongation. One is the resistance to separation especially at small intermicrofibrillar angles where the bonded length is large. The other is the resistance to movement along one another especially when the intermicrofibrillar angle with cell axis is small. There is also the possibility of resistance due to interlamellar bonds and restriction of longitudinal movement of microfibrils.

It is important to realize that the degree of microfibril reorientation discussed here may not often occur in mature walls due to the presence of thickenings and pits in the wall. However at least small scale movement does occur and, as discussed, this is sufficient to drastically reduce the bonding between microfibrils.

It would seem that in the young cell resistance to elongation may be greatest at the inner surface of the wall. A transverse microfibril orientation has been shown to favour elongation due to a high degree of radial resistance to turgor. This is possibly because widening of the cell requires not only a high degree of movement of microfibrils along one another but because this movement would take place with a small angle and therefore a high degree of bonding between microfibrils.

If the wall protein is directly involved in preventing microfibril separation through covalent bonding to the microfibrils then a bonding longitudinal to the cell axis and

and at right angles to the microfibrils would seem likely, unlike in the model of Keegstra et al.

This is consistent with the occurrence of microfibril bound protein reported in this work. However, when one considers the multiplicity of cross-links in the complex it should not matter greatly whether the reversible auxin sensitive bond is actually directly to the microfibril or between microfibril-attached components. Auxin or acid induced slippage could thus be envisaged as either between matrix components or between microfibril and matrix. In actual fact both could probably occur.

From the wall model it is clear that apposition is the only sterically feasible way of adding cellulose microfibrils to the wall, unless some major reorganization occurs. It is therefore fitting that the innermost microfibrils should have the most effect on cell growth and that the cell should therefore fairly rapidly be able to influence cell elongation. Using benzimidazole Probine (1965) was able to alter microfibril deposition and cell expansion markedly within a few hours. It is of interest to quantitatively assess the amount of deposition required to affect growth and the time required for this.

The wall model proposed here has important implications for the penetration of substances, particularly of high molecular weight, into the wall. Furthermore as enzymes have been found to alter wall properties it is of interest to know whether or not these are in fact acting on the same region of the wall as that acted upon by the cell during the induction of cell elongation. Ruesink (1969) found that treatment of live

Avena coleoptiles with high conentrations of cellulase did not increase the growth rate, although the extensibility was increased. Also, pronase is capable of removing a large proportion of the wall protein without drastically affecting wall properties. Possibly some of the penetration of high molecular weight substances takes place through pits, and these may be the areas which are attacked by the enzymes without greatly affecting the strength of the wall.

There seems to be little evidence for the outer and inner regions contributing in different ways to the mechanical properties of the wall, although the meaning of extensibility measurements in terms of wall structure is at the moment very poorly understood. Measurements of D P (plastic compliance) do not appear to be closely correlated with growth rate (Cleland, 1971; Chapter I this thesis). In lupin hypocotyl there is a rapid increase in elongation rate after the addition of IAA although D.P. does not increase markedly until about 40 minutes after auxin application (Penny et al., 1972).

Although extensive pronase treatment does not alter the mechanical properties of Avena coleoptile cell walls to any great extent its action in removing 90% of the wall proline is accompanied by some loosening of the wall similar to that induced by low pH. This does not necessarily mean that 90% of the regions of extensin chains which bear cross-links have been removed. The amino acid results of Chapter VI suggest uneven distribution of serine along the peptide chain. There is evidence that extensin is in fact resistant to enzymatic degradation where polysaccharide attachments are present (Brysk and Chrispeels, 1972). Thus while much of the extensin

molecule may be removed from the wall it is possible that most of the portions contributing to the physical unity of the extensin complex remain, so that the overall crosslinking within the wall is not reduced as drastically as proline or protein removal would suggest.

A difficulty in the system is deciding whether more than one protein is involved. It is perhaps significant that extensin fragments examined have differ in amino acid compositions from both the hydroxyproline-rich cytoplasmic fractions (extensin precursors?) isolated from various plants and the total bound cell wall protein. Some fragments examined appear to be greatly enriched in hydroxyproline. Either the wall contains covalently bound protein other than the hydroxyproline-rich polymer or else the polymer is of quite variable composition along its length.

The evidence on which the wall model given in Fig.14 is based is at the moment fairly inconclusive so that some work is necessary before the model can be regarded as much more than a working hypothesis.

One of the most important subjects for investigation is the nature of the alkali resistant protein and its association with the cellulose microfibrils.

It is important to examine the 24% KOH resistant protein especially in view of the fact that while it appears to be tightly bonded to the microfibrils it has a low level of serine. This raises the possibility of bonding through the hydroxyprolylarabinose or some other amino acid to polysaccharide. A separation of the protein residue from 24%

KOH extracted walls could possibly be achieved using cuprammonium salts if 6M·GTC. proves inadequate. Solutions of polysaccharide in such solvents have been submitted to gel chromatography. Such a method should, in the light of the present results, be attempted at about 0°C under nitrogen. Hopefully it would allow the isolation of microfibril-bound protein without disruption of covalent bonds - thus any protein-bound polysaccharide should remain intact. An added minor advantage in this system is that cuprammonium salts will form blue complexes with protein thus allowing movement of the protein to be followed, and possibly an accurate direct measure of protein in column eluates to be made. Even large fragments of extensin should be obtainable from walls, with or without acid stripping, by this method.

The release of extensin from the wall may require not only cleavage of the galactosylserine linkage but also scission of the polypeptide chain. If the extensin is joined to the microfibrils other than through galactosylserine alkali should not remove the hydroxyproline from the wall if the treatment is carried out in the presence of borohydride. Borohydride reduces the dehydropeptide intermediate of β-elimination so that instead of the peptide bond being cleaved, alanine is produced and the peptide chain remains intact. If treatment of the walls with alkali in the presence of borohydride fails to release extensin some bonding to the microfibrils other than through galactosylserine is suggested.

In the model proposed here for lupin hypocotyl cell walls pectins are not covalently linked into the extensin-polysaccharide complex as in the model of Keegstra *et al*.

because they can easily be extracted with oxalate or neutral detergent. However polyuronide has not been shown to be absent from detergent or oxalate extracted wall. This is significant in relation to the extraction of hemicellulose at 0°C with 10% KOH. Pectins have been shown to be extremely labile to alkali (Neukom and Deuel, 1958) and will depolymerize even at neutral pH. Alkaline degradation of polysaccharided proceeds by a stepwise elimination of residues from the reducing end. Glycosidic bonds within the chain are generally alkali resistant. However, polygalacturonic acid behaves like an oxidized polysaccharide in that glycosidic linkages within the chain are readily split. It is possible that the release of xylan by 0°C 10% KOH is due to cleavage of polyuronide. However, it is noteworthy that xylan is not extracted by neutral detergent even though pectins have been found to be extremely sensitive to elevated temperatures in neutral solution (Albersheim, 1959). Also Bailey and Kauss (in preparation) find that if alkali extraction is applied to cell walls of mung bean hypocotyl without initial depectination then while hemicellulose and glycoprotein is still dissolved by 10% KOH at 0°C and room temperature repectively the pectic polyuronide all remains associated with the wall. It seems that the depectination treatments may render pectins susceptible to alkali. The use of pectinases in conjunction with 6M·GTC and pronase should help elucidate the involvement of the 0°C 10% KOH soluble hemicellulose in the wall complex. Should endopectinase alter the characteristics of extraction of HO xylose and not HRT xylose a polyuronide bound between HO xylan and the remainder of the cell wall is likely. A similar

approach has already been used by Keegstra et al.

