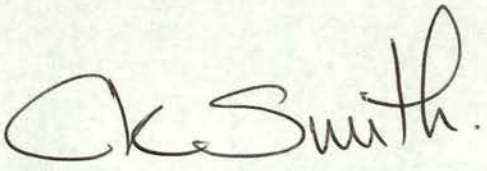
A Naturally Occurring Oligosaccharin Secreted by Rose Cells into their Suspension Culture Medium

Christina Kimmer Smith

A thesis presented in fulfillment of the requirements for the degree of Doctor of Philosophy, The University of Edinburgh, 1998.

Declaration

I hereby declare that this thesis was composed by myself and work contained herein is my own, except where otherwise stated.



Christina Kimmer Smith

Edinburgh, 1998.

Acknowledgements

I would like to thank Steve Fry for all his help, advice and support throughout this project. Also thanks to all the other people in the lab particularly Janice Miller, Joyce Laird and Nancy Steele for all their technical assistance, without which this project would have taken a great deal longer.

Apologies to my flatmate for all she as had to put up with over the years, there are better times to come.

To my parents.

Abstract

In recent years it has become apparent that the plant cell wall can act as a source of signalling molecules. Fragments of the complex polymers which make up the cell wall can be detected by the plant cell and initiate various responses. These range from initiation of defence responses to influencing the action of growth regulators and controlling morphogenesis. At present there is a shortage of information regarding the extent to which such biologically active oligosaccharides, termed oligosaccharins, occur *in vivo*.

To address this issue I analysed spent culture medium from plant cell suspension cultures for the presence of naturally occurring oligosaccharides. A potential oligosaccharin was purified from the spent culture medium of rose (*Rosa* sp.) cells. The oligosaccharide was analysed to determine its structure and bioassays were performed to determine whether it possessed biological activity.

The oligosaccharide was purified by anion exchange chromatography, gel permeation chromatography and HPLC. Its structure was determined by paper chromatography, HPLC and NMR and found to be a trisaccharide: α–D-mannopyranosyl-(1→4)-α-D-glucuronopyranosyl-(1→2)-*myo*-inositol (Man-GlcA-Ins). It accumulated in the culture medium at a steady rate between 6 and 19 days after sub-culturing to reach a concentration of 0.8 μg ml^{-1}. Use of the radiolabelled Man-[^{14}C]GlcA-Ins revealed no turnover of the oligosaccharide by the cells.

Man-GlcA-Ins was found to be able to inhibit the incorporation of L-[U-^{14}C]leucine into acid precipitable proteins in rose cells when presented to the cells at a concentration of 10^{-5} - 1.0 μg ml^{-1}, but was without effect on the uptake of the amino acid. It was also able to induce the formation of an acid resistant product of L-[U-^{14}C]phenylalanine

metabolism in rose cells when presented at a concentration of 10^{-6} - 1.0 μg ml^{-1}. It was found to be without effect on the gibberellin induced elongation of pea stems and on the proteins produced by the rose cells in its presence.

Following the pattern of accumulation of Man-[^{14}C]GlcA-Ins relative to that of the ^{14}C-polymers in the spent culture medium after presentation of D-[6-^{14}C]glucuronic acid suggested that Man-GlcA-Ins was formed by breakdown of an existing precursor molecule rather than by *de novo* synthesis. A possible source of Man-GlcA-Ins is a group of lipids termed the phytoglycolipids.

Abbreviations

Ara	Arabinose
BAW	Butanol : acetic acid : water, (12 : 3 : 5)
d.p.	Degree of polymerisation
Da	Daltons
EAW	Ethyl acetate : acetic acid : water, (1 : 1 : 1)
GA_3	Gibberellic acid
GalA	Galacturonic acid
GAX	Glucuronoarabinoxylans
Glc	Glucose
GlcA	Glucuronic acid
HPLC	High performance liquid chromatography
Ins	Inositol
M2-M7	Maltose oligosaccharides (maltose to maltoheptaose)
Man	Mannose
MES	2-[N-Morpholino]ethanesulphonic acid
Phe	Phenylalanine
PI	Proteinase inhibitor
PIIF	Proteinase inhibitor inducing factor
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
Rha	Rhamnose
R_T	Retention time
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
Tyr	Tyrosine
XET	Xyloglucan endotransglycosylase
Xyl	Xylose

Table of Contents

1. Introduction

The plant cell wall forms the outermost frontier of the cell. Its properties enable it to cope with a multitude of roles and it is able to adapt to ever-changing conditions as the plant develops and encounters its environment. Its roles are structural, providing support and texture to the plant as a whole, as well as to individual organs; it shapes the appearance of the plant and dictates its physical form. It can act as a barrier to pathogens and is able to throw up extra protection when the plant encounters its enemies. It also acts as a conduit for water and solutes as they are transported through the plant and as a food storage area. New evidence from recent years suggests that the cell wall is also a source of signalling molecules. Fragments of its structurally diverse and complex polysaccharides can be detected by the plant cell and are able to initiate a range of responses. In this way the cell wall may not only be the outermost perimeter of the plant cell but also its outermost sensory/signalling area. How this structurally diverse compartment is composed and how it responds to its environment will be dealt with in this review, as will the evidence that the cell wall is indeed a source of signalling molecules and what actions these may exert and how.

1.1 The Primary Cell Wall

The primary cell wall of plants is a complex three dimensional structure composed primarily of polysaccharides. These can be divided into the crystalline phase, composed of cellulose, and the matrix polysaccharides which are interwoven with the crystalline phase. The matrix polysaccharides form a large and very structurally diverse group of compounds. They are often characterised by their extractability from the cell wall; thus pectins are extracted with hot dilute acid or chelating agents. Following depectination the hemicelluloses are extracted with

increasing concentrations of alkali, eventually leaving insoluble cellulose (Brett and Waldron, 1990). In addition to polysaccharides the cell wall also contains an number of structural proteins such as extensins. (For reviews of cell wall structure see; McNeil *et al.*, 1984; Varner and Lin, 1989; Brett and Waldron, 1990; Carpita, 1996).

1.1.1. Components of the Primary Cell Wall

1.1.1.1. Cellulose

Cellulose is the main structural polysaccharide of the primary cell wall and is the main source of mechanical strength in herbaceous plants. It makes up approximately 20-30% of the primary cell wall and is composed of unbranching chains of β-(1→4)-D-glucose with a d.p. of up to 4500 per molecule (McNeil *et al.*, 1984). Its long chains can hydrogen bond together, forming microfibrils. It is the high tensile strength of these microfibrils, laid down in different planes within the cell wall that is the basis of the mechanical strength exhibited by cellulose in the cell wall.

1.1.1.2. Pectin

The pectic polysaccharides consist of homogalacturonan, a polymer of α-(1→4)-D-galacturonic acid residues, which may be esterified with methyl groups. At intervals L-rhamnose residues may interrupt the sequence, as is the case more frequently in rhamnogalacturonan-I (RG-I) (McNeil *et al.*, 1984). RG-I consists of alternating α-2-linked rhamnose and α-4-linked galacturonic acid residues with as many as 300 of each in a molecule. The rhamnose residues may be branched carrying residues of L-arabinose and D-galactose. Rhamnogalacturonan-II is a complex branched polysaccharide containing residues not normally found in other cell wall polysaccharides e.g. apiose and 3-deoxy-D-mannital-octulosonic acid (McNeil *et al.*, 1984).

1.1.1.3. Hemicelluloses

The hemicelluloses are a diverse group of polysaccharides, the most abundant of which is xyloglucan in dicotyledonous plants and xylan in graminaceous plants. Xyloglucan has a backbone of β-(1→4)-D-glucose, bearing on the 6-C position side chains of α-D-xylose, β-D-galactose-(1→2)-α-D-xylosyl; or α-L-fucosyl-(1→2)-β-D-galactosyl-(1→2)-α-D-xylosyl (McNeil *et al.*, 1984; Fry *et al.* 1993). These side chains often occur in repeats forming hepta- or nonasaccharide units. Xylans have a backbone of β-(1→4)-D-xylose with various substituents such as of α-4-*O*-methylglucuronic acid or α-linked arabinose (McNeil *et al.*, 1984).

Other less abundant hemicelluloses include glucuronomannan, the β-(1→3)/β-(1→4) glucans, as well as arabinogalactans, callose, mannans, galactomannans and glucomannans (Brett and Waldron, 1990).

1.1.1.4. Glycoproteins

There are 5 main classes of proteins in the cell wall. The extensins are hydroxyproline rich glycoproteins, characterised by a repeating serine-hydroxyproline$_4$ motif with the hydroxyproline residues bearing between 1 to 4 arabinose residues (Showalter, 1993). There are considerable variations in the structure of extensins from different plants, with different repeating motifs e.g. threonine-hydroxyproline rich glycoproteins in the graminaceous monocot maize (*Zea*).

Glycine rich proteins are found both in the cell wall and in the cytoplasm and have a structure consisting of up to 70% glycine with alanine and serine also present. Proline rich proteins are often characterised by a penta peptide repeat unit of Pro-Pro-X-Y-Lys where X and Y may be valine, tyrosine, glutamic acid, or histidine. The solanaceous lectins are a class of proteins with a structure similar to extensins but with a high carbohydrate content and are characterised by their ability to bind N-acetylglucosamine. The arabinogalactan proteins

are rich in hydroxyproline, serine, alanine, threonine and glycine with side chains of D-galactose and L-arabinose (Showalter, 1993).

1.1.2. Composition of the Primary Cell Wall

Any attempt to describe the way in which the components detailed above come together to form a cell wall can be only a generalisation. The exact composition of the cell wall depends on the species of plant, the type of tissue and which external environmental conditions the plant is and has been exposed to. However the work of Zablackis *et al.,* (1995), who sequentially extracted the cell wall polysaccharides from *Arabidopsis thaliana* leaves, can be used to give an indication of what may be contained in a typical dicotyledonous primary cell wall. They found the cell wall to consist of rhamnogalacturonan-I (11%), rhamnogalacturonan-II (8%), homogalacturonan (23%), xyloglucan (20%), glucuronoarabinoxylan (4%), cellulose (14%) and protein (14%).

However even within a given species this may not be the whole story. Flax (*Linum usitatissimum*) was found to possess differences in cell wall composition within the plant. Cellulose content in the roots and the shoot tip varied from 40% to 16% of the cell wall mass respectively and xylose content varied from 60-70% in the roots to 30% in the leaves (Gorshkova *et al.*, 1996). This means that the type of organ in a plant must be taken into account when describing cell wall composition.

Other differences in cell wall composition may be of developmental origin. *Arabidopsis thaliana* root cell walls contain polysaccharide epitopes, recognised by monoclonal antibodies; the location of these epitopes was found to vary depending on the developmental stage of the root cells (Freshour *et al.*, 1996). Thus an RG-I epitope was found in all cells in the mature root, except for 1 mm at the root tip. An arabinosylated β-(1→6)-galactan epitope was found in the cells of all root tips except those of lateral roots and a fucosylated epitope was found in all cells.

Molecular biology is currently beginning to shed light on the importance of cell wall composition on the ability of a plant to carry out its normal activities. An *Arabidopsis* cell wall mutant, deficient in fucose, has been identified (Reiter *et al.*, 1993). This contains less than 2% of wild type fucose concentrations in the aerial parts of the plant and 40% of wild type fucose levels in the roots. It is distinguishable by its dwarfed growth, reduced apical dominance and shorter petioles (Reiter *et al.*, 1993). Further mutants of *Arabidopsis thaliana* with reduced fucose, rhamnose and arabinose levels have also been identified though none showed obvious phenotypic changes (Reiter *et al.*, 1997).

The extent to which such altered cell wall monosaccharide composition affects the ability of the cell wall components to perform their roles and for the cell wall to maintain its integrity is at the present level of knowledge uncertain. It is possible that biosynthetic pathways are diverse enough to cope and to compensate as has been shown to be the case in fucose deficient mutants. These were found to replace L-fucose with the structurally similar L-galactose and thus still produce polysaccharides able to perform their normal roles (Zablackis *et al.*, 1996). It is also possible that the cell wall through its great structural diversity contains enough redundancy for other components to compensate should one fail. Alternatively it is possible that even small changes have profound effects on the ability of plants to survive and that many cell wall mutations are lethal.

1.1.3. Interactions between Cell Wall Components

The components of the cell wall do not exist in isolation but form complex interactions. Therefore the cell wall should be viewed as an integrated structure and not solely from the point of view of its individual components. It owes many of its properties to interactions between polymers, both of the same and of different classes.

Cellulose is a good example of the way in which polysaccharide molecules can interact. Cellulose chains hydrogen bond together forming microfibrils, which in turn form a strong network providing mechanical support to the cell. In addition, cellulose microfibrils hydrogen bond to the hydroxyl groups on xyloglucan. This can be observed when xyloglucan is added to carboxymethyl cellulose, as an increase in viscosity as the two polysaccharides interact (Hayashi *et al.*, 1987). Further, electron microscopy has revealed that xyloglucan molecules exist either tightly bound to cellulose or span the space between cellulose microfibrils thereby crosslinking and binding the cellulose network (Carpita and Gibeaut, 1993). This means that xyloglucan molecules act as bridges between cellulose fibrils crosslinking them for extra support and making the cellulose network less extensible.

Pectic polysaccharides can form gels through calcium bridges between different chains. Non-esterified pectin chains have a regular "egg-box" structure which in the presence of calcium can join to form blocks or gels, cross-linking the negatively charged strands of the pectin molecules (Jarvis, 1984). Such gels are important in determining the texture of fruit and changes in the degree of esterification and calcium levels may alter the size and abundance of the gels and subsequently alter the texture of an organ, as is the case during fruit ripening (Jarvis, 1984).

Covalent cross-links in the cell wall include extensin cross-links through isodityrosine and di-isodityrosine. Isodityrosine has been proposed to be a covalent crosslink in the cell wall and has been shown to be present at a concentration of 9-half cross links per extensin molecule in potato calli (Fry, 1982). This amount is sufficient to postulate a role for isodityrosine in the crosslinking of cell wall polymers (Fry, 1986b). Di-isodityrosine, consisting of four oxidatively coupled tyrosine residues may also act as a cross link between polypeptides of extensin (Brady *et al.*, 1996).

Ester-links are possible through uronic acids and uronic acids esterified to non-methyl groups have been found, though not identified (Brown and Fry, 1993). Feruloylated polysaccharide residues such as feruloylated arabinose or coumaroylated xylose have the potential to cross-link polysaccharides (Ishii, 1997). This may be through feruloyl esters to sugar hydroxyl groups or to another feruloyl group to form diferulate. This can be demonstrated when water soluble arabinoxylan forms gels in the presence of peroxidase and hydrogen peroxide. Diferulate bonds may effectively stop the growth of the cell wall by rendering it inextensible.

Some generalisation of the way in which cell walls are composed has been proposed by Carpita and Gibeaut (1993). They identify two types of cell walls. In type I walls the cellulose-xyloglucan network is embedded in the matrix molecules and the two are considered to be separate domains. The structural proteins are considered a third and independent domain. In type II cell walls the cellulose network is interlinked by glucurononarabinoxylans (GAXs), with the degree of binding to cellulose being dictated by the frequency of sidechains on the GAX molecule which tends to decrease as cell elongation progresses. Type I and II cell walls predominate in dicotyledonous and monocotyledonous plants respectively.

The primary cell wall is a complex structure both through the diversity of its components as well as through their interaction. It is these factors which allow the cell wall to be adaptable and respond to the demands placed on it by the plant regardless of the species of plant or the conditions in which it exists.

1.2. The Cell Wall Environment

The primary cell wall, which is composed of a network of polymeric material as described in the previous sections, is by no means a static environment. The cell wall is constantly changing as new polymers are

formed, interact with existing ones and are modified as the plant grows, encounters its environment and places new demands on the cell wall.

Bathing the cell wall polymeric network is the apoplastic fluid, which apart from the cells of the root endodermis, is a system of liquid continuous throughout the plant. In terms of studying the components of apoplastic fluid a common approach has been the use of suspension cultured cells, in which the culture medium acts as an extended apoplast and is easily recoverable in large volumes. In this way the culture medium of tobacco (*Nicotinia tabacum*) was found to contain an arabinoglucuronomannan (Aspinall *et al.*, 1989) and in a more detailed study *Nicotiana plumbaginifolia* culture medium has been found to contain arabinoxyloglucan, galactoglucomannan, arabinogalactan, xylan, arabinoglucuronomannan and galacturonan (Sims and Bacic, 1995). It is possible that these may be artifacts of cells growing in culture but alternatively it is possible that the synthesis and turnover of the cell wall may lead to polysaccharides being present in the apoplast and which are not closely associated with the cell wall. Their function is unknown.

Primary cell wall biosynthesis is an ongoing process in the primary cell wall and can take two forms. Either polysaccharides are synthesised at the cell wall by glycosyltransferases located at the membrane, as is the case with cellulose and callose or alternatively polysaccharides may be formed in the Golgi apparatus and transported to the cell membrane in targeted vesicles (Gibeaut and Carpita, 1994). The exact way in which polysaccharides are then integrated into the cell wall and the extent to which they are further modified is largely unknown.

In the case of xyloglucan an enzyme has been identified which is able to incorporate low molecular weight xyloglucan or xyloglucan oligosaccharides into large molecular weight xyloglucan (Smith and Fry, 1991). Xyloglucan endotransglycosylase (XET) is one way in which a plant may integrate newly synthesised xyloglucan polymers into the existing xyloglucan network (Thompson *et al.*, 1997). It may also be

through XET's ability to cut and reform xyloglucan and hence the xyloglucan-cellulose network that the cell wall is able to creep and gradually extend without loosing its structural integrity as the cell grows. XETs have since been cloned and their lack of homology with the endo-glucanases show them to be a separate class of cell wall enzyme activity (De Silva *et al.*, 1993). A role for XET in growth is supported by the fact that XET activity varies along a pea stem, peaking above the third internode and below the apical region, the regions of fastest growth (Fry *et al.*, 1992). In a later study, De Silva *et al.* (1994) confirmed this finding, by use of antibodies for XET to demonstrate that XET levels are highest in the growing regions of the plant.

Many other enzyme activities have been purified from the cell wall, although this is not necessarily synonymous with enzymes acting *in vivo*. Actual enzymic activity can be demonstrated microscopically, by changes in cell walls such as formation of end plates in xylem vessels, believed to be associated with cellulase activity (Fry, 1995). Alternatively enzyme activity in the cell wall may be demonstrated by the metabolic reactions which are observed, such as crosslinking of cell wall polymers. Isoperoxidase in castor bean (*Ricinus communis*) shows an increase in activity in the presence of an elicitor of lignin biosynthesis (Bruce and West 1989). As lignin is present in the cell wall this would suggest that this was the site of action of the enzyme.

Another source of enzymic alteration of the cell wall is the presence of pathogenic enzymes released as part of the invasion process. For instance *Cladosporium cucumerinum* when growing on cucumber cell walls was found to produce both pectinases and cellulases and was able to utilise the cell walls as its sole carbon source (Skare *et al.*, 1975). During invasion there may be extensive degeneration of the cell wall; cell wall lysing activities have been purified from apoplastic fluid of tomato (*Lycopersicon esculentum*) infected with *Fulvia fulva*, indicating that pathogenic invasion may cause cell wall lysis (Aldington and Fry,

1992). The cell wall is a rich source of enzymes. This means that there is potential for large scale alteration of the cell wall, although where and when many enzymes act is still largely unknown.

The composition of the cell wall may be altered e.g. by the presence of elicitors. On presentation of a fungal elicitor to bean (*Phaseolus vulgaris*) suspension cultures there were rapid changes in the cell wall composition (Bolwell *et al.*, 1985). The hemicellulosic, cellulosic, protein and phenolic components all showed changes. For instance there was loss of glucose from the hemicellulosic fraction with a subsequent change in xylose/glucose ratio and a 22 fold increase in phenolic bound material to that fraction. The cell wall also showed accumulation of cellulose and hydroxyproline.

Cell wall insolubilisation acts as a defense reaction in plants and extensin may accumulate on pathogenic invasion and wounding (Bowles, 1990). Extensin in tomato cell walls is insolubilised by the action of a yeast elicitor and within 1 hour of presentation of elicitor, extensin extractability had decreased by more than half (Brownleader *et al.*, 1997). When tomato cells were presented with either 1 mM H_2O_2 or fungal elicitors there was a decrease in the amount of extractable isodityrosine but an increase in di-isodityrosine, the tetramer of tyrosine, over a period of 2 hours (Brady and Fry, 1997), indicating increased crosslinking of extensin.

Ripening produces perhaps the most dramatic changes in the cell wall. One commonly reported change is the increased extractability of pectic polysaccharides. This may be as a result of breakdown of pectic polysaccharides by endo-polygalacturonases or less commonly by pectins being turned over and new pectin, usually highly esterified, being produced e.g. in apples (Jarvis, 1984). These changes greatly affect the extent to which pectic gels are able to form and hence have considerable effects on the texture of the plant as is clearly noticeable as fruit ripens. Sakurai and Nevins (1997) found that when Avocado (*Persea americana*

Mill) ripened the softening of the fruit was accompanied by an increase in water extractable uronic acids, that fraction increased by 38% on the first day after transfer to a higher temperature to induce ripening.

XET has also been implicated in the changes occurring in the cell wall during fruit development and ripening. Percy *et al.*, (1996) found that the activity of XET varied in apple (*Malus domestica*) and kiwi (*Actinida deliciosa*), with activity being highest approximately 2 weeks after anthesis then decreasing rapidly. However the pattern of XET activity was not found to be consistent for the two fruits and hence it is possible that the action of XET on the cell wall of these two fruits is different. Therefore the role of XET and its effect on the cell wall during ripening is still unclear.

Other environmental factors can also affect cell wall composition, e.g. water stress in wheat (*Triticum aestivum*) coleoptiles led to a decrease in accumulation of cellulose and hemicellulose in the cells whereas other polysaccharides such as β-(1→3),(1→4) glucans and arabinoxylans were unaffected (Wakabayashi, *et al.*, 1997).

These examples illustrate just a few of the events which take place in the cell wall and give an indication of the dynamic nature of this part of the plant. It is in this environment that biologically active fragments of the cell wall are believed to be formed. It is through the diversity of these different processes, which can result in breakdown of the cell wall, and the multitude of polymers on which they can act that the potential for a highly specific signalling process comes about. As the products of a certain set of events such as a pathogenic invasion may by unique, such cell wall fragments if they are detectable by the cell may act as highly specific messengers of the events taking place at the outermost limits of the cell.

1.3. Biologically Active Oligosaccharides

As described in the previous section it is clear that the cell wall of a plant is an active area with many processes and changes ongoing throughout the lifespan of the primary cell wall. An additional role for the cell wall has been proposed in which cell wall polymers are degraded and the resulting fragments detected by the plant and acting as signals for plant responses. In such a scenario the cell wall acts as a source of biologically active oligosaccharides, termed oligosaccharins (Albersheim *et al.* , 1983). Such a role would account for the great structural diversity in the cell wall components, which seems excessive in light of the structural role that the cell wall performs. On the other hand if the cell wall were a source of signalling molecules this diversity would only serve to provide an even more specific set of signals.

One of the first cases in which an oligosaccharide was found to posses signalling ability was in the case of spent culture medium from the plant pathogen *Phytophthora megasperma*. Although free of pathogen, the culture medium was able to elicit the accumulation of the phytoalexin glyceollin in soybean (*Glycine max*) cotyledons (Ayers *et al.*, 1976a). Phytoalexins are an important defence mechanism against pathogenic invasion and early accumulation of phytoalexins may determine the outcome of a pathogenic invasion (for reviews see: Darvill and Albersheim, (1984) & Albersheim and Valent, (1978)). From the spent culture medium of *Phytophthora megasperma* two elicitors were purified and determined to be of carbohydrate nature, predominantly 3-linked glucans (Ayers *et al.*, 1976a).

The origin of these carbohydrate elicitors was narrowed down when purified cell walls from *Phytophthora megasperma* were subjected to heat treatment. One compound which was released by this treatment was found to be structurally similar to the elicitor purified from the spent culture medium (Ayers *et al.*, 1976b and c). When applied to suspension

cultured soybean cells it was found to elicit the accumulation of glyceollin in the cells as well as increase the activity of phenylalanine ammonia-lyase, an enzyme involved in the production of glyceollin (Ebel *et al.*, 1976). The elicitor produced from *Phytophthora megasperma* cell walls was similar in all the races analysed and so it must be assumed that it is not responsible for race-specific defense reactions.

The ability of components of the fungal cell wall to elicit defence reactions in plants is very structure specific. Purified *Phytophthora megasperma* cell walls were subjected to acid hydrolysis and six elicitor inactive and one elicitor active oligosaccharides were produced, all found to consist of 3,6-linked glucopyranosyl residues (Sharp *et al.*, 1984a/b). The biologically active heptasaccharide was the first demonstration of an oligosaccharide acting as a signalling molecule in plants. Chemical synthesis of oligo-β-glucosides showed the following motif as essential in elicitation of phytoalexin accumulation in soybean cotyledons (fig. 3.1.1.):

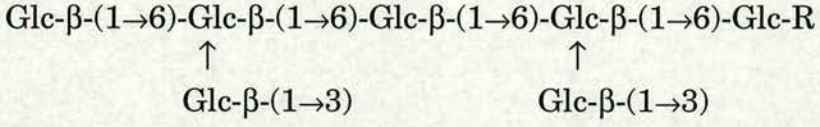


Fig. 1.3.1. Structure of biologically active heptasaccharide (R = reduced residue, in this case glucitol).

There were great differences in the levels of elicitor activity depending on changes in the structure, for instance two branched glucose residues adjacent to each other on the backbone produced a substantial reduction in the response (Cheong *et al.* 1991a).

Many other oligosaccharides derived from the cell walls of fungi have been found to be able to elicit activity in plants: elicitors prepared from stem rust (*Puccinia graminis*) were able to increase PAL activity in wheat (Moerschbacher *et al.* 1989). More recently partial cell wall hydrolysis products from the cell walls of *Vitis vinifera* were shown to be

able to induce an increase in the number of fronds in *Lemna minor* (Campbell *et al.*, 1995), an example of a non-defence related response.

These finding show that the fungal cell wall can provide oligosaccharins which in turn are detected by the plant and can act as signals for activating defence reactions. These oligosaccharides are present *in vivo* (Ayers *et al.,* 1976a) and their activity is very structure specific (Sharp *et al.* , 1984 a/b). It seems logical that plants would have evolved as many possible ways of detecting pathogenic invasion as possible. It is therefore highly plausible that plants should respond to fragments of the cell wall of pathogens as the generation of such fragments is likely during pathogenic invasion. The next question was whether the plant cell wall could also act as a source of oligosaccharin signalling molecules.

When soybean cell walls were subjected to partial acid hydrolysis several elicitors were found amongst the products. As had been the case with the *Phytophthora megasperma* elicitors, these were able to initiate glyceollin accumulation in soybean cotyledons (Hahn *et al.* 1981). Structural analysis of one of the elicitors showed it to be a dodecamer of α-(1,4)-D-galacturonic acid (Nothnagel *et al.* 1983). These findings demonstrated clearly that the plant cell wall is also a source of signalling molecules. This means that the complexity seen in cell wall composition amongst both plants and fungi might allow the cell wall to be a source of latent signalling molecules, which when released by enzymic cleavage of polysaccharides can be detected be the cell and initiate relevant responses.

One common approach to studying the effects of cell wall oligosaccharides which possess biological activity has been the partial hydrolysis of purified cell walls and bioassays of the resulting products (purified or crude). Thus, cell wall fragments from soybean and duckweed (*Lemna gibba*) were found to be able to inhibit flowering and promote

vegetative growth in *Lemna gibba* (Gollin *et al.* 1984). Fragments of strawberry (*Fragaria vesca* L.) cell walls were found to promote differentiation in strawberry calli (Bios, 1992). Acid hydrolysis products of sycamore (*Acer pseudoplantanus*) cell walls were able to affect the growth rate of mung bean (*Vigna radiata*) hypocotyls and wheat coleoptile segments (Mutaftsciev *et al.* 1993). In this case the type of response depended on the concentration of the cell wall fragments being tested. At above 10^{-5} mg ml^{-1} they caused a decrease in the growth rate but below 10^{-6} mg ml^{-1} were able to enhance growth (Mutaftsciev *et al.* 1993). Oligosaccharins derived from sycamore cell walls were able to inhibit the incorporation of L-[U-^{14}C]leucine into acid precipitable proteins without affecting the uptake of the radiolabelled precursor (Yamazaki *et al.*, 1983) and oligogalacturonides also from the cell walls of sycamore were able to induce the accumulation of shikonin - a naphthoquinone pigment - in *Lithospermum erythrorhizon* (Tani *et al.*, 1992).

This body of work demonstrates that the plant cell wall can act as a source of regulatory molecules and that these can act as signals, detected by the plant and used to initiate a variety of responses, ranging from defence related responses to affecting growth and differentiation, (for reviews see: Bishop and Ryan, 1987; Aldington *et al.*, 1991; Aldington and Fry, 1991; Ryan and Farmer, 1991 & Fry *et al.*, 1993). This has led to the concept of the oligosaccharin, a cell wall derived oligosaccharide with the ability to act as a signal to the plant cell (Albersheim *et al.*, 1983).

1.3.1. Hemicellulosic Oligosaccharides

Although the hemicelluloses are a diverse group of polysaccharides, apart from the xyloglucan oligosaccharides it has not yet yielded many oligosaccharins. In promising studies xylanase purified from *Trichoderma viride* grown on xylan was found to be able to induce ethylene biosynthesis and necrosis in tobacco leaves (Bailey *et al.* 1990). As both are responses to pathogenic invasion it was suggested that xylanase

might be producing biologically active oligosaccharides which were subsequently detected by the plant. In addition when birchwood xylan was treated with *T. viride* xylanase and the resulting products analysed they were found to contain both a xylotriose and a xylotetraose fragment (Dean *et al.* 1991). However protoplasts, devoid of a cell wall, exhibited the same response to xylanase as did whole cells (Sharon *et al.*, 1993). Even the possibility that the cells might still contain a small amount of cell wall was ruled out by presentation of cell wall material to the protoplasts which resulted in no overall increase in ethylene production. It must therefore be assumed that xylanase exerts its action in some other way not involving the cell wall or breakdown products of cell wall components.

Other hemicellulosic oligosaccharins include a feruloylated arabinoxylan trisaccharide isolated from the hydrolysate of bamboo shoot cell walls (Ishii and Saka, 1992). It was found to inhibit the effect of auxin on the elongation of excised rice (*Oryza sativa*) lamina joints. Loss of the feruloyl side chain left the oligosaccharide without biological effect which brings into question whether this can be considered an example of a hemicellulosic oligosaccharin.

Galactoglucomannan derived oligosaccharides prepared from poplar (*Populus monilifera*) were found to inhibit the 2,4-dichlorophenoxyacetic acid (2,4-D) stimulated elongation of pea (*Pisum sativum*) stem segments and spruce (*Picea abies*) seedlings (Auxtova, *et al.*, 1995). This response showed another typical oligosaccharin characteristic namely an optimum concentration and very high sensitivity. The oligosaccharins were detected at between 10^{-5} and 10^{-10} M with peas being more sensitive to the oligosaccharides than spruce.

Recently a semi-purified fraction containing xylooligosaccharides isolated from the wood of *Betula platyphylla* was found to cause cell elongation of cells of *Pinus radiata* grown in culture (Ishii and Teasdale,

1997). However, the pure neutral fraction was not found to be active and it is uncertain whether acidic xylooligosaccharides are necessary for activity or whether there are other active components in the test material.

1.3.1.1. Xyloglucan Oligosaccharides

When digested with a glucanase, xyloglucan yields a variety of xyloglucan oligosaccharides. They all possess a β-D-glucose backbone with side chains of α-D-xylose; β-D-galactose and α-L-fucose may also be present (Fry, *et al.*, 1993). This group of oligosaccharides has provided the most detailed information regarding the physiology of the oligosaccharin response and this evidence is the strongest in support of the role of oligosaccharides as signalling molecules.

One xyloglucan fragment (fig. 1.3.1.), a nonasaccharide (XXFG), produced by cellulase digestion of extracellular polysaccharides from suspension cultured sycamore cells was found to be able to inhibit the 2,4-D induced elongation of pea stem segments (York *et al.*, 1984).

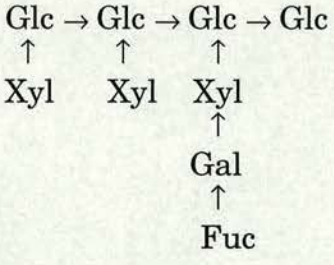


Fig. 1.3.1. Biologically active xyloglucan oligosaccharide; XXFG (Section 1.1.1.3.).

A concentration of 10^{-8} M was found to produce the greatest inhibition with a bell shaped profile of inhibition of elongation relative to concentration of XXFG. This ruled out a simple cytotoxic effect as

inhibition was lost at higher concentrations. The validity of this response is considerable as the work has been independently repeated on several occasions (McDougall and Fry, 1988; McDougall and Fry, 1989 & Hoson and Masuda, 1991).

These findings led to the hypothesis that XXFG acts as a feedback regulator of auxin activity. The low pH optimum endo-glucanases which are induced by auxin may result in xyloglucan hydrolysis and production of XXFG which in turn will inhibit and regulate auxin activity (York *et al.*, 1984).

Auxin is believed to cause cell wall loosening through an efflux of H^+ into the cell wall environment which in turn may activate low pH optimum cellulases. To determine whether the xyloglucan oligosaccharide anti-auxin effect was due to XXFG's ability to block the auxin induced efflux of H^+ the bioassay was repeated at low pH (Lorences *et al.*, 1990). If XXFG were blocking the release of protons, performing the bioassay at a low pH (4.5) would circumvent the action of XXFG; however this was found not to be the case and clearly XXFG exerts its action in another way, which remains unknown.

Another feature of the xyloglucan oligosaccharin response is the great structural specificity required of the oligosaccharin. Loss of the Fuc-Gal moiety from the molecule (leaving the heptasaccharide XXXG) results in the loss of the ability of XXFG to inhibit auxin stimulated pea stem segment elongation (York *et al.*, (1984). FG, a fucosylated pentasaccharide was found to inhibit auxin stimulated growth but free L-fucose or methyl-α-L-fucopyranoside were both without activity (McDougall and Fry, 1989). Loss of the xylose residue on the glucose at the non-reducing terminus was found to have no effect on the inhibitory nature of the molecule, as was reduction of the reducing terminal glucose (Augur *et al.*, 1993).

When fucose deficient mutants of *Arabidopsis* were first discovered it was assumed that they would be unable to produce XXFG. This would imply that the oligosaccharin was not essential for the viability of the plant. It is intersting to note that these mutants have since been shown to replace L-fucose with L-galactose and are a source of a nonasaccharide bearing an L-galactose residue (XXJG rather than XXFG) (Zablackis *et al.,* 1996). This oligosaccharide has almost the same growth regulatory properties as XXFG. Therefore it is not as yet possible to determine the effect of the absence of this oligosaccharin in plants (Fry, 1996).

In a twist to the tale, xyloglucan oligosaccharides were also found to be able to promote growth in certain cases. When pea stem segments were incubated in the presence of a mixture of xyloglucan oligosaccharides, XXXG, XXLG, and XLLG at 10^{-6} M these were found to possess auxin like activity and when tested individually XLLG was found to be the most active (McDougall and Fry 1990). Relative to the inhibition of auxin action, the auxin-mimicking response was found to occur at much higher concentrations and to be less structure specific, without the need for the fucose moiety.

XXFG has also been found to affect another growth regulator namely gibberellic acid. At a concentration optimum of 0.1 nM XXFG inhibited the elongation of etoliated pea shoots in the presence of 10^{-5} M GA_3 (Warneck and Seitz, 1993). The response was measurable with 0.01 nM XXFG, but at higher concentrations (100 nM) the oligosaccharide seemed to promote growth in the GA_3 treated plants. XXFG was also found to have an inhibitory effect on the endogenous growth of the excised pea shoots, with a concentration optimum of 1 nM XXFG but showed no growth promotion at higher concentrations. XXXG was without effect in either bioassay.

In addition to their growth promoting and inhibiting effects, xyloglucan oligosaccharides have been implicated in other effects. XXFG was found to decrease the viability of carrot (*Daucus carota*) regenerating

protoplasts at a concentration of 10^{-9} - 10^{-10} M (Emmerling and Seitz, 1990). XXFG has been found to elicit glycanohydrolase activities associated with the cell wall of bramble (*Rubus fruticosus)* (Joseleau *et al.*, 1992). A mixture of xyloglucan oligosaccharides was found to decrease the number of necrotic lesions caused by tobacco mosaic virus in cucumber (*Cucumis sativus*) cotyledons (Subikova *et al.*, 1994) and XXFG was also found to activate two peroxidase isoforms in pea epicotyls (Warneck *et al.*, 1996).

1.3.2. Pectic Oligosaccharins

Pectic oligosaccharins have proven to be able to initiate many biological effects. Rhamnogalacturonan-II is a source of oligosaccharins which have not been characterised. At a concentration of 200 μg ml^{-1} these were found to be able to inhibit the uptake of L-[U-^{14}C]leucine into tomato cells and incorporation into proteins (Aldington and Fry, 1994). Similar effects were found for proline, arginine, histidine and glutamate, whereas tyrosine and phenylalanine incorporation was slightly enhanced.

A mixture of oligomers (d.p. average 13), produced by digestion of galacturonan with a fungal endo-polygalacturonase (*Aspergillus niger*) were found to be able to inhibit the auxin induced elongation of pea stem segments at a total oligomer concentration of 2.0 x 10^{-4} M (Branca *et al.*, 1988); no attempts were made to purify or structurally characterise the oligomers responsible for activity however. This effect is akin to the action of both xyloglucan and galactoglucomannan oligosaccharides, which are also able to affect auxin induced growth in pea stem segments (Section 1.3.1.). Oligomers of galacturonic acid are able to induce ethylene production in pear (*Pyrus communis*) cell suspension cultures (Campbell and Labavich, 1991). Ethylene acts as a signal of many other plant responses including initiation of defence reactions. Another defence related action attributed to pectic oligosaccharins is the activation of enzymes involved in lignin synthesis and an increase in the overall lignin

content in castor bean (*Ricinus communis*) cells (Bruce and West, 1989). Galacturonan oligosaccharins released from cow pea (*Vigna unguiculata*) pods by the action of *Aspergillus niger* endo-polygalacturonase were able to induce necrosis when subsequently applied to the pods (Cervone *et al.*, 1987).

Through the use of a bioassay involving thin-cell-layer (TCL) explants of tobacco, pectic oligosaccharides have been found to possess the ability to regulate plant development. Endo-polygalacturonase-solubilised oligosaccharides from sycamore cell wall polysaccharides were found to be able to initiate morphogenic effects with respect to formation of root, callus and flower formation in the TCL system (Tran Thanh Van *et al.*, 1985). Subsequent work demonstrated that pectic oligosaccharides were able to inhibit root formation in root inducing media and in transition media pectic fragments at 10 $\mu g\ ml^{-1}$ were able to induce flowering (Eberhard *et al.*, 1989). All effects were dependent on growth factor and oligosaccharide concentrations. Marfa *et al.*, (1991) showed that purified α-(1,4)-linked oligogalacturonides with a d.p. of between 12 and 14 were able to induce flowers on TCL explants and that methyl esterification was not a requirement for activity.

1.3.2.1. Proteinase Inhibitor Inducing Factor

When subjected to mechanical wounding plants produce proteinase inhibiting factors. These prevent digestion of the cell wall by insects through inhibition of digestive enzymes. Infiltration of macerated leaf extract into tomato leaves was found to mimic this response and the search for a signal molecule was on (Green and Ryan, 1972; Ryan, 1974). This led to the purification of a proteinase inhibitor inducing factor (PIIF) from a water soluble extract of tomato leaves (Bishop *et al.*, 1984). The resulting galacturonic acid rich product when subjected to acid hydrolysis produced an α-(1-4)-D-galacturonic acid containing oligosaccharide with a d.p. = ~ 20. This could be further hydrolysed to produce a series of

oligogalacturonides, with the disaccharide α-D-galacturonosyl-(1-4)-D-galacturonic acid still able to induce PI. This is supported by the findings of Moloshok and Ryan (1989) who found that di- and tri-galacturonides, released enzymically from galacturonan, and supplied to the cut petioles of young tomato leaves were able to induce PI. Pectic fragments of mean d.p. = 15 were able to induce PI activity and in addition were found to be able to depolarise membranes in tomato (Thain *et al.*, 1990). This may act as a signalling mechanism to the cell.

Endopolygalacturonase was also found to cause an increase in the accumulation of proteinase inhibitor I in tomato leaves, with the boiled enzyme being inactive (Walker-Simmons, *et al.*, 1984). When polysaccharides were solubilised from the cell wall of sycamore cell suspension cultured cells RG-I but not RG-II or xyloglucan were able to induce accumulation of PI. The compositions of the sycamore RG-I and the tomato PIIF were not identical, with RG-I containing a higher percentage of arabinosyl and galactosyl residues, suggesting that the overall structure is not important to activity (Ryan *et al.*, 1981).

PI was found to accumulate in suspension cultures of tomato cells and coincided with depletion of sucrose in the medium (Walker-Simmons and Ryan, 1986). It accumulated to such an extent that by day 8 after subculturing PI accounted for 2% of the total protein of the cells. A pectic polysaccharide was found to accumulated in the culture medium immediately prior to the PI and that when purified that polysaccharide was able to induce PI activity in tomato cultures.

An oligogalacturonide PIIF of d.p. 20 was found to cause phosphorylation of a 29 and a 34 kDa protein in the plasma membrane of tomato (Farmer *et al.*, 1989). This is strong evidence that PIIF can initiate a signal transduction pathway from outside the cell, across the plasma membrane and into the cell where it can in turn activate synthesis of PI.

1.3.3. Other Oligosaccharins

Chitin fragments are known elicitors of responses in plants. Felix *et al.*, (1993) found that chitin oligomers of d.p. four or more were able to induce rapid alkalinisation of tomato culture medium at 10 mg l^{-1}. Chitosan affects the viability of rice (*Oryza sativa*) suspension cultured cells, causing a browning of the cultures as well as decreased viability when applied to freshly cultured cells (Masuta *et al.*, 1991). The effect was found to be closely linked with the growth state of the cells at the time of addition and 1 day after subculture the cells were found to be unaffected by addition of elicitor.

Feruloylated oligosaccharides are also active. Feruloylated arabinoxylan oligosaccharides (FAXX and FAXXX, a feruloylated tri- and tetrasaccharide respectively of arabinoxylan) were found to be able to inhibit gibberellin induced elongation of the second leaf sheath in dwarf rice at 85 and 69 nmol $plant^{-1}$. The arabinoxylan trisaccharide (AXX) was without activity at the same concentration levels (Ishii and Nishijima, 1995).

Algal laminaran has also been a source of oligosaccharins. A (1,3)/(1,6) mixed linkage linear β-glucan was purified from laminaran from *Eisenia bicyclis* and found to elicit flavanoid accumulation in lucerne (*Medicago sativa*) cotyledons (Kobayashi *et al.*, 1995).

The number of cases of oligosaccharin effects described above lends considerable weight to the idea that oligosaccharins may be important signalling molecules in plants. Just as important as information regarding such effects is an understanding of how such biological activity is brought about.

1.4. Physiology of Oligosaccharins

1.4.1. Generation of Response

Being predominantly covalent in nature cell wall polysaccharides are not readily broken down without the action of enzymes. However the cell wall is known to contain many endo-glucanase activities which means that the potential for formation of oligosaccharides is present (Fry, 1995). In addition there are many reports of fungal plant pathogens producing enzymes capable of lysing cell wall polysaccharides to release oligosaccharides many of which have been shown to be oligosaccharins (Aldington and Fry, 1992).

The source of oligosaccharins is generally believed to be cell wall polymers which is backed up by investigation of the origin of XXFG (McDougall and Fry, 1991). XXFG was found to accumulate in suspension culture medium of spinach following presentation of L-[^{3}H]fucose as were [^{3}H]polysaccharides. McDougall and Fry (1991) concluded that the kinetics of the accumulation of radiolabelled compounds and the lag in the detection of the [^{3}H]oligosaccharide relative to the [^{3}H]polysaccharides suggested that XXFG was produced from breakdown of an existing polysaccharide rather than by *de novo* synthesis.

Whereas XXFG was found to accumulate in healthy cells, when the apoplastic fluid of tomato was extracted from leaves infected with *Fulvia fulva*, it was found to contain proteins which could solubilise material from ^{14}C-labelled plant cell walls (Aldington and Fry, 1992). An incompatible infection yield more cell wall hydrolysing activity but both types of infection were able to release material which when fractionated was found to contain oligosaccharides of dp. 3-15.

Acidic oligosaccharides were found in the honeydew from aphids feeding on sorghum (*Sorghum bicolor*); as aphids are incapable of synthesising uronides, these were believed to be of plant origin

(Campbell, 1986). It is interesting to speculate on whether these may be signals for defence activation as seen in plants infected with sap feeding organisms (Campbell, 1986).

There are many cases of in which fungal cell wall lysing enzymes have been demonstrated to be able to elicit various responses in plants and it may be tempting to draw the conclusion that this is brought about by breakdown of cell wall polysaccharides to oligosaccharides which in turn act as oligosaccharins.

Endopolygalacturonase from *Rhizopus stolonifer* is able to elicit casbene synthase activity in castor beans (*Ricinus communis*). When the activity of the endopolygalacturonase was destroyed through modification of exposed carboxyl groups the elicitation effect was lost as well (Bruce and West 1982). As the modification was very mild, preserving the tertiary structure of the enzyme, it was assumed that the elicitation was a result of the enzyme activity. Bruce and West (1982) tested the ability of this galacturonase to release an elicitor by incubation with castor bean seedling cell walls and found that a heat stable elicitor fragment was released whose activity was lost by further digestion with endopolygalacturonase. This suggests that in this case the enzyme acted through production of oligosaccharins by breakdown of pectic material.

These findings are mirrored by Cervone *et al.* (1987) who found that polygalacturonase from *Aspergillus niger* was able to elicit necrosis in the pods of cow peas (*Vigna unguiculata*) as were the products of endo-polygalacturonase digestion of the pods and of galacturonan. Inactivation of the enzyme by heat resulted in the loss of activity again indicating the need for catalytic activity in the response. Endopolygalacturonic acid lyase purified from *Erwinia carotovora* acted as a heat labile elicitor of phytoalexin accumulation in soybean and solubilised heat stable elicitors with a 90% (w/v) uronosyl content (Davis *et al.*, 1984). Endopolygalacturonase was able to release oligogalacturonides from lemon (*Citrus limon*) seedling cell walls and the enzyme was found to

cause an increase in PAL activity and phytoalexin accumulation when applied to mechanically damaged seedlings (Rocco *et al.*, 1993). Even more specifically pathogenesis related proteins from soybean were found to contain an endo-(1,3)-β-glucanase which released oligosaccharides from laminarin (Ham *et al.*, 1995). These cases would suggest that cell wall lysing enzymes are able to generate oligosaccharins, however as the following cases show this assumption has to be used with caution.

There are cases in which it has been clearly shown that cell wall polysaccharide hydrolysing enzyme activities do not involve oligosaccharin mediated responses. When the action of the fungal enzyme mix Pectolyase (from *Aspergillus japonicus*) and the action of pectic oligosaccharides were compared on the production of ethylene in Valencia oranges (*Citrus sinensis*) both were able to elicit ethylene production (Baldwin, and Biggs, 1988). However the oligosaccharides showed very different patterns of ethylene elicitation. Acid hydrolysed pectic material showed a slower response but one which lasted after the Pectolyase response had started to decline. It may be argued that the use of pectic oligosaccharides and Pectolyase would give different concentrations of oligosaccharins in the plant tissue and hence a different response. Therefore different patterns of ethylene induction could still be taken to imply that the action of Pectolyase was through oligosaccharin production although the data would suggest otherwise.

Pectate lyase from *Aspergillus japonicus* and oligogalacturonides were both able to initiate alkalinisation of the culture medium when applied to suspension cultured tobacco cells and both imposed a refractory state after treatment, a period in which a second treatment failed to elicit a response (Rouet-Mayer *et al.*, 1997). However, treatment of the cells with oligogalacturonides followed by pectate lyase resulted in a second response, unlike what a second treatment of oligosaccharides would have. The same was observed when pectic lyase treatments were

followed by oligosaccharide treatments. These findings meant that the action of the enzyme and the oligosaccharides must be different and that the enzyme exerts its action in a way other than by production of oligosaccharins.

Strong evidence that fungal enzymes may act through production of oligosaccharins is the finding that a *Phaseolus vulgaris* protein can modulate the activity of an endopolygalacturonase from *Aspergillus niger* (Cervone *et al.*, 1989). When the polygalacturonase is incubated in the presence of galacturonan and endopolygalacturonase inhibiting protein (PGIP) the digestion products were larger, more elicitor-active and persisted for longer than in the absence of the PGIP, in which case the products were quickly converted to short oligosaccharides which were elicitor inactive. The effect was not confined to *Aspergillus* endo-polygalacturonase but was also seen with the *Fusarium moniliforme* enzyme. This suggests that plants are not only able to detect the by-products of pathogenic invasion but are able to modulate the process involved in pathogenicity to provide increased sensitivity to infection. This is certainly strong evidence for a role for oligosaccharins.

As much of the work done on oligosaccharins has involved the use of oligosaccharides prepared *in vitro* there is little evidence regarding the presence of oligosaccharins *in vivo* and much less regarding they way in which they may be formed. At present much of our understanding of the way in which oligosaccharides may be produced *in vivo* is through knowledge that the potential for their formation is present in the right location at the right time.

1.4.2. Signal Pathways

Many oligosaccharin mediated effects result in changes in cellular metabolism. This implies that a signal is transmitted from the cell wall environment, in which the oligosaccharin is generated, and into the cell where it can result in number of responses.

One frequent response of plants to oligosaccharins is the induction of enzymes. *Erwinia carotovora* was found to cause increased chitinase activity when inoculated into wounded tobacco as were pectic oligosaccharins (Broekaert and Peumans, 1988). The response was shown to involve *de novo* synthesis of the enzyme which means that oligosaccharins are able to regulate protein synthesis. This also meant that the oligosaccharin signal was transmitted from outside the cell, across the cell membrane and into the cell.

It has been suggested that oligosaccharins are able to bind to membrane receptors as demonstrated by Schmidt and Ebel (1987). They found fungal β-glucans of a d.p. of 20-25, which as well as being able to elicit phytoalexin accumulation were able to bind to membranes purified from soybean, with the highest binding seen in roots. The binding was structurally specific and the radioactive ligand was displaced in the presence of excess un-labelled ligand. Only other β-linked glucans were able to displace the ligand.

A study by Yoshikawa *et al.*, (1983) found that mycolaminaran, an inducer of phytoalexin synthesis in soybeans, was able to bind to membrane fractions of soybean and found to be associated with the plant plasma membrane fraction. The binding site was found to be heat labile and susceptible to incubation with proteolytic enzymes. No other tested carbohydrates were able to compete for binding.

Cheong and Hahn (1991) found that a radio-iodinated hepta-β-glucoside-tyramine conjugate could be detected bound to membranes from soybean. The highest affinity was found for membranes in the roots as had been the case with other fungal elicitors. Binding was inactivated by treatment of the membranes with proteases indicating that the ligand was of protein nature and the oligosaccharide bound to the membranes at elicitor concentrations. Again the binding was structure specific with oligosaccharides which were structurally similar to

the glucoside being able to compete with the radiolabelled oligosaccharide to a greater degree than oligosaccharides with a different spatial arrangement or n-acetyl substituted.

Similarly a glycoprotein isolated from the germ-tube walls of *Puccinia graminis* and which is an elicitor of lignification and PAL induction in wheat (*Triticum aestivum*) was found to bind to membrane preparations from wheat (Kogel *et al.*, 1991). The use of radiolabelled elicitor and non-radiolabelled elicitor showed that there was competition for binding in the presence of unlabelled elicitor and the number of binding sites was estimated at 250 pmol per mg of plasma membrane protein. Cosio *et al.*, (1990) solubilised a protein of approximate Mr = 300 000 from soybean membranes which retained its β-glucan binding activity. Together these studies provide compelling evidence that oligosaccharins can be detected by cell membrane receptors which is a common way in which signals may cross the cell membrane if the actual signal molecule is not transported across the plasma membrane. This is not to say that all oligosaccharin responses may be translocated in this fashion, but the specificity often seen with receptor binding does compare well with the structural specificity which has been seen to be a requirement in many oligosaccharin responses.

An alternative signal pathway to receptor binding is influx of calcium as following addition of a fungal preparation from *Fusarium oxysporum* to larch (*Larix decidua*) cell cultures (Bach and Seitz, 1997). The fungal preparation also induced PAL activity and lignin deposition. Elicitor preparations from two fungal pathogens *Colletotrichum lagenarium* and *Peronospora parasitica* were found to be able to depolarise membranes of melon (*Cucumis melo*) and maize (*Zea mays*) roots in a concentration dependent manner with an optimum concentration of ~ 100 μg glucose equivalent ml^{-1} (Pelissier *et al.*, 1986). The depolarisation was sustained for as long as the elicitor was present

but was reversible on its removal. Oligogalacturonides have been shown to elicit an increase in cystolic free calcium in carrot cells *(Daucus carota)*, with the greatest increases at the periphery of the cell (Messiaen *et al.*, 1993). These mechanism may constitute alternative signal pathways to membrane receptor binding.

As many transmembrane receptor molecules possess protein kinase activities, a secondary effect following binding of a ligand to a receptor is the phosphorylation or de-phosphorylation of proteins within the cell. This in turn may be the start of a signalling pathway or cascade leading to control of transcription. An elicitor from *Phytophthora megasperma*, known to elicit phytoalexin accumulation in soybean, was presented to soybean cells which had been fed [^{32}P]orthophosphate and the changes in phosphorylation of proteins was determined by 2 dimensional SDS-PAGE (Grab *et al.*, 1989). Several proteins were found to change phosphorylation state; in particular a protein with a Mr of 69000 was found to be dephosphorylated. The activity of the effector was reduced by treatment with pectinase, cellulase or alkaline phosphatase.

Oligogalacturonides were found to activate phosphorylation of threonine residues in a 34 kDa plasma membrane protein present in both tomato and potato (Farmer *et al.*, 1991). An elicitor derived from the cell walls of *Phytophthora megasperma* was found to initiate phosphorylation of a 45 kDa protein within one minute of addition of the elicitor and continued phosphorylation of the protein was dependent on the continued presence of the elicitor (Dietrich *et al.*, 1990).

There are many examples of the ability of oligosaccharins and elicitors to induce enzyme production. An elicitor released from the cell walls of *Phytophthora megasperma* was able to cause an increase in the accumulation of ACC synthase (the first enzyme in the ethylene biosynthetic pathway) in parsley cell cultures (*Petroselinum hortense*) (Chappell *et al.*, 1984a).

Chappell and Hahlbrock (1984b) showed that cell cultures when exposed to a fungal elicitor showed increased messenger RNA accumulation of the enzymes involved in the phenylpropanoid and furanocoumarin pathway. This means that an elicitor prepared from the cell walls of *Phytophthora megasperma* is able to affect transcription of genes. No attempts were made to purify the elicitor and hence it is not certain that it is indeed an oligosaccharide. Using an elicitor from *Colletotrichum lindemuthianum* Cramer *et al.*, (1985) were able to demonstrate induction of messenger RNA for phenylalanine ammonia lyase, chalcone synthase and chalcone isomerase, all enzymes of the phenylpropanoid pathway.

When a fungal glucan preparation from *Phytophthora megasperma* was sprayed on to tobacco and the plants subsequently assayed for the presence of mRNA encoding plant defence genes, it was found that the gene encoding a glycine rich protein had been activated with levels increasing nine fold by 4 hours after application of the glucan preparation (Brady *et al.*, 1993). Roby *et al.*, (1991) found that elicitors from *Colletotrichum lagenarium* were able to induce transcription of a chitinase gene as was the ethylene precursor ACC.

Taken together these findings indicate that oligosaccharins present in the cell wall environment can be detected by the cell, possibly by receptor molecules, though other signal mechanisms exist. This in turn can lead to changes in cellular metabolism including transcription of genes and for example production of enzymes.

1.4.4. Transport

In many cases when an oligosaccharin signal is detected by the plant it may be advantageous to the plant if the signal acts throughout the whole plant and this may come about by translocation of the signal molecule. There are exceptions to this; for instance it would not be desirable for a necrosis inducing signal to be widely translocated, since it

is only required in the immediate neighbourhood of infection. The extent to which a signal is transmitted about the plant may depend on the mobility of the signal and the speed at which it is broken down. Thus, although a signal might travel about the plant, if it were turned over very quickly this would limit its spread.

Wounded plants produce a proteinase inhibitor inducing factor (PIIF). Baydoun and Fry (1985) tested the ability of pectic oligosaccharides to move away from the site at which they were applied through a cut in the leaf. When ^{3}H-pectic oligosaccharides (d.p. 6) were presented at the wound site of tomato leaves along with [^{14}C]sucrose there was found to be little movement of tritiated material into the plant, less than 0.1% of that applied as compared with ~10% of sucrose. This suggests that in translocation of the proteinase inhibitor inducing factor the pectic oligosaccharides may be a primary signal and it is a secondary signal which is responsible for translocation of the response.

Warneck *et al.*, (1998) found that [^{3}H]XXFGol travelled acropetally in excised pea shoots with radioactivity detectable in the plumule following application in medium at the cut end of the stem. When analysed some of this radioactive material was found to have been degraded to glycitol but with [^{3}H]XXFGol also detectable. This means that a biologically active oligosaccharide is able to move through a plant and shows the possibility of translocation of an oligosaccharin signal. As with our knowledge concerning the occurrence of oligosaccharins *in vivo* there is very limited information regarding the way in which oligosaccharins may be translocated throughout a plant. Although there is the potential for molecules to be transported throughout a plant by way of the phloem system and the translocation stream, our knowledge of the way in which for instance phloem elements are loaded makes it uncertain whether oligosaccharins, which are sometimes of considerable size, can enter the translocation pathways.

1.4.5. Breakdown

Just as important as the ability of oligosaccharides to act as biological signalling molecules is the ability of these molecules to be broken down when their usefulness has been exceeded. If this does not happen it means that the plant will continually perceive the signal relayed by the oligosaccharin and will not be able to return to its native state, ready for a new signal. XXFG is a biologically active oligosaccharide which has provided a great deal of insight into the oligosaccharin physiology. It also illustrated one way in which plants may deal with oligosaccharins. When [^{3}H]XXFG was presented to rapidly growing spinach (*Spinacia*) cells the oligosaccharide was found to become incorporated into polymer (by the action of XET) (Baydoun and Fry, 1989). After 48 hours, 13% of the oligosaccharide was found to have been incorporated into polymeric material, as determined by changing retention time during gel permeation chromatography.

Cline and Albersheim (1981) showed that a mixture of cell wall enzymes extracted from the cell walls of soybean was able to decrease the glyceollin inducing activity of a high molecular weight (> 100 000) glucan elicitor extracted from *Phytophthora megasperma* by as much as 85%. Gel permeation chromatography of the resulting elicitor fraction showed it to have molecular weight range of 1000 - 100 000 with only 4% greater than 100 000.

A more short-term way of dealing with an oligosaccharin signal may be through insensitivity. Campbell and Labavich (1991) found that when cultured pear cells were presented with pectic oligomers there was a period of insensitivity to a second addition of oligosaccharides within 90 minutes of the first elicitation. This may prevent overloading a signal pathway before the oligosaccharin can be broken down.

The ability of XXFG to inhibit auxin induced elongation of pea stems is lost on cleavage of the terminal fucose residue. Augur *et al.*

(1992) found xyloglucan α-fucosidase activity could be solubilised from pea epicotyls and this was able to release the fucose residue from XXFG. The specific activity of this enzyme was largely unchanged in the presence of auxin which is known to increase the activity of xyloglucan degrading glucanases and the authors concluded that the fucosidase was expressed constitutively.

Once again we are faced with limited data to explain what may happen to oligosaccharins once their usefulness has been outlived. However it is clear that it is essential that an oligosaccharin must be broken down to maintain the ability of the signal pathway to respond to another signal at a later time and as described several mechanisms do exist.

1.5. Natural Occurrence

There is not much evidence for the presence of oligosaccharins *in vivo* and this is an area of research which requires more effort to validate the oligosaccharin concept. Despite the large body of evidence showing the physiological effects of oligosaccharides studies mean little without supporting evidence showing that oligosaccharins are present *in vivo*.

The apoplast is the area of the plant in which plant cells will first come into contact with a pathogen and pathogenic products. It is possible to extract the apoplastic fluid from whole plants and analyse it. In this fashion the apoplastic fluid from tomato was extracted after infection with *Fulvia fulva* and race-specific elicitors of necrosis were purified (De Wit *et al.*, 1988). Aldington and Fry (1992) showed that the apoplast contained cell wall lysing activity following infection of tomato with *Fulvia fulva*.

Brecht and Huber (1988) found that when enzymically active cell walls from ripe tomatoes were extracted a pectic fraction was released which when vacuum infiltrated into pre-climacteric tomatoes caused an

in increase in ethylene release by the fruit. The released pectic fraction was fractionated into oligomers on Bio-Gel P-2 and the d.p. >8 and 4-6 pools were both active. This is compelling evidence for the involvement of oligosaccharins in fruit ripening as it demonstrates both activity and presence.

With the many biological activities assigned to the oligogalacturonides detection of these oligosaccharins *in vivo* is very relevant. Garcia-Romera and Fry (1997) were unable to detect oligogalacturonides (limit of quantitation = 50 µg/l) when the culture medium from rose cells was analysed after presentation of D-[6-^{14}C]glucuronic acid to the cells despite the incorporation of radiolabel into polymeric anionic material. They also failed to detect the accumulation of ^{14}C-galacturonic acid, the product of oligogalacturonide turnover. This means that the exo-polygalacturonase enzymes detected in the spent culture medium and the cell wall are not acting on the galacturonan in the cell wall.

Schroder and Knoop (1995) found that tobacco culture medium contained a viability factor which affected the viability of tobacco cells, though they failed to determine its structure. Mature green tomato pericarps were found to contain a series of galacturonic acid containing oligomers similar to the molecules generated when citrus pectin was acid hydrolysed (Melotto *et al.*, 1994). These were water soluble and extracted by ethanol precipitation. Individual peaks were bioassayed and found to elicit ethylene production when applied to tomato pericarp discs. Structural analysis found these compound to contain material with a neutral sugar content and so could not be considered to be purely a series of oligogalacturonides.

Medium from 7-9 day old cultured *Zinnia elegans* mesophyll cells when applied to freshly isolated cells in culture was found to have a profound effect on the size of the tracheary elements with cell are a

varying from between 1000 - 2000 μm^2 in the controls to 7000 μm^2 in the treated cells (Roberts *et al.,* 1997). They proposed that an oligosaccharide able to bind to Concanavalin A and of less than 1 kDa was responsible for this activity.

Zabotina *et al.*, (1995) found that sap produced by buffer solubilisation of pea shoots contained oligosaccharides able to inhibit auxin induced elongation of pea stem segments as well as containing the major xyloglucan oligosaccharide composition of glucose, xylose and fucose. A soluble oligosaccharide fraction from pea stems was also found to be able to control root development in a buckwheat thin cell layer system and to exhibit anti auxin activity with loss activity through cellulase digestion (Zabotina *et al.*, 1996). Although these findings strongly suggest that oligosaccharins are present in the pea shoots no conclusions can be drawn as to the location of these oligosaccharins, they may be derived from the cell wall environment or the cytoplasm.

UV irradiation can be used to model the induction of phytoalexins normally associated with the initiation of defence reactions. When red bean cells (*Vigna angularis*) were irradiated, the culture medium was found to contain an elicitor able to stimulate PAL activity (Philips *et al.*, 1992). The active principle was partially purified but NMR and mass spectroscopy failed to characterise the compound other than to identify it as a complex oligosaccharide whose structure was not consistent with its being an oligogalacturonide but which was found to contain uronic acid residues.

Perhaps the best known biologically active oligosaccharide has been detected *in vivo* following its initial identification amongst the hydrolysis products of xyloglucan. XXFG was detected in cell suspension cultures of spinach after presentation of [^{3}H]arabinose and [^{3}H]fucose and found to accumulate to a steady state of 4.3×10^{-7} M (Fry, 1986a).

1.6 Conclusions

There is compelling evidence that the structural complexity of the plant cell wall is present for purposes other than purely structural roles. It appears that many of the processes which occur in the cell wall environment result in turnover of the cell wall and formation of oligosaccharides. The structural complexity of the oligosaccharin parent molecules and the specificity in the processes which cause oligosaccharins to be produced means that different circumstances can lead to unique oligosaccharins products. This in turn leads to a high degree of specificity in signals derived from the cell wall of plants. In this way oligosaccharins allow the cell wall to act as a sensory/signalling compartment and to alert the cell to the processes going on outside it.

Our knowledge of this process is based largely on the use of oligosaccharides produced by breakdown of cell wall polysaccharide and the screening of the resulting products for biological activity. This can be a very hit and miss process and no doubt many oligosaccharides which have been tested for biological activity and dismissed as oligosaccharins have simply not hit the right biological assay. Also there seems to be a reluctance to continue the work once a biologically active oligosaccharide has been detected and to try to answer some of the questions which arise from the discovery of a biologically active oligosaccharide, such as whether it occurs *in vivo*, how it is produced and how it exerts its action. To try and answer some of these questions the work described in this thesis details experiments aimed at detecting oligosaccharides present *in vivo* and to determining their structure, biological activity and details about their origin and fate.

2. Materials and Methods

2.1 General Methods

2.1.1. Chemicals

All chemicals were of at least standard laboratory grade (AnalaR). Radiochemicals were obtained from either Amersham International or Sigma Chemicals. D-[6-^{14}C]glucuronic acid was synthesised by Sigma Chemicals according to the method of Sowden (1952).

2.1.2. Rose Cell Suspension Culture

Culture medium for maintenance of "Paul's Scarlet" rose (*Rosa* sp.) cell suspension cultures was prepared as follows (Fry and Street, 1980). Culture medium was prepared in bulk according to table 2.1.2.1. and the pH adjusted to 6.1 with 1.0 M NaOH. Aliquots of culture medium (400 ml or 50 ml) were transferred to 1 L flasks or 250 ml flasks respectively. Flasks were sealed with cotton wool/muslin bungs and covered with aluminium foil before being autoclaved (121°C, 15 psi, 19 min).

Cultures were maintained at 25°C on orbital shakers, at a speed sufficient to keep the cells in suspension and prevent any settling to the bottom of the flasks. The cells were sub-cultured fortnightly by dividing the culture between four flasks containing fresh medium. All manipulations were carried out aseptically.

Table 2.1.2.1. Rose cell suspension culture medium.

Component	Concentration (g l^{-1})
$CaCl_2$	0.074
KH_2PO_4	0.14
KCl	0.75
$NaNO_3$	0.85
$MgSO_4.7H_2O$	0.25
$MnSO_4.4H_2O$	0.001
$ZnSO_4.7H_2O$	$5.0x10^{-4}$
KI	$1.0x10^{-4}$
$CuSO_4.5H_2O$	$2.0x10^{-5}$
H_3BO_3	$2.0x10^{-4}$
$CoCl_2.6H_2O$	$1.0x10^{-5}$
$Na_2MoO_4.2H_2O$	$2.0x10^{-5}$
$FeCl_3.6H_2O$	$5.4x10^{-3}$
$Na_2EDTA.2H_2O$	$7.4x10^{-3}$
2,4-Dichlorophenoxyacetic acid	$1.0x10^{-3}$
Kinetin	$5.0x10^{-4}$
Glucose	20.0

2.1.3. Analysis of Carbohydrates

2.1.3.1. Acid Hydrolysis

a) Trifluoroacetic Acid Hydrolysis

Hydrolysis was performed by dissolving or suspending the sample in 2.0 M TFA and heating at 120°C for 1 hour in a sealed screw-capped Pyrex tube (Fry, 1988).

b) Formic Acid Hydrolysis

Formic acid hydrolysis was performed by dissolving or suspending the sample in 75% (v/v) formic acid and heating at 100°C for 5 hours in a sealed screw-capped Pyrex tube. After hydrolysis the sample was diluted by addition of 4.0 M TFA to a final concentration of 2.0 M TFA and heated again for 1 hour at 120 °C (Aspinall, 1982).

2.1.3.2. Separation of Carbohydrates

a) Paper Chromatography

Monosaccharides and oligosaccharides were separated by descending paper chromatography. Samples were dried onto Whatman 3MM paper as spots or streaks and developed in either of the following solvent systems for approximately 16 hours (Fry, 1988):

Butan-1-ol : acetic acid : water, (12 : 3 : 5, v:v:v), (BAW).
Ethyl acetate : acetic acid : water (1 : 1 : 1, v:v:v), (EAW).

Monosaccharide markers were prepared from a commercial source (Sigma Chemicals) and oligomeric markers of galacturonic acid were prepared by hydrolysis of polygalacturonic acid (2.0 g) with 100 mg pectinase (*Erwinia* sp.) in pyridine acetate buffer (25 mM, pH ~ 4.7). The mobility of external markers was assessed on both edges of the paper to account for the possibility of the solvent front moving at an angle to the vertical. The products were partially purified by gel permeation chromatography and analysed by paper chromatography (solvent system EAW).

Mobility of a compound during paper chromatography was quantified as its relative mobility (R_F):

$$R_F = \frac{\text{Distance moved by compound}}{\text{Distance moved by solvent front}}$$

In the event of the solvent front having moved beyond the end of the paper a marker was used to replace the solvent front in the equation.

b) Paper Electrophoresis (pH 3.5)

Uronic acids and uronic acid containing oligosaccharides were separated according to their relative charge/mass ratio by paper electrophoresis, pH 3.5 (Wright and Northcote, 1975). Electrophoresis was performed in a chromatography tank containing white spirit coolant and with platinum electrodes placed in running buffer (acetic acid : pyridine : water (10:1:189, v:v:v)). Samples were dried onto Whatman no. 1 paper as spots or streaks, and the paper was wetted with half-strength running buffer and lightly blotted. The samples were electrophoresed at 3 kV and approximately 100 mA, for 1.5 hours, with Orange G as a visible marker.

The mobility of external markers was assessed on both edges of the paper to account for the possibility of the solvent front moving at an angle to the vertical. Mobility of a compound during paper electrophoresis was quantified as follows:

$$m_{marker} = \frac{\text{(distance migrated: compound) - (distance migrated: glucose)}}{\text{(distance migrated: marker) - (distance migrated: glucose)}}$$

c) Gel Permeation Chromatography

Oligosaccharides were separated by gel permeation chromatography on Bio-Gel P-2 columns of either 270 ml or 780 ml bed volume, depending on the sample volume (no greater then 5% of the column bed volume). The sample was eluted with pyridine : acetic acid : water (1 : 1 : 25, v:v:v, pH ~ 4.7) buffer.

Desalting of oligosaccharides was carried out on a Sephadex G10 column (150 ml bed volume). The sample was eluted with pyridine : acetic acid : water (1 : 1 : 25, v:v:v, pH ~ 4.7) buffer.

Desalting and separation of polysaccharides was carried out on a Sephadex 4B column (150 ml bed volume). The sample was eluted with pyridine : acetic acid : water (1 : 1 : 25, v:v:v, pH ~ 4.7) buffer.

All columns were calibrated using a mixture of Blue Dextran and cobalt chloride to determine the void and included volumes respectively and the K_{av} quantified as follows:

$$K_{av} = \frac{\text{Elution volume of compound - void volume}}{\text{Included volume - void volume}}$$

d) Anion Exchange Chromatography

Anionic oligosaccharides were exchanged on a Dowex-1 column (100 ml or 3 ml bed volume). The columns were washed with 2.0 M sodium acetate (pH 5.0 with glacial acetic acid) followed by 10 mM pyridine acetate (1 column bed volume of each), before the sample was added. Neutral material was washed through the column with 10 mM pyridine acetate (1.5 bed volume). Pyridine acetate was used to elute oligosaccharides retained on the column, either as a 1.0 M solution to elute without fractionation or as a series of dilutions between 10 mM and 1.0 M to fractionate the anionic material.

e) Dionex HPLC

Oligosaccharides and monosaccharides were separated by Dionex HPLC on a CarboPac PA1 column (4 x 250 mm); alditols were separated on an MA1 column (4 x 250 mm). The sample (20 µl) was injected onto the column and eluted with sodium hydroxide or sodium hydroxide/sodium acetate eluents. Different eluent profiles were used depending on the size and charge of the sugars contained in the sample.

Relative retention time for a compound was defined by its retention time (R_T) relative to two markers eluting before and after the compound in question:

$$R_{\text{marker 1-marker 2}} = \frac{R_T \text{ Compound} - R_T \text{ Marker 1}}{R_T \text{ Marker 2} - R_T \text{ Marker 1}}$$

Eluent Programme 1

Eluent Programme 1 was run on the PA1 column. The sample was eluted by a 40 minute gradient of 50 mM sodium acetate/100 mM sodium hydroxide to 100 mM sodium acetate/100 mM sodium hydroxide. 5 Minutes isocratic 100 mM sodium acetate/100 mM sodium hydroxide, a 35 minute gradient to 500 mM sodium acetate/500 mM sodium hydroxide, and 5 minutes isocratic 500 mM sodium acetate/500 mM sodium hydroxide. The column was re-equilibrated with 50 mM sodium acetate and 100 mM sodium hydroxide for 10 minutes. The flow rate was 1 ml min^{-1} except when running 500 mM sodium hydroxide when the flow was reduced to 0.8 ml min^{-1}.

Eluent Programme 2

Eluent programme 2 was run on the PA1 column and the sample eluted with 20 mM sodium hydroxide/10 mM sodium acetate isocratically for 10 minutes, 10 minutes isocratic 50 mM sodium acetate/100 mM sodium hydroxide, a 30 minute gradient to 100 mM sodium acetate/100 mM sodium hydroxide, 5 minutes isocratic 100 mM sodium acetate/100 mM sodium hydroxide, a 25 minute gradient to 500 mM sodium acetate/500 mM sodium hydroxide, and 15 minutes isocratic 500 mM sodium acetate/500 mM sodium hydroxide. The column was re-equilibrated with 20 mM sodium hydroxide/10 mM sodium acetate for 10 minutes. The flow rate was 1 ml min^{-1} except when running 500 mM sodium acetate/500 mM sodium hydroxide, when the rate was

0.8 ml min^{-1}. 0.5 M sodium hydroxide post column addition base was run at a flow rate of 0.5 ml min^{-1}.

Eluent Programme 3

Eluent programme 3 was run on the PA1 column and the sample eluted with 30 minutes water, a 20 minute gradient to 800 mM sodium hydroxide, and 5 minutes isocratic 800 mM sodium hydroxide. The column was re-equilibrated with water for 10 minutes. The flow rate was 0.8 ml min^{-1}, with 0.5 M sodium hydroxide post column addition base, at a flow rate of 0.5 ml min^{-1}.

Eluent Programme 4

Eluent programme 4 was run on the PA1 column and the sample eluted with 5 minutes 10 mM sodium hydroxide, 25 minutes water, a 40 minute gradient to 800 mM sodium hydroxide, and 5 minute isocratic 800 mM sodium hydroxide. The column was re-equilibrated with 10 mM sodium hydroxide for 10 minutes. The flow rate was 1 ml min^{-1}, with 0.5 M sodium hydroxide post column addition base, at a flow rate of 0.5 ml min^{-1}.

Eluent Programme 5

Eluent programme 5 was run on the MA1 column and the sample was eluted with isocratic 520 mM sodium hydroxide and a flow rate of 0.4 ml min^{-1}.

2.1.3.3. Detection of Carbohydrates

a) Radiolabelled Sugars

Sugars contained on paper, following paper chromatography or paper electrophoresis, were detected by scintillation counting in a Beckman LS 5000 or LS 6500 liquid scintillation counter. The paper was

wetted in either non-Triton scintillant (0.5% 2,5-diphenyloxazole, 0.05% 1,4-di-2-(5-phenyl-oxazolyl)-benzene in toluene) or Optiscint Hisafe scintillant (Wallac Chemicals) prior to scintillation counting. Alternatively the paper was autoradiographed by exposing it to X-ray film and developing the film after a suitable time period.

If the sample was needed for further analysis after scintillation counting, the scintillant was washed off with toluene and the paper allowed to air dry. It was then placed in the barrel of a plastic syringe, wetted with distilled water, centrifuged (1500 *g* for 5 min) and the eluent collected. The wash was repeated 3 times to ensure complete elution.

Aqueous samples were scintillation counted in either Triton scintillant (0.33% 2,5-diphenyloxazole, 0.033% 1,4-di-2-(5-phenyl-oxazolyl)-benzene in toluene : Triton X-100, (2:1, v:v)) in the ratio 10 : 1 scintillant to sample volume or in Optiphase Hisafe (Wallac Chemicals) in the ratio 10 : 1 scintillant to sample volume.

b) Staining of Non-radioactive Reducing Sugars

Reducing sugars and oligosaccharides contained on paper, following paper chromatography or electrophoresis, were stained using aniline hydrogen phthalate (Fry, 1988). The paper was dipped through a solution of phthalic acid (16 g) in acetone, diethylether and water (490 ml, 490 ml and 20 ml respectively) containing 0.5% aniline and left to air dry before being heated at 105°C for 5 minutes. Hexoses stained brown, pentoses red and uronic acids stained orange.

Aqueous carbohydrates were assayed by the phenol/sulphuric acid assay. The sample (0.4 ml) was mixed with 10 µl aqueous 80% phenol and 1.0 ml concentrated sulphuric acid, the samples were left for 10 minutes to develop and the absorbance was determined at 490 nm (Dubois *et. al.*, 1956).

c) Dionex HPLC

Dionex HPLC was used to detect monosaccharides, oligosaccharides and alditols after separation on the CarboPac PA1 or MA1 columns. The sugars were detected by a pulsed amperometric detector fitted with a gold electrode.

2.2 Oligosaccharides in Spent Culture Medium

The accumulation of naturally occurring oligosaccharides in the spent medium of various plant cell suspension cultures was investigated by Dionex HPLC and radiolabelling. Purification methods were developed to enable further studies of the structure and biological activity of the oligosaccharides.

2.2.1. Oligosaccharides in Spent Culture Medium of Rose Cells

Spent culture medium from rose cells (800 ml) was harvested two weeks after sub-culturing. The cells were filtered off on 53 μm nylon gauze and the extracellular polymers precipitated with 50% ethanol at 4 °C overnight. The precipitate was filtered off on 53 μm nylon gauze and the ethanol removed by rotoevaporation. The spent culture medium was loaded onto a 780 ml Bio-Gel P-2 column and eluted with pyridine : acetic acid : water (1:1:25, v:v:v) and the fractions were freeze dried.

2.2.1.1. Carbohydrate Content of Bio-Gel P-2 Fractions

Before freeze drying, a portion of each fraction (0.1 ml) was assayed by the phenol/sulphuric assay to determine its carbohydrate content.

2.2.1.2. Oligosaccharides in Bio-Gel P-2 Fractions

The freeze dried Bio-Gel P-2 fractions were resuspended in 1.0 ml of distilled water, filtered through a 0.45 μm nylon filter device and analysed by Dionex HPLC (Eluent Programme 1).

2.2.2. Uronic Acid Containing Oligosaccharides in Spent Medium of Rose Cells

In order to radiolabel uronic acid containing oligosaccharides, rose cells were presented with D-[6-^{14}C]glucuronic acid (55 mCi mmol^{-1}). The radiolabelled precursor (3.7 MBq) was autoclaved in 5 ml fresh rose culture medium, one week old rose culture (2.0 ml) was added to the medium and the culture incubated under standard growth conditions for 7 days. All manipulations were carried out aseptically.

2.2.2.1 Separation of Oligosaccharides in Spent Culture Medium

After 7 days the cells were filtered off on 53 μm nylon gauze and the spent culture medium was loaded on a 270 ml Bio-Gel P-2 column and eluted with pyridine : acetic acid : water (1:1:25, v:v:v). A portion of each fraction was scintillation counted in Triton scintillant.

The Bio-Gel P-2 fractions were freeze dried, resuspended, dried onto Whatman 3MM paper and developed in solvent system EAW overnight. The paper was dried and autoradiographed for 1 week.

2.2.3. Purification of Oligosaccharides

2.2.3.1. Purification of Trisaccharide

a) ^{14}C-Trisaccharide

Radiolabelled trisaccharide was purified from spent culture medium of rose cells following presentation of D-[6-^{14}C]glucuronic acid. The radiolabelled precursor, 150 kBq, was filter sterilised through a

sterile 0.45 μm nylon filter device into 1 week old rose culture (50 ml) and the culture was incubated under standard growth conditions for 1 week.

The spent culture medium was harvested by filtering off the cells on 53 μm nylon gauze, dried onto 3 sheets of Whatman 3MM and developed in solvent system BAW. This separated the ^{14}C-trisaccharide from the unused D-[6-^{14}C]glucuronic acid remaining in the culture medium. The $GalA_3$ external marker region of the chromatogram was eluted, loaded on one sheet of Whatman 3MM and developed in solvent system EAW and the $GalA_3$ region was eluted.

To check the purity of the final ^{14}C-trisaccharide preparation, a portion of the product was analysed by paper chromatography in solvent system BAW and the paper scintillation counted in Optiscint Hisafe scintillant.

b) Non-radioactive Trisaccharide

The trisaccharide was extracted from 800 ml of 2 week old rose culture. The cells were removed by filtering the culture on 53 μm nylon gauze and the extracellular polymers were removed by alcohol precipitation with 50% ethanol overnight at 4°C. The precipitate was removed by filtering on 53 μm nylon gauze and the ethanol removed by rotoevaporation.

The spent culture medium was anion exchanged on a Dowex-1 column (100 ml bed volume). After addition of the sample the column was washed with 150 ml of 10 mM pyridine acetate to remove the unused glucose from the culture medium, while the trisaccharide was retained on the column. Negatively charged material was then eluted with 1.0 M pyridine acetate and 150 ml of eluate collected and dried by rotoevaporation.

The anion exchanged oligosaccharides were separated by gel permeation chromatography on Bio-Gel P-2 (270 ml bed volume column). The trisaccharide was detected in the eluted fractions by analysing a

small portion of each fraction (0.1 ml) between K_{av} = 0.7 and 0.9 by the phenol/sulphuric assay. The trisaccharide was detectable as a peak of increased absorbance at K_{av} = 0.8.

The final trisaccharide preparation was desalted on a Sephadex G10 column and detected in the eluted fractions by analysing a small portion of each fraction between K_{av} = of 0.0 and 0.4 by the phenol/sulphuric assay. The trisaccharide was detectable as a peak of increased absorbance with a K_{av} = 0.25.

The extraction procedure was developed and followed by Dionex HPLC (Eluent Programme 2) to check the purity of the final product and to ensure minimisation of loss at any stage of the extraction. All pyridine acetate was removed by rotoevaporation. Where a freeze dried product was required the sample was resuspended in water and freeze dried.

2.2.3.2. Calibration of Dionex PAD Detector

In order to conserve stocks of trisaccharide a maltotriose stock solution was calibrated to determine its PAD detector response relative to a trisaccharide solution. Stock solutions were prepared by accurately weighing out purified trisaccharide and commercially available maltotriose (Sigma) and preparing solutions at 1.0 mg ml^{-1}. These were diluted to 100, 50, 25 and 12.5 μg ml^{-1} and analysed by Dionex HPLC (Eluent Programme 1).

2.2.3.3. Heptasaccharide

A putative heptasaccharide was extracted from 800 ml of 2 week old rose culture. The cells were removed by filtering the culture on 53 μm nylon gauze and the extracellular polymers were removed by alcohol precipitated with 50% ethanol overnight at 4°C. The precipitate was removed by filtering on 53 μm nylon gauze and the ethanol removed by rotoevaporation.

The spent culture medium was loaded on a Bio-Gel P-2 column (780 ml bed volume) and eluted. The fractions were freeze dried and a portion of each fraction in the region of K_{av} = 0.0 - 0.4 was analysed by

Dionex HPLC (Eluent Programme 1). The fractions found to contain the heptasaccharide ($R_{GalA\text{-}GalA2}$ = 0.68) were pooled.

The volume was reduced in a rotoevaporator and the sample was anion exchanged on a Dowex-1 column (3 ml bed volume). After addition of the sample the column was washed with 2.0 ml 10 mM pyridine acetate and the sample eluted with a series of dilutions of pyridine acetate (2 ml) as follows: 78 mM, 0.156, 0.313, 0.625, 1.25 and 2.5 M. The putative heptasaccharide eluted between 0.313 - 0.625 M pyridine acetate.

The fractions were freeze dried and resuspended and a portion was analysed by Dionex HPLC (Eluent Programme 1) to determine the purity of the final product.

2.2.4. Accumulation of Trisaccharide in Spent Culture Medium

To determine the concentration to which the trisaccharide accumulated in the spent culture medium with time, 2 week old rose culture was sub-cultured into fresh medium as described. Aliquots of the culture (30 ml) were transferred aseptically to sterile flasks and grown under normal growth conditions.

At intervals up to 26 days after sub-culturing, 2 cultures were harvested, transferred to graduated tubes and centrifuged at 1500 *g* for 5 minutes to determine the packed cell volume. The supernatant was collected, dried in a rotoevaporator and resuspended in 1.0 ml of distilled water. The samples were analysed by Dionex HPLC using Eluent Programme 2.

2.2.5. Turnover of ^{14}C Trisaccharide *In Vivo*

Purified ^{14}C-trisaccharide was filter sterilised through a 0.45 μm nylon filter device into 7 day old rose culture or fresh culture medium as a control, to give a final concentration of 425 cpm ml^{-1}. Aliquots (1 ml)

were transferred to sterile 5 ml Petri dishes and incubated under standard growth conditions. All manipulations were carried out aseptically.

At intervals of 3, 24, and 72 hours two cultures were collected by centrifugation and the cells washed twice with 0.4 ml water. The spent culture medium and the washings were pooled and analysed by paper chromatography in solvent system EAW. The controls containing no cells were dried straight onto the paper. The radioactivity was determined by scintillation counting in Optiscint Hisafe scintillant.

2.2.7. Oligosaccharides in Other Plant Suspension Cultures

Zea, *Festuca* and *Acer* cell cultures were kindly supplied by Mrs. Janice Miller. They were maintained in the following media:

Table 2.2.7.1. *Festuca* culture medium (pH 5.7).

Component	Concentration (g l^{-1})
Murashige and Skoog basal salts (Sigma, M5524)	4.4
Nicotinic acid	0.5
Aneurine-HCl	0.1
Pyridoxine-HCl	0.5
Glycine	2.0
Glucose	20.0

Table 2.2.7.2. *Zea* culture medium (pH 5.6).

Component	Concentration (g l^{-1})
Murashige and Skoog basal salts (Sigma, M5519)	4.7
2,4-D	$2x10^{-3}$
Sucrose	20.0

Table 2.2.7.3. *Acer* culture medium (pH 6.4).

Component	Concentration (g l^{-1})
KCl	0.75
$MgSO_4.7H_2O$	0.25
$NaNO_3$	0.85
NaH_2PO_4	0.13
$CaCl_2.2H_2O$	0.074
$ZnSO_4.7H_2O$	$1.0x10^{-3}$
H_3BO_3	$1.1x10^{-3}$
$MnSO_4.4H_2O$	$1.0x10^{-4}$
$CuSO_4.5H_2O$	$3.0x10^{-5}$
KI	$1.0x10^{-5}$
$NaFe(EDTA)_2$	$1.5x10^{-3}$
Thiamine HCl	$1.0x10^{-3}$
Calcium pantothenate	$2.5x10^{-3}$
Choline chloride	$5.0x10^{-4}$
2,4-D	$1.0x10^{-3}$
L-Cysteine HCl	0.01
Glucose	20.0

Cultures were harvested one and two weeks after sub-culturing and the cells filtered off on 53 μm nylon gauze. Extracellular polysaccharides were removed by alcohol precipitation with 50% ethanol overnight at 4°C. The precipitate was filtered off on 53 μm nylon gauze and the filtrate dried in a rotoevaporator. The samples were resuspended and analysed by Dionex HPLC (Eluent Programme 2), with controls of fresh culture medium.