The use of degradative enzymes has the advantage that there is a high degree of specificity attainable and applied to wall fragments in conjunction with chromatography should be useful in confirming the presence of suspected bonds and associations.

The biosynthesis of the wall glycoprotein-polysaccharide complex is of interest in that the wall properties could depend on the rates and degree of formation of certain of the links between its constituent polymers. Certainly the rapidity of auxin induced elongation makes it unlikely that auxin "induces" immediate formation or cleavage of covalent bonds. However the potential of the wall to respond and its elongation rate under the steady state conditions generally found could be controlled by the crosslinking to the glycoprotein.

If extensin is active in the control of elongation it is important to know whether the number of cross-links per unit length of extensin is constant, and if not, what conditions will lead to a change in the number. It should be possible, using β-elimination as a measure of serine glycosylation, to measure any change in the ratio of glycosylated to non-glycosylated serine with stage in growth. Using radioactive serine short term changes, perhaps in response to auxin, should be apparent.

It now seems that two different linkage systems can be regarded as determining the physical properties of the wall. They are (1) covalent cross-links of the glycoprotein-polysacchoride complex and (2) polar interactions which act to rigidify the wall as a whole.

Probably the covalent cross-links assume increasing importance as the wall ages and confer an irreversible rigidity on the matrix. The polar interactions on the other hand would be primary consolidation forces in the young matrix, and being non-covalent would be susceptible to changes in their environment such as lowering of pH.

It is plausible that auxins have their primary effect on cell wall development by influencing the rate at which the covalent cross-linkage occurs between the separate polymers of the wall. Probably distinct from this is the short term auxin effect apparently due to a lowering of the pH in the cell wall brought about by the action of an ATP dependent hydrogen ion pump in the plasmalemma (Hager et al., 1971). Such a drop in pH will disrupt the interpolymer associations upon which gel formation is dependent. Keegstra et al. have suggested that hydrogen bonding between the cellulose microfibrils and the wall xyloglucan is pH dependent and thus the rate of creep of the xyloglucan along the microfibril will be determined by the efflux of H^+ ions into the wall.

Such a system can explain the rapidity of the action of auxins and low pH upon the wall. How important this effect is in the intact plant where a fluid matrix exists in young tissues is not yet certain, and how generally the young wall matrix is affected is of interest. If only the inner surface of the wall needs to be acted upon a pump in the plasmalemma might be quickly effective. (It is relevent that the pH at which polygalacturonic acid chains become protonated is similar to that at which wall loosening occurs). Perhaps a general disaggregation and reorientation of matrix polymers occurs

during elongation of the cell wall until such time as the incorporated extensin cross links to prevent it. Then will disruption of the extensin in young non-elongating wall restore the ability to respond to IAA or dilute acid? It may be possible to directly test this using treated cell walls surrounded by young living tissue.

There remain many interesting questions to be answered about cell walls, and with constantly improving techniques it should be possible to make rapid progress in the next few years and to sift and collate the relevancies from the large amount of data so far published on the cell wall.

APPENDIX A

INCORPORATION OF C^{14}-ARABINOSE AND C^{14}-GALACTOSE INTO VARIOUS FRACTIONS OF LUPIN HYPOCOTYL CELL WALLS

INTRODUCTION: Extensin has a postulated role in controlling cell wall extensibility by covalent cross-linking with the wall carbohydrates (Lamport, 1965). Evidence for cross links to arabinose through hydroxyproline (Lamport, 1967) and to galactose via serine (Lamport, Katona and Roerig, 1973) has been provided. However, there is also evidence that extensin is incorporated into the wall with arabinose oligosaccharides attached and then becomes linked to the galactan (Brysk and Chrispeels, 1972). If this occurs it is of interest to gain some idea of the source within the wall of the polysaccharides that become associated with the extensin after its transport into the wall.

One method of investigating this is to pulse label the wall and try to determine whether the level of a particular labelled sugar increases in the extensin-rich fraction at the expense of another fraction. Such cell wall turnover involving the apparent movement of label between different fractions has been reported by a number of workers (Lamport, 1970). It has not yet been investigated in relation to hydroxyproline rich fractions of the cell wall extracted with alkali.

In an earlier experiment it was shown that hot barium hydroxide solution was able to remove most of the cell wall arabinose during hydrolysis of the wall protein. Thus the degradation of extensin results in the release of the arabinose

linked to it.

Because at the time it seemed likely that the protein bound arabinose formed part of a protein linked araban it was considered that if the linkage of the polysaccharide and protein was formed within the wall, then it could be the site of control of wall extensibility, as suggested by Lamport (1970). Furthermore, upon bonding to the extensin the polysaccharide probably changes its solubility from that typical of a pectin to a form insoluble in pectin solvents. This being the case it should be possible to detect a metabolic turnover between the two fractions with label from C^{14}-arabinose moving from the ammonium oxalate soluble (pectic) fraction of the wall to the barium hydroxide extracted portion of the residue. An examination of turnover between two such fractions was conducted and is reported below.

It has been shown that much of the hemicellulose (HO) but little hydroxyproline can be extracted by 10% KOH at $0^{o}C$ (Chapter IV). Subsequent extraction with 10% KOH at room temperature ($22^{o}C$) will remove nearly all the hydroxyproline and some additional hemicellulose (HRT). If the extensin complex is fabricated within the wall it is possible that the carbohydrate portion of the complex is derived from hemicellulose which in becoming linked to extensin is rendered insoluble in the $0^{o}C$ 10% KOH, but is removed, along with the extensin, by the room temperature treatment. If this is the case a movement of label from HO to HRT might be apparent. Furthermore, if the extensin is incorporated with arabinose oligosaccharides attached and then becomes linked to galactan a movement of predominantly C^{14}-galactose into this HRT and a

corresponding decrease in the pectic and/or HO fractions might be seen. With these points in mind the incorporation of C^{14}-arabinose and C^{14}-galactose into various wall fractions was followed. The results of the experiment are reported below.

The two pulse-chase experiments were conducted at different times. The first was done at a stage when the hydroxyproline-arabinose linkage was thought to be responsible for the binding of extensin to the cell wall, and before the publication of evidence for a galactosylserine bond (Lamport, Katona and Roerig, 1973). The second experiment was carried out after the discovery of galactosylserine in extensin fragments had been made public. It was done between the discovery that most of the hemicellulose could be removed independently of hydroxyproline by extraction with 10% KOH at $0^{o}C$ and the application of $0^{o}C$ and room temperature alkaline extraction sequences to lower and upper sections of hypocotyl. It should therefore be regarded as preliminary. There was insufficient time either for the method to be applied to lower and upper sections or for any more than a limited number of labelling experiments to be done. But although a high degree of confidence cannot be placed in the results some points emerge which suggest that the approach is worth pursuing.