2.3. Structural Analysis

The trisaccharide purified from spent rose culture medium, either in its D-[6-^{14}C]uronic acid form or as the non-radiolabelled form, was subjected to structural analysis.

2.3.1. Paper Electrophoresis (pH 3.5)

Purified ^{14}C-trisaccharide (250 cpm) was dried onto Whatman no. 1 paper and analysed by paper electrophoresis at pH 3.5. The paper was dried and the radioactivity was determined by scintillation counting in non-Triton scintillant.

2.3.2. Acid Hydrolysis

2.3.2.1. TFA Hydrolysis

Purified ^{14}C-trisaccharide (250 cpm) was hydrolysed by 2.0 M TFA and the products were dried directly onto Whatman no. 1 paper and electrophoresed at pH 3.5. The paper was dried and the radioactivity was determined by scintillation counting in non-Triton scintillant.

2.3.2.2. Formic Acid Hydrolysis of ^{14}C-Trisaccharide

Purified ^{14}C-trisaccharide (3000 cpm) was hydrolysed by 75% formic acid, followed by 2.0 M TFA and the products were dried onto Whatman 3MM paper and developed in solvent system BAW. The radioactivity was determined by scintillation counting in non-Triton scintillant. Each of the products was analysed in turn:

a) Peak 1

The peak of radioactive material nearest the origin of the chromatogram was eluted from the paper and re-chromatographed in solvent system EAW and the paper scintillation counted in non-Triton scintillant.

b) Peak 2

Radioactive material which co-migrated with the external uronic acid markers was eluted from the paper, dried onto Whatman no. 1 paper with an internal marker of glucuronic acid and electrophoresed (pH 3.5). The radioactivity was determined by scintillation counting in non-Triton scintillant.

c) Peak 3

Material which co-migrated with the glucuronolactone marker was eluted from the paper and saponified as follows. The solution was made up to 50 mM NaOH using 100 mM NaOH and incubated at 20 °C for 10 minutes then neutralised with glacial acetic acid. This was dried onto Whatman no. 1 paper immediately and electrophoresed at pH 3.5; the radioactivity was determined by scintillation counting in non-Triton scintillant.

2.3.2.3. Formic Acid Hydrolysis of Non-Radiolabelled Trisaccharide

The purified non-radioactive trisaccharide was subjected to formic acid hydrolysis, the acid was evaporated off in a Speedvac (Genevac Ltd. UK.) and the products were analysed by Dionex HPLC (Eluent Programme 3). The products were identified by inclusion of internal markers.

2.3.3. Attempted Reduction of Trisaccharide

Purified trisaccharide (0.5 mg) was reduced by 0.5 M $NaBH_4$ in 1.0 M ammonia (0.5 ml) for 4 hours after which the reaction was stopped by addition of glacial acetic acid. The trisaccharide was cation exchanged on a 1.5 ml Dowex-50 column previously washed with 1.0 M HCl (aq). The sample was eluted with 3 ml of water, dried in the Speedvac and re-dried in 90% methanol, 10% acetic acid 5 times to remove boric acid as

volatile methyl ester. The trisaccharide was hydrolysed by 2.0 M TFA and analysed by Dionex HPLC (Eluent Programmes 4 and 5).

2.3.4. Nuclear Magnetic Resonance

NMR was kindly performed by Dr. Ian Sadler, Department of Chemistry, The University of Edinburgh.

2.4. Biosynthesis of Trisaccharide

2.4.1. Accumulation of Man-[^{14}C]GlcA-Ins and ^{14}C-Polymers

Rose cells (9.7 g) were collected on sterile muslin 5 days after sub-culturing, and added to 800 ml fresh culture medium. D-[6-^{14}C]Glucuronic acid, was filter sterilised through a 0.45 μm filter device directly into the culture. Aliquots of the culture (2.0 ml) containing 7.0 kBq of radiolabelled precursor, were added to 5.0 ml Petri dishes and incubated under standard growth conditions. All manipulations were carried out aseptically.

At time intervals of 0 to 31 hours, two cultures were harvested by filtering off the cells on 53 μm nylon gauze and washing them with 2 x 1.0 ml of water. The spent culture medium and the washings were pooled, analysed by paper chromatography (solvent system EAW) and scintillation counted in non-Triton scintillant.

The extracellular ^{14}C-polymers were quantified from the amount of chromatographically immobile material at the origin. The D-[6-^{14}C]glucuronic acid remaining in the culture medium was determined from the amounts of radioactivity at the glucuronic acid and glucuronic acid lactone marker positions.

To quantify the Man-[^{14}C]GlcA-Ins trisaccharide, the $GalA_3$ position was eluted and re-chromatographed (solvent system EAW), the paper

scintillation counted and the radioactivity in the $GalA_3$ region of the chromatogram determined. The ^{14}C-trisaccharide could not be detected in the first chromatogram owing to the "tail" from the peak of unused radiolabelled precursor which overlapped with the $GalA_3$ position.

2.4.2. Acid-Resistant Disaccharide

2.4.2.1. ^{14}C-Extracellular Polymers and ^{14}C-Cells

Following presentation of D-[6-^{14}C]glucuronic acid to the rose cultures, the cells and extracellular polymers (the origin of the paper chromatogram) which had been presented with the radiolabelled precursor for 25 hours were subjected to acid hydrolysis with 2.0 M TFA. The products were analysed by paper chromatography (solvent system EAW) and the radioactivity was determined by scintillation counting in non-Triton scintillant. [^{14}C]GlcA-Ins disaccharide (produced by 2.0 M TFA hydrolysis of authentic Man-[^{14}C]GlcA-Ins trisaccharide) was used as a marker and material found to co-migrate was eluted.

The ^{14}C-disaccharide material was dried on to Whatman no. 1 paper, electrophoresed (pH 3.5) with authentic [^{14}C]GlcA-Ins marker and the radioactivity determined by scintillation counting in non-Triton scintillant.

2.4.2.1. Purified Cell Walls

Rose cells were collected 5 days after sub-culturing by filtering the culture on 53 μm nylon gauze and freeze drying the cells. The freeze dried cells (7 g) were resuspended and stirred overnight in glacial acetic acid : 80% phenol (1:2.5, v:v) to remove proteins, and the cell walls were collected by centrifugation. The cell walls were resuspended in 100 ml glacial acetic acid : 80% phenol (1:2, v:v) and stirred overnight. The cell walls were collected and the supernatant was assayed for the presence of proteins by addition of 50 μl 10% ammonium formate and 5 ml acetone to

1.0 ml of the supernatant. A precipitate indicated the presence of protein. The extraction was repeated until no more protein could be detected in the supernatant. The cell walls were washed in 70% ethanol.

Starch was removed by resuspending the de-proteinated cell walls in 90% DMSO and stirring overnight. The cell walls were collected by centrifugation and the supernatant tested for starch by addition of a drop of potassium iodide/iodine solution (blue/black precipitate with starch). The extraction was continued until no more starch was detected. The final cell wall preparation was washed with excess 70% ethanol then excess acetone and left to air dry.

Extracellular polysaccharides were collected from the spent culture medium by precipitation with 80% ethanol at 4°C overnight followed by centrifugation 1500 *g*, 10 minutes.

The purified cells walls were extracted according to Zablackis *et al.,* (1995). Purified cell walls (0.5 g) were resuspended in 25 mM pyridine acetate buffer (15 ml, pH 5.2) and incubated with 50 mg polygalacturonase (*Erwinia* sp.) at 25 °C overnight. The cell walls were collected by centrifugation and the extracted polysaccharides were removed with the supernatant. The cell walls were digested twice more then de-esterified by adjusting the pH to 12 with 0.1 M NaOH for 4 hours at 4 °C and digested again with polygalacturonase.

Hemicelluloses were extracted with 0.01; 0.02; 0.3; 0.045; 0.06; 0.08; 0.1; 0.2; 0.3; 0.45; 0.6; 0.8; 1; 2 and 3 M KOH containing 0.3% $NaBH_4$, and a final extraction of 4.0 M KOH containing 4.0% H_3BO_3.

All extracts were filtered through a 0.8 μm nylon filter device, dialysed overnight and freeze dried. All extracted polysaccharides and the remaining residue were acid hydrolysed with 2.0 M TFA and the products dried in the Speedvac before analysis by Dionex HPLC (Eluent Programme 4).

2.4.3. Breakdown of ^{14}C-Polysaccharides by Rose Cells

Rose cells were presented with D-[6-^{14}C]glucuronic acid (2.2.3.1.a) and the cell walls purified as described (2.4.3.1.). ^{14}C-Polysaccharides were extracted by stirring the cell walls in 50 mM CDTA (pH 7.5) overnight, the cell walls were collected by centrifugation and the extracted ^{14}C-polysaccharides removed with the supernatant. A portion of the supernatant was assayed for ^{14}C by scintillation counting in Optiphase Hisafe scintillant and the extraction repeated until no more radioactivity was detected. The cell walls were extracted with 8.0 M urea (pH 7.5 with 50 mM HEPES), NaOH (6 M containing 1% $NaBH_4$) NaOH (6 M containing 4% H_3BO_3). In all cases the extraction was repeated until no more radioactivity was detected in the supernatant.

Extractions from each treatment were pooled, desalted on a Sephadex 4B column (150 ml bed volume) and eluted with pyridine : acetic acid : water (1:1:25, v:v:v). A portion of each fraction was scintillation counted in Optiphase Hisafe scintillant. Fractions were pooled and freeze dried.

The freeze dried ^{14}C-polysaccharides were resuspended in 0.25 ml of water, added to 6 day old rose culture or fresh culture medium (10 ml) in 9 cm Petri dishes and incubated under standard growth conditions for 24 hours. The cells were filtered off on 53 μm nylon gauze, washed with 2 x 1.0 ml of water and pooled. The spent culture medium plus washings were dried onto Whatman 3MM paper and developed in solvent system EAW. The radioactivity was determined by scintillation counting in Optiscint Hisafe scintillant.

2.5 Biological Effects

2.5.1. Effect of Man-GlcA-Ins on GA_3 Mediated Elongation of Excised Pea Shoots

Progress peas (*Pisum sativum*) were grown in the dark, in moist vermiculite for 6 days at 21 °C. The stems were cut under water to a constant length of 3.9 cm, as measured from the apical hook, and incubated for 0.5 hours in medium containing 50 mM KH_2PO_4, 6% sucrose, 0.2% Streptomycin at pH 6.1. The peas were mixed to ensure randomisation before being transferred to test-tubes containing fresh incubation medium (1 ml), 2.5 x 10^{-5} M GA_3 and Man-GlcA-Ins trisaccharide in the range of 1 x 10^{-7} to 2 x 10^{2} µg ml^{-1} (Warneck and Seitz, 1993). Controls containing no GA_3/no trisaccharide and GA_3/no trisaccharide were also prepared. 20 Shoots were used for each treatment with one stem per tube. All manipulations were carried out under green light. The tubes were loosely sealed with aluminium foil and incubated in the dark for 24 hours at 21 °C, before the pea shoots were measured to determine the increase in length.

2.5.2. Effect of Man-GlcA-Ins on Uptake and Incorporation of Radiolabelled Amino Acids

Rose cell culture (7 day old) was filtered through muslin and the filtrate, containing a fine cell suspension was used. Aliquots (300 µl) were added to 100 µl 50 mM MES buffer, pH 6.0 (with 1.0 M NaOH) in 5 ml Petri dishes and incubated under standard growth conditions for 1 hour. The Man-GlcA-Ins trisaccharide was added as a 100 µl solution in the range of 1.0 x 10^{1} - 1.0 x 10^{-7} µg ml^{-1} with water blanks as controls. The cells were left for a further hour before 0.9 kBq (20 µl) of radiolabelled precursor was added. After 3 hours, uptake of the radiolabelled precursor by the cells was measured by filtering the culture on glass micro fiber

filters (GF/A, Whatman) and washing the filters with 2 x 5 ml of deionised water. The filters were dried and scintillation counted in Optiscint Hisafe scintillant. To determine incorporation into polymers cultures were prepared as for determination of uptake but the proteins were precipitated by addition of 5.0 ml cold, 10% trichloroacetic acid to cultures. There were left at 4 °C overnight, before collecting the proteins on glass microfibre filters.

Five repeats at each trisaccharide concentration were carried out and 10 controls containing no trisaccharide were also performed. The Petri dishes were mixed before addition of the trisaccharide/water blanks to ensure randomisation.

2.5.3. Effect of Man-GlcA-Ins on Proteins in Rose Cells

2.5.3.1. Bioassay

Rose cells were collected on 53 μm nylon gauze 4 days after sub-culturing. They were washed with fresh culture medium (50 ml) before resuspending to their original volume in fresh culture medium (50 ml). Aliquots of the culture (600 μl) were added to 200 μl of 50 mM MES buffer, pH 6.0 (1 M NaOH) in 5 ml Petri dishes and incubated for 1 hour under standard growth conditions. The Man-GlcA-Ins trisaccharide was added in a final concentration range 1.0×10^{1} - 1.0×10^{-7} μg ml^{-1} in 200 μl water and the cultures were incubated for either 4 or 24 hours under standard growth conditions.

Controls containing no Man-GlcA-Ins trisaccharide were performed and the cultures were mixed before addition of trisaccharide to ensure randomisation.

2.5.3.2. Proteins in the Spent Culture Medium.

After incubation in the presence of trisaccharide for either 4 or 24 hours the cells and the spent culture medium were separated by

centrifugation for 2 minutes at 1500 *g* and the supernatant/spent culture medium removed and filtered on 0.2 μm nylon filter device.

2.5.3.3. Water-Extractable Proteins

To extract water soluble proteins the cells were washed with 3 x 0.4 ml water before resuspending in 200 μl water and sonicating for 30 seconds on ice. Insoluble material was pelleted by centrifugation for 4 minutes and the supernatant was removed, filtered on 0.2 μm nylon filter devices.

2.5.3.4. Proteins in the Cell Wall

To clean the cell wall of cytosolic material the cell walls were washed in 3 x 100 μl of water before resuspending in 250 μl of water. The cell walls were ground in a glass on glass homogeniser (10 strokes) in 250 μl buffer containing 80 mM glycylglycine, 2% Triton-X-100 and 1 M KCl, pH 7.5. The cell walls were collected by centrifugation and washed 3 times in buffer containing 40 mM glycylglycine (Atwell and Ap Rees, 1986).

All three preparations were suspended in sample buffer (31.25 mM Tris-HCl (pH 6.8), 5% glycerol, 2.5% β-mercaptoethanol, 1% SDS and 0.001% bromophenol blue) and boiled for 5 minutes.

2.5.3.5. Estimation of Protein Content

The spent culture medium, the cell contents and the cell walls were all resuspended in sample buffer and the protein content of the samples determined by assaying with amido black. 200 μl of the sample was made up to 1 ml with 10% acetic acid, 90 % methanol containing 1% amido black and shaken by hand. A pellet was collected by centrifugation for 5 minutes, then washed twice with 10% acetic acid, 90% methanol and left to air dry. The pellet was resuspended in 0.2 M sodium hydroxide and the

absorbance measured at 615 nm (method supplied by Dr. Nancy Steele, The University of Edinburgh).

2.5.3.6. Analysis of Proteins by SDS-PAGE

A 10% resolving gel (42.3% distilled water, 25% 1.5 M Tris-HCl (pH 8.8), 0.1% SDS, 31.3% acrylamide (30% w/v) : biS acrylamide (0.8% w/v) (37.5 : 1), 0.05% ammonium persulphate and 0.05% TEMED) was poured and allowed to set. The stacking gel (61% distilled water, 25% 0.5 M Tris-HCl (pH 6.8), 0.1% SDS, 13% acrylamide (30% w/v) : biS acrylamide (0.8% w/v) (37.5 : 1), 0.05% ammonium persulfate and 0.1% TEMED) was poured on top and allowed to set, before the samples were loaded. Loading volumes were inversely proportional to the absorbance at 615 nm with a maximum volume of 30 µl. The gel was electrophoresed for 1.5 hours at 25 mA and 175 mV.

2.5.3.7. Visualisation of Proteins

SDS-PAGE gels were stained with silver stain after fixing the gel in 50% ethanol, 10% acetic acid for 30 minutes immediately after electrophoresis. The gel was then rehydrated in 5% ethanol, 1% acetic acid for 15 minutes followed by 3 x 5 minute washes in de-ionised water. The gel was placed in $Na_2S_2O_3.5H_2O$ (0.2 g l^{-1}) for 1 minute, washed in water (30 sec) and left in $AgNO_3$ (2 g l^{-1}) containing 750 µl l^{-1} formaldehyde (75% (v/v)) for 20 minutes. The gel was washed well with distilled water and developed in Na_2CO_3 (60 g l^{-1}) containing 500 µl l^{-1} formaldehyde (75% (v/v)) and $Na_2S_2O_3.5H_20$ (0.004 g l^{-1}). When the gel was sufficiently developed the reaction was stopped by addition of excess 5% acetic acid (method supplied by Dr. Nancy Steele, The University of Edinburgh).

2.5.4. Effect of the Man-GlcA-Ins on Phenylalanine Metabolism in Rose Cells

2.5.4.1. Bioassay

Rose cells were collected on 53 μm nylon gauze 4 days after sub-culturing. They were washed with fresh culture medium before resuspending to their original volume in fresh culture medium. Aliquots of the culture (600 μl) were added to 200 μl 50 mM MES buffer, pH 6.0 (1 M NaOH) in 5 ml Petri dishes and incubated for 1 hour under standard growth conditions. The Man-GlcA-Ins trisaccharide was added in 200 μl water to give a final concentration of 1.0×10^{1} - 1.0×10^{-7} μg ml^{-1} and the cultures left for a further hour. L-[U-^{14}C]Phenylalanine (0.45 kBq) was added (10 μl) and the cultures were left for either 4 or 24 hours.

Controls containing no trisaccharide were carried out as well. The cultures were mixed before addition of trisaccharide to ensure randomisation.

2.5.4.2. Protein Hydrolysis

After 4 or 24 hours the cells were collected by centrifugation and washed once with 6.0 M HCl (0.5 ml, to reduce the unused radiolabelled precursor added to the paper chromatogram). They were resuspended in 0.5 ml of 6.0 M HCl and hydrolysed at 110°C for 20 hours. The acid was dried off in a Speed Vac and the acid hydrolysis products were resuspended in 200 μl water, dried onto Whatman 3MM paper and developed in solvent system BAW. The radioactivity was determined by scintillation counting in Optiscint Hisafe scintillant. The amino acid markers were stained by dipping through acetone containing 0.5% ninhydrin and heating at 105°C for 5 minutes.

2.5.4.3. Analysis of Inducible Acid-Resistant Material

The compound with R_{Phe} = 0.26 was eluted from the paper with 1.0 M ammonia and paper electrophoresed at pH 2.0. Paper electrophoresis was performed in a chromatography tank containing white spirit coolant, with platinum electrodes in a running buffer of formic acid : acetic acid : water (1 : 4 : 45, v:v:v). The sample was dried onto Whatman no. 1 paper and the paper wetted with running buffer and lightly blotted. The paper was electrophoresed for 45 minutes at a voltage of 3 kV and a current of approximately 100 mA. Methyl green was used as a visible marker. The radioactivity was determined by scintillation counting in Optiscint Hisafe scintillant and the markers were stained by ninhydrin.

3. Results

3.1. Oligosaccharides in Spent Media

Two approaches were employed to detect naturally occurring oligosaccharides in the spent media from plant cell suspension cultures. One approach involved feeding cells one of a number of radiolabelled precursors of cell wall biosynthesis and assaying the spent culture medium for the presence of radiolabelled oligosaccharides. Use of radiolabelled precursors which are incorporated into selected monosaccharide residues means that this approach can provide a certain amount of structural information regarding which sugars are present in an oligosaccharide. Alternatively the spent culture medium was analysed by Dionex HPLC which allowed indiscriminate detection of oligosaccharides in the culture medium present at above the quantitation threshold for the method. This approach gave an indication of how many oligosaccharides were present in the spent culture medium and their relative abundance. These two approaches allowed the accumulation of oligosaccharides in culture media to be followed and oligosaccharides to be purified and used for further study.

3.1.1. Oligosaccharides in the Spent Medium of Rose

Rose culture medium (800 ml) was collected 2 weeks after sub-culturing, its contents were separated by gel permeation chromatography using Bio-Gel P-2 and the fractions analysed by Dionex HPLC.

3.1.1.1. Size Distribution of Carbohydrates

The Bio-Gel P-2 fractions were analysed by the phenol/sulphuric assay to assess their relative carbohydrate content. Fig 3.1.1.1. shows that the void and the included volumes contained large amounts of carbohydrates, corresponding to material at or above 1.8×10^3 and below

1 x 10^2 Da respectively. The void volume (K_{av} = 0) material probably consisted of small polysaccharides, not removed by alcohol precipitation and large oligosaccharides. Unused glucose from the culture medium will have eluted immediately before the included volume (K_{av} = 1) and probably accounted for the majority of the carbohydrate detected there. The fact that carbohydrate material appears to elute after the included volume may be due to overloading of the column and the fact that acidic carbohydrates are slightly retained on Bio-Gel P-2 resin. Oligosaccharides eluted in the region between K_{av} = 1 and 0.

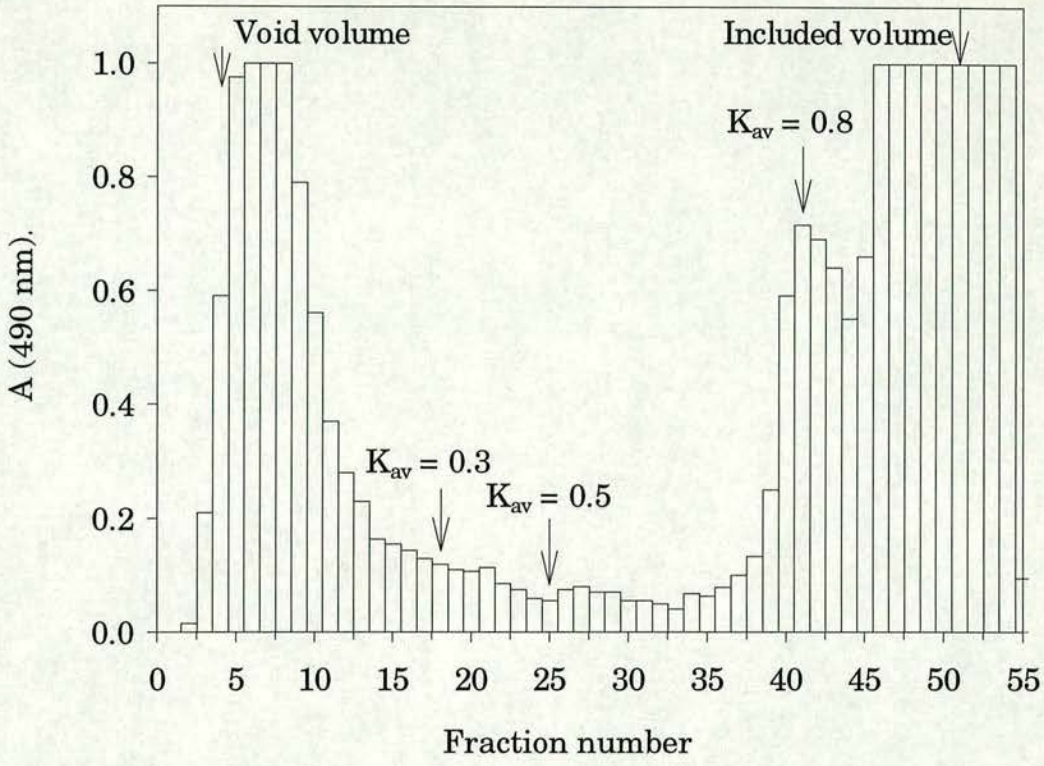


Fig. 3.1.1.1. Size distribution of the carbohydrate content from spent culture medium of rose as determined by gel permeation chromatography on Bio-Gel P-2.

3.1.1.2. Oligosaccharides in Bio-Gel P-2 Fractions

To determine whether any oligosaccharides could be detected in the fractions, these were analysed by Dionex HPLC (Eluent Programme 1) which resolves malto-oligosaccharides up to a d.p. of at least 7 and oligogalacturonides up to d.p. of at least 9 (fig. 3.1.1.2.a.). Fresh culture medium was analysed as a control to ensure any oligosaccharides detected were products of the culture and not derived from the growth medium (fig 3.1.1.2.b). Fig. 3.1.1.3. shows two Bio-Gel P-2 fractions analysed by Dionex HPLC. These chromatograms contain the oligosaccharides detected in other fractions, in varying relative amounts, collected from the Bio-Gel P-2 column and analysed.

At K_{av} = 0.3, the Bio-Gel P-2 fraction (fig. 3.1.1.3.a) was found to contain an oligosaccharide with a retention time of approximately 66 minutes. This corresponded to a relative retention of $R_{GalA\text{-}GalA2}$ = 0.7. With a K_{av} in the top third of the mass range on Bio-Gel P-2 this would suggest a d.p. of between 7 and 10 although no calibration of the Bio-Gel P-2 column was performed. In addition the K_{av} of a compound is dependent not only on size but also the overall shape of the molecule; hence K_{av} can only be an estimate of the size of a molecule. The highly branched xyloglucan oligosaccharides are found to elute later than expected for linear molecules which would indicate a lower d.p. than is actually the case. Negatively charged uronic acids are also retained slightly on Bio-Gel P-2, hence eluting later than expected.

The retention time during Dionex HPLC placed the oligosaccharide either in the range of neutral oligosaccharides greater than maltoheptaose or in the region of the negatively charged molecules, but the oligosaccharide did not co-elute with any of the known markers though without structural information no size determination can be made from its relative retention time.

Other carbohydrate compounds were found to be present, though, except for the oligosaccharide at R_T = 12-13 minutes, they were all present in small amounts and were not studied further, though they may be worth examining in future. Material which eluted before the maltotriose marker was not considered as this region is heavily contaminated with material from the culture medium.

The Bio-Gel P-2 fraction at K_{av} = 0.5 (fig. 3.1.1.3.b) contained an oligosaccharide with a retention time of approximately 12-13 minutes and an $R_{M3\text{-}M4}$ = 0.2. This was the most abundant of the oligosaccharides detected. It was present in earlier Bio-Gel P-2 fractions, though at lower concentrations, which may be due to overloading of the column and poor resolution. The fractions after K_{av} = 0.5 continued to show increasing concentrations of the oligosaccharide, but it was not possible to determine the K_{av} of the oligosaccharide, as later fractions, nearing the included volume, were viscous and difficult to prepare for Dionex HPLC. It must therefore be assumed that the K_{av} of this compound is greater than 0.5, and no assumptions can be made about its d.p. from this experiment.

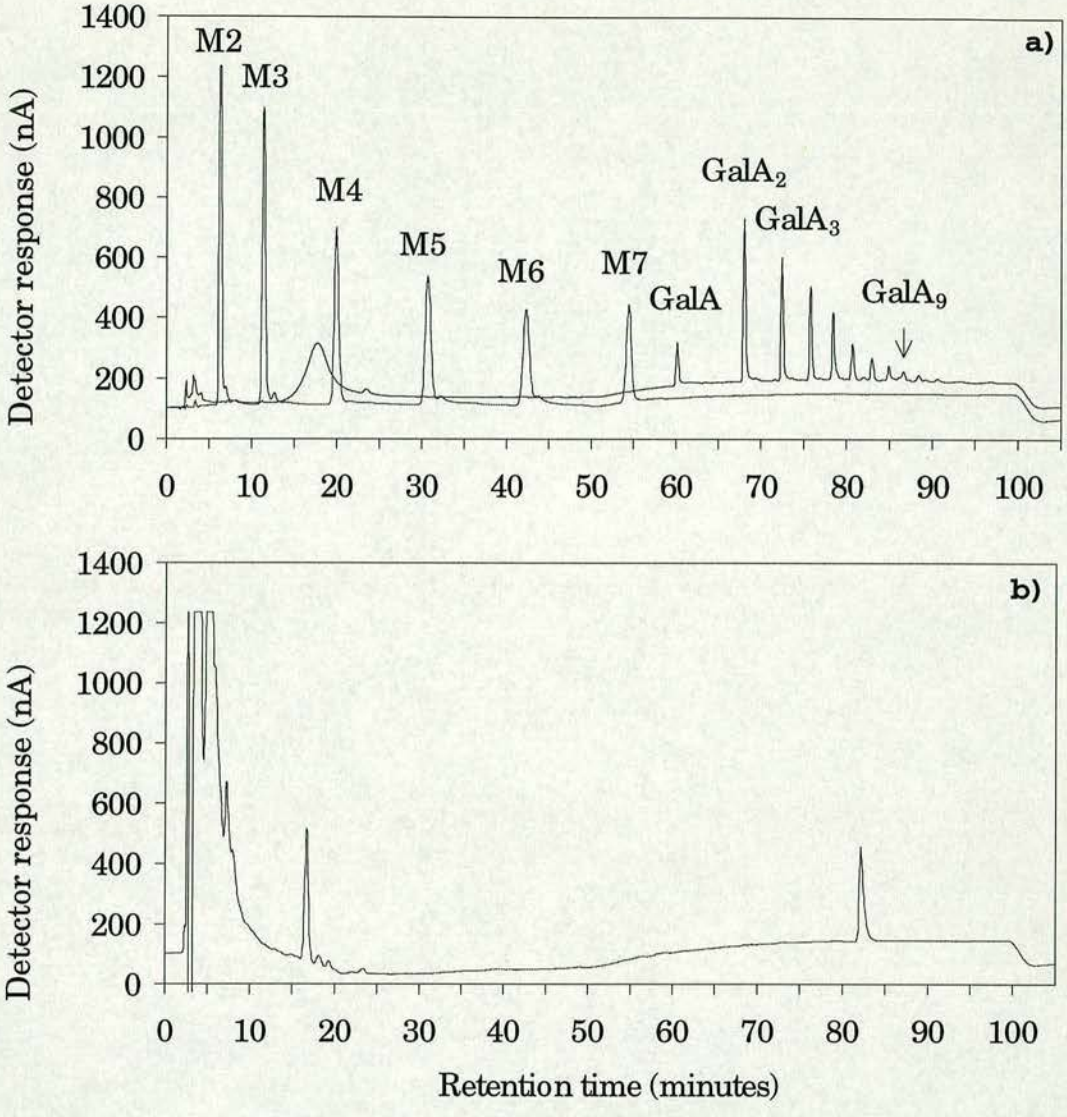


Fig. 3.1.1.2. Dionex HPLC (Eluent Programme 1) analysis showing separation of malto-oligosaccharides (M2 - M7) and oligogalacturonide markers (a) and the components of fresh rose culture medium (b).

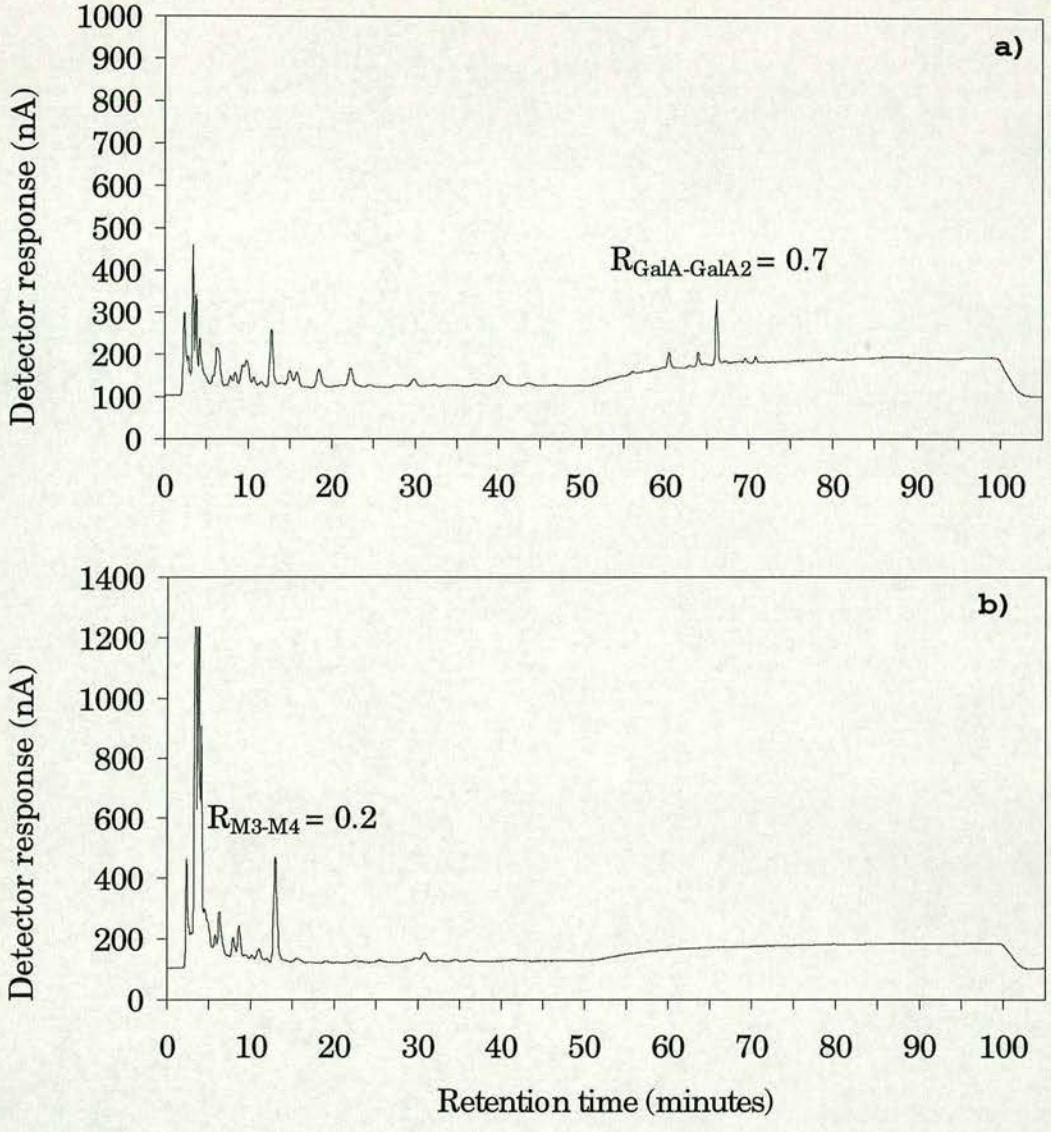


Fig. 3.1.1.3. Dionex HPLC analysis of spent culture medium of rose analysed by gel permeation chromatography. Fractions at K_{av} = 0.3 (a) and 0.5 (b) are shown.

3.1.2. Uronic Acid Containing Oligosaccharides in Spent Culture of Rose

In order to detect uronic acid containing oligosaccharides, rose cells were presented with D-[6-^{14}C]glucuronic acid, and the spent culture medium was analysed by gel permeation chromatography and paper chromatography.

3.1.2.1. Radioactive Compounds

A portion of each of the Bio-Gel P-2 fractions was scintillation counted and fig. 3.1.2.1. shows the total radioactivity in each fraction. As expected there was radioactive material present at the void volume; this consisted of material with a molecular weight in excess of 1800 Da and so included small soluble polysaccharides and large oligosaccharides. There was a peak of radioactivity at the included volume and two other peaks eluting immediately before and after the included volume. Glucuronic acid lactone moves as a neutral sugar whereas glucuronic acid is retained slightly on Bio-Gel P-2; hence these probably accounted for the included volume peak and the later peak respectively.

The oligosaccharide region of the column contained radioactivity, although apart from the peak of material at K_{av} = 0.8 no discrete peaks were present. The material at K_{av} = 0.8 appeared to correspond to the material detected in section 3.1.1.1. where non-radiolabelled spent culture medium was analysed by phenol/sulphuric assay following Bio-Gel P-2 (fig. 3.1.1.1).

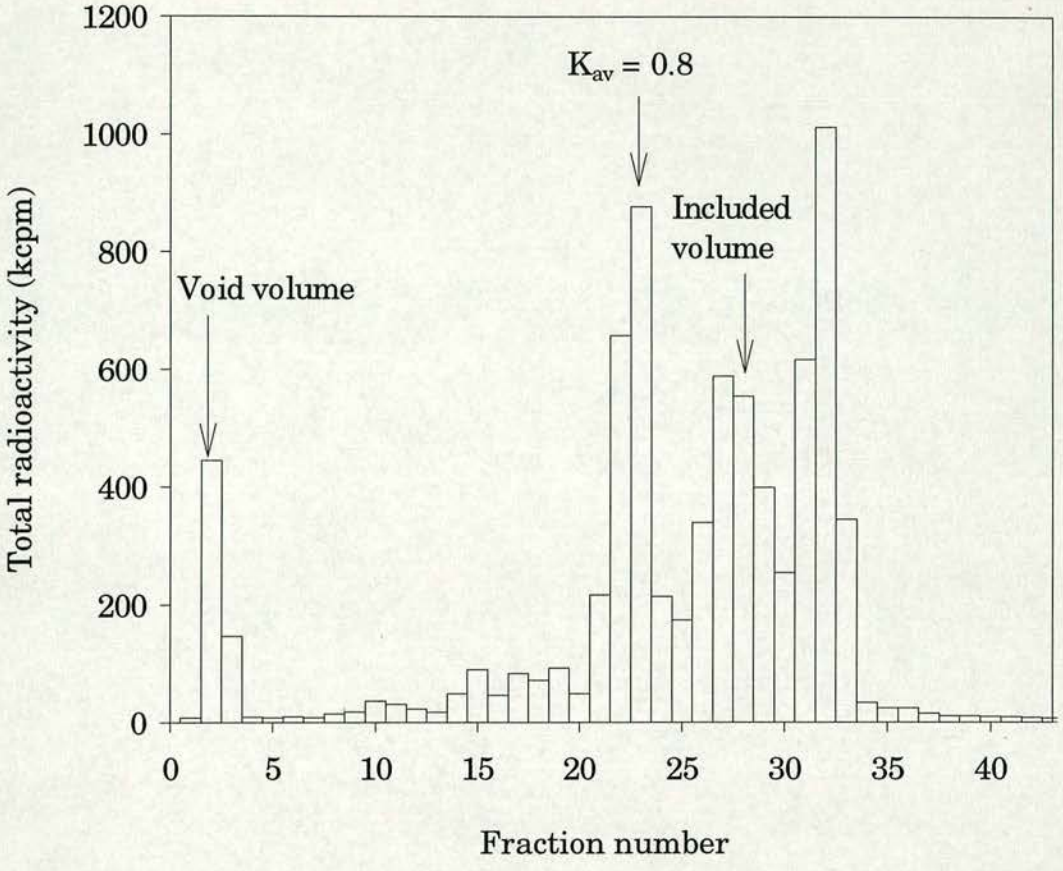


Fig. 3.1.2.1. Radioactivity in gel permeation chromatography fractions from spent culture medium of rose following presentation of D-[6-^{14}C]glucuronic acid to the cells.

3.1.2.2. Uronic Acid Containing Oligosaccharides

To separate and detect ^{14}C-oligosaccharides in the Bio-Gel P-2 fractions these were freeze dried, resuspended and analysed by paper chromatography (solvent system EAW), which separates oligosaccharides on the basis of size and resolves oligogalacturonides up to a d.p. of at least 8. The paper was autoradiographed for 1 week.

Radioactive material was detected at the origin of the void volume fractions as expected as polysaccharide material is chromatographically immobile in this solvent system. Unexpectedly there was material at the origin of the paper chromatogram in all the fractions right through to the included volume; this may be a "tail" on the void volume due to overloading of the column but this appears to be unlikely considering the low levels of radioactivity detected in the fractions immediately behind the void volume. The nature of this material remains unknown.

The K_{av} = 0.8 radioactive material was found to have co-migrated with an external marker of $GalA_3$ (autoradiogram not shown). The area of the chromatogram was eluted and approximately 43 kcpm of radioactive material was recovered. Although this material co-eluted with a large peak of radioactivity (fig. 3.1.2.1.) during gel permeation chromatography, the amount recovered when this compound was eluted was insufficient to account for all the radioactivity in that peak. As with the other fractions, there was radioactivity at the origin of the paper chromatogram, which must account for most of the radioactivity of that peak.

3.1.2.3. Correlation of ^{14}C- and Non-radiolabelled Oligosaccharides

A portion (2000 cpm) of the radioactive $GalA_3$ co-migrating material was analysed by Dionex HPLC (Eluent Programme 1). The eluted material was collected in a fraction collector and scintillation counted

(Fig. 3.1.2.2.). There was a delay between the Dionex detector and the fraction collector owing to the length of tubing between them, this also accounts for the tailing of the radioactive peak. The radioactive material was detected by Dionex HPLC as expected of a carbohydrate and appeared to co-elute (R_T = 11.74 minutes) with the oligosaccharide detected when spent culture medium was analysed by Dionex HPLC in section 3.1.1.2. Although no maltose markers were analysed at the time the similarity in retention time (within one minute on separate days of analysis) and the absence of other peaks of similar retention time suggests that the two compounds might be the same.

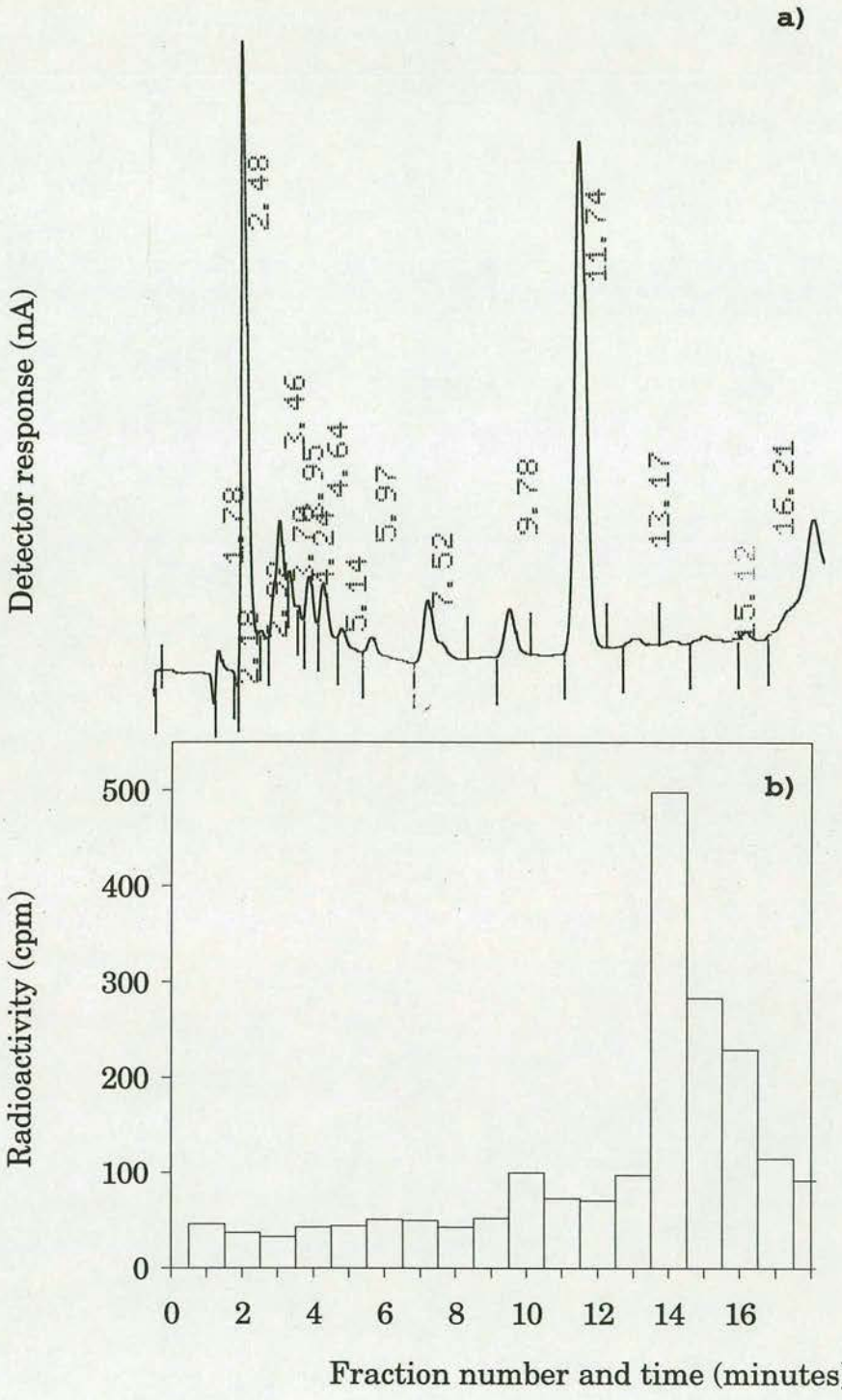


Fig. 3.1.2.2. Dionex HPLC analysis (Eluent Programme 1) of radioactive, $GalA_3$ co-migrating material, detected by paper chromatography following presentation of D-[6-^{14}C]glucuronic acid to rose cells (a) and determination of radioactivity in fractions collected from Dionex HPLC (b).

3.1.3 Purification of Oligosaccharides

All attempts to purify the putative heptasaccharide were unsuccessful as other peaks continued to coelute (fig. 3.1.3.4.).

3.1.3.1. Purification of Trisaccharide

a) ^{14}C Trisaccharide.

Presentation of 150 kBq D-[6-^{14}C]glucuronic acid to 7 day old rose culture and the purification of the ^{14}C-trisaccharide from the spent culture medium, led to 4000 cpm of trisaccharide being recovered. A portion of the product was analysed by paper chromatography (solvent system BAW) and the paper scintillation counted. The only radioactive product was found to co-migrate with $GalA_3$, as expected of the trisaccharide.

b) Non-Radiolabelled Trisaccharide.

The rose trisaccharide was extracted from spent culture medium of rose 2 weeks after sub-culturing. The spent culture medium was alcohol precipitated before anion exchange; this step removed extracellular polysaccharides and other polymeric material which otherwise affected column chromatography; fig. 3.1.3.1.b,c. shows that there was no loss trisaccharide at this step. After drying off the ethanol, the spent culture medium was anion exchanged in order to separate the negative trisaccharide from the large amount of unused glucose in the culture medium. Fig. 3.1.3.2a. shows the reduction in the large glucose peak at approximately 6 minutes by anion exchange as seen by Dionex HPLC analysis; note the presence of the other oligosaccharide (R_T = 65 minutes) detected in 3.1.1.2 which shows that this too is an acidic oligosaccharide.

After Bio-Gel P-2 chromatography the remaining glucose was almost completely removed and any remaining was lost after desalting on Sephadex G10 (fig. 3.1.3.2b,c.).

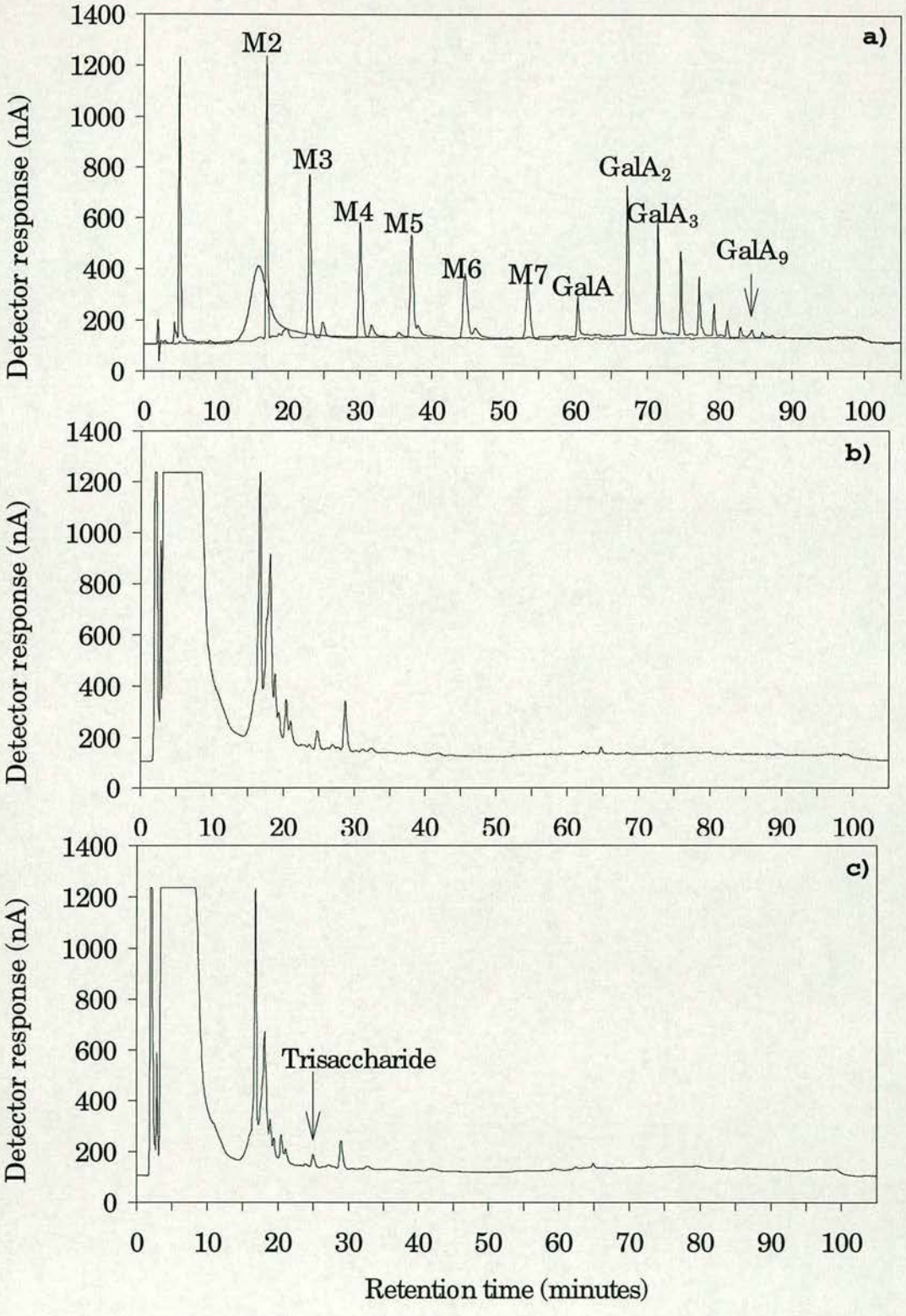


Fig. 3.1.3.1. Dionex HPLC (Eluent Programme 2) of extraction procedure for rose trisaccharide, showing markers (a), spent culture medium (b) and spent culture medium following alcohol precipitation (c).

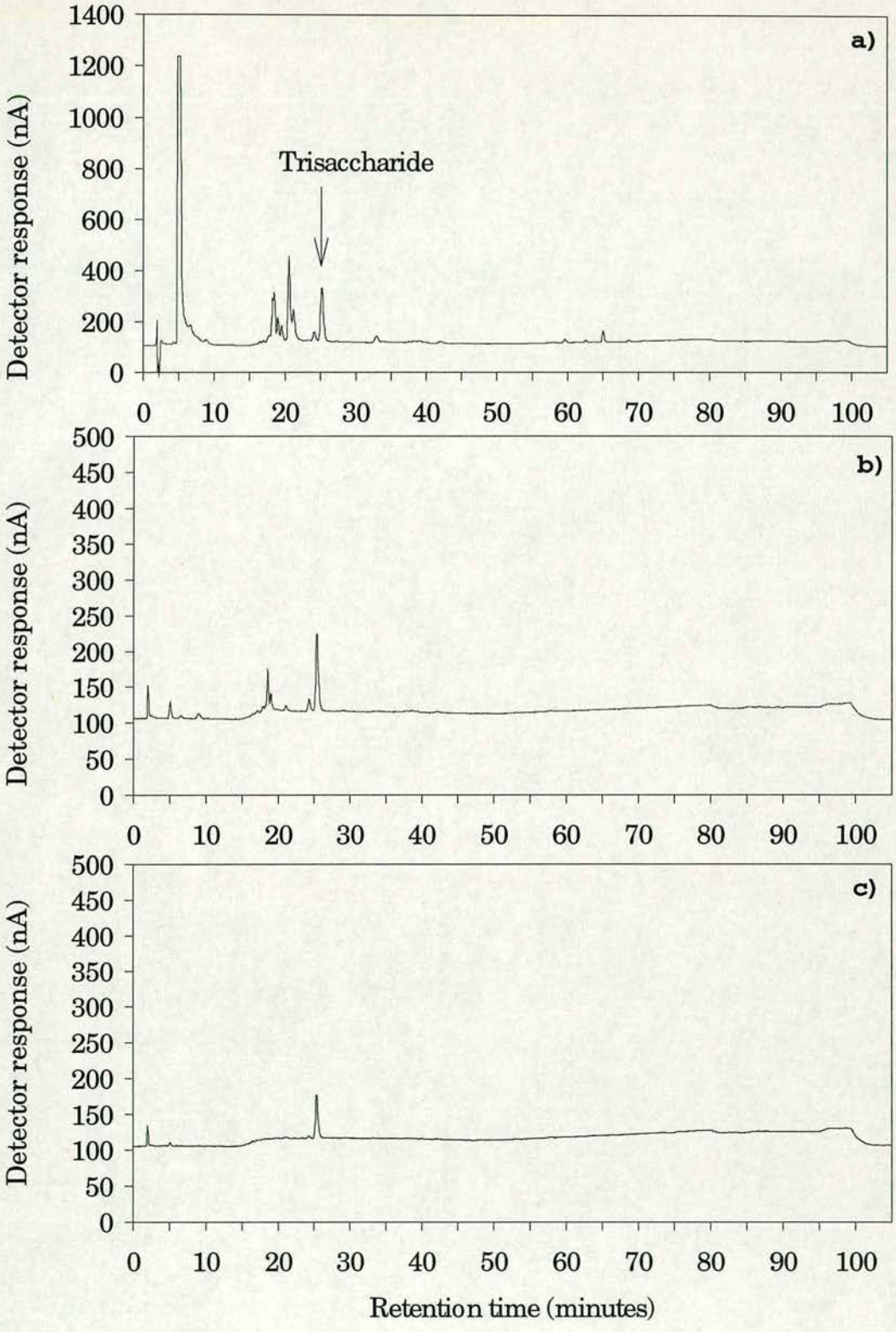


Fig. 3.1.3.2. Dionex HPLC (Eluent Programme 2) analysis of the extraction procedure of the rose trisaccharide, showing the effect of anion exchange (a), Bio-Gel P-2 (b) and the Sephadex G10 (c) purification.

The desalting step also removed the remaining impurities in the trisaccharide preparation. The small peak at approximately 2.5 minutes is a feature of the injection. (Note that the analysis of the spent culture medium and the ethanol precipitation (fig. 3.1.3.1.) samples are at half the concentration of the other analyses (fig. 3.1.3.2.)). There was some loss of yield in the extraction due to the presence of the small peak of material eluting immediately before the trisaccharide (e.g. fig. 3.1.3.2.b). This impurity also chromatographed close to the trisaccharide and in the desalting step it was necessary to discard some fractions containing trisaccharide as they contained the other compound as well. An extraction process starting with 800 ml of two week old culture medium typically yielded approximately 0.5 mg of trisaccharide.

3.1.3.2. Calibration of Dionex HPLC PAD Detector Response

In order to calculate concentrations of trisaccharide when performing Dionex HPLC analysis without having to use up limited trisaccharide stock, the Dionex PAD detector response relative to the trisaccharide and to maltotriose was determined. A maltotriose stock solution was calibrated by direct comparison of a trisaccharide stock solution at 4 concentrations. Maltotriose was found to give a mean 12% lower PAD response compared with the trisaccharide (fig. 3.1.3.3.). This maltotriose stock solution was used to calculate future Dionex HPLC analyses of trisaccharide concentrations with analysis of 4 concentration levels with duplicate samples at each level.

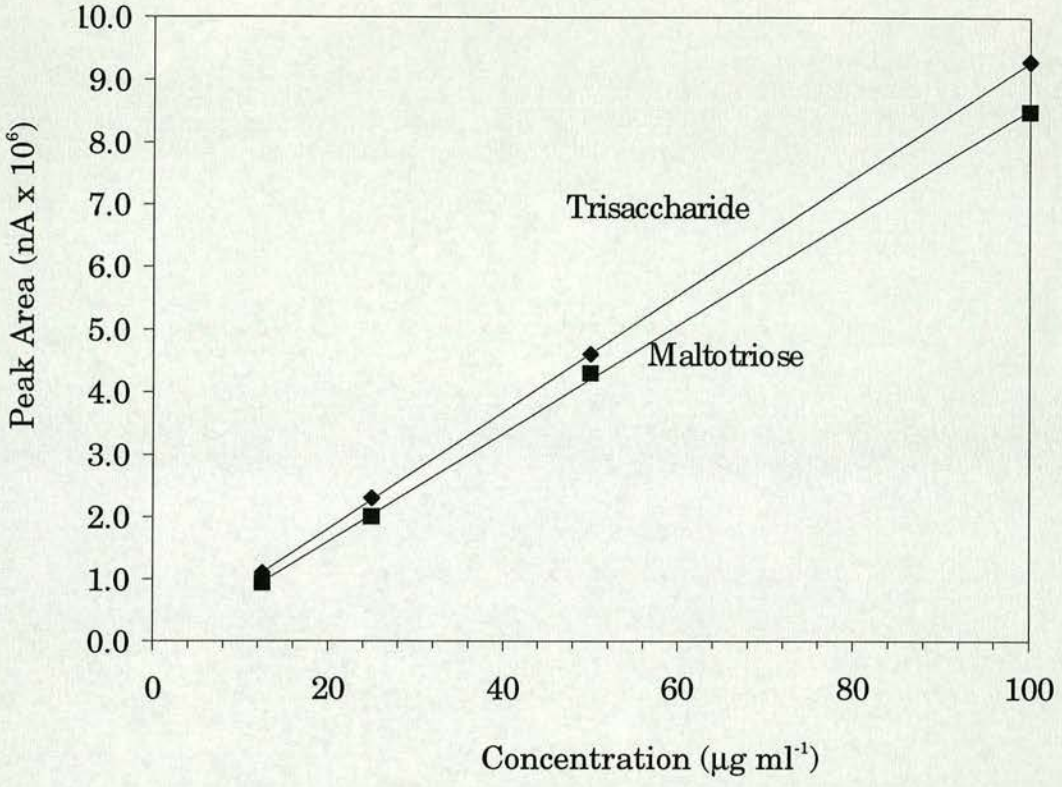


Fig. 3.1.3.3. Comparison of Dionex (Eluent Programme 2) PAD response (peak area) to maltotriose and trisaccharide.

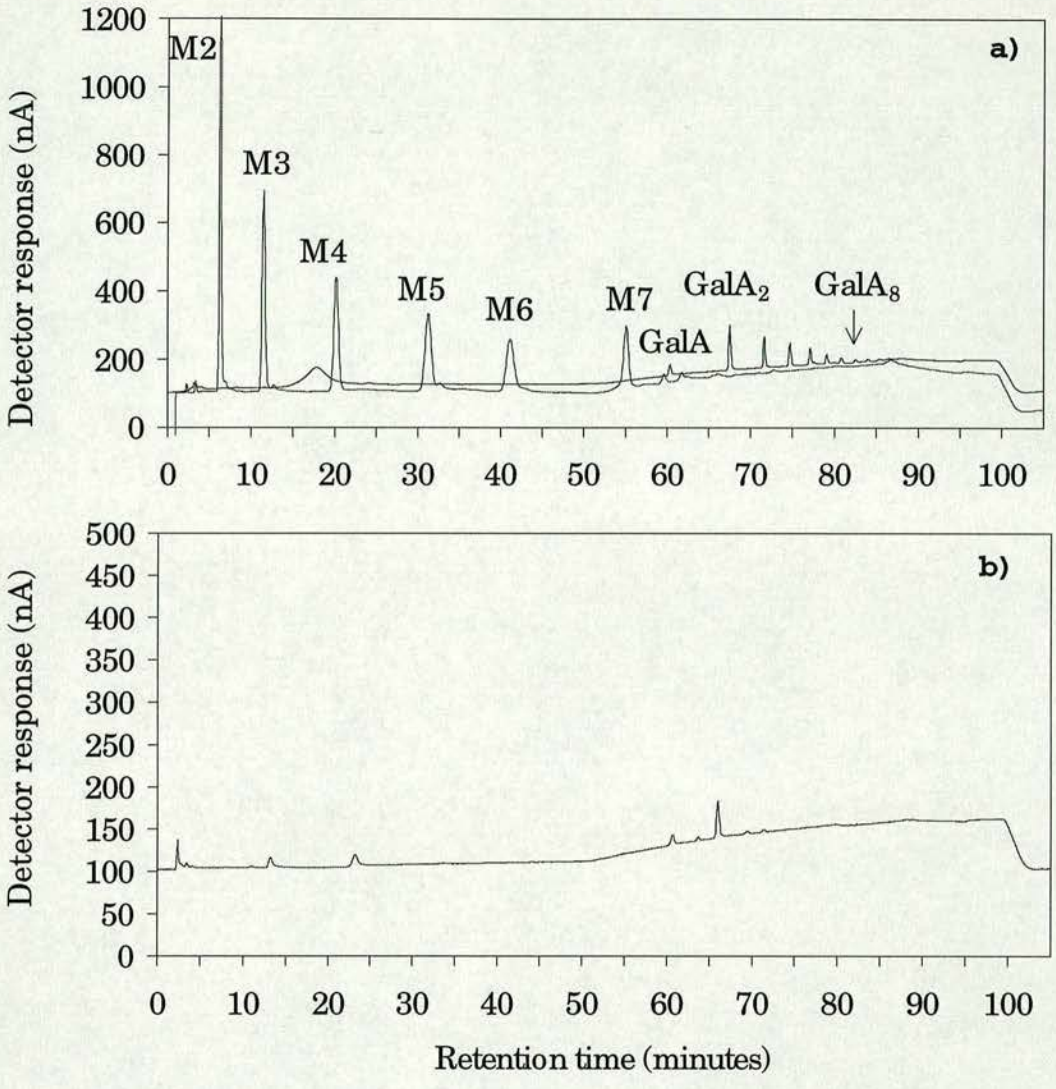


Fig. 3.1.3.4. Dionex HPLC analysis (Eluent Programme 1) of the purification procedure of the putative heptasaccharide from spent culture medium of rose by anion exchange chromatography, showing markers (a) and effect of anion exchange (b). Putative heptasaccharide at R_T = ~ 66 minutes.

3.1.4. Accumulation of Trisaccharide

3.1.4.1. Growth of the Culture

The rate of growth of the rose culture was determined by measuring the packed cell volume (PCV) over a period of time. Fig. 3.1.4.1. shows the packed cell volume from the time of sub-culturing and for 26 days thereafter. There was a lag period in cell growth in which the PCV remained close to the level at the time of sub-culturing (approximately 0.03 ml ml^{-1}) and which lasted for 6 days. From 6 to 19 days after inoculation, the culture grew at a constant rate with an increase in PCV to approximately 0.54 ml ml^{-1}. Between 19 and 26 days there was a slight slowing down in the rate of growth possibly as the glucose substrate was depleted.

3.1.4.2. Rate of Accumulation of Trisaccharide

The accumulation of the trisaccharide was measured by Dionex HPLC. Fig. 3.1.4.2. shows the components of the spent culture medium at 4 and 14 days after sub-culturing. At 4 days no trisaccharide was detectable but by 6 days post inoculation trisaccharide was detected at 0.03 $\mu g\ ml^{-1}$. This increased in line with the growth of the culture, accumulating at a constant rate until 22 days after sub-culturing (0.8 $\mu g\ ml^{-1}$). At this point there was a decrease in the rate of accumulation and possibly a slight decrease in the overall concentration of trisaccharide in the culture perhaps due to turnover of the trisaccharide.

The Dionex analysis also showed that the slowing down in rates of growth and trisaccharide accumulation corresponded to the decrease in glucose (R_T = ~8 minutes) remaining in the culture medium (fig. 3.1.4.2.).

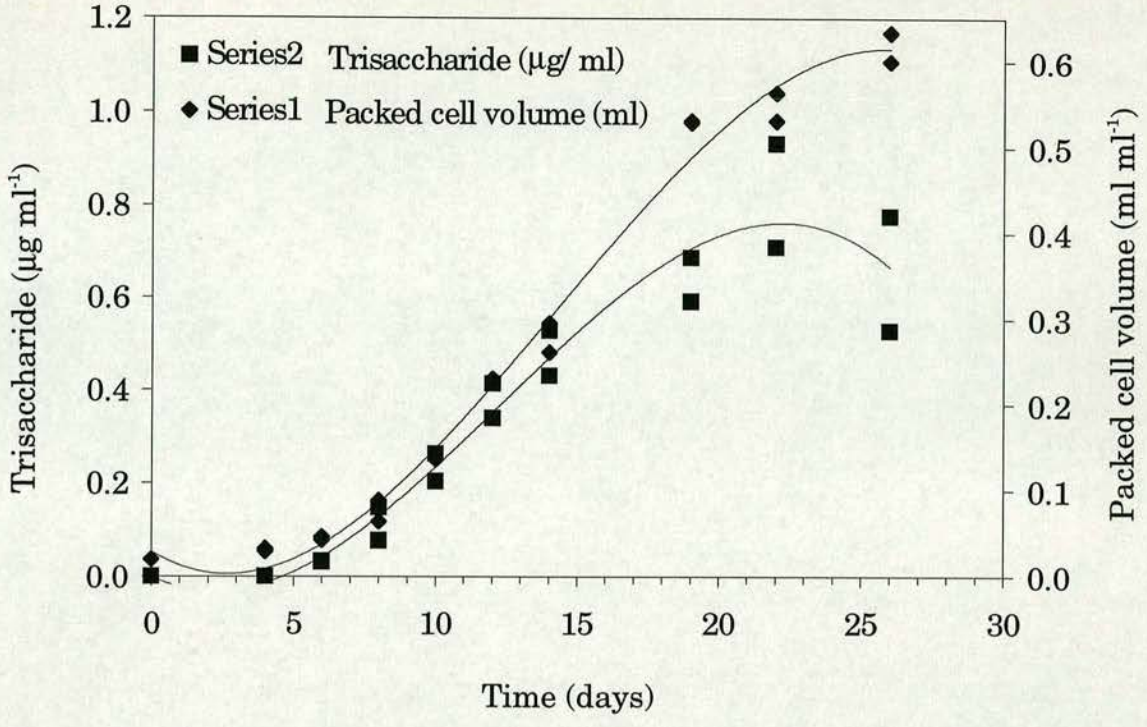


Fig. 3.1.4.1. Levels of trisaccharide and packed cell volume of rose cell culture for 26 days following sub-culturing.

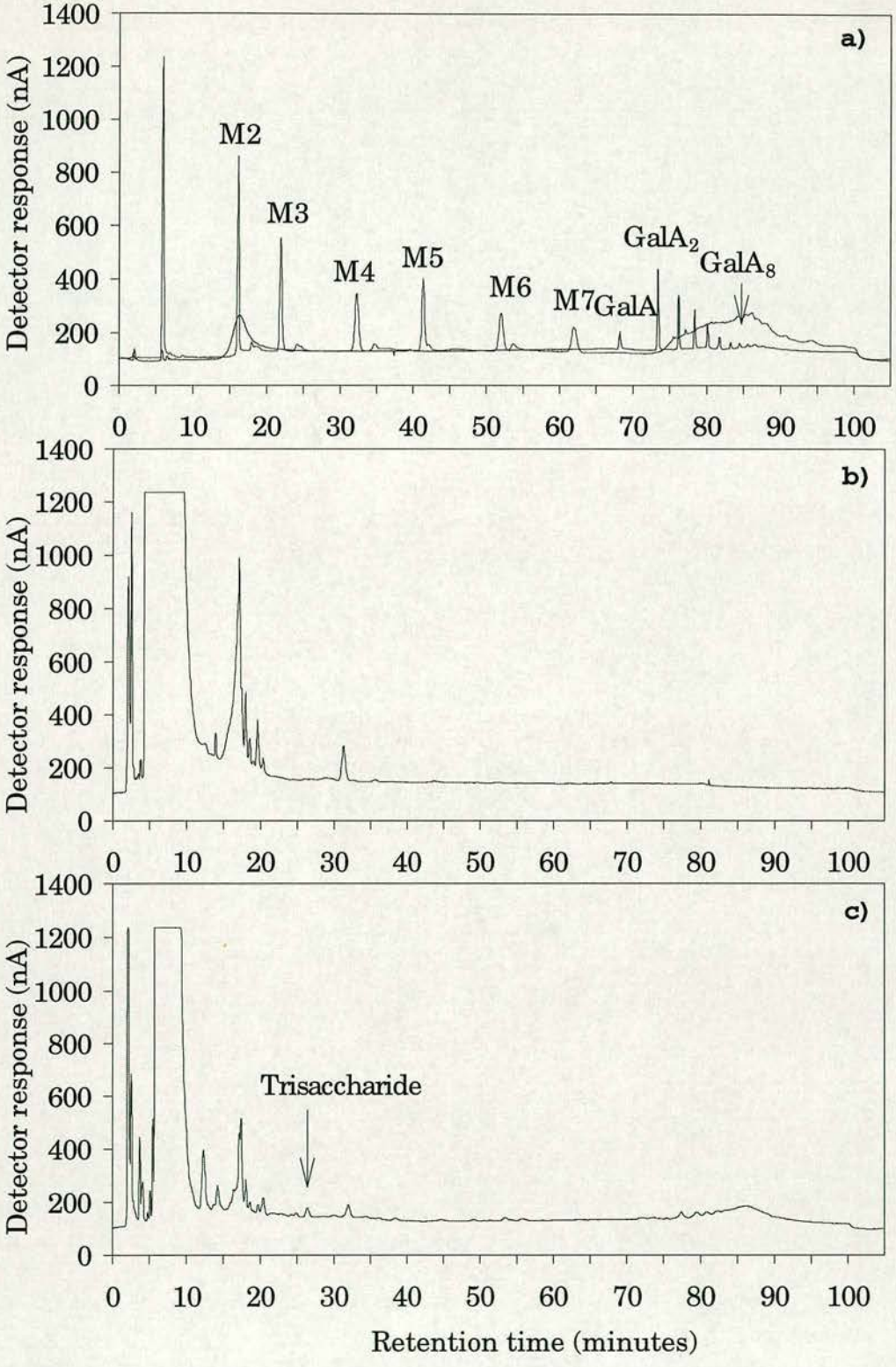


Fig. 3.1.4.2. Dionex HPLC analysis (Eluent Programme 2) to determine levels of trisaccharide in spent culture medium. Markers (a) culture medium at 4 days (b) and 14 days (c) after inoculation.

3.1.6. Turnover of ^{14}C-Trisaccharide

The ability of rose cells to break down the trisaccharide was determined by presentation of purified ^{14}C-trisaccharide to rose cells and analysis of the radioactivity remaining in the culture by paper chromatography (solvent system EAW). This method allowed not only the amount of trisaccharide remaining in the culture to be determined, but also changes in d.p. due to breakdown or crosslinking to be detected.

Fig. 3.1.6.1. shows that there was essentially no loss of ^{14}C-trisaccharide as the culture grew from 7 days to 10 days after sub-culturing. A smaller amount of trisaccharide was recovered from the cultures containing cells than from the controls, but this may have been due to incomplete washing of the cells. The difference between the amount of trisaccharide recovered from the controls and the cell cultures was constant throughout the experiment, which lends weight to the difference being of experimental origin.

There appeared to be no change in the structure of the trisaccharide as determined by paper chromatography (solvent system EAW); throughout the experiment the trisaccharide continued to co-migrate with the $GalA_3$ external marker.

This experiment was performed when the cells were growing rapidly as seen in fig 3.1.4.1. and over a period of 72 hours there was essentially no breakdown of trisaccharide. If sufficient radiolabelled trisaccharide had been available it would have been interesting to repeat the experiment late in the life of the culture, when fig. 3.1.4.1. suggested that there might be a slight turnover of the trisaccharide.

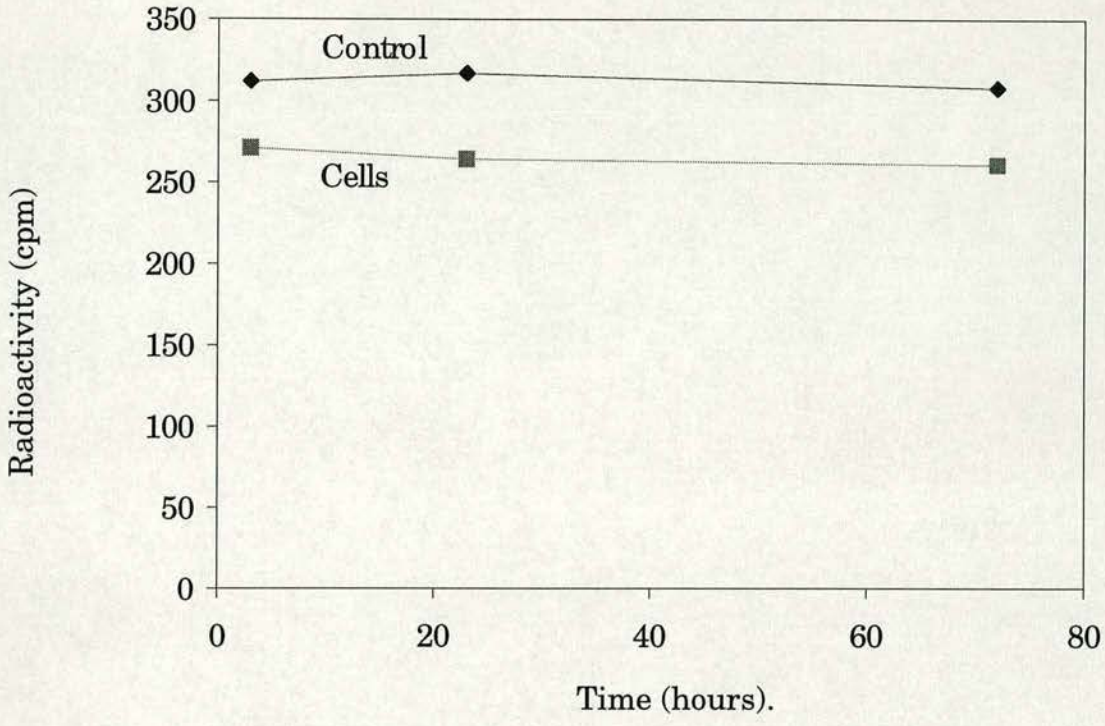


Fig. 3.1.6.1. Turnover of the ^{14}C-trisaccharide by rose cells *in vivo*, as determined by paper chromatography (solvent system EAW) of spent culture medium from rose cells following presentation of ^{14}C-trisaccharide.

3.1.7. Oligosaccharides from Other Plant Suspension Cultures

To determine whether the oligosaccharides discovered in the spent culture medium of rose were present in the spent culture medium of other cultures, *Acer* (dicotyledonous plant), *Festuca* (monocotyledonous plant) and *Zea* (monocotyledonous plant) cell suspension cultures were analysed by Dionex HPLC (Eluent Programme 2), with authentic trisaccharide as a marker (fig. 3.1.7.1.).

3.1.7.1. Spent Cell Suspension Culture Medium of *Acer*

The spent *Acer* culture medium contained material which co-eluted (R_T = ~ 26 minutes) with the trisaccharide detected in spent rose medium; however, co-elution cannot be used as absolute identification (fig. 3.1.7.2.). The heptasaccharide seen in rose culture was not detected. There was a large peak at R_T = 20 minutes; this appeared to be of low d.p., eluting before maltotriose but may be worthy of further study.

3.1.7.2. Analysis of the Spent Culture Medium of *Festuca*

Spent culture medium of *Festuca* did not contain any material which co-eluted with the rose trisaccharide nor with the putative heptasaccharide (fig. 3.1.7.3.). There was accumulation of material at R_T = 15 - 25 minutes between weeks one and two, all eluting before maltotriose, and poorly resolved. It was also not clear whether this was present in the fresh culture medium as this contained a large peak at this position, which disappeared in the inoculated medium, possibly having been used up by the cells.

3.1.7.3. Analysis of the Spent Culture Medium of *Zea*

Spent culture medium of *Zea* did not contain material co-eluting with either the rose trisaccharide or putative heptasaccharide (fig. 3.1.7.4.). There were small peaks of material near the maltotriose marker but

similar material was also present in the control. One peak which showed an increase between weeks one and two (R_T = 23 minutes) may be worth investigating although the amount present is low.

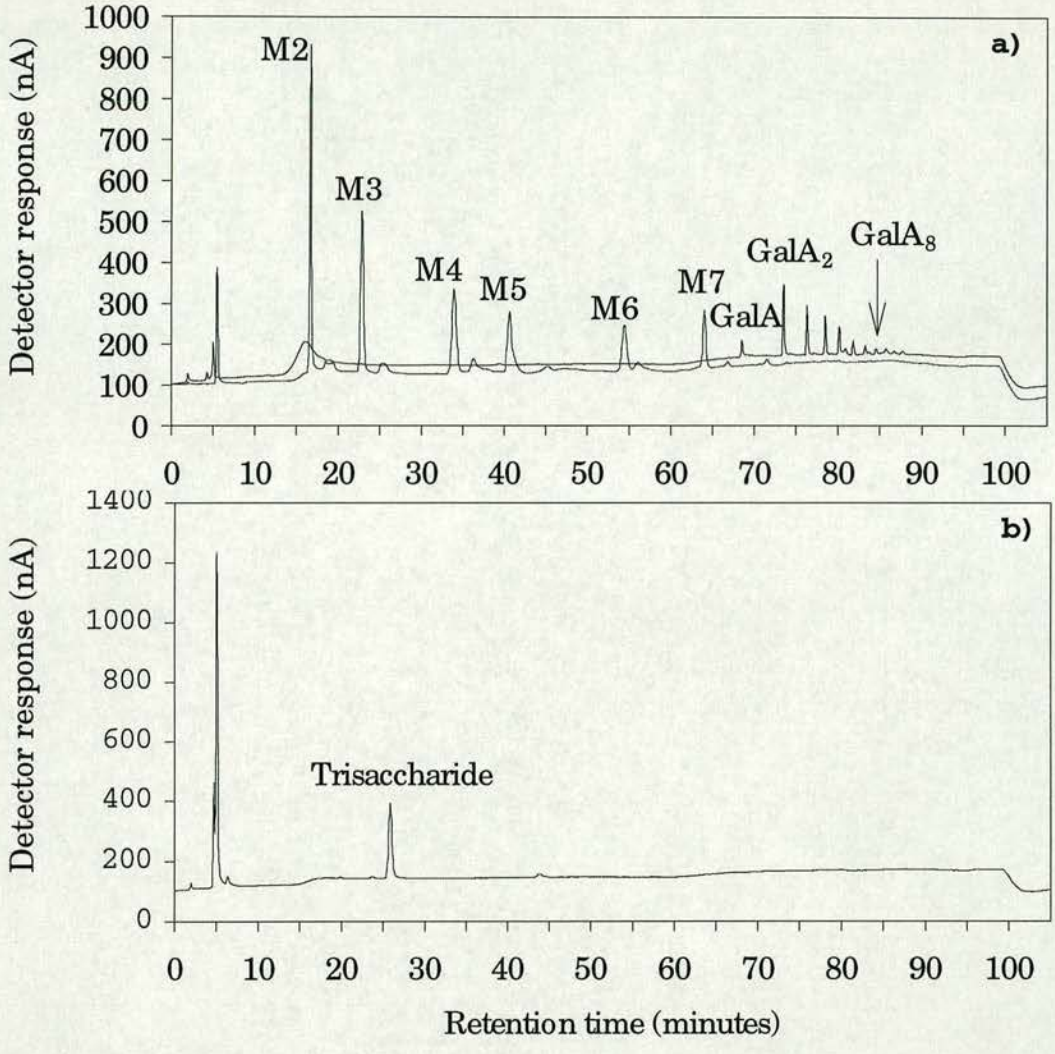


Fig. 3.1.7.1. Dionex HPLC analysis of authentic oligosaccharides (Eluent Programme 2), showing maltose and oligogalacturonide markers (a) and trisaccharide (b).

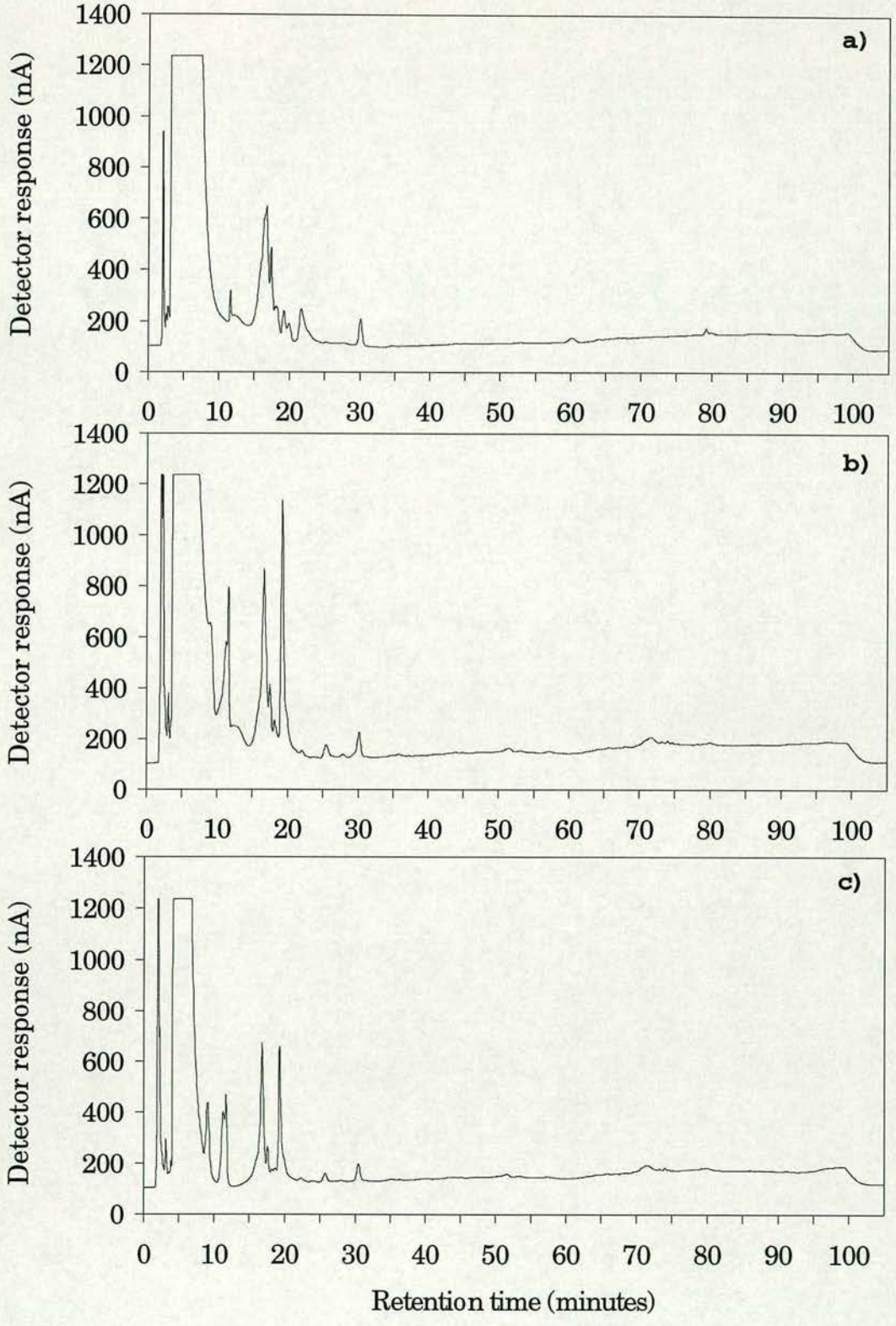


Fig. 3.1.7.2. Dionex HPLC analysis (Eluent Programme 2) of the spent culture medium of *Acer*, showing fresh culture medium (a), one week old culture (b) and two week old culture (c).

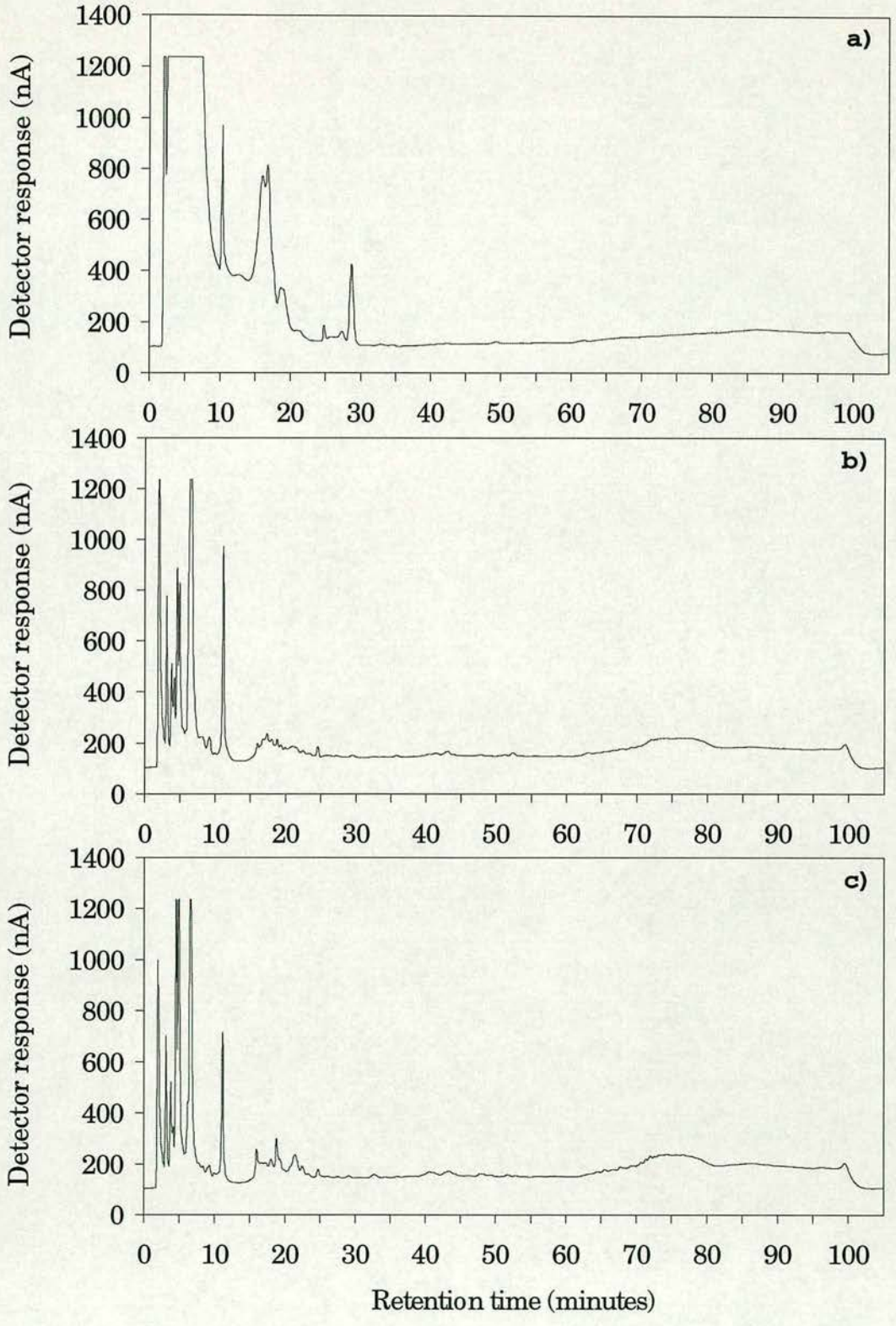


Fig. 3.1.7.3. Dionex HPLC analysis (Eluent Programme 2) of the spent culture medium of *Festuca*, showing fresh culture medium (a), one week old culture (b) and two week old culture (c).