RESULTS AND DISCUSSION

(i) Extraction of C^{14}-Arabinose labelled Hypocotyls with $Ba(OH)_2$

Hypocotyl sections were incubated in C^{14}-arabinose for $1\frac{3}{4}$ hours and then given a chase in cold arabinose for 5 hours. During the chase arabinose incorporated into the wall in molecules unattached to extensin and as part of the pectin

fraction could become linked to the wall protein and thus rendered insoluble in pectin solvents. To test this labelled sections from before and after the chase were ground and extracted with 80% ethanol to remove free arabinose and then under reflux with 0.5% ammonium oxalate to remove pectins and other water soluble wall components. The extensin was then removed by a subsequent extraction with 0.43 N.$Ba(OH)_2$ at 90°C.

The residual hemicellulose was dissolved with N·H_2SO_4 under reflux and the residue hydrolysed with 72% H_2SO_4. The fractions were neutralized where necessary and their radioactivity counted. The results are shown in Table XXVI where the percentage of total incorporated counts in each fraction is given after correction for counting efficiency.

There is some indication of turnover, especially between the oxalate and $Ba(OH)_2$ soluble fractions. The difference of approximately 5% in the percentage incorporated into the $Ba(OH)_2$ soluble fraction from chased and non-chased tissue is fairly high when one considers the relatively short duration of chase. However, if the extensin polysaccharide complex is formed due to linkage of extensin to pectic polysaccharides this rate is not surprising, as it would theoretically take only one cross-link from wall bound extensin to any pectic polymer to render that polymer insoluble in oxalate. Furthermore, should the oxalate soluble polymers themselves become cross-linked within the wall the effect of a bond to extensin will be magnified. Some time after the completion of this experiment Keegstra, Talmadge, Bauer and Albersheim(1973) published a model for sycamore cell walls in which the pectic polysaccharides were extensively cross-linked to other

TABLE XXVII. DISTRIBUTION OF C^{14}-L-ARABINOSE IN FRACTIONS OF LABELLED LUPIN HYPOCOTYL BEFORE AND AFTER CHASE WITH NON-RADIOACTIVE L-ARABINOSE

Fraction*	Incorporated Counts			
	After Pulse		After Chase	
	Counts in Fraction	% of total	Counts in Fraction	% of total
0.5% ammonium oxalate (100°C) soluble	203,470	46.7	160,000	39.2
0.43N. Ba(OH) (90°C) soluble	117,690	27.2	132,660	32.5
1.N H_2SO_4 (100°C) soluble	109,360	25.1	108,170	26.5
Residue ("Cellulose")	4,790	1.1	7,750	1.9

* Fractions obtained from sequential extraction with the solvents named.

polysaccharides and extensin. In the present experiment the formation of this complex means that a shift in label would apply to arabinose side chains on any linked polymer, as well as on araban.

The working hypothesis in the second labelling experiment is that turnover between two hemicellulose fractions is occurring. The design of the present experiment probably is such that turnover between $0^{o}C$ and room temperature 10% KOH soluble hemicellulose would not be detected.

It is well to remember that in this experiment any molecules extracted by the 0.5% oxalate are collectively termed pectic substances. This is a sufficiently loose definition to allow the inclusion of those polymers typical of other wall fractions, but so recently transported to the wall that they have not yet become linked to it. Thus part of the pectin which apparently becomes linked to the $Ba(OH)_2$ soluble fraction could be extensin which bears arabinose oligosaccharide side chains but is not yet incorporated into the glycoprotein-polysaccharide complex. Karr (1972) has isolated a cytoplasmic enzyme system which catalysed the glycosylation of extensin. Although the precursor fraction of extensin appears to be only a small proportion of the total wall glycoprotein (Chrispeels, 1969), because it is a precursor it will have a relatively high specific radio-activity after a pulse of labelled C^{14}-L-arabinose.

Chromatographic analysis showed that some conversion to xylose had occurred, but as xylose is virtually absent from the oxalate and $Ba(OH)_2$ soluble fractions this should not affect the results. Furthermore C^{14}-xylose was shown

chromatographically to be very low in these fractions. Nearly all the counts involved in the oxalate and $Ba(OH)_2$ fractions are therefore from C^{14}-L-arabinose and the change in distribution of counts must involve arabinose-containing polymers. The present experiment has shown the suitability of C^{14}-L-arabinose for use in labelling experiments on cell walls. Substantial amounts of C^{14}-xylose were found in the $1N \cdot H_2SO_4$ soluble fraction.

The percentage change in counts shown in Table XXVIIis not necessarily a true reflection of transfer of label between polysaccharides as there is a loss of about 6.3% of the total counts incorporated which could account for much of the change in labelling observed. This is possibly due to the activity of polysaccharidases as suggested by the work of others (Lamport, 1970).

Autolysis of isolated Zea mays coleoptile cell walls has been shown to occur to the extent of 10% loss of glucan in 8 hours (Lee, Kivilaan and Bandurski, 1967) and growing pea sections are reported to be capable of losing 30% of incorporated label in 24 hours (MacLachlan and Young, 1962). On the other hand Roberts and Butt (1968, 1969) using root tips of maize reached the conclusion that galactose or glucuronic acid is essentially stable once incorporated.

(ii) Incorporation of C^{14}-Arabinose or C^{14}-Galactose into $0^{\circ}C$ and $22^{\circ}C$ 10% KOH soluble fractions

To see whether there is any movement of label from the hydroxyproline-free $0^{\circ}C$ 10% KOH soluble fraction (H0) to the hydroxyproline-rich $22^{\circ}C$ 10% KOH soluble fraction (HRT) of the wall sections were pulse-labelled for 1 hour with either

C^{14}-L-arabinose or C^{14}-D-galactose and analysed immediately after incubation in the C^{14}- containing medium and after six and twentyfour hours chase in non-radioactive media. The incubated sections were ground in 80% ethanol to remove unincorporated labels and then submitted to an alkaline fractionation basically as already described.

The ground walls were refluxed with neutral detergent to remove cytoplasmic and pectic materials, followed by 10% KOH at $0^{o}C$ and finally by 10% KOH at room temperature ($22^{o}C$) to remove most of the wall protein along with that hemicellulose-B not extracted at $0^{o}C$. The detergent and KOH extracts (neutralized) were dialysed and an aliquot taken for scintillation counting. The results are tabulated in Table XXVIII and XXIX and drawn graphically in Fig.19.

The distribution of label in the hemicellulose and residue (that is excluding the neutral detergent extract) is shown in Table XXX. The changes are in the same direction as shown in Table XXIX but the differences are accentuated.

There are several changes in labelling that occur during the chase. They are percentagewise

(a) a decrease in neutral detergent soluble label after 6 hours, changing to a slight increase over the 6 hour level after 24 hours

(b) a decrease in $0^{o}C$ 10% KOH soluble label (arabinose and galactose) with time

(c) a slight increase in $22^{o}C$ 10% KOH soluble C^{14}-arabinose but not C^{14}-galactose with time

(d) a slight increase in C^{14}-galactose and a much greater increase in C^{14}-arabinose in the residue.

TABLE XXVIII. INCORPORATION OF C^{14}-L-ARABINOSE AND C^{14}-D-GALACTOSE INTO TOTAL * CELL WALL OF LUPIN HYPOCOTYL

Isotope and Sugar	Time of Analysis**	Total d.p.m. Applied ($X10^{-3}$)	d.p.m. Incorporated ($X10^{-3}$)	Percentage Incorporation
C^{14}-L-Arabinose	1	2,542	74.7	2.94
C^{14}-L-Arabinose	6	2,065	51.9	2.51
C^{14}-L-Arabinose	24	1,709	57.6	3.37
C^{14}-D-Galactose	1	2,077	35.4	1.70
C^{14}-D-Galactose	6	2,194	44.4	2.03
C^{14}-D-Galactose	24	2,111	37.2	1.76

* Includes neutral detergent extractable fraction

** Sections pulsed during first hour only, thereafter chase in cold L-arabinose solution.

TABLE XXIX. INCORPORATION OF C^{14}-L-ARABINOSE AND C^{14}-D-GALACTOSE INTO VARIOUS FRACTIONS OF TOTAL CELL WALLS OF LUPIN HYPOCOTYL

Isotope and Sugar	Time of* Analysis	Neutral Detergent Extract		0°C 10% KOH Extract		22°C 10% KOH Extract		Residue "Cellulose"		Total d.p.m.** ($X10^{-3}$)
		d.p.m. ($X10^{-3}$)	% total	d.p.m. ($X10^{-3}$)	% total	d.p.m. ($X10^{-3}$)	% total	d.p.m. ($X10^{-3}$)	% total	
C^{14}-L-Arabinose	1	14.3	47.9	10.8	36.3	0.54	1.82	4.19	14.0	29.8
C^{14}-L-Arabinose	6	9.56	28.2	8.37	35.4	0.81	3.45	4.85	20.6	23.6
C^{14}-L-Arabinose	24	11.96	36.2	8.07	24.5	1.99	6.04	10.98	33.3	33.0
C^{14}-D-Galactose	1	6.69	41.2	2.13	16.1	0.31	1.92	6.61	40.7	15.7
C^{14}-D-Galactose	6	6.75	34.4	2.77	14.1	0.56	2.88	9.47	48.4	19.6
C^{14}-D-Galactose	24	6.35	38.4	1.67	10.2	0.325	1.96	8.23	49.7	16.6

* Sections pulsed during first hour only. Thereafter chase in non-radioactive L-arabinose and D-galactose solutions.

** All values of d.p.m. per gm. fresh weight of tissue

TABLE XXX. INCORPORATION OF C^{14}-L-ARABINOSE AND C^{14}-D-GALACTOSE INTO NEUTRAL DETERGENT INSOLUBLE FRACTIONS OF LUPIN HYPOCOTYL CELL WALLS

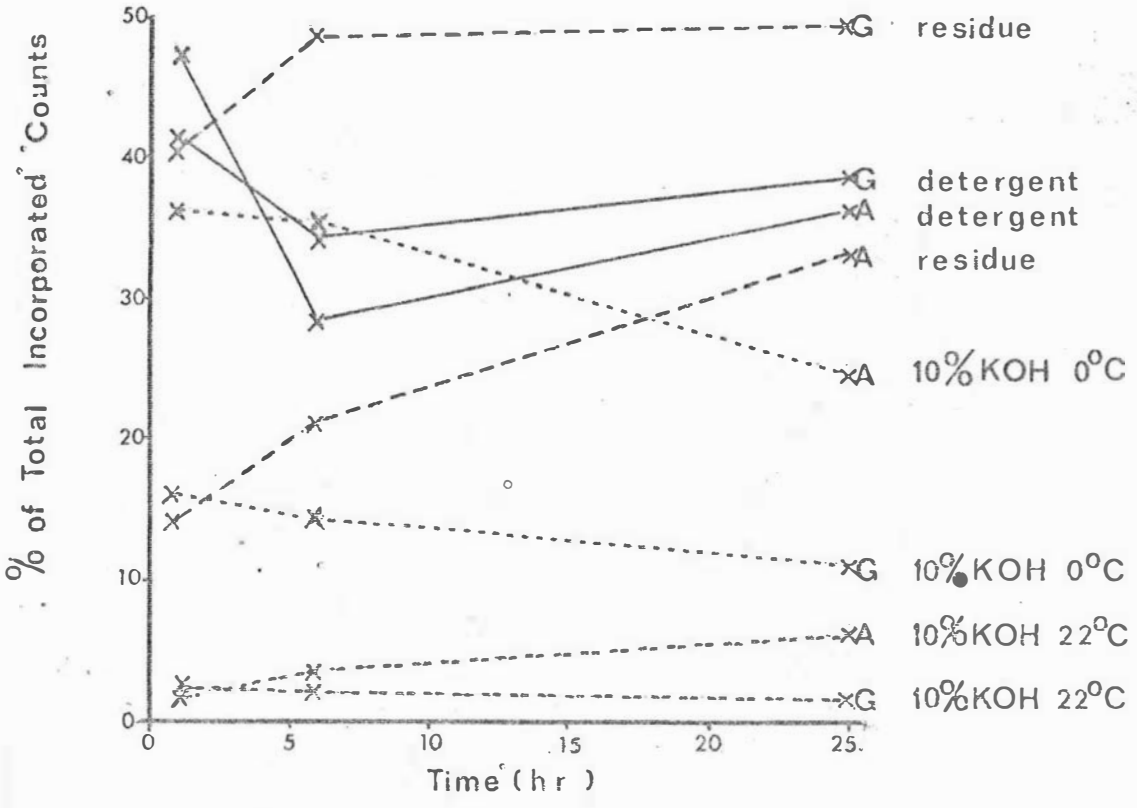
Sugar and Isotope	Time of Analysis*	Incorporation into fractions as a percentage of sum of counts in the three fractions at each time			Sum of counts in the three fractions at each time**
		$0^{o}C$ 10% KOH Extract (HO)	$22^{o}C$ 10% KOH soluble (HRT)	Residue	($X10^{-3}$)
C^{14}-L-Arabinose	1	69.6	3.4	26.9	19.68
C^{14}-L-Arabinose	6	59.6	5.8	34.7	14.34
C^{14}-L-Arabinose	24	38.3	9.5	52.2	17.77
C^{14}-D-Galactose	1	27.5	3.3	69.3	9.83
C^{14}-D-Galactose	6	21.7	4.4	74.0	13.90
C^{14}-D-Galactose	24	16.5	3.17	80.3	10.71

* Time from start of incubation which includes a 1 hour pulse in radioactive sugar

** d.p.m. per gram fresh wgt of tissue

FIG 19

INCORPORATION OF C^{14}-L-ARABINOSE OR C^{14}-D-GALACTOSE INTO FRACTIONS OF LUPIN HYPOCOTYL CELL WALLS



(a) is rather surprising as it suggests that the solubility of some pectic material decreases after six hours but then increases again. This suggests the possibility of linkages forming between the pectic and non-pectic polysaccharides, and, contrary to the results of others from turnover studies (Katz and Ordin, 1967), the passage of label from the hemicellulose to the pectin. The nature of molecules involved if this occurs is of interest. Greater resolution of this fraction could reveal that a pectic polysaccharide is acting as a precursor to a hemicellulose polymer, a situation similar to that already reported by Stoddart and Northcote (1967), while a hemicellulosic polymer is having its linkage to the insoluble portion of the wall cleaved. The former would have a relatively short half life as a precursor. The pulse of label could therefore pass quite quickly into the hemicellulose fraction where a gradual release of the incorporated label occurs.