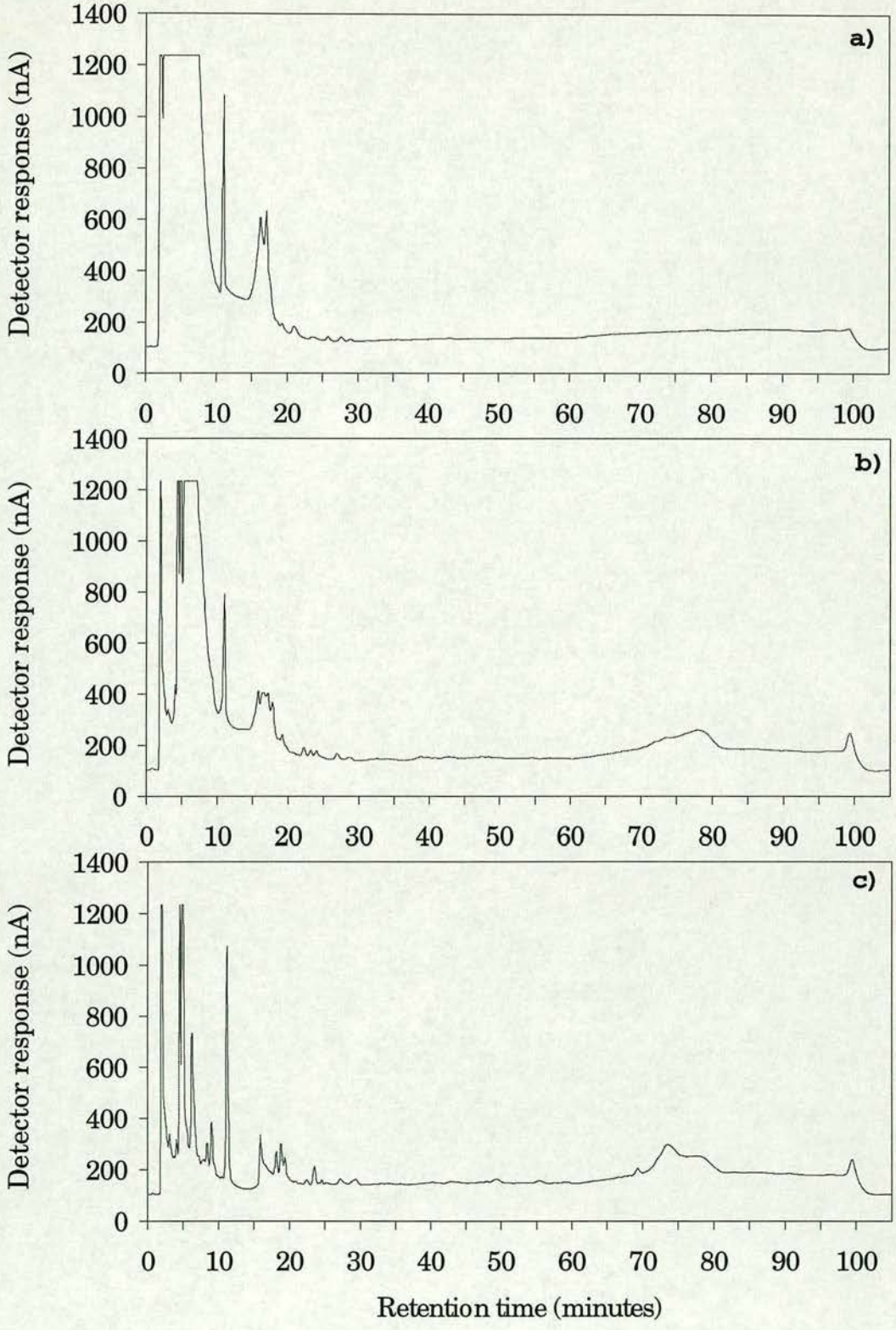


Fig. 3.1.7.4. Dionex HPLC analysis (Eluent Programme 2) the spent culture medium of *Zea*, showing fresh culture medium (a), one week old culture medium (b) and two week old culture medium (c).

3.1.8. Discussion of Results

Spent culture medium from rose cell suspension cultures was analysed by partial purification on a Bio-Gel P-2 column and by Dionex HPLC of the resulting fractions. The spent medium was found to contain two main oligosaccharides, one eluting in the region of a heptasaccharide and one in the region of the included volume though the exact K_{av} was not established. When the cells were presented with D-[6-^{14}C]glucuronic acid, whose ^{14}Carbon is specifically incorporated into uronic acids, a radiolabelled oligosaccharide was detected in the spent culture medium. This was purified by paper chromatography and compared with the smaller oligosaccharide detected in non-radiolabelled culture medium. They were found to show close retention time similarity during Dionex HPLC analysis. Unfortunately no non-radiolabelled oligosaccharide was available at the time and it was not possible to analyse both together. The putative trisaccharide was purified from spent culture medium with the aim of determining its structure and assess it for biological activity. It was not possible to purify the putative heptasaccharide within the time available.

The accumulation of the oligosaccharide was followed in the culture medium with time and it was found to increase in concentration at a steady rate between days 6 and 19 after sub-culturing. After day 19 there was a decrease in the rate of accumulation and possibly a slight turnover with an oligosaccharide concentration in the medium by day 22 of 0.8 $\mu g\ ml^{-1}$. The pattern of accumulation closely followed that of the growth of the culture as determined by measuring the packed cell volume.

Use of the radiolabelled oligosaccharide showed that the cells did not break down the oligosaccharide over a period of 72 hours during the part of the growth cycle in which they were growing at their fastest rate. It is possible however that at other times in the lifecycle of the culture

this may be different. Not enough radiolabelled material was available to investigate this.

When other cell suspension cultures were analysed to determine which oligosaccharides, if any, were present in the spent culture medium, only *Acer* was found to contain an oligosaccharide which showed co-migration with the trisaccharide detected in rose; however, it was not purified from the medium and further analysed. *Zea* and *Festuca* were not found to contain any oligosaccharides in any amounts that it would have be possible to purify in sufficient quantities for analysis within the time available.

3.2. Structural Analysis of Trisaccharide from Spent Culture Medium of Rose

Rose cell suspension cultures were found to produce a uronic acid containing putative trisaccharide which accumulated in the spent culture medium. This trisaccharide was analysed to determine its structure.

3.2.1. Paper Electrophoresis (pH 3.5)

In order to determine whether the trisaccharide was an oligogalacturonide e.g. $GalA_3$, it was subjected to paper electrophoresis at pH 3.5. Negatively charged carboxylic acid groups on uronic acid cause electrophoretic movement towards the anode at pH 3.5. The migration rate is proportional to the charge/mass ratio of the compound. This technique will separate individual uronic acids e.g. galacturonic and glucuronic acid as well as oligosaccharides containing uronic acids.

Approximately 250 cpm of purified ^{14}C-trisaccharide was loaded onto Whatman no. 1 paper with an internal marker of galacturonic acid. The compounds were electrophoresed at pH 3.5, the radioactivity was determined by scintillation counting and the markers were visualised by staining with aniline hydrogen phthalate. The oligogalacturonides were found to be poorly resolved; $GalA_2$ just separated from the larger and more mobile oligogalacturonides and $GalA_3$ to $GalA_6$ were unresolved (fig. 3.2.1.1.).

A single peak of radioactivity was found to have migrated away from the origin. This mobility confirms that the oligosaccharide contained a negatively charged group probably a uronic acid as expected following incorporation of D-[6-^{14}C]glucuronic acid. The trisaccharide did not co-migrate with any of the oligogalacturonide markers, being considerable less mobile, with an m_{GalA} = 0.82. This meant that the trisaccharide possessed a lower charge to mass ratio than $GalA_3$. This is

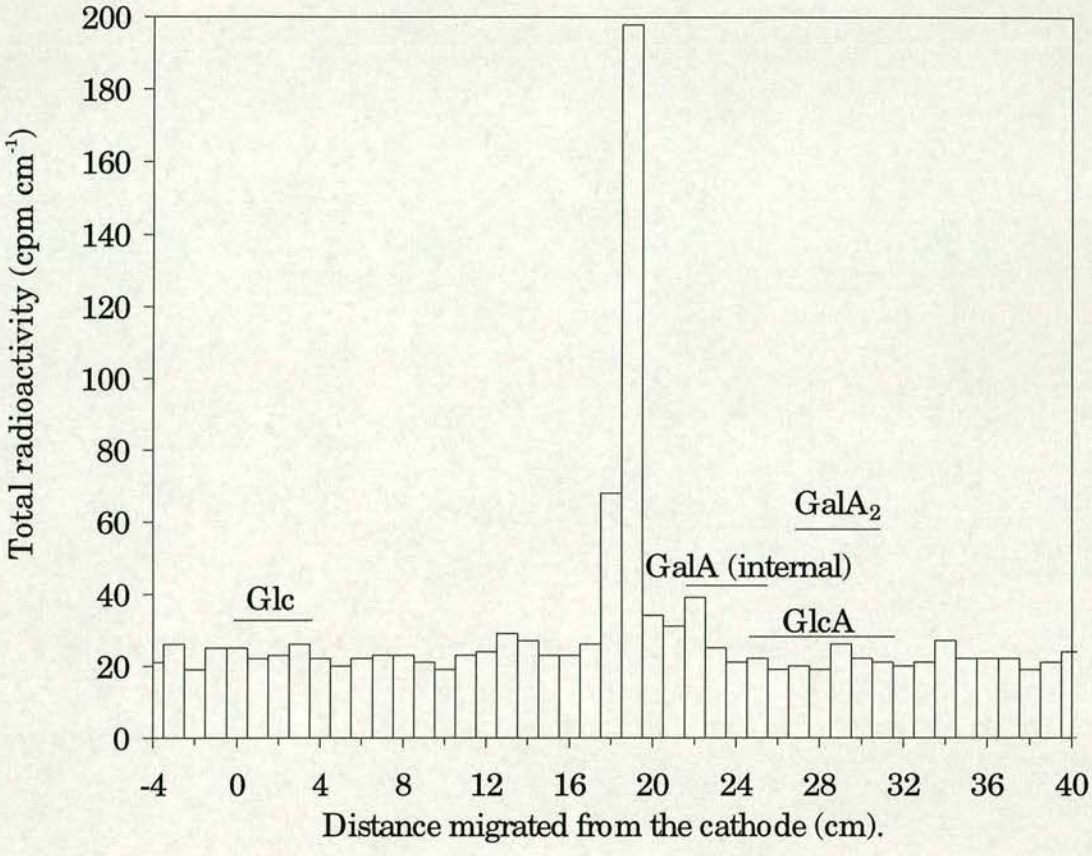


Fig. 3.2.1.1. ^{14}C-Trisaccharide analysed by paper electrophoresis (pH 3.5) to determined its charge/mass ratio relative to oligogalacturonide markers.

consistent with the trisaccharide containing neutral residues as well as uronic acid residues.

3.2.2. Analysis of Trisaccharide by Acid Hydrolysis

In order to determine the monosaccharide composition of the trisaccharide, it was subjected to acid hydrolysis and the products were identified by paper chromatography, paper electrophoresis (pH 3.5) and Dionex HPLC.

3.2.2.1. TFA Hydrolysis of ^{14}C-Trisaccharide.

To determine the susceptibility of the trisaccharide to acid hydrolysis, the purified ^{14}C-trisaccharide was hydrolysed by 2.0 M TFA and the products were separated by paper electrophoresis (pH 3.5). Fig. 3.2.2.1. shows the only radioactive product of the hydrolysis, which had an m_{GalA} value of 0.91 as compared with 0.82 for the original trisaccharide. It appeared that 2.0 M TFA produced a compound with a higher charge/mass ratio during electrophoresis, although the value, being less than 1 argues against a compound consisting purely of uronic acid residues. As none of the original trisaccharide remained and no uronic acid products were detected, this was consistent with the complete hydrolysis of the trisaccharide and the release of a non-radioactive neutral residue, not detectable in this experiment. It would appear that a compound smaller than the original trisaccharide was formed which possessed a higher charge to mass ratio than its parent compound e.g. a disaccharide containing a uronic acid and a neutral residue linked by an acid resistant bond.

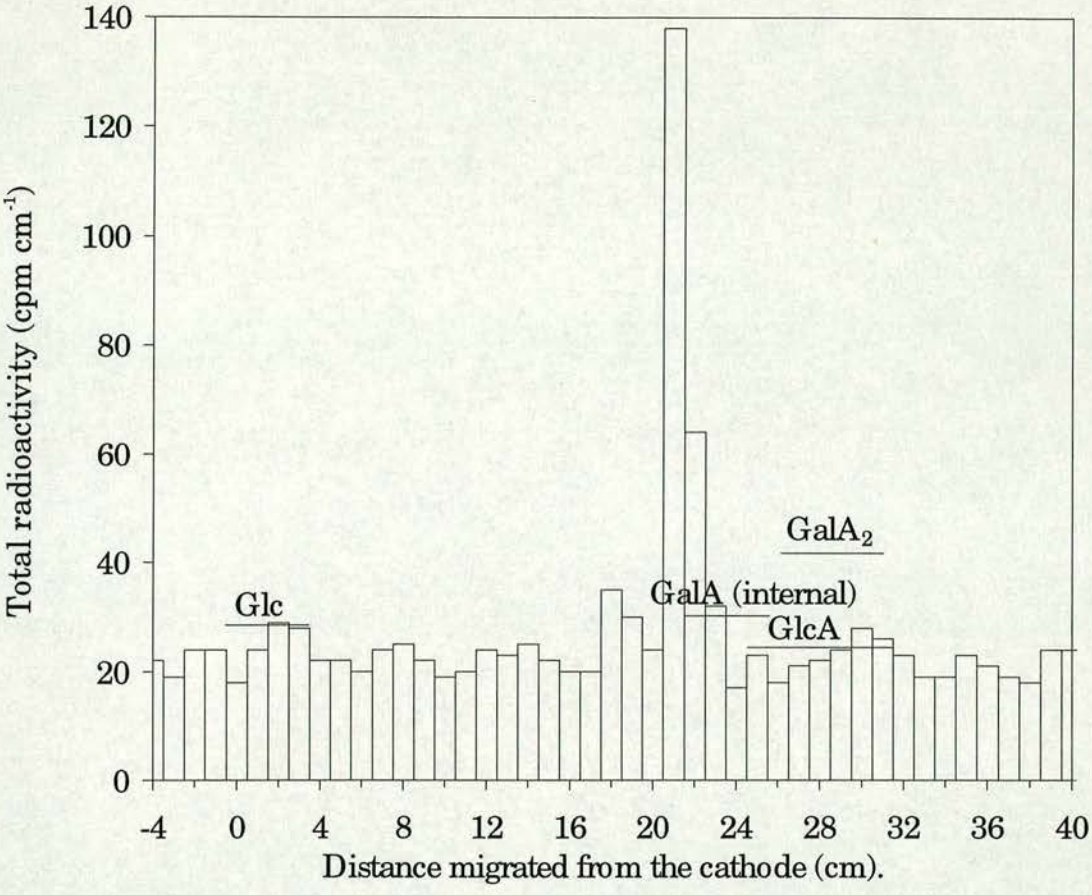


Fig. 3.2.2.1. ^{14}C-Trisaccharide analysed by paper electrophoresis (pH 3.5) following hydrolysis with 2.0 M TFA.

3.2.2.2. Analysis of the ^{14}C-Trisaccharide by Formic Acid Hydrolysis

As the trisaccharide had proved to be acid resistant, more severe conditions were used to try to hydrolyse it. The ^{14}C-trisaccharide was hydrolysed by 75% formic acid and the products were analysed by paper chromatography (solvent system BAW). The led to the detection of three radiolabelled products with none of the original compound remaining (fig. 3.2.2.2.). A radiolabelled peak of material was found to have migrated just ahead of the $GalA_3$ marker; this may be the putative disaccharide detected following TFA hydrolysis (section 3.2.2.1.). There was a peak of radioactivity at the uronic acid position (galacturonic and glucuronic acid are not resolved using this solvent system), and there was a peak near the position of the lactone of glucuronic acid. All three products were eluted for further analysis.

a) Peak 1

When the putative ^{14}C-disaccharide peak was eluted, and analysed by paper chromatography (solvent system EAW), with a marker of authentic ^{14}C-trisaccharide, it confirmed that this was not the original trisaccharide (fig. 3.2.2.3.). This is consistent with the acid-stable ^{14}C-disaccharide believed to be formed after TFA hydrolysis.

b) Peak 2.

The uronic acid peak was eluted and analysed by paper electrophoresis (pH 3.5), where it showed exact co-migration with an internal marker of glucuronic acid (Fig. 3.2.2.4.). As the trisaccharide was efficiently radiolabelled by addition of D-[6-^{14}C]glucuronic acid to the rose cells it can be assumed that the glucuronic acid in the trisaccharide is D-glucuronic acid.

c) Peak 3.

The final product, which had co-migrated with glucuronic acid lactone in the original analysis, was saponified to convert the lactone to the free acid and the product paper electrophoresed (pH 3.5). As was the case with the uronic acid product, this compound showed co-migration with glucuronic acid (Fig. 3.2.2.5.).

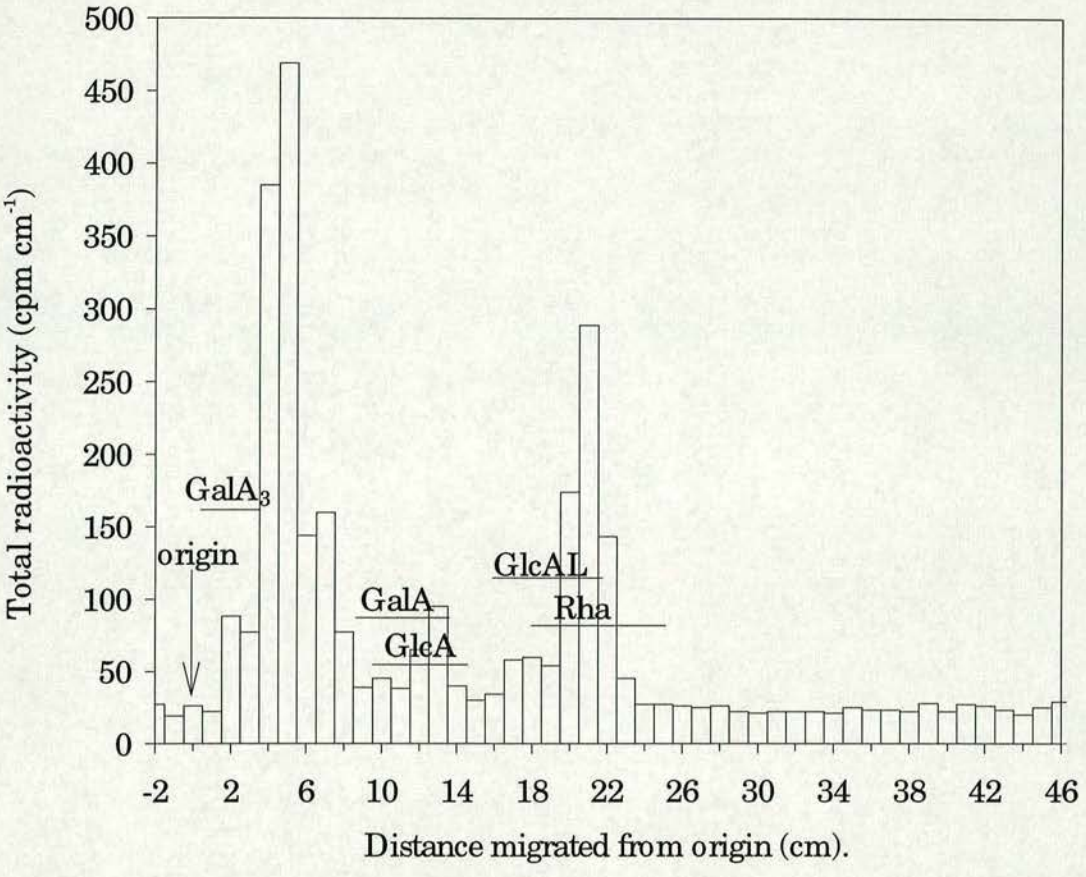


Fig. 3.2.2.2. Paper chromatogram (solvent system BAW) showing the products of ^{14}C-trisaccharide following formic acid hydrolysis.

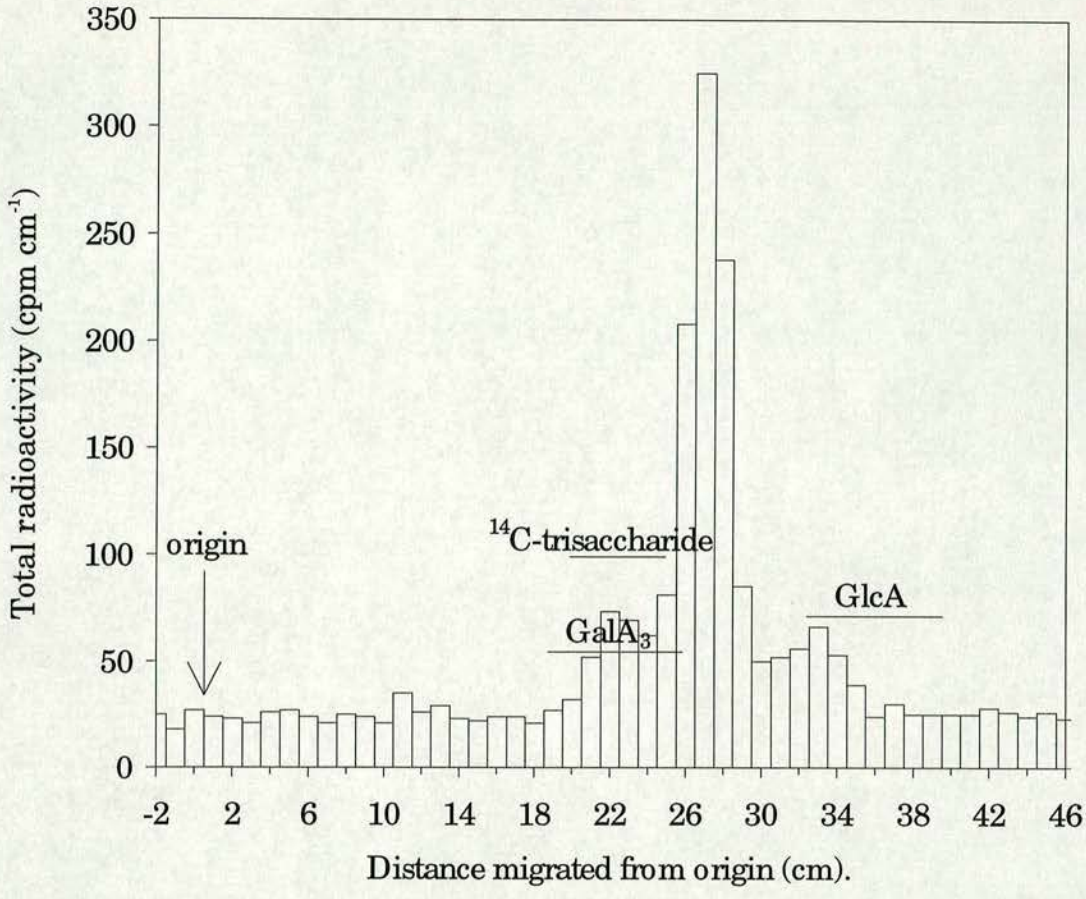


Fig. 3.2.2.3. Paper chromatogram (solvent system EAW) of the largest radioactive product released by formic acid hydrolysis of the ^{14}C-trisaccharide.

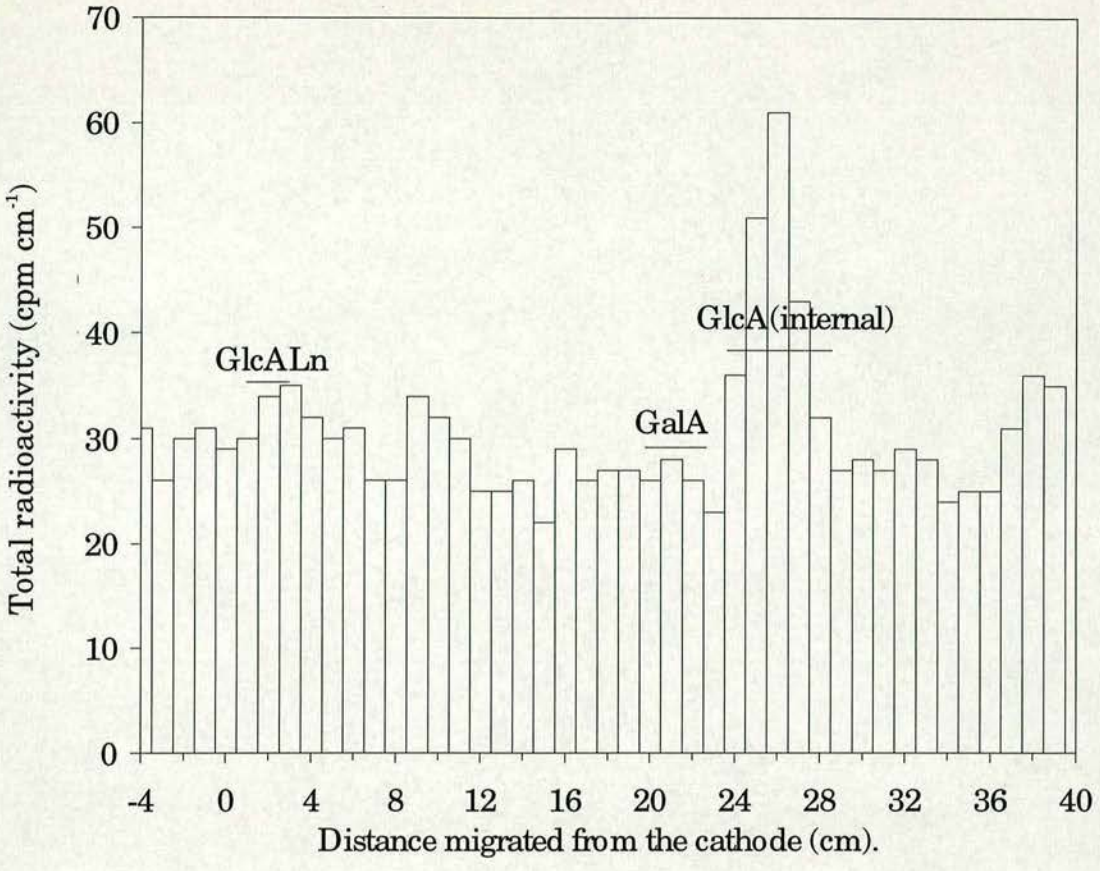


Fig.3.2.2.4. Paper electrophoresis (pH 3.5) of the ^{14}C-uronic acid product formed by formic acid hydrolysis of ^{14}C-trisaccharide.

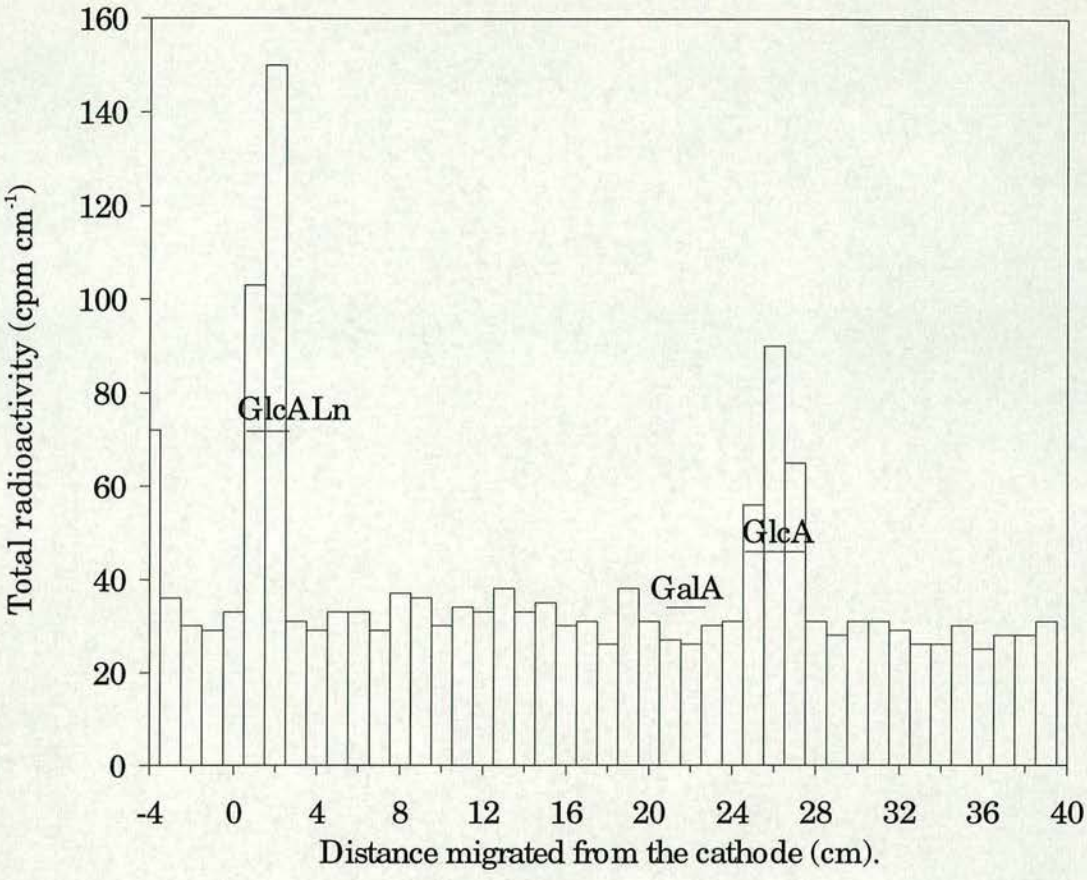


Fig. 3.2.2.5. Paper electrophoresis (pH 3.5) of the glucoronolactone co-migrating product of formic acid hydrolysis of ^{14}C-trisaccharide. The eluted compound was saponified immediately prior to electrophoresis.

3.2.2.3. Formic Acid Hydrolysis of Non-Radiolabelled Trisaccharide

To identify the non-radioactive residues of the trisaccharide, non-radioactive purified trisaccharide was hydrolysed by formic acid and the products analysed by Dionex HPLC (Eluent Programme 3). Fig. 3.2.2.6.b. shows the three products formed. One appeared to co-migrate with glucuronic acid as expected from the ^{14}C-trisaccharide analysis and a larger peak was detected close to the galacturonic acid marker; they were both in the region of the chromatogram where negatively charged sugars were expected. Their location was consistent with the findings of the previous acid hydrolysis, that glucuronic acid and a negatively charged disaccharide were the hydrolysis products. Another sugar was detected at R_T = 32 minutes which was in the region associated with neutral monosaccharides.

To identify these products, a mixture of internal markers were added to the sample. A mix of arabinose, galactose, xylose, galacturonic acid and glucuronic acid confirmed the identity of the uronic acid, as glucuronic acid marker and the proposed glucuronic acid peak in fig.3.2.2.6. were seen to co-elute (fig. 3.2.2.7.a.). The sugar at R_T = 25 minutes (fig. 3.2.2.7.a.) was identified as mannose by addition of mannose to the sample (fig. 3.2.2.7.b.). No standard was available for the disaccharide to confirm whether the peak at R_T = ~ 50 minutes was the same material as the putative disaccharide detected following acid hydrolysis of ^{14}C-trisaccharide.

Dionex HPLC analysis thus showed that the trisaccharide contained mannose and glucuronic acid, with mannose being released by acid hydrolysis and the disaccharide being partly broken down to give glucuronic acid and another unknown product. Note that the difference in retention time of mannose in fig. 3.2.2.7.a,b are a result of preparation of

fresh eluents between the two analyses, which were performed on separate days.

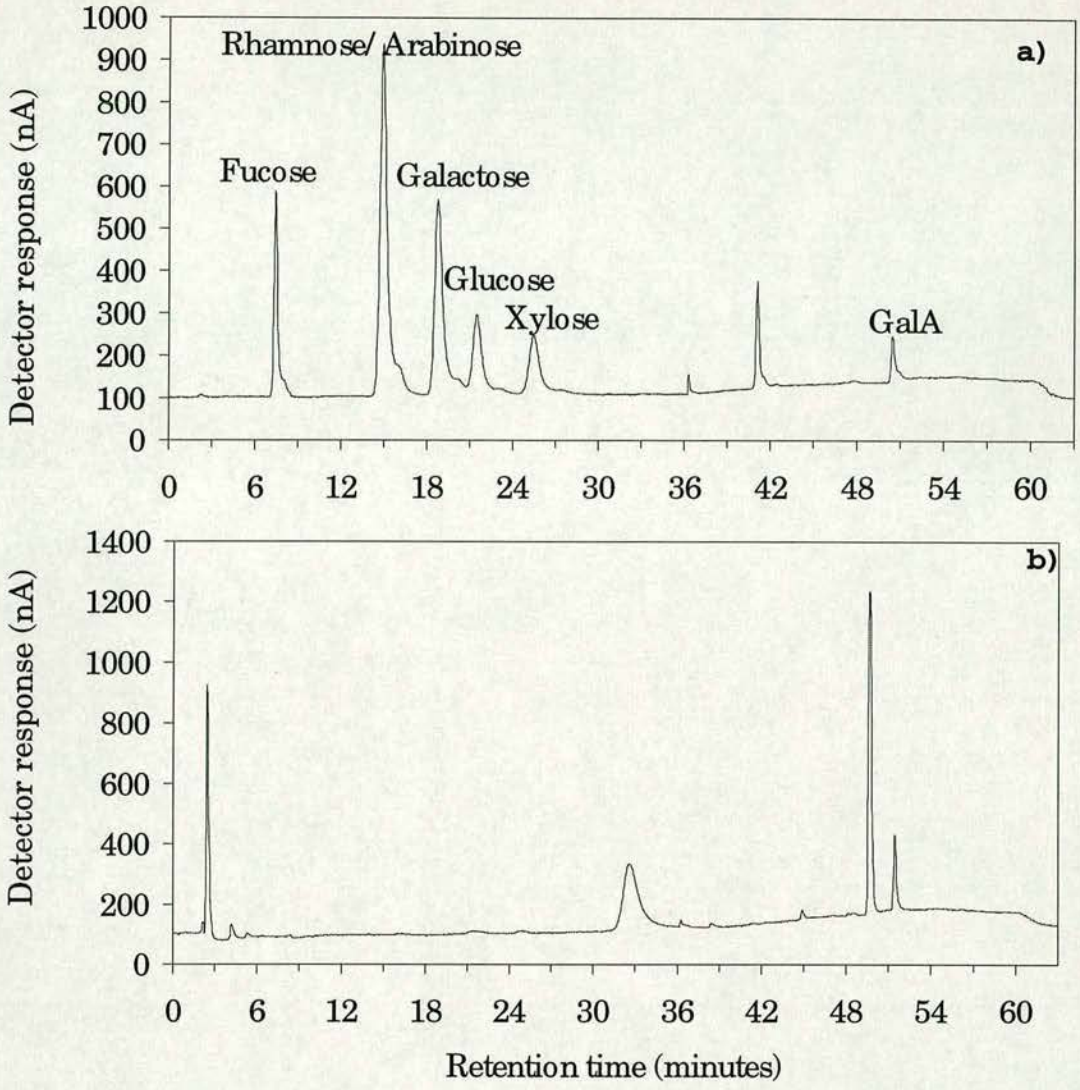


Fig. 3.2.2.6. Dionex HPLC analysis (Eluent Programme 3) showing markers (a) and the products of formic acid hydrolysis of the purified trisaccharide (b).

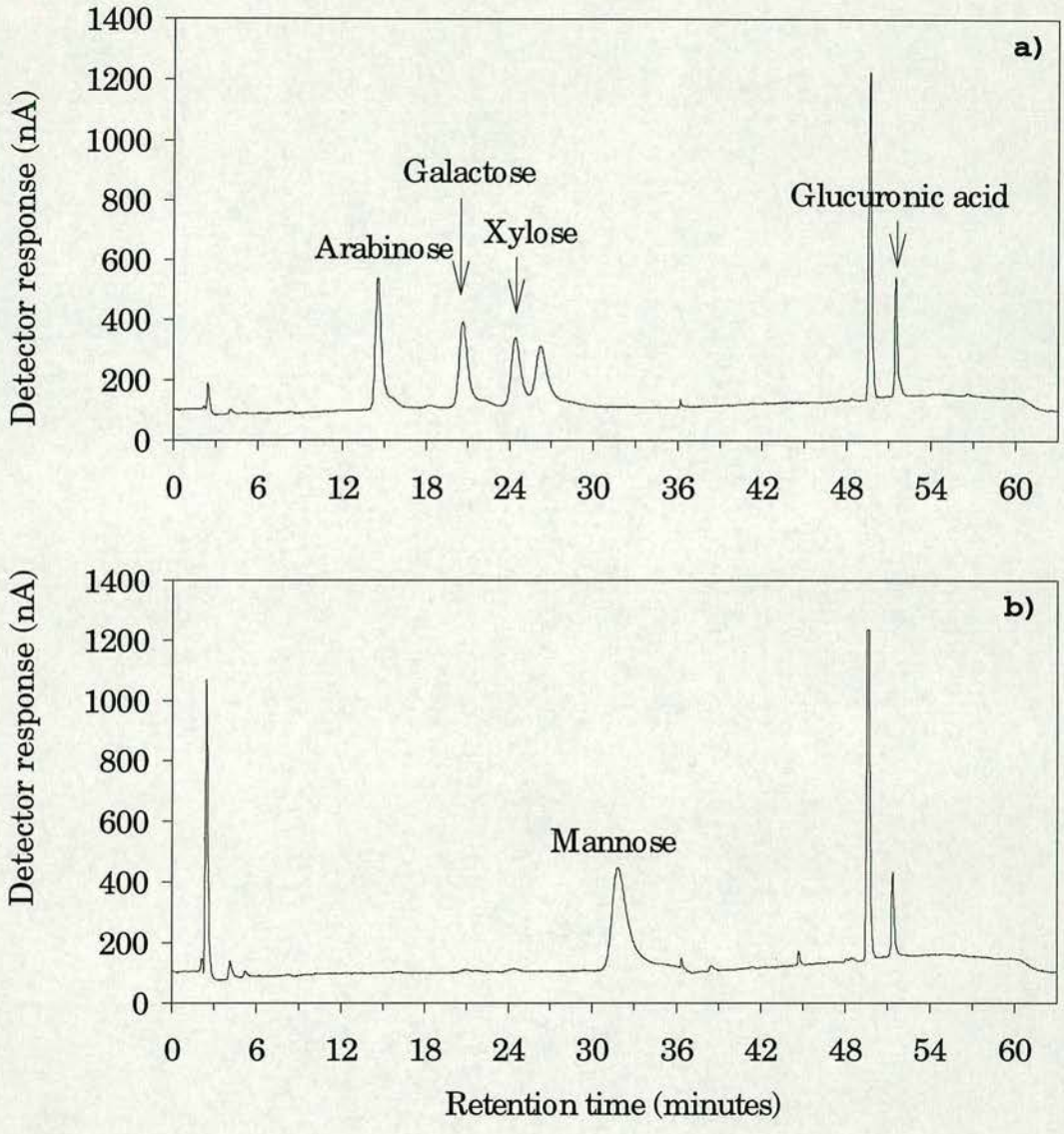


Fig. 3.2.2.7. Dionex HPLC analysis of the products of purified trisaccharide following formic acid hydrolysis. Internal markers of arabinose, galactose, xylose and glucuronic acid (a) or mannose (b) were added.

3.2.3. Attempted Reduction of Trisaccharide

To attempt to determine the identity of the residue at the reducing terminus of the trisaccharide, purified non-radioactive trisaccharide was reduced by sodium borohydride to convert the reducing terminus into an alditol.

Acid hydrolysis of the trisaccharide had shown it to contain mannose and glucuronic acid. Glucuronic acid at the reducing terminus would be converted to gulonic acid on reduction and mannose would be converted to mannitol. The sample was analysed by Dionex HPLC using both the PA1 and MA1 columns, to separate monosaccharides and sugar acids as well as alditols.

Fig. 3.2.3.1. shows the results of hydrolysing the trisaccharide and the reduced trisaccharide and separating the products by Eluent Programme 4 on the PA1 column. Glucuronic acid and mannose were present before and after reduction and no gulonic acid was detected in either; there appeared to be no difference between the acid hydrolysis products of the non-reduced and the reduced trisaccharide. There does appear to be a small peak of mannitol present in (b) however, this early part of the column, very close to the void, is prone to contamination. Note the contaminant glucose and galactose at 15-20 minutes and the smaller contaminants of rhamnose and arabinose at 10-13 minutes: all were present in blank controls. The contamination could be removed by extra cleanup steps of the hydrolysis tubes.

The acid hydrolysis products of the reduced trisaccharide as well as the native trisaccharide were analysed by Eluent Programme 5 using the MA1 column (fig. 3.2.3.2.). This did not reveal mannitol, but rather mannose and no other peaks within the alditol range were present. There was a small peak that co-eluted with inositol and a large peak of material which eluted immediately before inositol and remains unidentified. There appeared to be no difference between the products of the reduced and the non-reduced trisaccharide. Note the glucose contaminant.

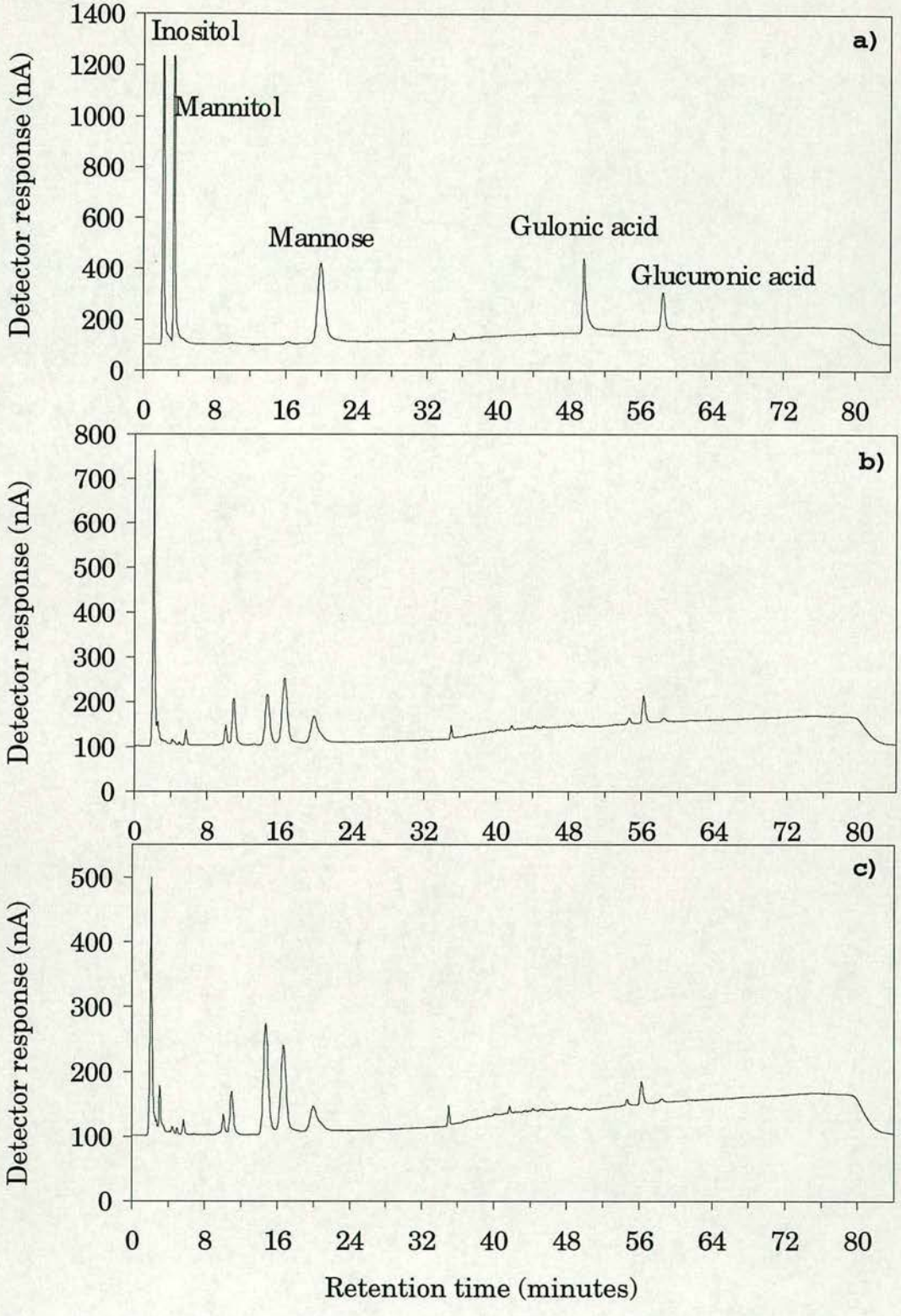


Fig. 3.2.3.1. Dionex HPLC analysis (Eluent Programme 4) on PA1 column showing markers (a), and the hydrolysis products of non-reduced trisaccharide (b) and of reduced trisaccharide (c).

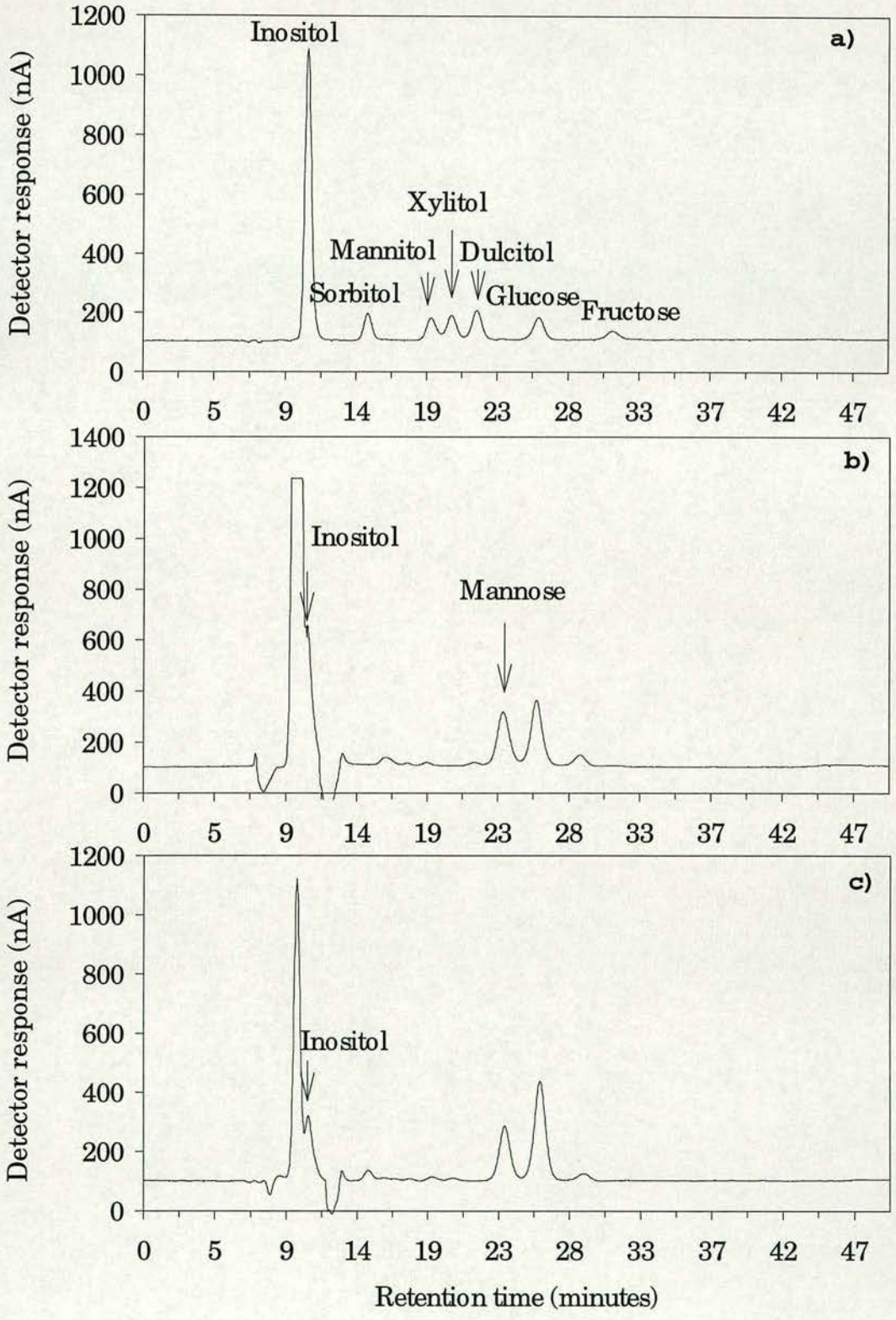


Fig. 3.2.3.2. Dionex HPLC analysis (Eluent Programme 5) on MA1 column showing markers (a), and the hydrolysis products of the non-reduced trisaccharide (b) and of reduced trisaccharide (c).

3.2.4. Nuclear Magnetic Resonance

The NMR spectra were kindly obtained by Dr. Chandralal Hewage and interpreted by Dr. Ian Sadler, Department of Chemistry, The University of Edinburgh. The standard 1-D single pulse 600 MHz proton spectrum (fig. 3.2.4.1.) shows 18 protons (all OH signals having exchanged in D_2O used as solvent) excluding the residual HOD signal after suppression. The resonances are labelled in order of increasing chemical shift (A-T, except I and O). Only two signals were attributable to anomeric protons (S and T) and only one (D) couples to three other protons. In part of the spectrum the resonances from 10 protons (B-M) overlap to varying extents and special techniques were necessary to separate the signals.

A series of 1-D TOCSY spectra (fig. 3.2.4.2.) established that only three separate spin systems, were present, indicating the presence of a non-reducing trisaccharide. As expected, the 2-D TOCSY spectrum (fig. 3.2.4.3.) confirmed these three spin systems. The individual proton multiplets were sufficiently well resolved to allow identification of the carbohydrate residues. One residue (I) was consistent with an α-linked D-mannose unit, a second (II) consistent with an α-linked D-glucuronic acid and the third (III) consistent with a *myo*-inositol residue. Most of the resonances were assigned from coupling patterns in these 1-D spectra and any ambiguities were resolved by the 2-D DQFCOSY spectrum (fig. 3.2.4.4.).

Carbon-13 chemical shifts were assigned from the 1-bond proton-carbon correlation (HMQC) spectrum (fig. 3.2.4.5.). The carbon shifts corresponding to residue (I) compared well with those of α-D-mannoside. C-1 showed a high frequency shift of about 0.5 ppm compared with free mannose. The carbon shifts corresponding to residue (III) lie in the same range (71 - 75 ppm) as those for *myo*-inositol. Alkylation of one oxygen normally results in a chemical shift increase of 8-10 ppm of the

corresponding ring carbon. As the shift of C-2 in this residue is markedly higher in residue (III) than in *myo*-inositol, this suggests that the linkage is at C-2.

The mannose (1-4) glucuronic acid link was demonstrated by three-bond correlations in the 2-D long-range proton-carbon HMBC spectrum (fig. 3.2.4.6.) between Man[H-1] and GlcA[C-4] and between Man[C-1] and GlcA[H-4]. This was supported by NOE enhancement of GlcA[H-4] on irradiation of Man[H-1].

The glucuronic acid (1-2) inositol link was confirmed by the following data: (a) A four bond correlation in the 2-D long-range COSY spectrum between GlcA[H-1] and Ins[H-2], (fig. 3.2.4.7.). (b) three-bond correlations in the 2-D long-range proton-carbon HMBC spectrum between GlcA[H-1] and Ins[C-2] and between GlcA[C-1] and Ins[H-2]. This was supported by NOE enhancement of GlcA[H-1] on irradiation of Ins[H-2] (figs. 3.2.4.8. and .9)

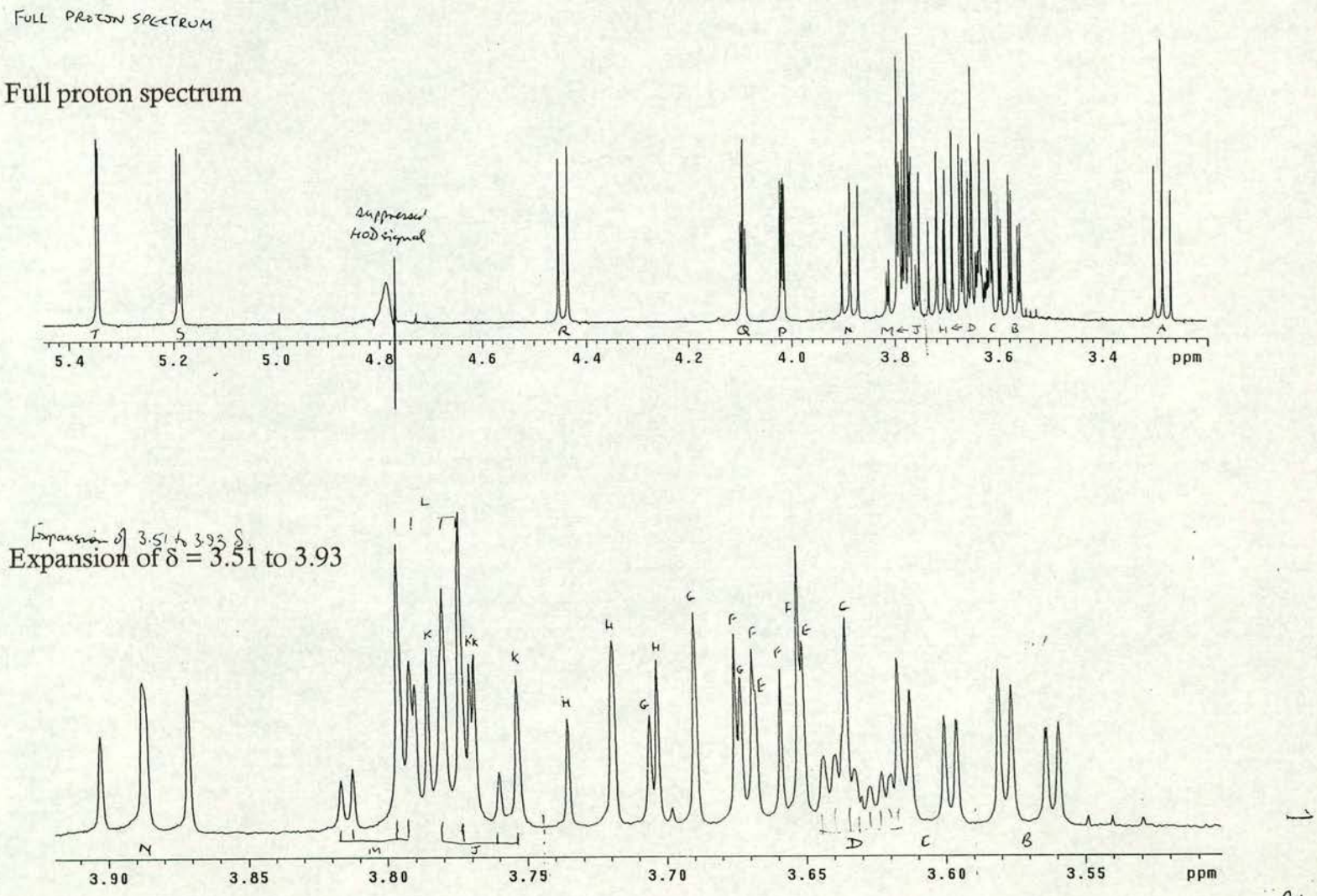


Fig. 3.2.4.1. 1-D single pulse 600 MHz proton spectrum of trisaccharide.

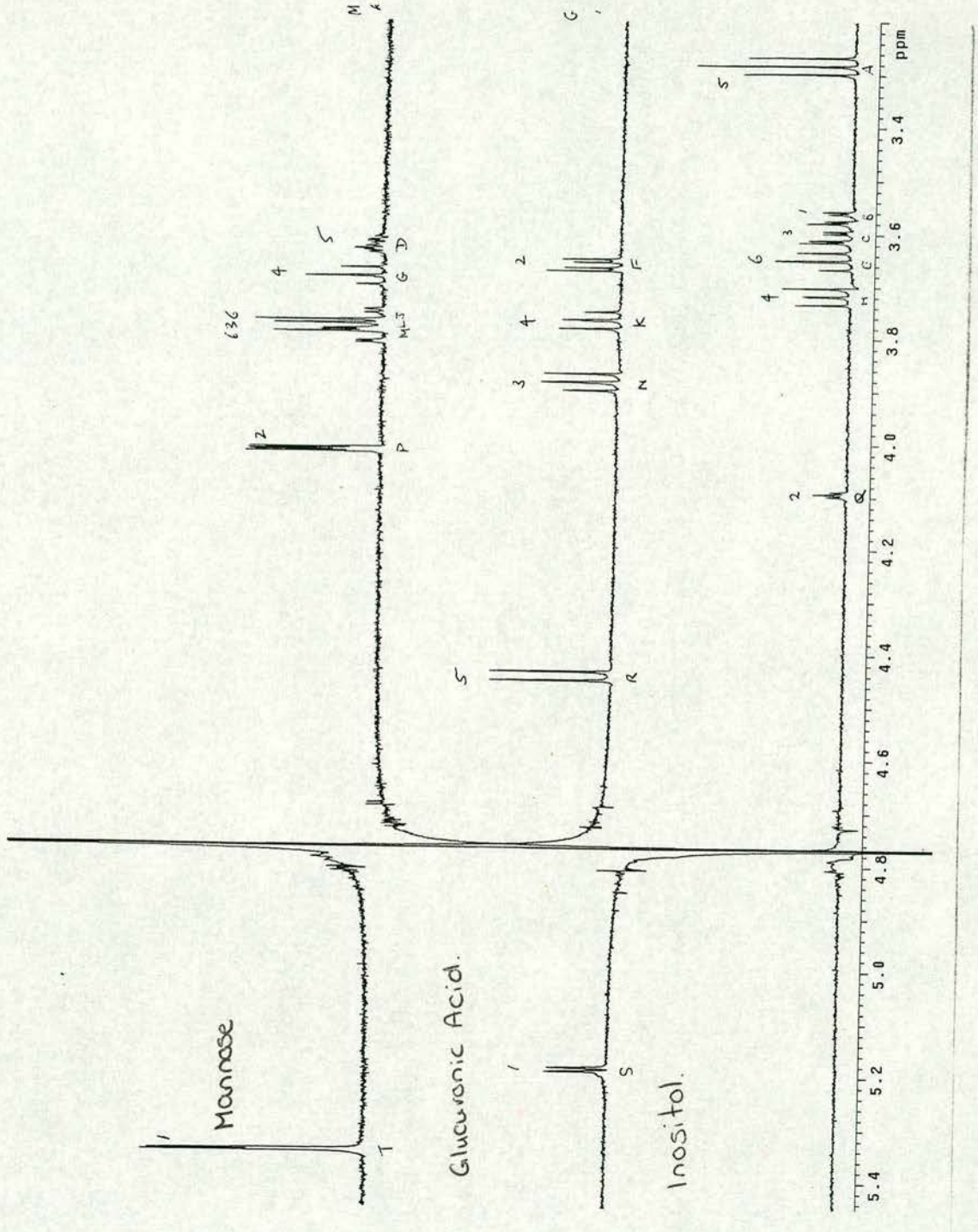


Fig. 3.2.4.2. 1-D TOCSY spectra showing individual residues in trisaccharide.

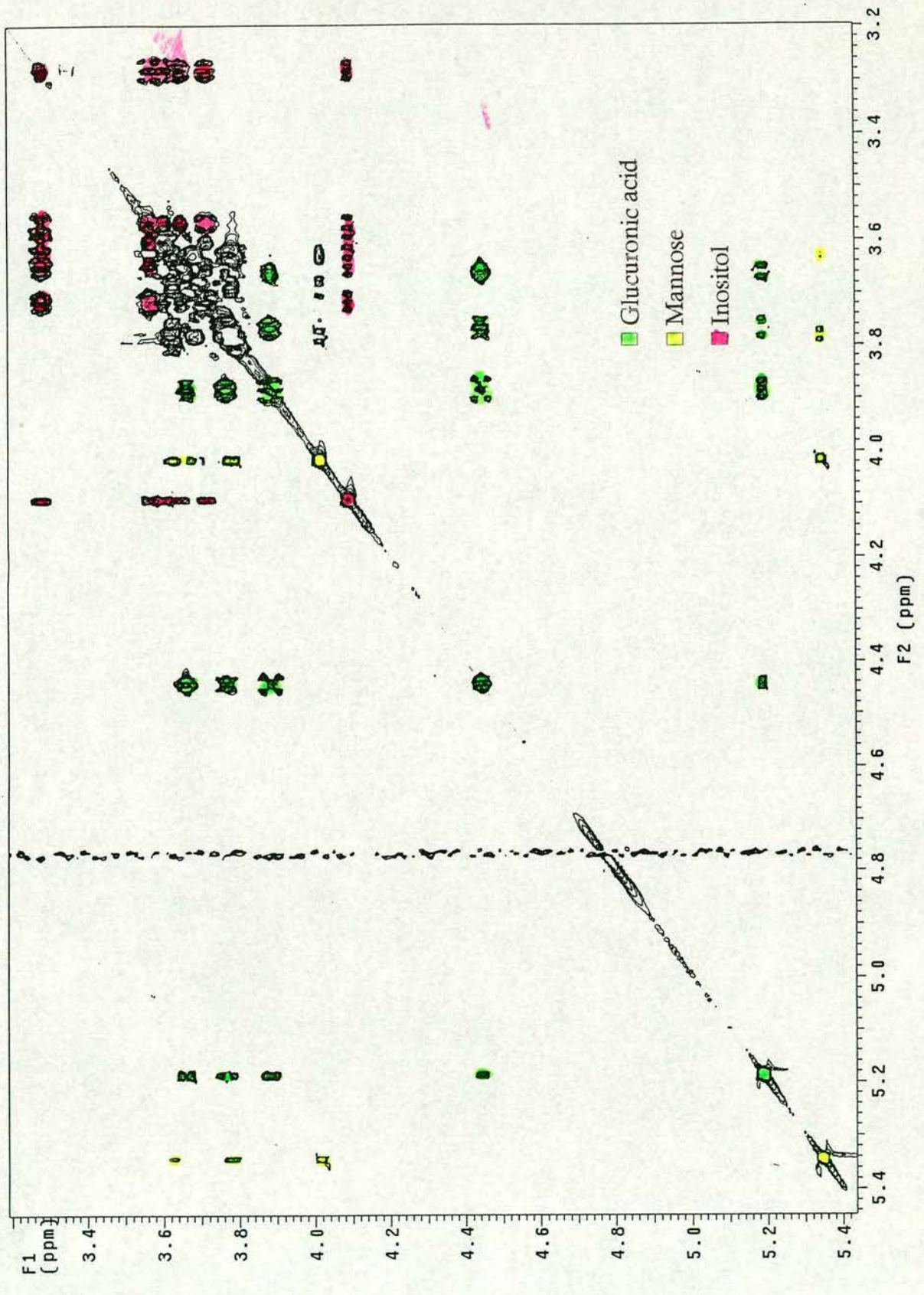


Fig. 3.2.4.3. 2-D TOCSY spectrum of trisaccharide.

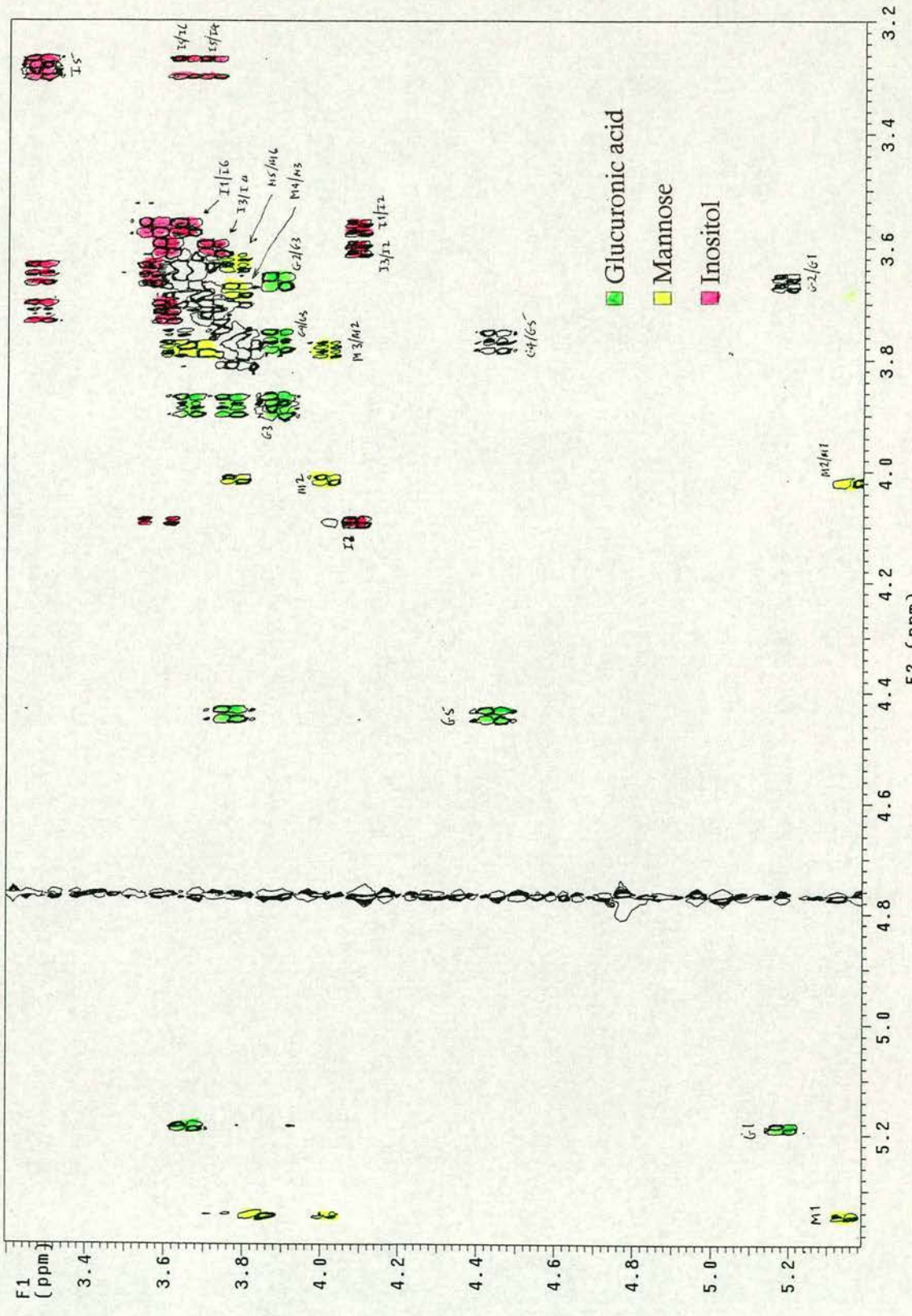


Fig. 3.2.4.4. 2-D DQFCOSY spectrum of trisaccharide.

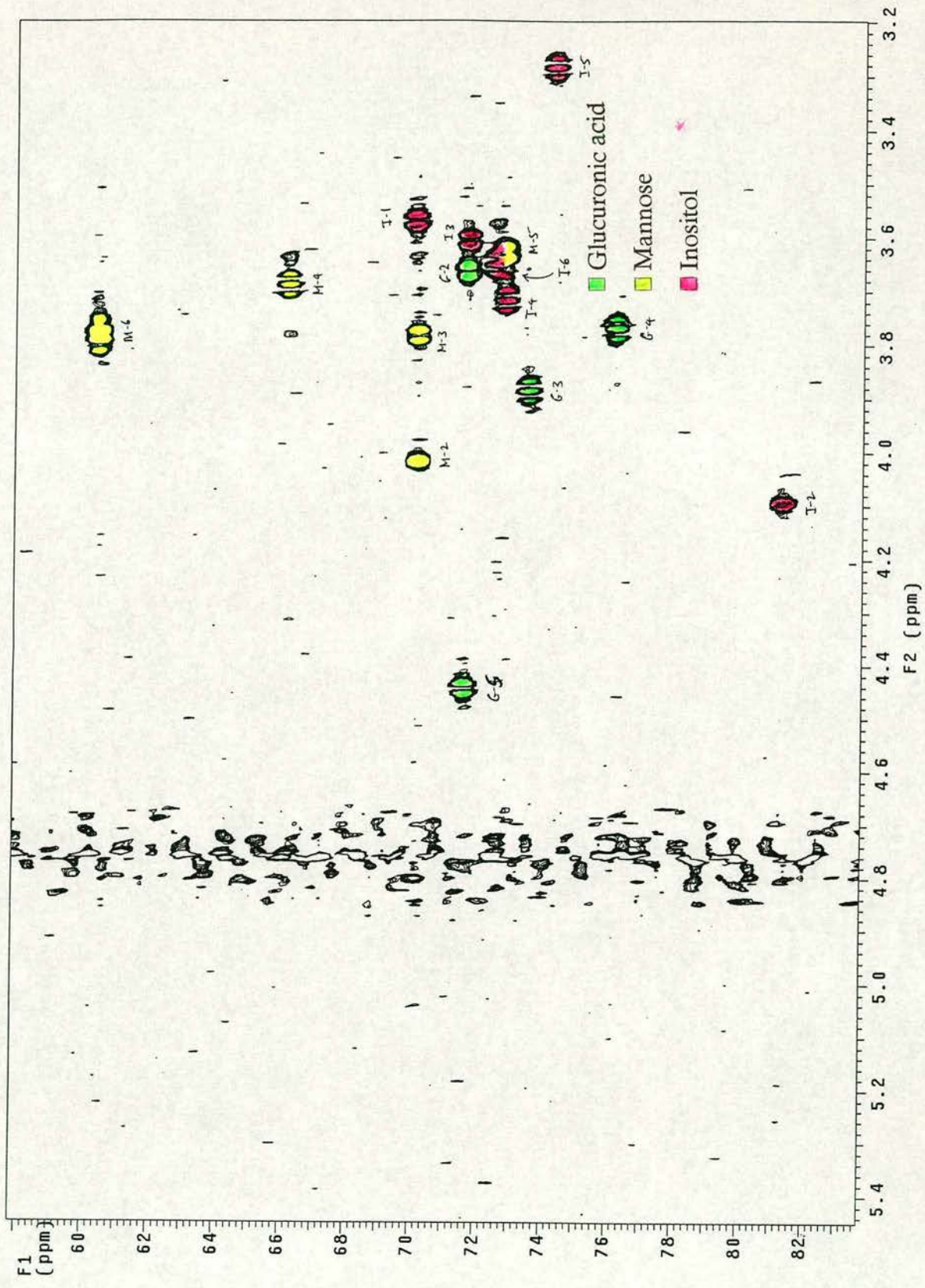


Fig. 3.2.4.5. 1-Bond proton-carbon correlation (HMQC) spectrum of trisaccharide.

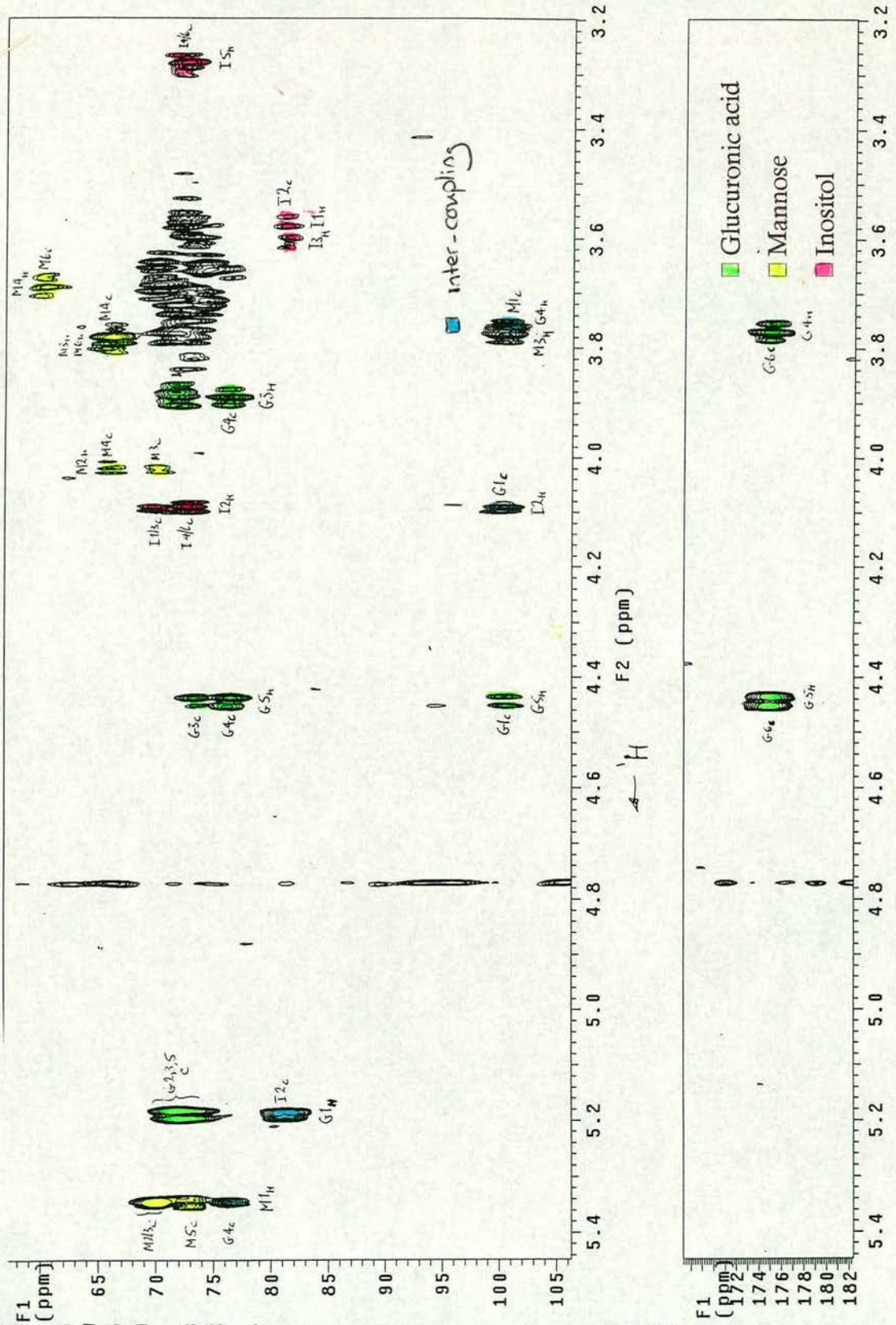


Fig. 3.2.4.6. 2-D long-range proton-carbon HMBC spectrum of trisaccharide.

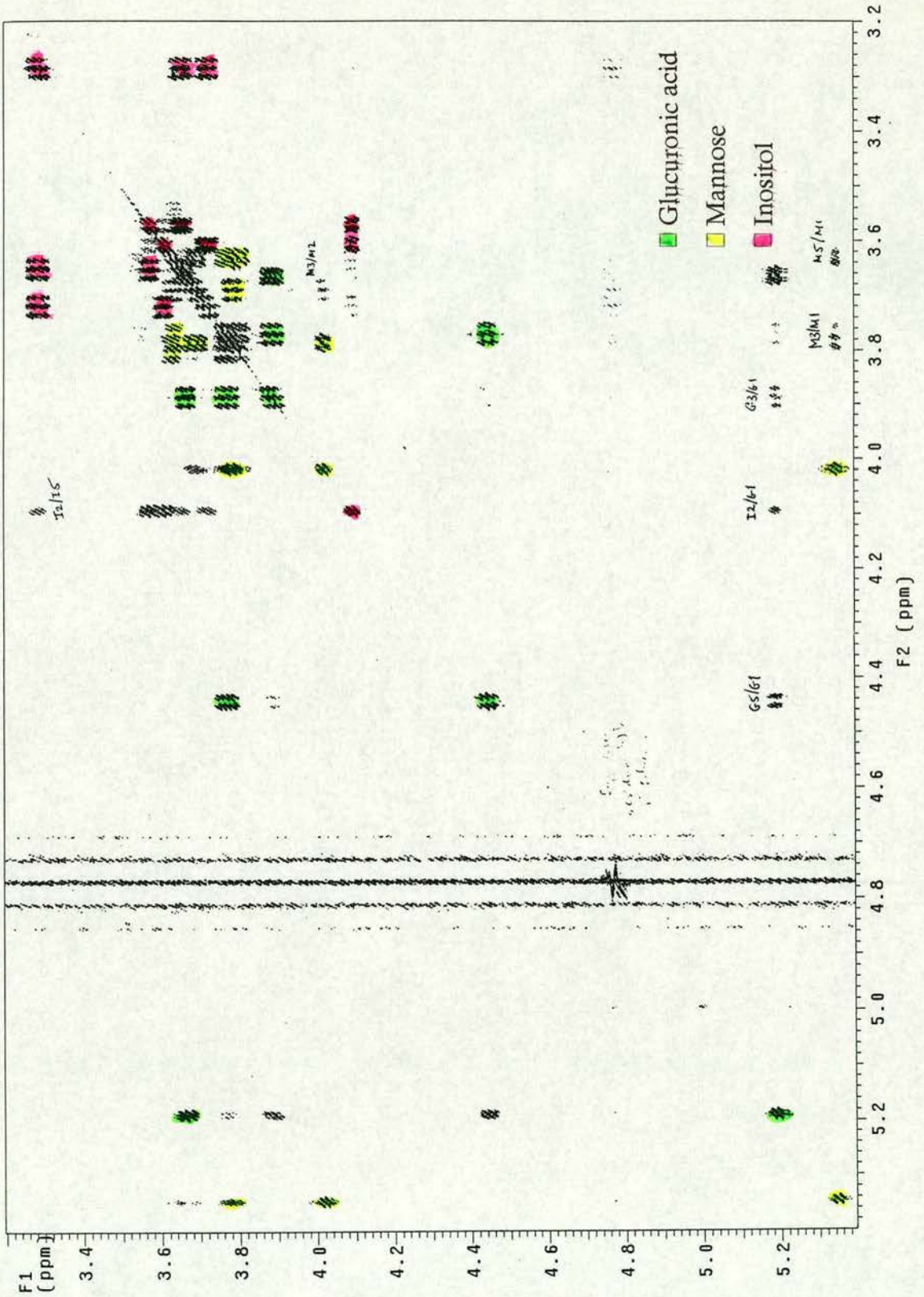


Fig. 3.2.4.7. 2-D long-range COSY spectrum between GlcA[H-1] and Ins[H-2].

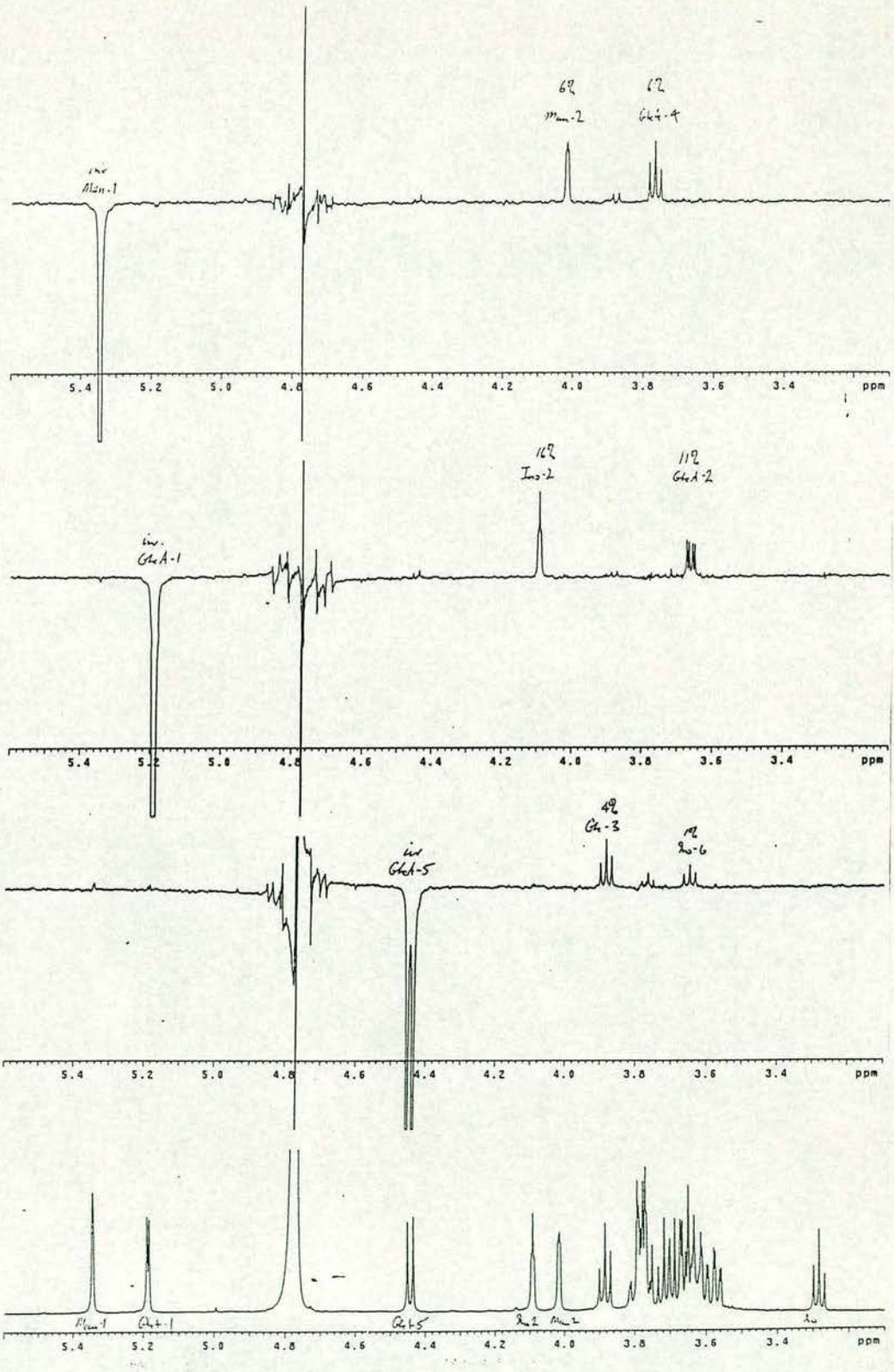


Fig. 3.2.4.8. NOE enhancement of GlcA[H-1] on irradiation of Ins[H-2].

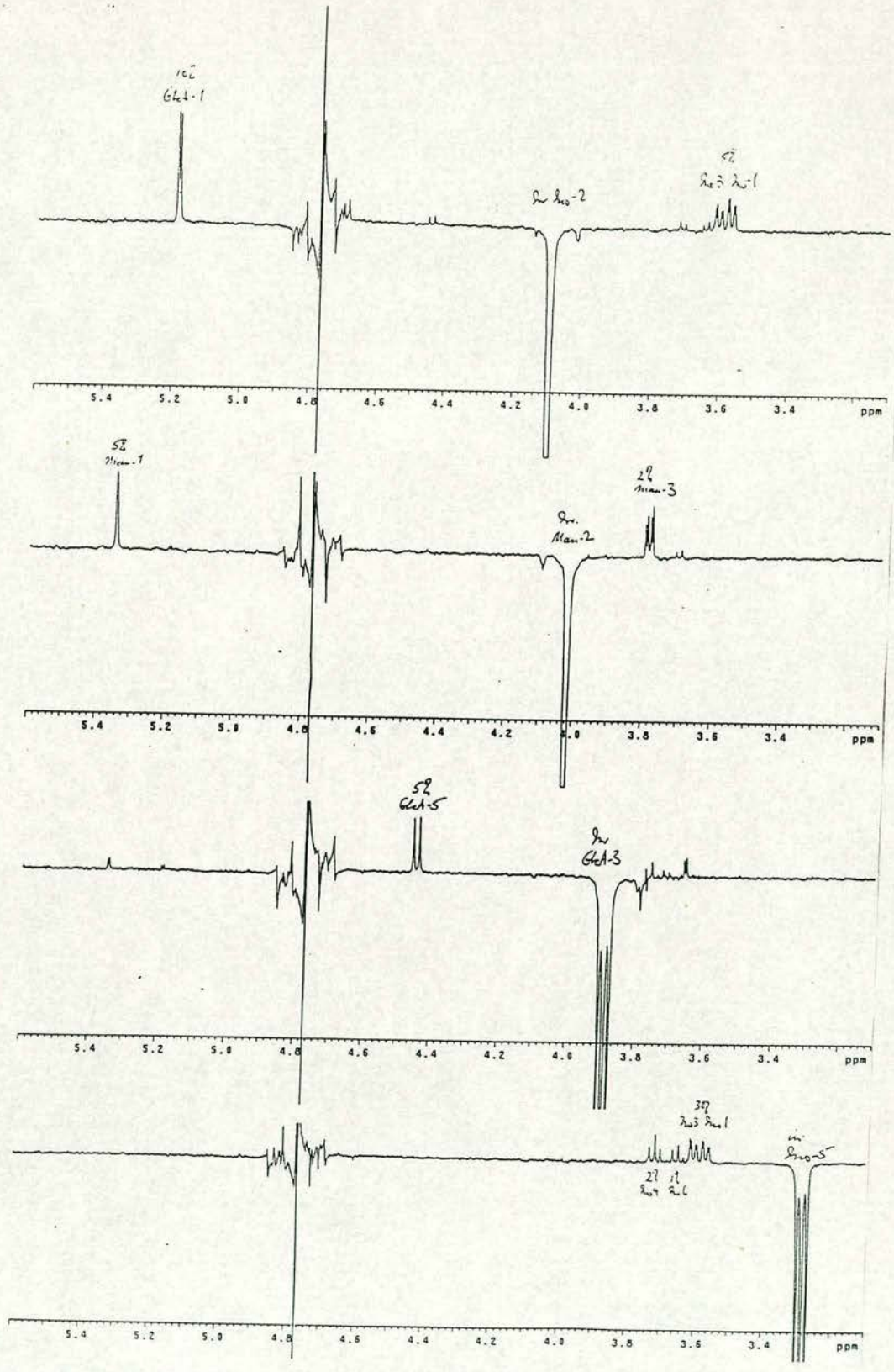


Fig. 3.2.4.9. NOE enhancement of GlcA[H-1] on irradiation of Ins[H-2].

3.2.5. Discussion of Results

The naturally occurring oligosaccharide detected in the spent culture medium of rose was analysed and found to be a trisaccharide with the following structure: α–D-mannopyranosyl-(1→4)-α–D-glucuronopyranosyl-(1→2)-*myo*-inositol. Whereas mannose and glucuronic acid are regularly found in cell wall polysaccharides this is not the case for inositol. The uronic acid component glucuronic acid was identified by paper chromatography and paper electrophoresis of the radiolabelled trisaccharide. The neutral sugars mannose and inositol were determined by Dionex HPLC of the non-radiolabelled purified trisaccharide. The linkage and conformation was determined by NMR analysis, which also confirmed the monosaccharide composition. In addition, these experiments confirm that the radiolabelled oligosaccharide found in spent culture medium after feeding D-[6-^{14}C]glucuronic acid to rose cells and the non-radiolabelled oligosaccharide purified from the spent culture medium are one and the same (section 3.1.2.3.). This trisaccharide is believed to be a novel compound (fig. 3.2.4.10).

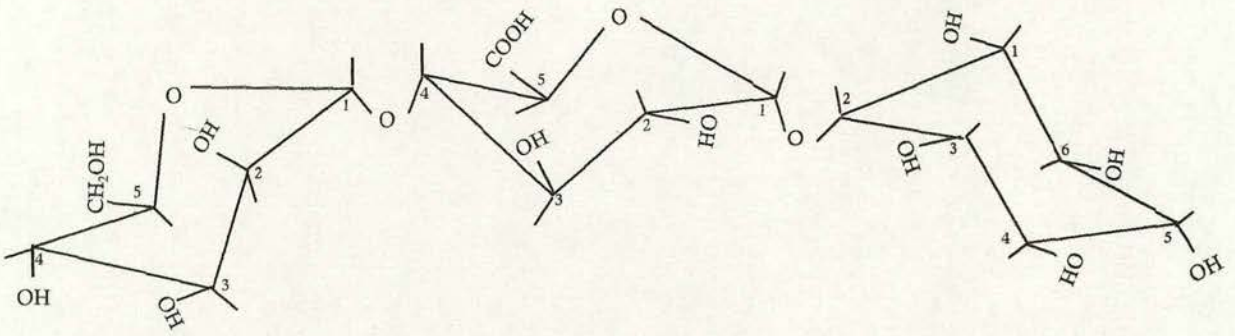


Fig. 3.2.4.10. Structure of the novel trisaccharide α-D-mannopyranosyl-(1→4)-α-D-glucuronopyranosyl-(1→2)-*myo*-inositol secreted by rose cells into their suspension culture medium.