A decrease in HO C^{14}-arabinose and C^{14}-galactose with time suggests turnover, but into which fraction cannot be determined from these results. Certainly it would be consistent with points raised in the discussion in previous chapters if the label present in the HO became incorporated into HRT with time, as a result of the junction of extensin and arabinogalactan *in vivo*. However the extent of labelling achieved coupled with the heterogeneity observed in the fractions is not sufficient to allow any indication of this to be deduced.

Similarly only small changes are to be seen in the 22°C 10% KOH soluble fraction over 24 hours. If the small increase in this fraction in C^{14}-arabinose derived label compared with

that from C^{14}-galactose is real then it is consistent with the incorporation of extensin into this fraction with arabinose but not galactose attached (Brysk and Chrispeels, 1972), and it also suggests that if such is the origin of the arabinose then incorporation of a pulse of C^{14}-arabinose takes place over a relatively long time period, otherwise the increase in C^{14} in the fraction would be rather abrupt. It is possible that linkage of galactose and not arabinose had occurred within the wall, but by bonds susceptible to 0°C 10% KOH and which once cleaved would release the galactose containing molecule from the extensin complex into the 0°C 10% KOH soluble extract. Unfortunately we do not know the time taken for various molecules to become stabilized within the wall and therefore do not really know how closely a pattern of labelling relates to the distribution of molecules suggested by the fractionation results presented earlier. To determine that larger scale labelling experiments enabling specific activities at various times to be calculated will be required.

There is a prolonged increase in C^{14}-arabinose derived label compared with that from C^{14}-galactose in the 22°C 10% KOH extracted residue ("cellulose"). The galactose C^{14} appears to remain fairly constant in level. This suggests that galactose passes into tightly-bound polymer (possibly galactoglucomannan, which is 10% KOH insoluble) in the 10% KOH insoluble fraction fairly directly or else that the galactose entering equals the galactose leaving. Therefore, if more than one galactose containing polymer exists in this fraction a high degree of turnover (as defined in the introduction) could be occurring. However, if this were the case, one would expect that over a 24

hour period exhaustion of the precursor would result in an overall loss from the fraction, unless there is a contribution from turnover of a number of other fractions within the wall. Some studies have shown that incorporated label tends to accumulate in the less soluble fractions of the wall with time (Katz and Ordin, 1967), whereas one study involving pulse-chase labelling could provide no evidence for breakdown of labelled polymers after incorporation into the wall (Roberts and Butt, 1969). The galactose label in the residue may reside largely in the galactoglucomannan, and if so it would appear to be fairly stable.

The data presented here suggests that although transformations of labelled polymers within the wall may occur, they are either too gradual, or too confined to a small number of molecules within a mixture to be detected with certainty by the methods employed here. Which molecules are involved can only remain speculation until they are positively identified, and it seems that this demands chromatographic and electrophoretic or such methods of high resolution.

There are several aspects of this experiment which make interpretation of the results difficult. Firstly, we do not know the extent to which the fact that the sections are ceasing elongation will effect the results. One might expect there to be relatively little synthesis of extensin in the first hour (hour of incubation) compared with at later times.

There is also the problem of sugar interconversions (Hassid, 1967). The galactose glucose transformation is likely to be the most important in this case and has probably resulted in much non-galactose label in the C^{14}-galactose incubated sections.

The C^{14} in the residue is particularly high in this case. That it is also high in the "cellulose" fraction from the C^{14}-L-arabinose incubations does however suggest that some of this label could be attached to fragments of extensin remaining with the cellulose. Amino acid analyses show the presence of tightly bound protein in 10 and 24% KOH extracted cell walls. Much of the label in the neutral detergent fractions may also have been derived from cytoplasmic molecules metabolically related to C^{14}-glucose derived from C^{14}-galactose. Few such molecules (lipids, proteins, etc.) are not in some way related to glucose.

A very serious limitation in this experiment, which casts doubt on the worth of the data presented is the presence of considerable variation in the total counts recovered. This is unlikely to be due to variation in uptake or to respiratory degradation of the label. The most likely point of loss would be in the dialysis. It is particularly noticeable in the 6 hour arabinose analysis, where there is an inconsistent drop in the neutral detergent label. Otherwise the labelling patterns in the arabinose incubations are fairly consistent.

Clearly there is much improvement required in this experiment including the use of methods of higher resolution than used here or elsewhere in this thesis.

EXPERIMENTAL

(a) $Ba(OH)_2$ extraction

Tissue. Hypocotyls were grown under the conditions described in Chapter I until 4 cm in height. The upper 0.5 cm were excised and floated on Tris-maleate buffer pH6.1, 0.02 M

in a petri dish for 30 minutes. Prior to incubation sections were removed from the dish and gently blotted dry with tissue paper before weighing.

Radioisotopes

Incubation. Groups of about 20 sections were weighed and placed in small petri dishes under the conditions in which the hypocotyls were grown, along with a medium consisting of distilled water (4.0 ml), tris-maleate (0.5 ml, pH6.1, 0.2M) and C^{14}-L-arabinose (0.5 ml, 10 µc/ml). The dishes were gently agitated with a slowly rotating reciprocating shaker during the incubation, which lasted 1 hour 45 minutes. At the end of this time half the groups of sections were filtered on a wad of glass wool and washed with distilled water. Three lots of sections were then dropped into hot ethanol to kill them while the remaining three groups were given a chase incubation in cold arabinose (0.02 M).

Chase incubations were carried out under the same conditions as the labelling, as follows. The rinsed sections were placed in dishes containing 20 ml of 0.05 M arabinose solution 0.02 M in tris-maleate pH 6.1 and agitated as before. The solution was renewed after 5, 10, 20, 45 and 90 minutes and the incubation allowed to continue for 5 hours. At the end of this time the sections were drained and dropped into hot ethanol to kill them.

(b) 10% KOH extraction

Tissue. Hypocotyls were grown as before but until about 6 cm in length. The top 1 cm were excised and pooled on 0.025 M phosphate buffer pH 6.3 for about 30 minutes.

Incubation. Groups of 20 - 30 sections were blotted dry and weighed prior to placing in small flasks for incubation. The incubating medium consisted of 5 ml of 0.025 M phosphate buffer pH 6.3 containing either 1 μc of C^{14}-L-arabinose or 0.5 μc of C^{14}-D-galactose. The incubations were allowed to proceed for 1 hour. At the end of this time groups of sections were drained, rinsed, and either killed by putting into hot ethanol or given a chase incubation for 6 or 24 hours in 25 ml of the same phosphate buffer pH 6.3 but containing 2% sucrose. The non-radioactive media were changed after 10, 20 and 30 minutes and then half hourly three times. After 6 and 24 hours groups of sections were killed by putting them into hot ethanol.

Extractions. To minimize error due to losses during extraction the quantity of walls to be extracted was increased by the addition of ten times the quantity of similar but unlabelled walls.

(a) $Ba(OH)_2$ extraction.

Sections were ground in a pestle and mortar and then washed for 3 hours in 6 changes of 25 ml of 80% ethanol. At this and subsequent stages in the processing of the walls they were collected on a filter pad by millipore filtration. The $0^{o}C$ 10% KOH extraction was performed by stirring the walls in 20 ml of 10% KOH (precooled to $0^{o}C$) in a nitrogen flushed stoppered flask standing on ice in a cold room. After 14 hour the contents of the flask were filtered on Whatman No.1 filter paper in the cold room and the supernatant neutralized slowly with 50% acetic acid. The residue was re-extracted at $22^{o}C$ for 18 hours and the extract similarly neutralized. Both extracts were dialysed overnight. The residue was hydrolysed

with 72% H_2SO_4 in the same way as the $Ba(OH)_2$ treated residue (part i).

Scintillation Counting

A 1 ml aliquot of each of the aqueous extracts was placed in 10 ml of scintillation fluid and counted on a Packard Tri-Carb model 2009 Liquid Scintillation Spectrometer. The scintillation fluid used consisted of Toluene:Triton X-100 (2:1) in which the toluene contained 0.4% (w/v) PPO and 0.1% (w/v) POPOP.

APPENDIX B

HYDROLYTIC ACTIVITY IN LUPIN HYPOCOTYL CELL WALLS

INTRODUCTION: Central to certain theories of cell wall elongation is the idea of hydrolytic activity within the wall rendering it extensible through the cleavage of polymer chains (Matchett and Nance, 1962; Fan and MacLachlan, 1966; Masuda, 1968). There is now little doubt that polysaccharidases exist within the wall, and it has been claimed (Masuda and Wada, 1967) that exogenous application of such enzymes will result in loosening of walls to which they are applied, although one cannot claim that the latter point is very relevant to the *in vivo* situation.

If hydrolysis of wall components occurs it is of interest to know whether any specific polymers are cleaved. To this end a variety of hydrolytic activities were assayed on a number of polysaccharides, and cell wall fractions and the hydrolytic activity of fresh cell walls was also tested.

The results are reported below.

RESULTS AND DISCUSSION

To gain an idea of some of the hydrolytic activities present in the cell wall a variety of substrates were incubated with cell walls obtained from grinding whole young hypocotyls in ice cold buffer. The carbohydrate substrates are listed in Table XXXI and include fractions isolated from the lupin hypocotyl. It was of interest to see whether or not any of these fractions were particularly susceptible to

TABLE XXXI. POLYSACCHARIDASE ACTIVITY OF LUPIN HYPOCOTYL CELL WALL PREPARATION

Substrate, and principle linkage of same	Activity*
Cell wall (autolysis)	-
Cell wall boiled	-
Arabinoxylan-B	-
Hemicellulose-A	-
Hemicellulose-B_{10}**	-
Hemicellulose-B_{24}**	-
24% KOH insoluble wall	-
cellobiose (1-4)	++++
lichenin (1-4)(1-3)	++
laminarin (1-3)	++++
starch (1-6)	+
sucrose (1-4)	++++
cellulose (1-4)	-

* Activity measured from intensity of monosaccharide spots vizualized on the chromatography paper after reaction with alkaline Ag NO_3

** B_{10} and B_{24} refer to hemicellulose-B extracted with 10% KOH and 24% KOH respectively

the hydrolytic activity of hypocotyl wall preparation as such fractions could thus contain polymer closely involved in determining wall plasticity. The results are shown in Table XXXI.

In case proteolytic activity was present and acted on the protein component of the cell wall protein-carbohydrate complex, the activity of cell wall preparations on the general proteolytic substrate Azocoll was tested. This is a particulate substrate and is a conjugate of collagen and a dye. It is fortuitous that the wall protein appears to be similar to collagen in both structure and function. The proteolytic activity is determined by measuring the intensity of colour due to dye released into solution from the particles of Azocoll. Because such a substrate is particulate it would possibly not have access to wall proteolytic enzymes which would in effect also be particle bound. Therefore the wall preparation was degraded with a potent cellulase and a pectinase, and a mixture of the two, prior to incubation with Azocoll.

The results indicate that while substrates of short chain length and (1-4) and (1-3) linkage are susceptible, there is very little attack on the fractions from the wall. It is perhaps relevant that these fractions have been thoroughly dialysed and are probably free of any short chain material. Thus if the degree of activity present depends on the number of chain endings such materials as cellobiose (2 monosaccharide units), laminarin (20 monosaccharide units) will yield a large

amount of monosaccharide. In this context it is also relevant that the cell walls are reported to contain exo- as well as endo-polysaccharidase (Katz and Ordin, 1967), although it is difficult to reconcile the activity of the former with the suggested action of such enzymes in chain splitting during cell elongation. However if their action in shortening polysaccharide chains also reduces the hydrogen bonding capacity and thus reduces the number of junction zones possible for gel formation in the wall then they can conceivably have a role in determining the wall properties directly through their action on wall polymers. It does not seem likely that they are directly involved in the rapid response to auxin or low pH. Their precise function in wall metabolism is obscure and an important subject to be investigated.

Masuda and Wada (1967) were able to induce cell wall elongation by exogenous application of exo-β-(1-3) glucan hydrolase. Nevins (1972) has found that auxin application will lead to release of glucose from the all of Avena coleoptile and loss of a hemicellulose glucan, which seems to indicate an exo-glucanase activity which is particularly susceptible to auxin action. An exo-β-(1-4)-glucanase activity has been reported from Avena coleoptiles by Heyn (1969) and autolysis of the cell wall has for some time been known (Lee, Kivilaan and Bandurski, 1967; Lamport, 1970).

More definite results may possibly be obtained if the wall preparation is incubated for a longer time with the substrate. Certainly in vitro wall autolysis is very slow where it has been detected, and will probably give results more indicative of the in vivo situation than can be obtained by application of

exogenous substrates to wall preparations. However, had differential susceptibility to wall activity been detected the indications would have been worth investigating further.

Table XXXII shows that there was no indication of any proteolytic activity in the wall. Although cellulase plus cell wall did give a rather higher reading than the cellulase alone the fact that the effect on Azocoll of walls both boiled and unboiled prior to cellulase treatment suggests that a turbidity due to small fragments of the degraded walls was responsible for the higher readings.