3.3. Biosynthesis of Man-GlcA-Ins by Rose Cells

There are two main ways in which rose cells may produce Man-GlcA-Ins. These are *de novo* synthesis or breakdown of an existing polymer. To distinguish between these two routes, rose cells were presented with D-[6-^{14}C]glucuronic acid and the patterns of accumulation of ^{14}C-labelled extracellular polymeric material and Man-[^{14}C]GlcA-Ins were followed and compared. In the case of *de novo* synthesis it is assumed that the transit times of the radiolabelled precursor through the biosynthetic pathways of the plant are similar. The shortest route involves glucuronic acid (or galacturonic acid), converted GlcA-1-P, then to UDP-GlcA which is incorporated into the trisaccharide and other uronic acid containing compounds such as mannans and pectins and secreted. If this is the case it is assumed that the ^{14}C-trisaccharide and other ^{14}C-labelled compounds will be detectable at approximately the same time and show similar patterns of accumulation. If Man-GlcA-Ins were produced by breakdown of an existing cellular component then a pool of the ^{14}C-parent compound would have to accumulate before the breakdown product could be detected. This would result in a time delay before the appearance of the Man-GlcA-Ins trisaccharide relative to other ^{14}C-labelled compounds and possibly different patterns of accumulation.

A similar method was used to determine the route of biosynthesis of the biologically active oligosaccharide XXFG. Following presentation of the radiolabelled precursor [^{3}H]fucose, the pattern of accumulation of this [^{3}H]oligosaccharide as well as tritiated polysaccharides was followed and the synthesis was deemed to be through breakdown of an existing cell wall polysaccharide (McDougall and Fry, 1991).

3.3.1. Radiolabelled Components of Spent Medium

3.3.1.1. ^{14}C-Extracellular Polymeric material

Following presentation of D-[6-^{14}C]glucuronic acid to rose cells the spent culture medium was analysed by paper chromatography (solvent system EAW). Material which was chromatographically immobile in this solvent system was deemed to be polymeric in nature for the purposes of this study. Ten minutes after presentation of the radiolabelled precursor, ^{14}C-labelled material could be detected at the origin of the paper chromatogram when the spent culture medium was analysed. From 15 minutes and onwards there was an increase in the amount of radioactivity of this fraction and by 30 minutes it had reached approximately 1 kcpm per culture. Between 30 minutes and 10 hours the rate of accumulation was relatively constant and the total radioactivity in this fraction reached approximately 90 kcpm per culture. Between 10 and 25 hours the rate of accumulation decreased to zero and from 25 hours until the end of the experiment there was a decrease in the amount of polymeric radiolabelled material, possibly indicating turnover of polymers (fig. 3.3.1.1.).

3.3.1.2. D-[6-^{14}C]Glucuronic Acid

The pattern of accumulation of the extracellular ^{14}C-polymers was closely mirrored by the disappearance of radiolabelled precursor from the culture medium. This suggested that the decrease in the rate of ^{14}C-polymer accumulation was most probably a function of non-availability of radiolabelled precursor and not an indication of the overall growth pattern of the culture (fig. 3.3.1.1.). By the end of the experiment only approximately 33% of the radiolabelled precursor remained in the culture. The exact composition of the fraction is uncertain. It will have contained both the free acid, the lactone of D-[6-^{14}C]glucuronic acid as well as a small amount of L-[^{14}C]iduronic acid

and its lactone. These are products of the radiolabelling procedure. It is also possible that [^{14}C]galacturonic acid may have been released by breakdown of radiolabelled pectic polysaccharides, though this was not found to be the case according to Garcia-Romera and Fry (1997).

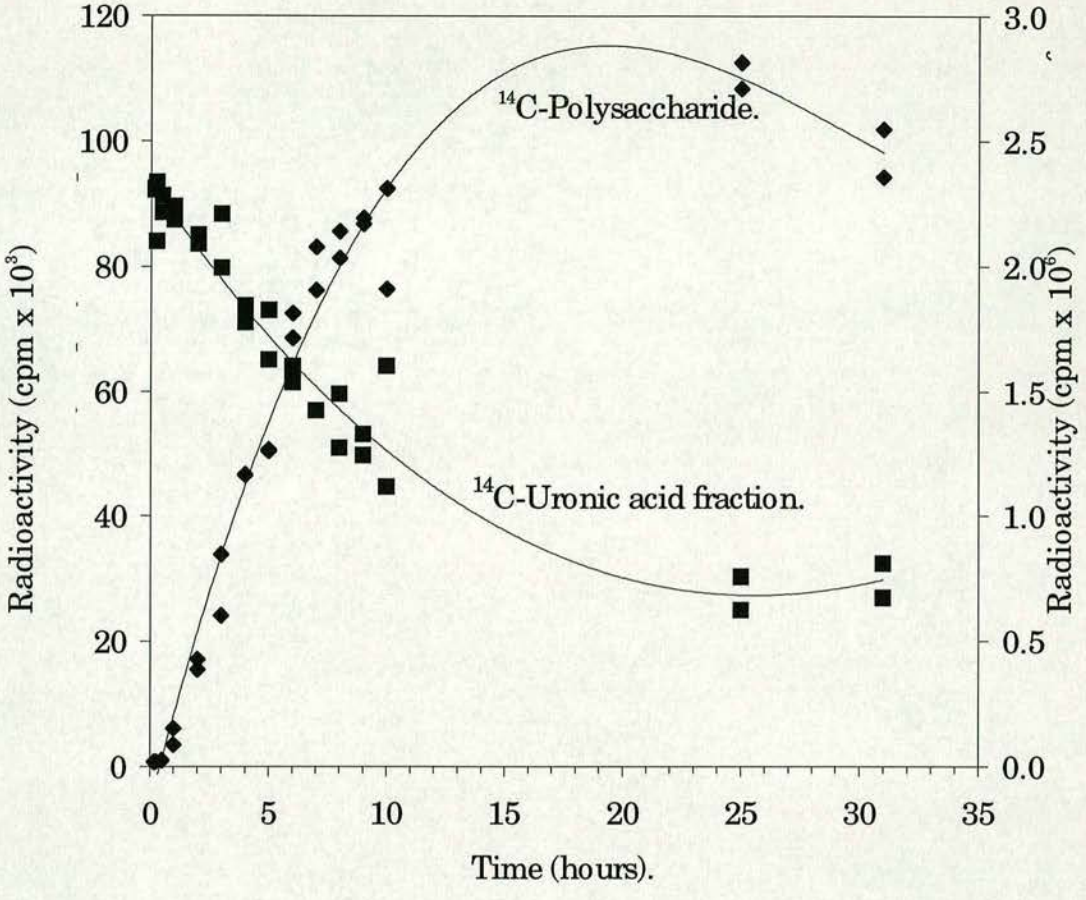


Fig. 3.3.1.1. Accumulation of extracellular ^{14}C-polymeric material and uptake of D-[6-^{14}C]glucuronic acid in the spent culture medium of rose cells following presentation of D-[6-^{14}C]glucuronic acid.

3.3.1.3. Man-[^{14}C]GlcA-Ins

Man-[^{14}C]GlcA-Ins was not detectable on the first chromatogram owing to the "tail" from the uronic acid position (fig. 3.3.1.2.), which overlapped with the $GalA_3$/trisaccharide position. When this region of the chromatogram was eluted and re-chromatographed the ^{14}C-trisaccharide could be detected and quantified (fig. 3.3.1.3.). The ^{14}C-trisaccharide was not detectable in the culture medium until 6 hours after presentation of the radiolabeled precursor. Between 6 and 31 hours the Man-[^{14}C]GlcA-Ins accumulated at a constant rate until the end of the experiment, by when approximately 1100 cpm was present per culture. Unlike with the ^{14}C-polymeric compounds there was no decrease in the rate of accumulation of Man-[^{14}C]GlcA-Ins as the radiolabelled precursor was depleted from the culture medium.

The patterns of accumulation of the ^{14}C-extracellular polymers and of the Man-[^{14}C]GlcA-Ins were very different following presentation of D-[6-^{14}C]glucuronic acid. The ^{14}C-polymers were secreted into the culture medium after just 15-30 minutes, which gives an indication of the minimum transit time of the radiolabel into the cell and through the cell wall biosynthetic pathways. As the radiolabelled precursor was depleted in the medium so the rate of accumulation of these compounds decreased, the two mirroring each other with essentially no lag time. On the other hand Man-[^{14}C]GlcA-Ins showed a distinct lag in emergence, being detectable in the culture medium 6 hours after presentation of the radiolabel and more than 5 hours after appearance of other radiolabelled compounds. The rate of accumulation of the Man-[^{14}C]GlcA-Ins did not seem related to the amount of radiolabelled precursor remaining in the culture medium.

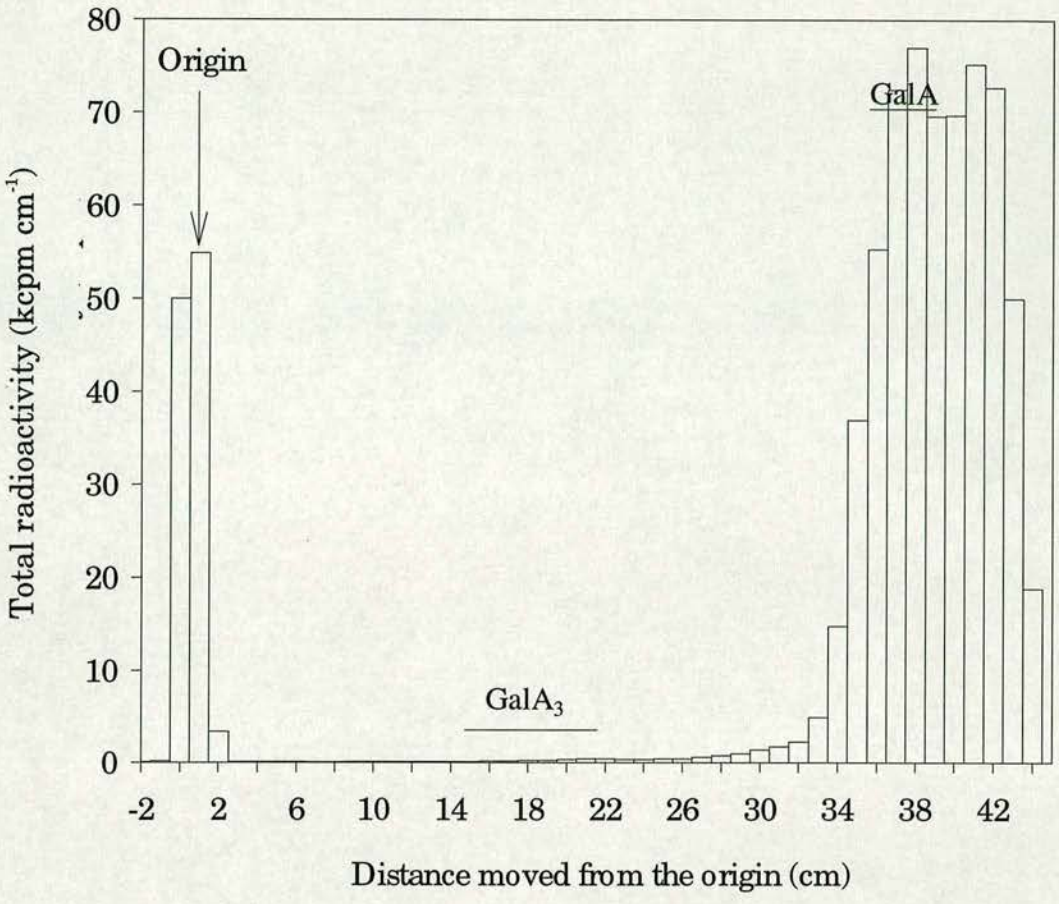


Fig. 3.3.1.2. Paper chromatogram (solvent system EAW) showing the ^{14}C-labelled components of spent culture medium of rose 25 hours after presentation of D-[6-^{14}C]glucuronic acid.

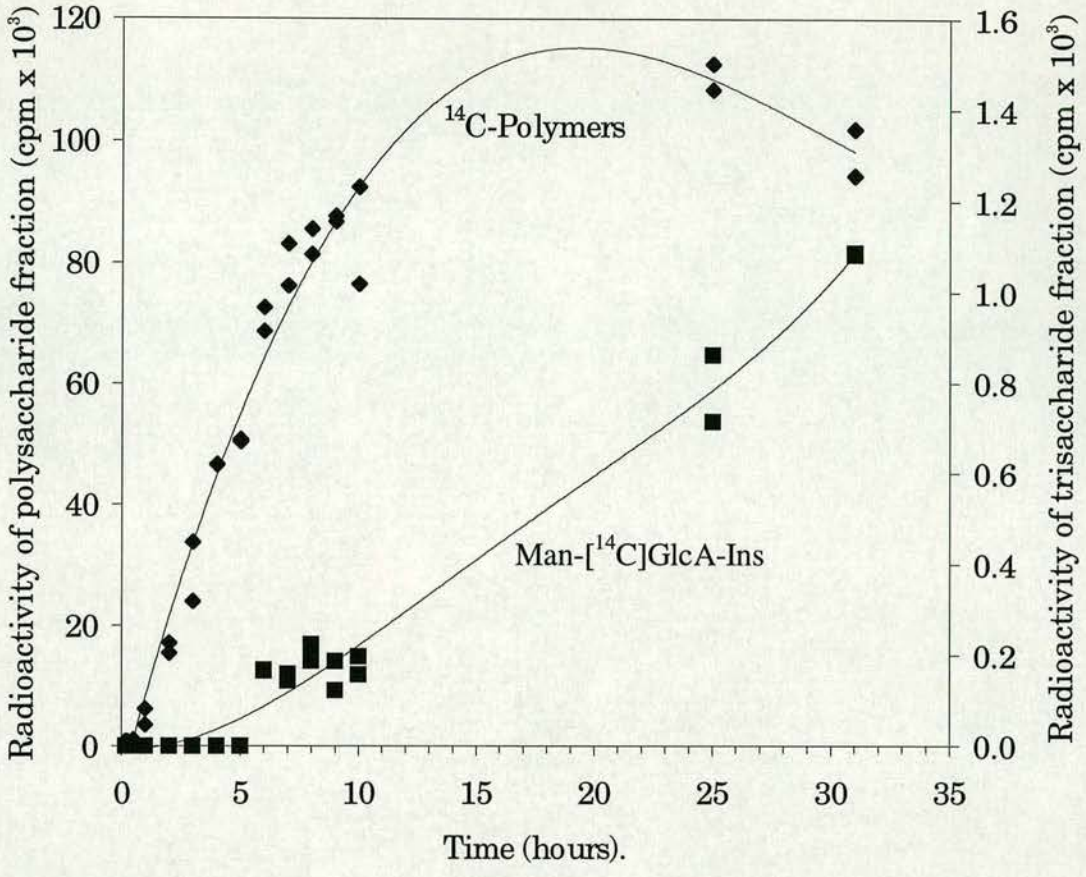


Fig. 3.3.1.3. Accumulation of Man-[^{14}C]GlcA-Ins and ^{14}C-extracellular polymers in the spent culture medium of rose cells after presentation of D-[6-^{14}C]glucuronic acid.

3.3.2. Parent Polysaccharide

The previous results suggest that Man-GlcA-Ins is produced by breakdown of an existing precursor in the cells. The acid resistant nature of Man-GlcA-Ins was used to try to devise an assay for the parent compound based on the fact that acid hydrolysis should release the acid resistant disaccharide unit (GlcA-Ins), which is detectable by Dionex HPLC and paper electrophoresis.

3.3.2.1. ^{14}C -Extracellular Polymers and ^{14}C-Labelled Cells

The ^{14}C-labelled cells and the ^{14}C-extracellular polymers from the time course labelling experiment (25 hours) were subjected to acid hydrolysis (2.0 M TFA) and the products separated by paper chromatography in solvent system EAW (fig. 3.3.2.1.). Both the extracellular polysaccharides and the cells were found to yield acid resistant, radioactive material which was detectable at the origin and as tri- and disaccharide material. In addition uronic acids were released (glucuronic acid and galacturonic acid are not resolved), as well as glucuronolactone. The lactone is indicative of glucuronic acid as galacturonic acid does not lactonise under these conditions.

The disaccharide material was eluted and analysed by paper electrophoresis (pH 3.5). Fig. 3.3.2.2. shows the migration of the disaccharides relative to the authentic [^{14}C]GlcA-Ins, as produced by 2.0 M TFA hydrolysis of purified Man-[^{14}C]GlcA-Ins. The disaccharide material from both the ^{14}C-extracellular polymers and the ^{14}C-labelled cells, eluted from the paper chromatogram, turned out to be two compounds when electrophoresed. However, neither showed exact co-migration with the authentic [^{14}C]GlcA-Ins disaccharide.

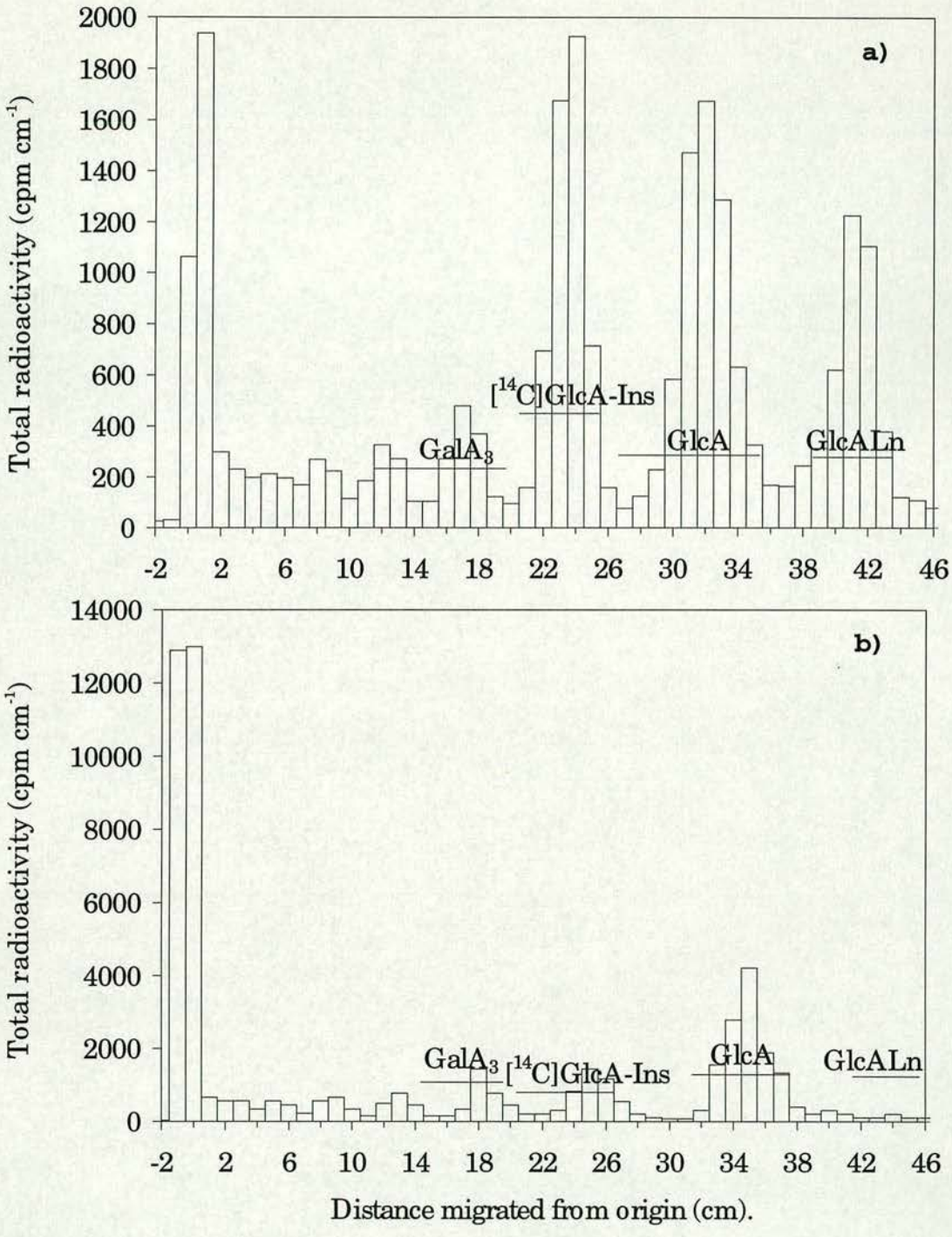


Fig. 3.3.2.1. Separation of the products of 2.0 M TFA hydrolysis of ^{14}C-labelled extracellular polymers (a) and ^{14}C-labelled cells (b) by paper chromatography (solvent system EAW).

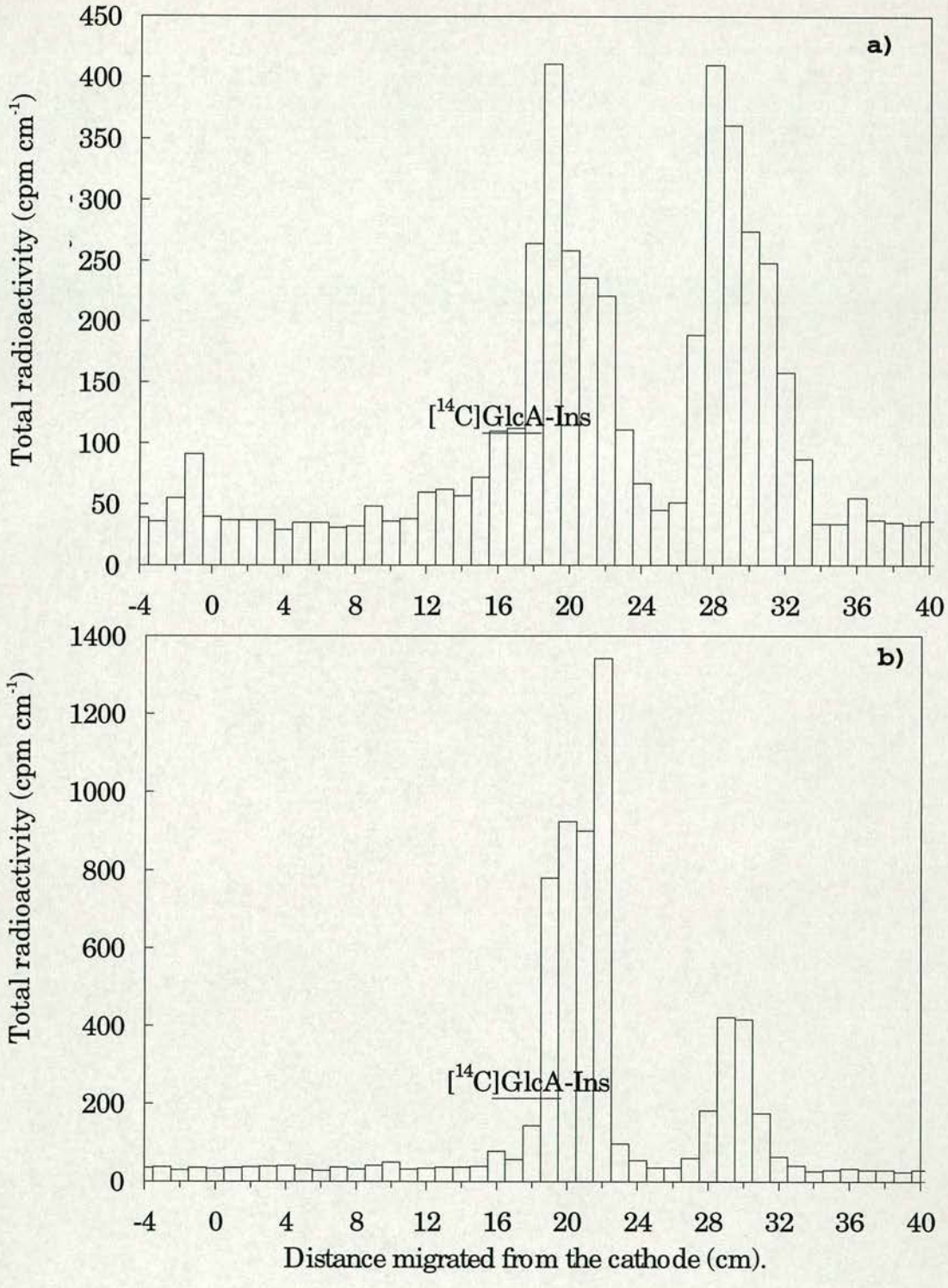


Fig 3.3.2.2. Paper electrophoresis (pH 3.5) of the acid resistant disaccharide produced by 2.0 M TFA hydrolysis of ^{14}C-extracellular polymers (a) and ^{14}C-labelled cells (b).

3.3.3.2. Disaccharide from Non-radiolabelled Rose Cells Walls

Purified rose cells walls were extracted sequentially to fractionate the polysaccharides. The extracted polysaccharides were dialysed to remove the extractant, freeze dried and hydrolysed by 2.0 M TFA in an attempt to produce the acid resistant disaccharide (GlcA-Ins). The products of the acid hydrolysis were analysed by Dionex HPLC (Eluent Programme 4) with authentic disaccharide marker produced by hydrolysis of purified trisaccharide with 2.0 M TFA. None of the extracted fractions from the purified rose cells yielded material which co-eluted with the authentic GlcA-Ins marker.

3.3.3. Breakdown of ^{14}C-Polysaccharides by Rose Cells

Rose cells which had been fed D-[6-^{14}C]glucuronic acid were purified and extracted and the ^{14}C-polysaccharides produced were desalted on Sephadex 4B. All extractants, except for $NaOH/H_3BO_4$, yielded radioactive material which when desalted on Sephadex 4B eluted at or near the void volume. These fractions were pooled, freeze dried and presented to rose cells or to fresh culture medium as a control (10 ml culture). The following amounts were added: CDTA solubilised material: 17 kcpm; urea solubilised material: 6.8 kcpm; $NaOH/NaBH_4$ solubilised material: 7 kcpm and residue: 3.4 kcpm. The culture and controls were incubated overnight under standard growth conditions and the spent culture medium was analysed by paper chromatography (solvent system EAW). The only radioactive material detected was at the origin as chromatographically immobile compounds.

3.3.4. Discussion of Results

When rose cells were presented with D-[6-^{14}C]glucuronic acid it was found that Man-[^{14}C]GlcA-Ins only accumulated in the spent culture medium following a delay of more than 5 hours after appearance of other ^{14}C-labelled compounds. The exact extent of the delay is uncertain as due

to the low amount of Man-[^{14}C]GlcA-Ins 5 hours may be the point at which the quantitation threshold was reached not the time at which the Man-[^{14}C]GlcA-Ins was first present in the culture. Extrapolation of results (fig. 3.3.1.3.) would suggest that the Man-[^{14}C]GlcA-Ins was present at 2 hours. This is entirely possible that the need to elute and re-chromatograph the trisaccharide will have reduced sensitivity in the experiment.

Perhaps even more compelling than the time delay is the pattern of the accumulation of the Man-[^{14}C]GlcA-Ins trisaccharide relative to the other ^{14}C-components of the spent culture medium. The trisaccharide continued to accumulate at a relatively constant rate, possibly even accelerating, after the accumulation of other ^{14}C-labelled compound was decreasing owing to depletion of their radiolabelled precursor in the culture medium. This suggests that the Man-GlcA-Ins trisaccharide does not exhibit as direct a precursor→product relationship with D-[6-^{14}C]glucuronic acid as could be the case were the trisaccharide synthesised *de novo*. Rather it would point to a relatively long-lived intermediate compound which acts as the parent of the trisaccharide and which had to accumulate in sufficient amounts before the radiolabelled product could be detected, accounting for the lag time. Also the amounts of this parent compound were sufficient to allow continued synthesis of the Man-[^{14}C]GlcA-Ins trisaccharide after the radiolabelled precursor of the parent compound had become a limiting factor in the synthesis of other ^{14}C-labelled compounds such as pectic polysaccharides.

As previous experiments (section 3.1) failed to reveal significant amounts of other uronic acid containing oligosaccharides it seems likely that the parent compound would be polymeric in nature. Unfortunately attempts to detect the parent compound failed. The acid resistant products of acid hydrolysis of both the cells and the extracellular material remaining after the time course labelling experiment failed to yield

3.4. Biological Activity of Man-GlcA-Ins

Man-GlcA-Ins detected in the spent suspension culture medium of rose was purified and bioassayed to determine whether it possessed biological activity and could be considered to be an oligosaccharin.

3.4.1. Effect on Gibberellic Acid-Mediated Elongation of Excised Pea Shoots

This bioassay had been successfully used to demonstrate biological activity of xyloglucan oligosaccharides (Warneck and Seitz, 1993). As expected there was a statistically significant increase in the length of the controls containing gibberellic acid (GA_3)/no trisaccharide relative to the no GA_3/no trisaccharide controls. However in the case of samples containing GA_3/trisaccharide no consistent, statistically significant effect of Man-GlcA-Ins on the action of GA_3 was detected in eight repeats of the experiment.

3.4.2. Effect on Uptake and Incorporation of Radiolabelled Amino Acids by Rose Cells

This bioasssay has been successfully used to demonstrate biological activity of RG-II (Aldington and Fry, 1994).

3.4.2.1. Effect on L-[U-^{14}C]Leucine Uptake and Incorporation

Fig. 3.4.2.1. shows the amounts of L-[U-^{14}C]leucine taken up by the cells rose cells. Less than 1% of the available radiolabelled amino acid was taken up by the cells during the experiment; hence, throughout the experiment, nonavailability of radiolabelled precursor is unlikely to have affected the results. The experiment was repeated four times and, although some treatments gave statistically significant effects, the results were not reproducible and there appeared to be no trend in the

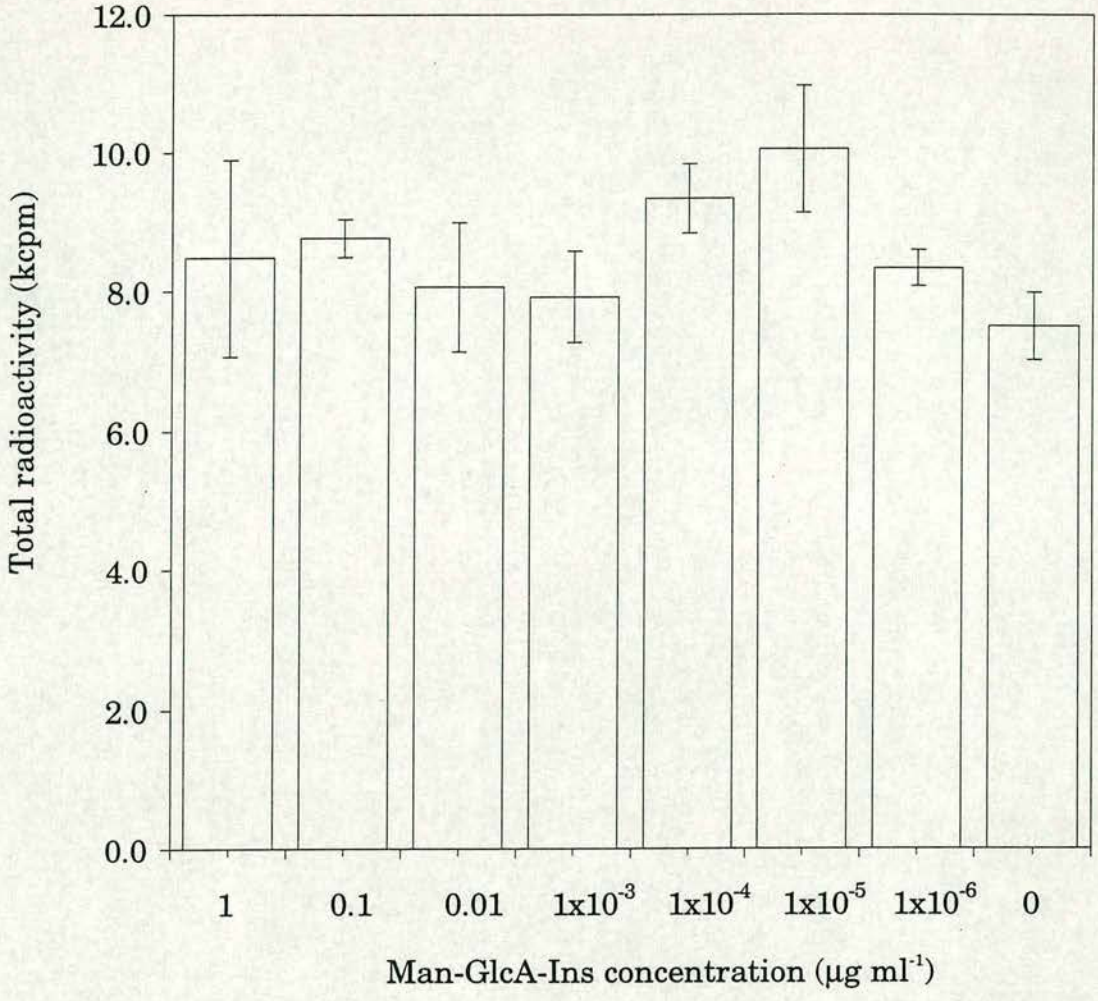


Fig. 3.4.2.1. Uptake of L-[U-^{14}C]leucine by rose cells in the presence of Man-GlcA-Ins trisaccharide at various concentrations. The intra-experiment standard error of mean for five repeats at each concentration is shown.

amount of radiolabelled amino acid taken up relative to the concentration of Man-GlcA-Ins.

Fig. 3.4.2.2. shows the amounts of ^{14}C-proteins which could be precipitated by 10% TCA after rose cells had been presented with the L-[U-^{14}C]leucine in the presence of Man-GlcA-Ins. Approximately 20% of the radiolabelled amino acid taken up by the cells was incorporated into proteins. There was a statistically significant and repeatable trend towards a decrease in the incorporation of the radiolabelled amino acid in the presence of Man-GlcA-Ins. The concentration response was in the range of 1 - 1 x 10^{-5} ng ml^{-1} of trisaccharide and the response was repeatable in five experiments performed on separate days. It appeared as though there might be a trend towards increased incorporation in the presence of lower concentrations of the Man-GlcA-Ins trisaccharide but this was not consistently repeatable. It was also not possible to determine whether the response was present at higher concentrations as insufficient amounts of Man-GlcA-Ins stocks were available to continue beyond 1 µg ml^{-1}.

3.4.2.2. Effect on L-[U-^{14}C]Phenylalanine and L-[U-^{14}C]Tyrosine Uptake and Incorporation.

No effect on either the uptake or the incorporation of either L-[U-^{14}C]phenylalanine or L-[U-^{14}C]tyrosine by rose cells in the presence of Man-GlcA-Ins was detected.

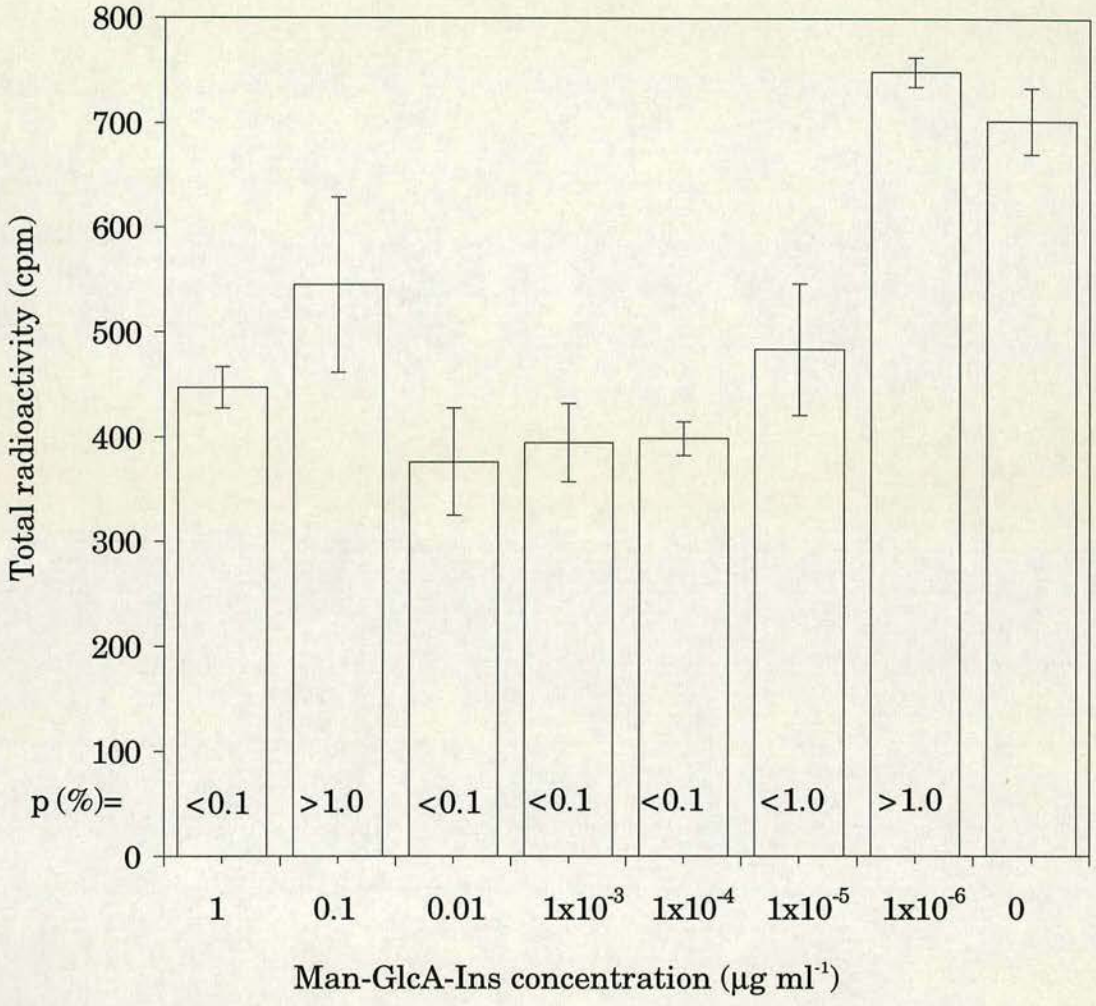


Fig. 3.4.2.2. Incorporation of L-[U-^{14}C]leucine into acid precipitable proteins by rose cells in the presence of Man-GlcA-Ins. The intra-experiment standard error of mean for 5 repeats at each concentration is shown, as is Student's t-test probability relative to the control.

3.4.3. Effect on Proteins Produced by Rose Cell

3.4.3.1. Proteins in the Spent Culture Medium

Fig. 3.4.3.1. shows the proteins present in the spent culture medium 4 and 24 hours after presentation of the Man-GlcA-Ins trisaccharide. There were several proteins present in the spent culture medium, with significantly more protein present after 24 hours especially at ~ 80 kDa. In two repeats of the experiment on two different days no differences could be detected between the proteins present or their relative amounts in the cultures treated with the Man-GlcA-Ins trisaccharide and the controls.

3.4.3.2. Proteins in the Cytosol

Fig. 3.4.3.2. shows the proteins extracted from the cytosol 4 and 24 hours after presentation of the Man-GlcA-Ins trisaccharide. This fraction was a rich source of proteins, but in two repeats of the experiment on two different days no differences could be detected between the proteins present or their relative amounts in the cultures treated with the Man-GlcA-Ins trisaccharide and the controls.

3.4.3.3. Proteins in the Cell Wall

Fig. 3.4.3.3. shows the proteins extracted from the cell wall 4 and 24 hours after presentation of the Man-GlcA-Ins trisaccharide. The proteins extracted from the cell walls were distinct from both those from the spent culture medium and those from the cytosol. In two repeats of the experiment on two different days no differences could be detected between the proteins present or their relative amounts in the cultures treated with the Man-GlcA-Ins trisaccharide and the controls.

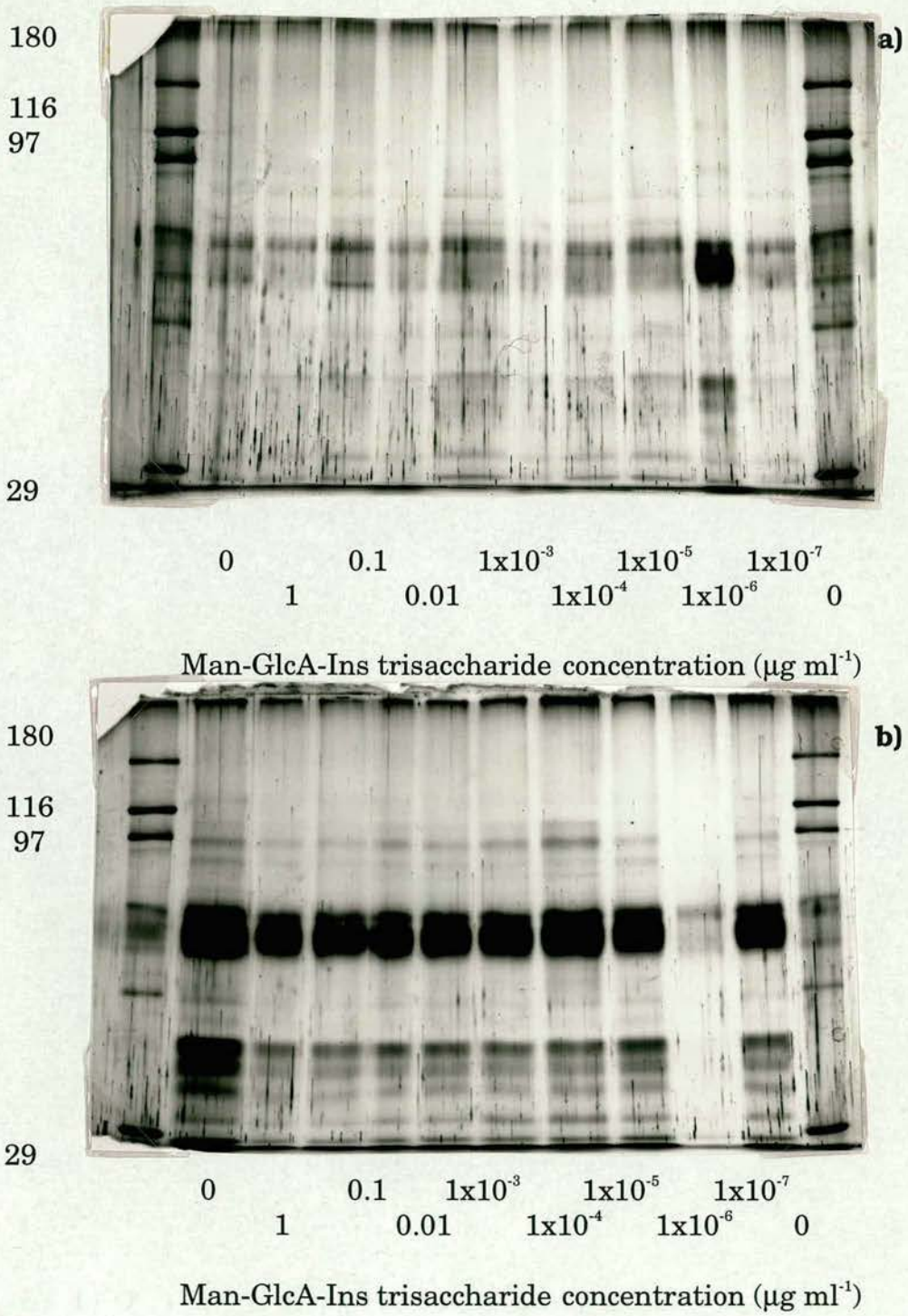


Fig. 3.4.3.1. Proteins in the spent culture medium of rose cells after presentation of the Man-GlcA-Ins trisaccharide for 4 (a) and 24 (b) hours.