It is perhaps not to be expected that protease is active in controlling wall properties, at least by hydrolysis of extensin. Extensin becomes increasingly important in quantity as the elongation slows (Cleland and Karlsnes, 1969). It would certainly be a wasteful process if this were a reflection on waning protease activity. Furthermore the high degree of cross-linking to polysaccharide would probably render extensin somewhat resistant to proteolysis. One could postulate that by varying the degree of cross-linking the extent of cleavage of the extensin-polysaccharide complex is controlled.

Perhaps a more reasonable hypothesis is that cross-linkage within the extensin complex sterically hinders polysaccharidase activity. In this case extensin may in fact contribute to the properties of the wall not only by virtue of the strength of the polypeptide chain but also by preventing the breakdown of carbohydrates and resulting wall loosening.

TABLE XXXII. PROTEOLYTIC ACTIVITY OF LUPIN HYPOCOTYL CELL WALLS AFTER VARIOUS TREATMENTS*

Tube	Cellulase (Worthington)	Cellulase (Trichoderma)	Pectinase (Sigma)	Cell Wall	Boiled Cell Wall	Reading**
1	+	-	-	-	-	120
2	-	+	-	-	-	1.5
3	-	-	+	-	-	14
4	-	-	-	+	-	5
5	-	+	-	+	-	7
6	+	-	-	+		150
7	-	-	+	+	-	12.5
8	-	+	+	+	-	15
9	-	-	-	-	+	5
10	+	-	-	-	+	150
11	-	-	-	-	-	1.5

* Cell walls pretreated with the enzymes for 4 hours at 30°C

** Spectrophotometer reading at 520 mμ a measure of proteolytic release of dye from collagen-dye complex.

EXPERIMENTAL

Hypocotyls (80 g) 5 cm in length were harvested and ground in iced buffer (Tris-HCl, pH 6.5) with Ballotini beads to give a thick paste. They were washed several times with iced buffer. The beads and walls were separated by slow centrifugation on a layer of carbon tetrachloride. The aqueous phase (containing the cell walls) was removed and spun at 20,000 g for 30 minutes and the cell wall pellet suspended in phosphate buffer pH 6.5 to form a pippetable slurry. Cycloheximide was added to this to a final concentration of 10 mg/ml.

Cell Wall Pretreatments. The cell wall preparation was used as such or after pretreatment of 4 ml aliquots with cellulase (20 mg) or pectinase (10 mg) or a mixture of the two (20 mg cellulase and 10 mg pectinase for 4 hours at 30°C).

Hydrolytic Activity of Cell Walls. (i) Carbohydrates - The specified substrate (5 mg) was put into a small test tube with 1 ml of cell wall preparation and a drop of toluene. The tubes were corked and incubated in darkness at 30°C for 16 hours. At the end of this time aliquots were taken from the tubes and spotted for chromatography.

(ii) Proteolytic substrate - Azocoll (Calbiochem general proteolytic substrate, 20 mg) was placed in a 15 ml conical centrifuge tube with 4 ml of cell wall preparation. In the case of pretreated cell walls the Azocoll was added to the pretreating incubation mixture in its own tube.

Tubes were allowed to incubate at room temperature for 20

minutes with occasional shaking then quickly filtered through a wad of glass wool. The filtrates were read on a Spectra bench spectrophotometer at 520 mμ.

Chromatography

The aliquots from incubation of carbohydrates and wall fractions with the cell wall preparation were spotted onto Whatman 1 mm paper. These were run for 10 hours in Ethyl Acetate:Pyridine:Water (12:5:4) and the monosaccharides vizualized with alkaline silver nitrate.

APPENDIX C

ATTEMPT TO FOLLOW PRODUCTION OF REDUCING END GROUPS WITHIN THE CELL WALL

If, as has been suggested (Matchett and Nance, 1962; Fan and MacLachlan, 1966; Masuda, 1968), cell wall elongation results in increased rupture of wall polysaccharides then it is theoretically possible to detect this by using a technique capable of giving a measure of any increase in end groupings. An attempt was therefore made to measure an increase in reducing sugars after application of IAA to lupin hypocotyl sections, followed by identification of wall fractions and sugars involved.

Sections 1 cm in length were excised from the upper end of 5 cm hypocotyls. These were then divided lengthwise and each half incubated in separate batches in Tris-maleate buffer pH 6.1 for 1 hour. One group was then made to 3×10^{-5} M in IAA and the incubation continued for 1½ hours. The sections were then dropped into 95% ethanol and washed in several changes of 80% ethanol. They were rinsed in absolute ethanol and dried overnight.

The sections were then reacted with 0.3 M Tritiated sodium borohydride at 4°C for 1 hour followed by 30 minutes at room temperature. Reaction was stopped by the addition of 0.02 N. HCl. The sections were rinsed in several changes of 80% ethanol and then fractionated. This involved extraction with 0.5% ammonium oxalate, 10% KOH and 24% KOH. Extracts were dialysed overnight and aliquots taken for scintillation

counting.

Although the sections were fairly heavily labelled there was no significant difference between the plus and minus IAA treatments, either in total label or distribution within the fractions.

At this stage the experiment was discontinued in favour of others in progress. Had differences been apparent the analyses would have been continued and an attempt made to separate and identify the labelled polyols derived from the reduced end groups, and thereby gain some idea of which bonds underwent increased cleavage due to IAA. This could then have lead to identification of the enzymes involved.

It is perhaps not surprising that no overall effect was noted, due to the large background of end groups already within the wall and because although accelerated cleavage might occur there will probably also be an increased rate of bond formation. Thus although the number of free endings may remain constant, the rate of breakage and reformation of bonds, and the rate of wall creep may be increased by IAA.

The experiment could perhaps be extended by reducing the cell wall with cold borohydride before auxin treatment, although the effect of this on the cell metabolism is likely to be damaging. If it is not, then an examination of the effect of borohydride on wall creep where bond formation could not reoccur would be interesting. Similarly, on the off chance that bond formation but not bond cleavage requires the production of ATP, it could be worthwhile to induce sections to elongate and then apply cyanide and look for an increase in end-groups over the non IAA control. This could be done in

conjunction with the cold borohydride reduction mentioned above.

As a means of overcoming the effect of cold borohydride on metabolism one could try surrounding living tissue with borohydride treated walls and then note changes in reducing power of the system with and without IAA. Such experiments however have the severe limitation that the plasmalemma and treated walls are at a greater distance from one another than in the intact plant, and that they are separated by the untreated cell wall which could, due to its proximity to the living cell, act as a sink for any factors responsible for wall plasticization.

The use of protoplasts or of partial protoplasts could overcome this difficulty, e.g. borohydride reduced coleoptile surrounding hemicellulose treated living coleoptile, or reduced coleoptile containing protoplasts within its cylinder. Although the working hypothesis has been that covalent bond cleavage occurs during wall elongation, this is not necessarily valid, as pH induced changes in gel structure could be responsible (Rees, 1969). The early responses to IAA (Penny, 1969; Nissl and Zenk, 1969; Evans and Ray, 1969; De la Fuente and Leopold, 1970) and low pH (Rayle and Cleland, 1970) in Avena coleoptile suggest this possibility. If there is more than one IAA effect action on covalent bonds is more likely to occur in the longer term IAA effect which does not become apparent for over 30 minutes (Baker and Ray, 1965).

APPENDIX D

EFFECT OF PRONASE ON CELL WALL FRACTIONS

In order to explore the associations between extensin and cell wall carbohydrate, walls with and without pronase treatment were submitted to an alkaline fractionation. It was felt that if any carbohydrate component was held into a cell wall fraction through association with the extensin extensive pronase treatment might result in an alteration of the relative amounts of fractions extracted from the wall. For instance, any polysaccharide which is bound through extensin to the arabinoxylan-B might after cleavage of the extensin enter the heteroglycan-B fraction.

As a preliminary investigation the following experiment was done on cell walls from 5 cm hypocotyl which had been ground in buffer and nonidet-90 detergent (Shell), washed in water, and dried after an ethanol rinse. To 200 mg of cell walls and 20 mg of Pronase in a large tube was added 35 ml water, 10 ml 0.05 M phosphate buffer and 5 ml cycloheximide (100 mg/ml). The tube was stoppered and agitated gently for 36 hours at 32°C. A control tube containing all of the components except pronase was similarly treated. The walls were filtered and then fractionated into 0.5% ammonium oxalate, 10% KOH and 24% KOH soluble fractions. Each of these was dialysed 24 hours against tap water and freeze dried and weighed.

No significant differences in weight between the plus and minus pronase treatments was found, possibly because of the

variation in weights of fractions from such a small amount of starting material was too great.

The 10% KOH extract was fractionated into the heteroglycan-B and arabinoxylan-B fractions as described in Chapter I of this thesis, and the latter fraction acid hydrolysed and analysed by quantitative chromatography as also described in Chapter I.

The results are shown in Table XXXIII, where the monosaccharide compositions are given relative to xylose.

Clearly there is a sharp decrease in arabinose (ca.60%) due to pronase treatment and a smaller (20%) decrease in galactose.

It is likely that this reflects the extent of binding of these sugars to the arabinoxylan-B through extensin. The results are consistent with the existence of arabinose and galactose in separate molecules in the complex.

The dialysability of the arabinose suggests the possibility that arabinose oligosaccharides of hydroxyproline are not attached to galactan as suggested by Lamport (1970), and that the galactan is attached to some other part of the molecule.

From a knowledge of the structure of arabinogalactans and of arabinose oligosaccharide side chains of extensin one can account for the relative proportions of arabinose and galactose lost by assuming that much arabinosylhydroxyproline was eliminated along with some arabinogalactan. Furthermore should the bulk of the arabinogalactan or galactan be bound to the remainder of the arabinoxylan-B fraction it will not be

TABLE XXXIII. MONOSACCHARIDE COMPOSITION OF ARABINOXYLAN-B FROM PRONASE TREATED AND NON-TREATED CELL WALLS

Treatment	Monosaccharide			
	Arabinose	Galactose	Glucose	Xylose
-Pronase	0.63	0.48	0.74	1
+Pronase	0.23	0.38	0.71	1

released therefrom by pronase.

The extent of deproteination has not yet been measured but judging from the loss of arabinose was quite extensive. The difference between the + pronase and - pronase treatments gives some indication that the 10% KOH extraction may not have extensively degraded the arabinoxylan-B as the effect of pronase is still very noticeable despite 24 hour dialysis. However, if the specificities of 10% KOH and pronase in peptide bond cleavage differ then the extents of degradation by each will be additive.

This work can possibly be extended and give more clear cut results by pronase treatment of arabinoxylan-B isolated from a larger quantity of hypocotyls than was used here. This used in conjunction with exopolysaccharidases should provide useful information on the interrelationships of polysaccharides and protein in the extensin complex. For instance, treatment of the arabinoxylan-B with specific exopolysaccharidases should give information on which of the molecules are bonded to xylan or extensin. By altering the sequence of use of specific enzymes a picture of wall or of wall fraction structure should be obtained.

APPENDIX E

THE EFFECT OF $\alpha\alpha'$-DIPYRIDYL ON C^{14}-ARABINOSE INCORPORATION INTO THE CELL WALL

Evidence has been provided that covalent cross links between wall polymers can develop within the wall (Northcote, 1967; Rubery and Northcote, 1970; Brysk and Chrispeels, 1972), and it now seems likely that the macromolecular nature of the wall is due largely to bonds formed between constituents after their incorporation.

At the time that this experiment was started it was known that the wall protein extensin contained arabinose and it was thought that through this the protein might be linked to polysaccharides (Lamport, 1970). Possibly this linkage would be to polymers which would before bonding to the protein be soluble in hot water and oxalate, or 10% KOH at 0°C, but through the bonding would become part of the 0°C 10% KOH insoluble wall.

A method to test this was presented in Appendix A where the movement of a pulse of label into the hydroxyproline-rich fraction was followed. This unfortunately has the drawback that until the bond between and label-containing polysaccharide that changes fractions and extensin is actually demonstrated the evidence is only indirect. A more direct approach although still not providing a definite answer is to observe how stopping the formation of a known carbohydrate-protein bond will alter the distribution of pulsed labels in the cell wall. By adding a reagent which prevents formation

of the bond, polysaccharide which normally would appear with the extensin might instead be extracted by more mild conditions.

Such experiments were attempted using the reagent $\alpha\alpha'$-dipyridyl which has been used to prevent the hydroxylation of peptide bound proline to hydroxyproline (Barnett, 1970; Chrispeels, 1972). It was hoped that its effect, due to chelation of ferrous ions, would be specific enough to enable the relationship of the hydroxylation of proline to cell wall biosynthesis to be investigated. Thus it was thought that where $\alpha\alpha'$-dipyridyl was present and the formation of hydroxyproline limited, polysaccharide which might usually become linked to hydroxyproline or arabinosyl-hydroxyproline would be unable to form this association and instead of ultimately requiring 10% KOH at room temperature to extract it would remain soluble in $0^{o}C$ 10% KOH or in pectin solvents.

A comparison of the fate of a pulse of label in the presence or absence of $\alpha\alpha'$-dipyridyl should therefore give an indication of the origin of the hydroxyproline linked polysaccharide. Furthermore, by varying the times of C^{14} pulse and $\alpha\alpha'$-dipyridyl application some indication of whether the polysaccharide must be freshly incorporated or can be already part of the wall to form a bond with extensin can be obtained.

In the few preliminary experiments carried out it was found that the $\alpha\alpha'$-dipyridyl over a range of concentrations had a fairly general inhibiting effect. This is not really surprising when one considers that its action is due to a chelating capacity. Moreover it became apparent from work in

other laboratories (Lamport, 1973; Keegstra et al., 1973) that the hydroxyproline-arabinose was probably not the main point of further linkage of extensin to cell wall polysaccharides.

However, the rationale can be applied to other bonds within the wall if suitable inhibitors of their formation can be found.

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Sometimes he thought sadly to himself, "Why?" and sometimes he thought, "Wherefore?" and sometimes he thought, "Inasmuch as which?" - and sometimes he didn't quite know what he was thinking about.

(Eeyore, in Winnie-the-Pooh
by A.A.Milne, 1926)