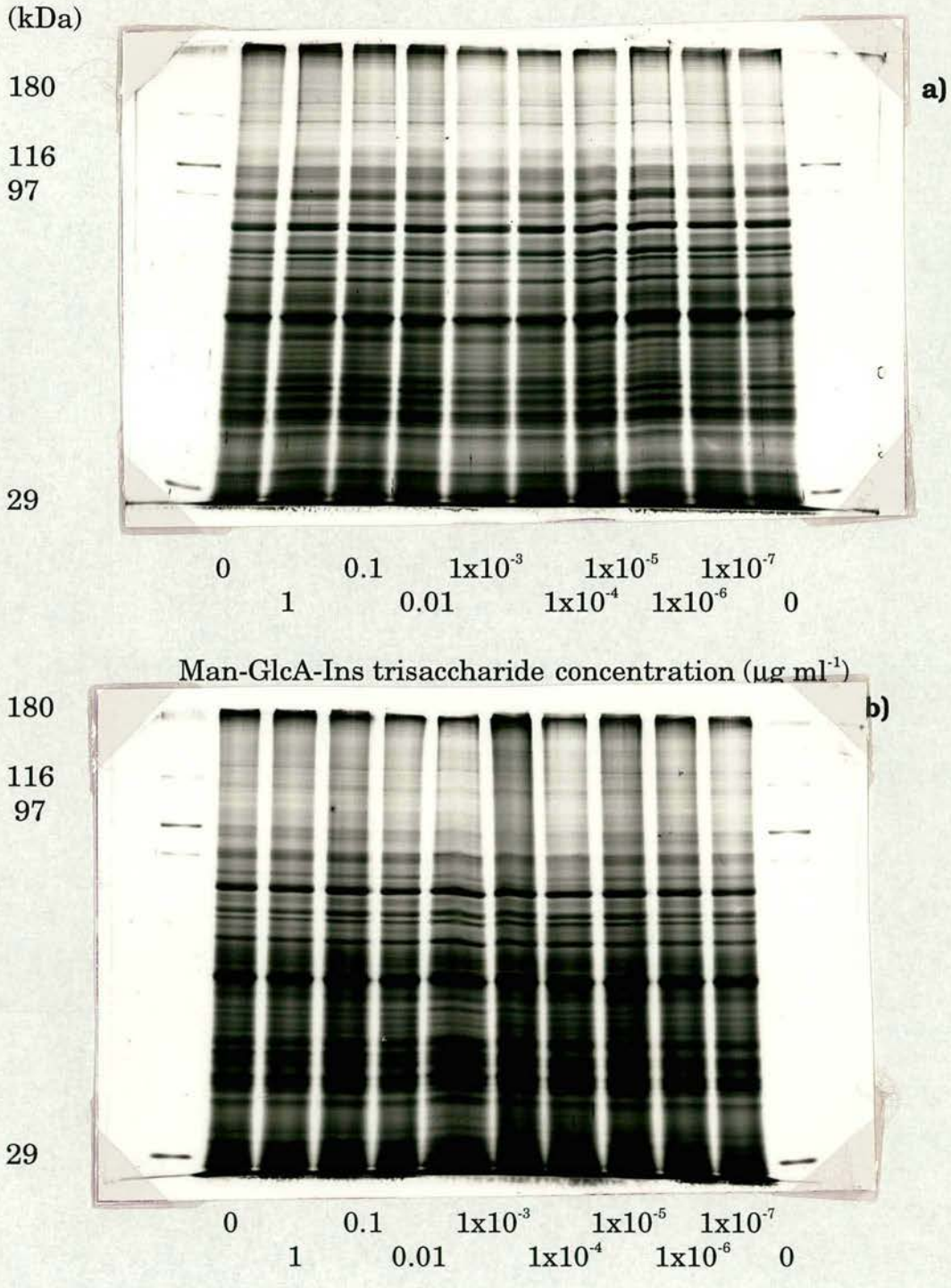


Fig. 3.4.3.2. Proteins of the cytosol of rose cells after presentation of the Man-GlcA-Ins trisaccharide for 4 (a) and 24 (b) hours.

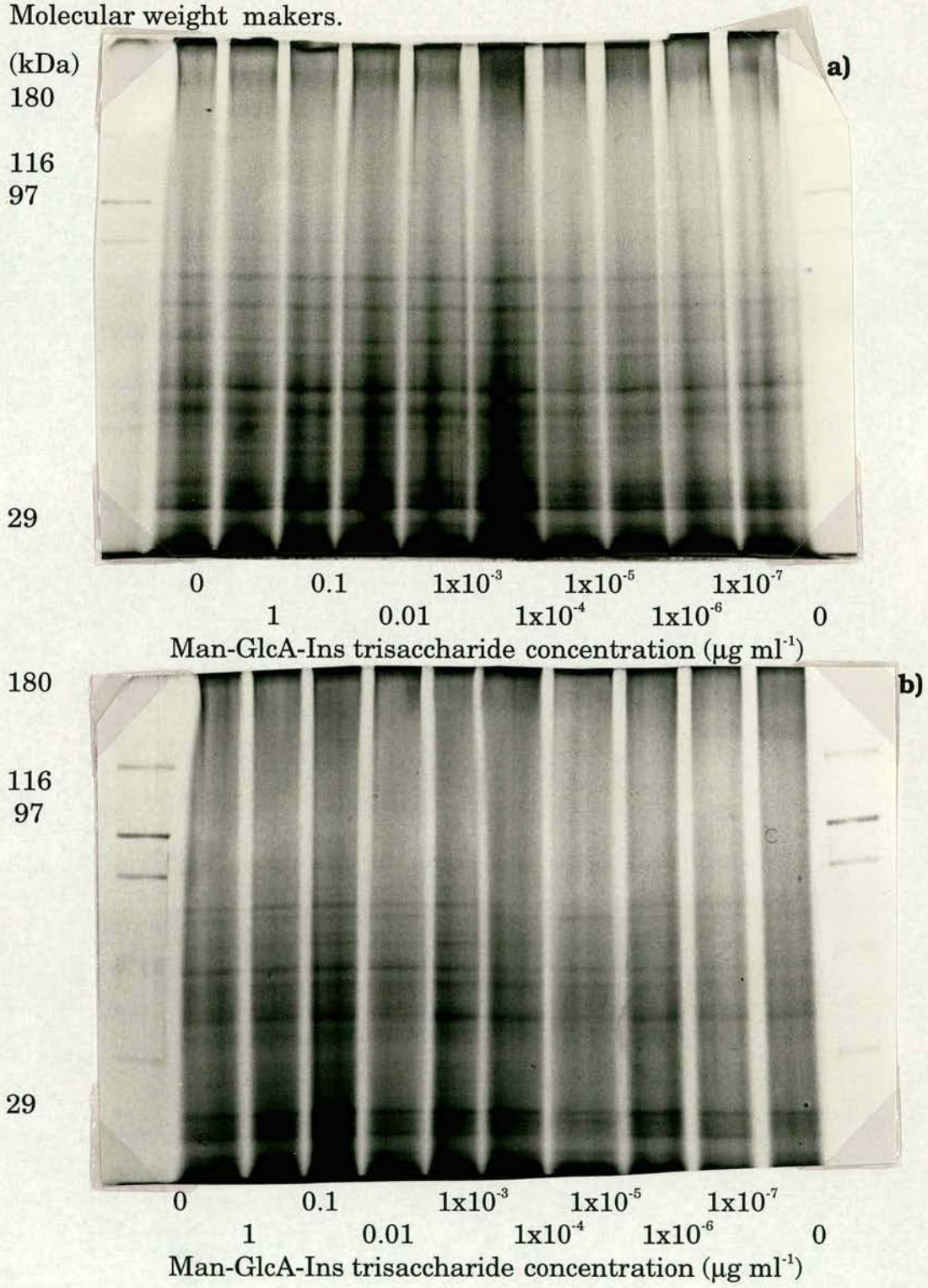


Fig. 3.4.3.3. Proteins in the cell wall of rose cells after presentation of the Man-GlcA-Ins trisaccharide for 4 (a) and 24 (b) hours.

3.4.4. Effect on Phenolic Metabolism and Protein Cross-linking in Rose Cells

Rose cells were fed L-[U-^{14}C]phenylalanine, which resulted in the radiolabelling of phenolic metabolites and proteins. The cells were presented with the Man-GlcA-Ins trisaccharide in the presence of the radiolabelled amino acid and after 4 and 24 hours the cells were collected and subjected to acid hydrolysis using 6.0 M HCl. This treatment hydrolysed proteins and the products were separated by paper chromatography (solvent system BAW), (fig. 3.4.4.1.). As expected there was a large peak of [^{14}C]phenylalanine: this may consist of phenylalanine taken up by the cells and incorporated into protein, then released on hydrolysis; it may be present free in the cytosol; and some may be derived from the spent culture medium and not removed by the washing. There was a peak of [^{14}C]tyrosine which was also expected as phenylalanine and tyrosine are interconverted by cellular metabolism. In addition there was material which showed a relative mobility to phenylalanine (R_{Phe}) = 0.45, which is the expected mobility of isodityrosine (Fry, 1988). If this is indeed isodityrosine, rose cells would appear to be a rich source. This material was also present in the trisaccharide free controls and hence does not appear to be present as a result of Man-GlcA-Ins.

In some of the trisaccharide treated cultures there was an additional peak of material with an R_{Phe} = 0.26 (fig. 3.4.4.2.) and which was not present in the controls. Fig. 3.4.4.3. shows the amounts of this material present after presentation of the Man-GlcA-Ins trisaccharide for a period of 4 and 24 hour. This compound was found to be present in cultures presented with the trisaccharide in the concentration range $1 - 10^{-6}$ µg.

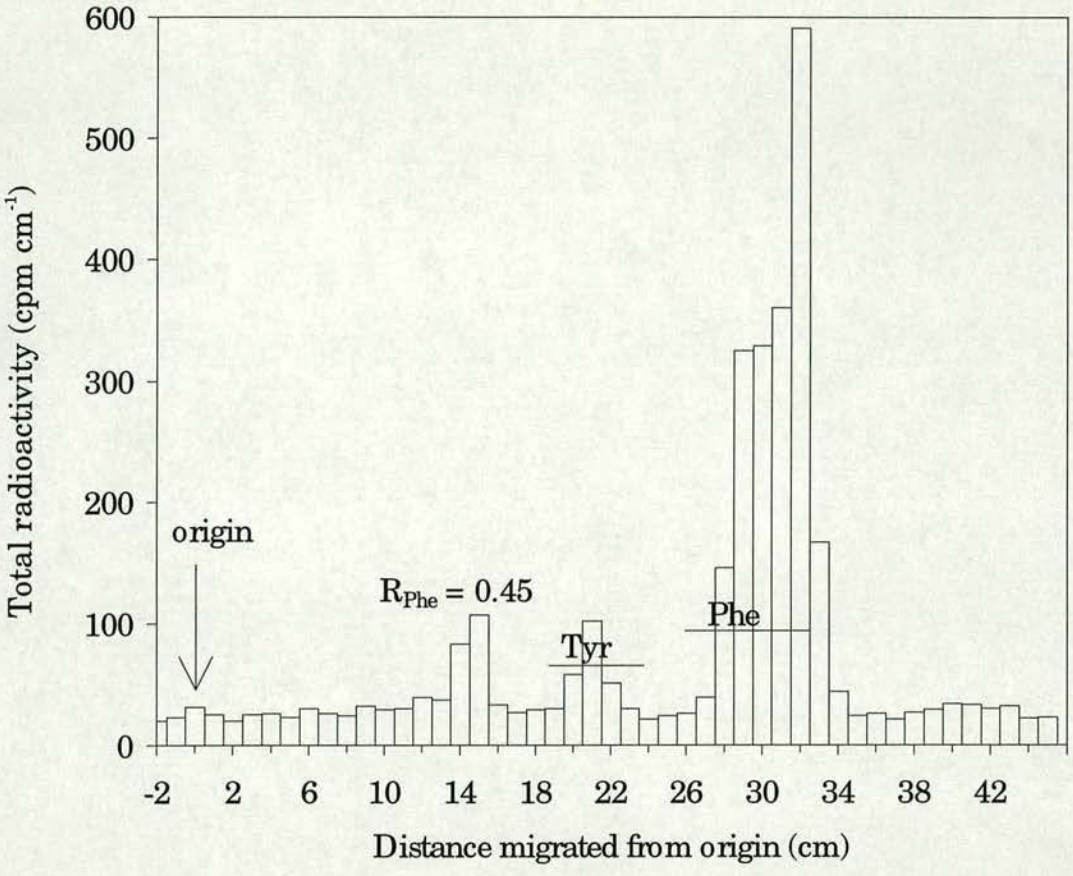


Fig. 3.4.4.1. Paper chromatogram (solvent system BAW) showing the products of acid hydrolysis of rose cells fed L-[U-^{14}C]phenylalanine for 24 hours, in the absence of Man-GlcA-Ins.

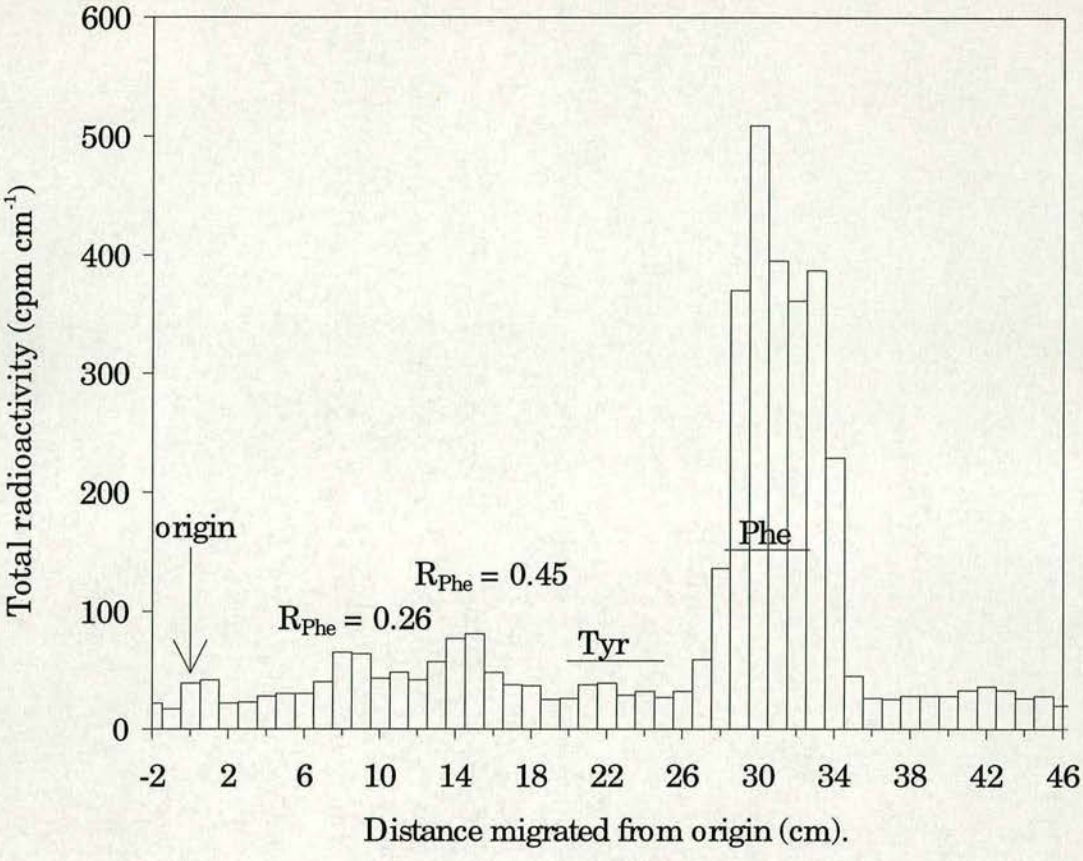


Fig. 3.4.4.2. Paper chromatogram (solvent system BAW) showing the products of acid hydrolysis of rose cells fed L-[U-^{14}C]phenylalanine for 24 hours in the presence of 0.1 μg ml^{-1} of Man-GlcA-Ins.

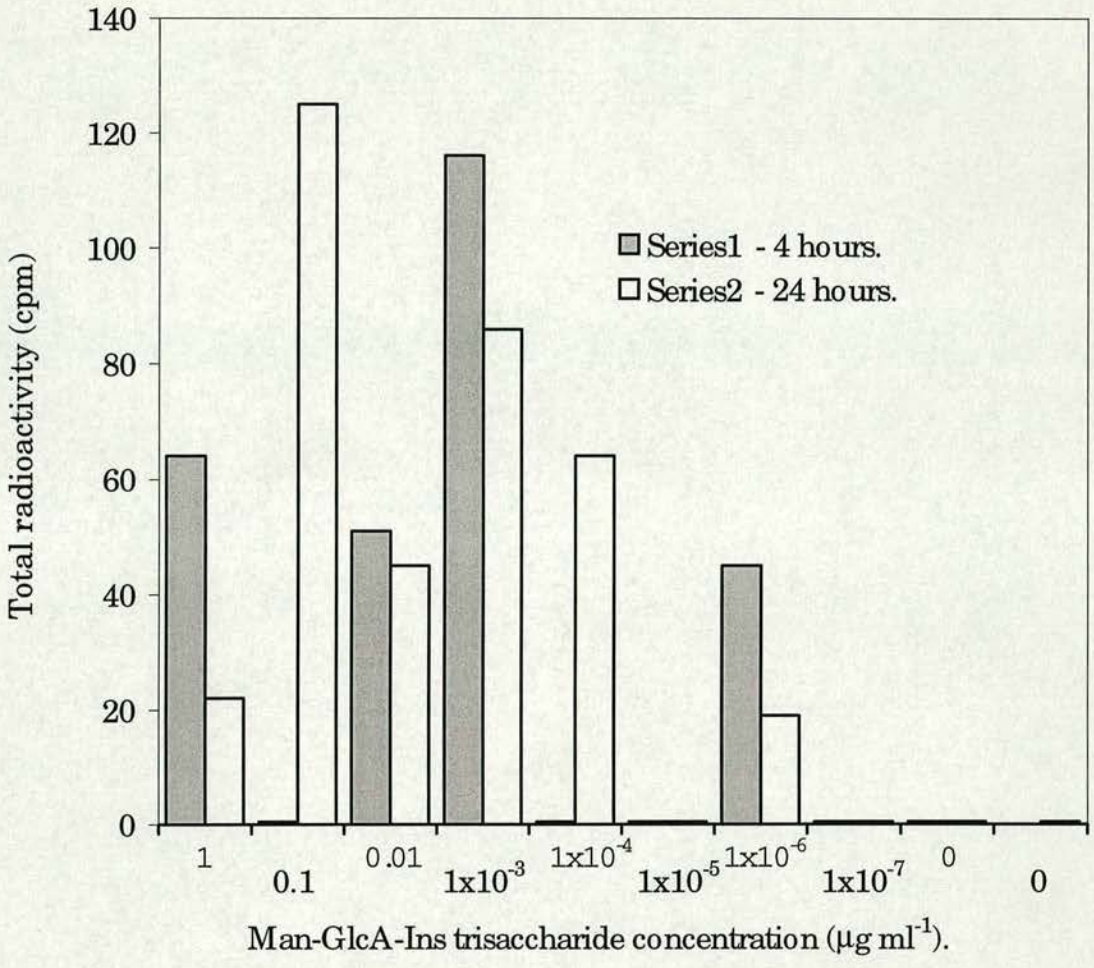


Fig. 3.4.4.3. Amounts of acid resistant compound (R_{Phe} = 0.26) present in rose cells fed [U-^{14}C]phenylalanine in the presence or absence of Man-GlcA-Ins for 4 or 24 hours.

3.4.4.2. Analysis of Compound with R_{Phe} = 0.26

To determine whether the acid resistant compound detected in rose cells presented with Man-GlcA-Ins was cationic, the compound was eluted from the paper chromatogram and subjected to paper electrophoresis at pH 2.0. At this pH all amino acids except cysteine possess a net positive charge and will move towards the cathode unlike non-amino acid phenolic compounds which are neutral. Fig. 3.4.4.4. shows that the compound possessed a net positive charge, moving towards the cathode, confirming that it is most probably amino acid in nature. It was less mobile, however, than either tyrosine or phenylalanine and less mobile than Tyr-Tyr.

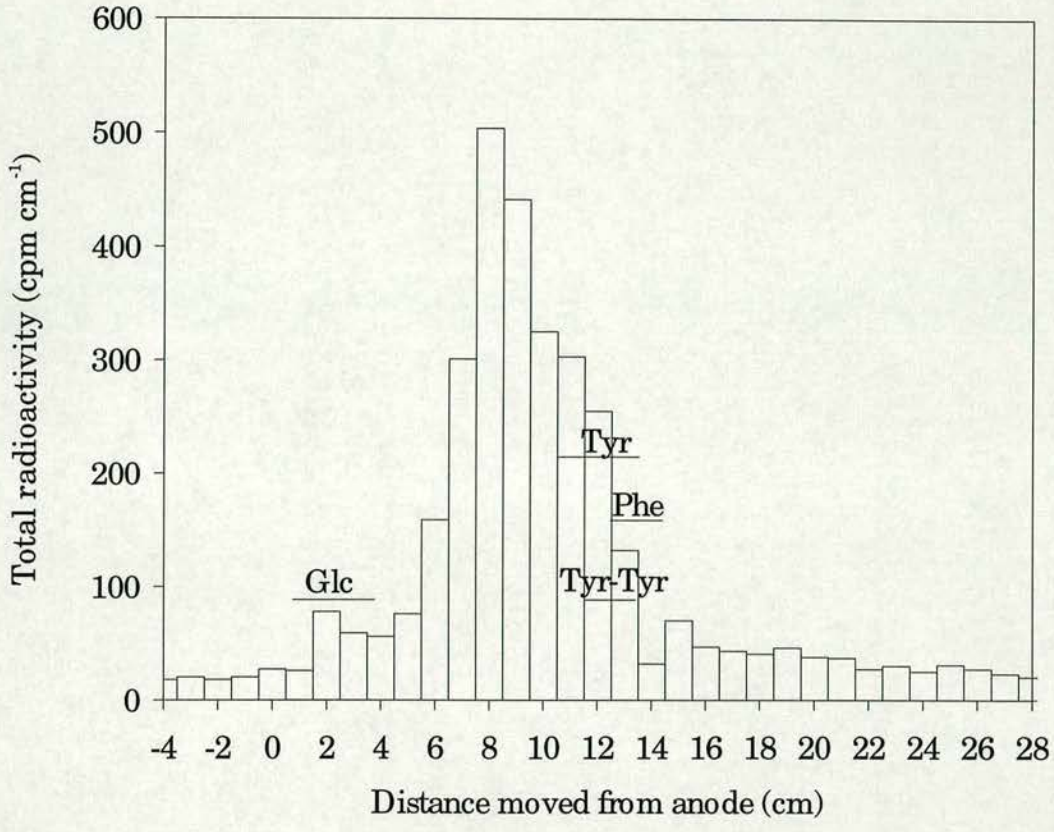


Fig. 3.4.4.4. Paper electrophoresis (pH 2.0) of the acid resistant compound detected in rose cells following presentation of L-[U-^{14}C]phenylalanine in the presence of Man-GlcA-Ins (0.1 μg ml^{-1}).

3.4.5 Discussion of Results

Out of 4 bioassays attempted, Man-GlcA-Ins was found to be without effect on two of the ones tested; namely effect on GA_3 mediated growth in pea stems and effect on the proteins produced by rose cells. Rose cells however were found to exhibit decreased incorporation of L-[U-^{14}C]leucine in the presence of the Man-GlcA-Ins trisaccharide. The effect showed a concentration optimum of between 1 - 10^{-5} ng ml^{-1}. In this experiment no attempts were made to remove endogenous trisaccharide present in the culture medium and this must therefore be taken into account when interpreting these results. It means that in theory the concentrations at which the trisaccharide was found to be biologically active are in addition to the amounts of trisaccharide present in the culture medium before the experiment started. It is possible that the positive results were a product of the experimental design, though this seems unlikely when considering that the uptake of the amino acid was unaffected although the experiments was performed in parallel. It is also possible that the production of a fine cell suspension altered the composition of the culture medium and that under certain conditions the trisaccharide may be broken down. Alternatively it is possible that the culture at the time was growing slowly and that the amounts of trisaccharide were lower than estimated in section 3.1.4.

Crosslinking and insolubilisation of cell wall polymers is a common response to many elicitors and therefore it is not surprising that the Man-GlcA-Ins trisaccharide was able to produce a change in the structure of the proteins in the cells and the formation of an acid resistant phenylalanine derivative. Preliminary experiments into the structural requirements of this response, though bioassays of the GlcA-Ins disaccharide, and mannose, glucuronic acid and inositol were inconclusive.

4. Discussion

4.1. Naturally Occurring Oligosaccharins

There is firm evidence in the literature that cell wall derived oligosaccharides, be they from plant or plant pathogen cell walls, can act as signalling molecules in plants (for reviews; Aldington and Fry, 1991; Ryan and Farmer, 1991). Much of this evidence has been accumulated through the lysis of cell wall components, often by fungal pathogenic enzymes which specifically cleave the cell wall to produce oligosaccharides, and bioassays of the products. There are now many cases of oligosaccharin effects and we have some insight into the possible ways in which they may exert their biological action, the ways in which they may be produced, broken down and transported although we still do not have a great deal of information regarding the extent to which they occur *in vivo*.

Oligosaccharins from cell walls are assumed to be associated with the liquid which surrounds the cell and infiltrates the cell wall. Although there are techniques for extracting the apoplastic fluid (De Wit *et al.*, 1988), this will only yield very small amounts of material and there are therefore practical difficulties involved in analysing such material and detecting oligosaccharides *in situ*. It is possible to make some extrapolations though. Aldington and Fry (1992) found cell wall lysing activity in tomato apoplastic fluid from plants infected with *Fulvia fulva*. From such a finding it is hard to assume that cell wall derived oligosaccharides would not be present as well, although fungal cell wall lysing activity does not necessarily translates into biologically active oligosaccharides (Sharon, 1993).

Another way of analysing for the presence of oligosaccharides is through the use of spent culture medium. When cells are grown in

suspension culture the culture medium acts as an extended apoplast and as it is easy to bulk up cultures so large amounts of "apoplastic" fluid can be easily obtained simply by filtering off the cells. It was in this way that rose cells were analysed during this project and in this way that an oligosaccharide was found to accumulate in the spent culture medium.

4.2. Naturally Occurring Oligosaccharides in the Spent Culture Medium of Rose

Two approaches were taken to identify oligosaccharides in the spent culture medium of rose cell suspension cultures. The culture medium was partially purified on a Bio-Gel P-2 column and Dionex HPLC was used to identify oligosaccharide in the fractions. This led to the identification of two oligosaccharides. One failed any attempts to purify it within the timescale of the project whereas the other and smaller oligosaccharide was successfully purified. This oligosaccharide, which showed chromatographic properties consistent with a trisaccharide, proved to incorporate D-[6-^{14}C]glucuronic acid when this was presented to the cells. The specificity of this radiolabelled precursor meant that the trisaccharide had to contain a uronic acid residue (Brown and Fry, 1993). The other residues, mannose and inositol, were identified through the use of paper chromatography, paper electrophoresis and Dionex HPLC. NMR analysis confirmed that this was a novel trisaccharide consisting of α-D-mannopyranosyl-(1→4)-α-D-glucuronopyranosyl-(1→2)-*myo*-inositol.

Man-GlcA-Ins accumulated to a concentration of 0.8 μg ml^{-1} (1.5 μM) in the spent culture medium of rose cells in a pattern which closely mirrored the increase in the packed cell volume of the culture. The rate of accumulation of the trisaccharide slowed down as the rate of growth of the culture decreased, which was possibly as a result of the carbon source, glucose, having been depleted in the culture medium. The

best known oligosaccharin found to occur *in vivo,* namely XXFG was found to accumulate to a concentration of 0.43 μM in spinach suspension cultures (Fry, 1986). It is known, however, that XET is able to incorporate XXFG into xyloglucan and it is possible that the lower levels of XXFG reflect a continued removal of the oligosaccharide by incorporation onto polymers. On the other hand there is no evidence that Man-GlcA-Ins is removed from the culture medium. The trisaccharide continued to accumulate for the duration of the experiment and for as long as the culture was growing, but there was no significant decrease in the concentration measured. When purified ^{14}C-trisaccharide was presented to the rose cells there was no decrease in the amount of the radioactivity nor in its chromatographic mobility indicating that while the cells were growing at their fastest rate there was no turnover of the trisaccharide over a 72 hour period. It is possible that in older cultures this would be different but unfortunately not enough radioactive trisaccharide was available to repeat the experiment with older cells.

The oligosaccharides in spent culture medium of rose cells was compared with the spent culture medium from *Acer*, *Festuca* and *Zea* cell suspension cultures and only *Acer* was found to contain an oligosaccharide which at least chromatographically appeared to be identical to the rose Man-GlcA-Ins trisaccharide. Nor did these other cultures yield a great deal of oligosaccharides in their spent culture medium. In general two sensitive methods, Dionex HPLC and radiolabelling, did not reveal a large amount of naturally occurring oligosaccharides. This may be in part due to the fact that when growing under normal condition i.e. not challenged by pathogens or subjected to environmental stresses oligosaccharides are not produced.

By definition if oligosaccharins are signalling molecules they are not likely to be constitutively expressed. This would then seem to cast doubt on the role of the Man-GlcA-Ins as a signalling molecule. It has been observed however that a pectic proteinase inhibitor inducing factor,

accumulates in the suspension culture of tomato cells coinciding with depletion of the carbon source, and that PI subsequently accumulated in large amounts (Walker-Simmons *et. al.*, 1984). It is possible that such patterns of accumulation are an artifact of the way in which plant cells in suspension culture are growing and they might not reflect the way in which an oligosaccharide might accumulate in whole plants.

One possible future approach to detecting oligosaccharides may be through re-creation of some of the conditions under which oligosaccharins are likely to be produced as signalling molecules. This could involve the screening of ripening fruit which has successfully been used to reveal oligosaccharins in tomato (Melotto *et. al.*, 1994) and plant material infected with pathogens (Aldington and Fry, 1992). Such conditions may yield an entirely different pattern of oligosaccharide presence and prove to be a rich source of oligosaccharins. It is also worth attempting to detect oligosaccharides in whole plants though this is experimentally difficult due to the small amounts of apoplastic fluid present relative to what is obtainable from suspension culture cells.

4.3 Man-GlcA-Ins

The structural analysis of the trisaccharide purified from suspension cultured rose cells was found to contain mannose, glucuronic acid and inositol in the following configuration:

α-D-mannopyranosyl-(1→4)-α-D-glucuronopyranosyl-(1→2)-*myo*-inositol.

This is believed to be a novel compound. The radiolabelling time course experiment in section 3.3. indicated that this compound was formed by breakdown of an existing polymer. The addition of D-[6-^{14}C]glucuronic acid to the culture medium of suspension cultured rose cells resulted in the radiolabelling of not only the trisaccharide but also of other carbohyrates, probably predominantly pectins, and this was monitored as chromatographically immobile material in the spent culture medium.

Chromatographically immobile material which was deemed to polymeric in nature for the purposes of the experiment started to accumulate in the culture medium within 30 minutes of presentation of the radiolabelled precursor and continued to accumulate until the precursor was depleted in the medium. The patterns of accumulation of product and depletion precursor were closely matched. On the other hand Man-GlcA-Ins only accumulated after a lag of approximately 5 hours and then continued to accumulate at a constant rate (or with slight acceleration) even after the accumulation of the other radiolabelled components of the culture medium had started to decrease owing to depletion of their precursor. This does not suggest a direct radiolabelled precursor → trisaccharide relationship only involving the intermediate stages necessary to convert glucuronic acid to GlcA-1-P to UDP-GlcA which could then be directly incorporated into the trisaccharide and secreted. There steps would all be very short lived. Rather the time delay suggests a longerlived intermediate compound. Such an intermediate compound would have to accumulate as its radiolabelled form before the ^{14}C-trisaccharide could be produced in sufficient quantities to be detectable and the intermediate might act as a reservoir of ^{14}C allowing the synthesis of the ^{14}C-trisaccharide to continue after the radiolabelled precursor had been depleted in the culture medium. It was in this fashion that XXFG was demonstrated to be formed by breakdown of an intermediate, proposed to be a polysaccharide rather than by *de novo* synthesis (McDougall and Fry, 1991).

The nature of this intermediate is another matter altogether. Whereas α-D-glucuronic acid is a common cell wall polysaccharide residue the same cannot be said of *myo*-inositol or α-D-mannose. Attempts to assay the intermediate molecule were unsuccessful. This may be due to low specific activity and amounts produced below the quantitation limit of the detection methods available. Alternatively, it is possible that

whereas the trisaccharide is hydrolysed to a glucuronic acid-inositol disaccharide the same is not the case for the intermediate. It may contain other acid resistant residues.

4.4 *Myo*-Inositol

The biggest question arising from the structural analysis of Man-Glca-Ins is what polymers it may have arisen from. To try to answer this it is worth looking into which polymers are known to contain *myo*-inositol. As this is not the case with any of the known cell wall polysaccharides it is necessary to look elsewhere. Within cellular metabolism *myo*-inositol is generally considered ubiquitous. It can accumulate to considerable levels in plants. In kiwi fruit (*Acinidia deliciosa*) up to 40% of the dry weight of the fruit at mid development may be inositol. The exact reason for this is uncertain; it is thought that it may be present to allow glucuronic acid synthesis as part of fruit development (Bieleski *et. al.*, 1997). *Myo*-Inositol is an intermediate in several metabolic pathways including one route of formation of glucuronic acid, through oxidation of inositol. It is present in many compounds including phytic acid and phosphatidylinositol (Loewus and Loewus, 1980). It is also part of the phosphosphingolipid group and intriguingly the following structure has been identified in maize (*Zea*);

α-D-glucosamine-(1→4)-α-D-glucuronic acid-(1→6)-*myo*-inositol-

(2←1)- α-D-mannose, with the inositol residue bearing a phosphoceramide (Carter *et. al.*, 1969). This structure is tantalisingly close to the structure derived for the trisaccharide. It appears that Man-GlcA-Ins may be a fragment of a lipid molecule rather than a cell wall polysaccharide. Some other structures have also been identified in plant phosphosphingolipids, several of which contain α-D-glucuronic acid, α-D-mannose and *myo*-inositol though none in the exact configuration seen in the rose trisaccharide (Laine and Hsieh, 1987).

Such lipids are present in plants and can be extracted e.g. from tomato suspension culture cells (Drobak *et. al.*, 1988) and there is evidence that they may be present in the membranes of plants. A monoclonal antibody was found which recognised a glycosyl inositol phospholipid present in nodules of roots in peas as well as in the non-leguminous carrot (Perotto *et al.*, 1995).

It is not possible to prove or disprove whether Man-GlcA-Ins which is produced by rose cells and accumulates in their spent culture medium is indeed a fragment of a phosphoshingolipid. If this is the case then the definition of an oligosaccharin may have to be extended to include oligosaccharides present in the cell wall environment rather then derived from the cell wall (Albersheim *et al.*, 1983). In this case the oligosaccharide is certainly present in the location where we would normally expect cell wall derived oligosaccharides to be present although it may have a different source e.g. the plasma membrane.

4.5 Oligosaccharin

It appears that, whatever the source of the oligosaccharide, it possesses biological activity. It is without effect on the gibberellin induced elongation of pea shoots and on the amount and types of proteins being produced by rose cells. It does appear to have an effect on the incorporation of L-[U-^{14}C]leucine into proteins. This bioassay has previously been used successfully on rhamnogalacturonan-II to demonstrate biological activity (Aldington and Fry, 1994). As mentioned, these results have to be interpreted with some care, however, as the absolute concentration of Man-GlcA-Ins to which the cells were subjected was uncertain.

That an oligosaccharide should be able to influence crosslinking in the cells/cell wall is to be expected. Many elicitors are known to alter the extractability of extensin (Brady and Fry, 1997; Brownleader *et. al.*,

1997), though unfortunately the nature of the acid resistant compound remains unknown.

What is also unknown is the extent to which the structure of Man-GlcA-Ins is important for its activity. It is known that the structure of oligosaccharins is crucial for activity and often loss of a single residue is sufficient for loss of activity (McDougall and Fry, 1989). Unfortunately, preliminary results aimed at testing whether the disaccharide (GlcA-Ins) and the monosaccharides (glucuronic acid, mannose and inositol) were able to produced the same effect were inconclusive.

Intriguingly Roberts *et al.,* (1997) have found but not identified a component of *Zinnia* culture medium which affects the growth of cells in culture. This compound was found to be less than 1 kDa in size and able to bind to ConA, indicative of mannose. It is interesting to speculate as to whether this compound and the trisaccharide detected in the spent culture medium of rose may be the same.

4.6 Conclusions

Rose cells are able to produce the oligosaccharide α-D-mannopyranosyl-(1→4)-α-D-glucuronopyranosyl-(1→2)-*myo*-inositol which accumulates in their suspension culture medium. It is believed that it arises by breakdown of a precursor molecule whose identity is unknown, but which may be a phosphosphingolipid. Man-GlcA-Ins is able to decrease the incorporation of L-[U-^{14}C]-leucine into proteins in rose cells as well as alter the products of L-[U-^{14}C]phenylalanine metabolism in rose cells. I hope that future work will explore further the nature of the biological effect for which it is believed to be responsible and its source within the plant cell. To further validate this oligosaccharide and extend our knowledge of biologically active oligosaccharides in general, attempts should be made to determine whether or not it is present in other plant species and whether it is present in whole plants.

5. References

Albersheim, P. and Valent, B., (1978). Host-pathogen interactions in plants. Plants when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. J. Cell Biol. **78**, 627-643.

Albersheim, P., Darvill, A.G., McNeil, M., Valent, B.S., Sharp, J.K., Nothnagel, E.A., Davis, K.R. and Yamazaki, N., (1983). Oligosaccharins: Naturally-Occurring Carbohydrates with Biological Regulatory Functions. In "Structure and Function of Plant Genomes" (O. Ciferri and L. Dure, eds.), pp 293-312, Plenum, New York. NATO ASI on the structure and function of plant genome, Lake Garda, Italy.

Aldington, S. and Fry, S.C., (1991). Oligosaccharins. Adv. Bot. Res., **19**, 1-101.

Aldington, S., McDougall, G.M. and Fry, S.C., (1991). Structure-activity relationships of biologically active oligosaccharides. Plant, Cell, Env., **14**, 625-636.

Aldington, S. and Fry, S.C., (1992). Plant cell wall-lysing enzymes in the apoplast of tomato leaves infected with *Fulvia fulva*. Can. J. Bot., **70**, 607-613.

Aldington, S. and Fry, S.C., (1994). Rhamnogalacturonan-II - a biologically active fragment. J. Exp. Bot., **45**, 287-293.

Aspinall, G.O., (1982). Chemical characterisation and structure determination of polysaccharides. In: The Polysaccharides, vol 1, eds G.O. Aspinall. Academic Press, New York.

Aspinall, G.O., Khondo, L. and Puvanesarajah, V., (1989). Selective fragmentation of methylated tobacco extra-cellular arabinoglucuronomannan by cleavage of β-D-glucopyranosiduronic acid units. Carbohydr. Res., **188**, 113-120.

Atwell, B.J. and Ap Rees, T., (1986). Distribution of protein synthesised by seedlings of *Oryza sativa* grown in anoxia. J. Plant Physiol., **123**, 401-408.

Augur, C., Yu, L., Sakai, K., Ogawa, T., Sinay, P., Darvill, A.G. and Albersheim, P., (1992). Further studies of the ability of xyloglucan oligosaccharide to inhibit auxin-stimulated growth. Plant Physiol., **99**, 180-185.

Augur, C., Benhamou, N., Darvill, A. and Albersheim, P., (1993). Purification, characterisation, and cell wall localisation of an α-fucosidase that inactivates a xyloglucan oligosaccharin. Plant J., **3**, 415-426.

Auxtova, O., Liskova, D., Kakoniova, D., Karacsonyi M. and Bilisics, L., (1995). Effect of galactoglucomannan-derived oligosaccharides on elongation growth of pea and spruce stem segments stimulated by auxin. Planta, **196**, 420-424.

Ayers, A.R., Ebel, J., Finelli, F., Berger, N. and Albersheim, P., (1976a). Host-pathogen interactions IX. Quantitative assays of elicitor activity

and characterisation of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. Plant Physiol., **57**, 751-759.

Ayers, A.R., Ebel, J., Valent, B. and Albersheim, P., (1976b). Host-pathogen interactions X. Fractionation and biological activity of an elicitor isolated from the mycelial wall of *Phytophthora megasperma* var. *sojae*. Plant Physiol., **57**, 760-765.

Ayers, A.R., Valent, B., Ebel, J. and Albersheim, P., (1976c). Host-pathogen interactions XI. Composition and structure of wall-released elicitor fractions. Plant Physiol., **57**, 766-774.

Bach, M. and Seitz, H.U., (1997). Elicitor-induced defence responses of a suspension-cultured woody plant (*Larix decidua*) and possible mechanisms of signal transduction. Can. J. Bot., **75**, 1243-1251.

Bailey, B.A., Dean, J.F.D. and Anderson, J.D., (1990). An ethylene biosynthesis-inducing endoxylanase elicits electrolyte leakage and necrosis in *Nicotiana tabacum* cv Xanthi leaves. Plant Physiol., **94**, 1849-1854.

Baldwin, E.A. and Biggs, R.H., (1988). Cell-wall lysing enzymes and products of cell wall digestion elicit ethylene in citrus. Physiol. Plant., **73**, 58-64.

Baydoun, E. A-H. and Fry, S.C., (1985). The immobility of pectic substances in injured tomato leaves and its bearing on the identity of the wound hormone. Planta, **165**, 269-276.

Baydoun, E, A-H. and Fry, S.C., (1989). In vivo degradation and extracellular polymer binding of xyloglucan nonasaccharide, a naturally occurring anti-auxin. J. Plant Physiol., **134**, 453-459.

Bieleski, R.L., Clark, C.J. and Klages, K.U., (1997). Identification of *myo*-inositol as a major carbohydrate in kiwi fruit, *Actinidia deliciosa*. Phytochemistry, **46**, 51-55.

Bishop, P.D., Pearce, G., Bryant, J.E. and Ryan, C.A., (1984). Isolation and characterisation of the proteinase inhibitor-inducing factor from tomato leaves. J. Biol. Chem., **259**, 13172-13177.

Bishop, P.D. and Ryan, C.A., (1987). Plant cell wall polysaccharides that activate natural plant defences. Meth. Enzymol., **138**, 715-724.

Bois, F., (1992). The influence of some natural cell-wall derived precursors of organogenesis and differentiationn of wild strawberry (*Fragaria vesca* L.) callus culture. Plant Cell, Tissue and Organ Culture, **28**, 91-96.

Bolwell, G.P., Robbins, M.P. and Dixon, R.A., (1985). Metabolic changes in elicitor-treated bean cells. Enzymic responses associated with rapid changes in cell wall components. Eur. J. Biochem., **148**, 571-578.

Bowles, D.J., (1990). Defence-related proteins in higher plants. Annu. Rev. Biochem., **59**, 873-907.

Brady, K.P., Darvill, A.G. and Albersheim, P., (1993). Activation of a tobacco glycine-rich protein gene by a fungal glucan preparation. Plant J., **4**, 517-524.

Brady, J.D., Sadler, I.H. and Fry, S.C., (1996). Di-isodityrosine, a novel tetrameric derivative of tyrosine in plant cell wall proteins: a new potential cross-link. Biochem. J., **315**, 323-327.

Brady, J.D. and Fry, S.C., (1997). Formation of di-isodityrosine and loss of isodityrosine in the cell walls of tomato cell-suspension cultures treated with fungal elicitors or H_2O_2. Plant Physiol., **115**, 87-92.

Branca, C., De Lorenzo, G. and Cervone, F., (1988). Competitive inhibition of the auxin-induced elongation of α-D-oligogalacturonides in pea stem segments. Physiol. Plant., **72**, 499-504.

Brecht, J.K. and Huber, D.J., (1988). Products release from enzymically active cell wall stimulate ethylene production and ripening in preclimacteric tomato (*Lycopersicon esculentum* Mill.) fruit. Plant Physiol., **88**, 1037-1041.

Brett, C. and Waldron, K., (1990). In Physiology and Biochemistry of Plant Cell Walls, 2nd edition. Unwin Hyman Ltd, London, UK.

Broekaert, W.F. and Peumans, W.J., (1988). Pectic polysaccharides elicit chitinase accumulation in tobacco. Physiol. Plant., **74**, 740-744.

Brown, J.A. and Fry, S.C., (1993). Novel *O*-D-galacturonoyl esters in the pectic polysaccharides of suspension cultures plant cells. Plant Physiol., **103**, 993-999.

Brownleader, M.D., McNally, P.E., Davies, G.E.A., Trevan, M. and Dey, P.M., (1997). Elicitor-induced extensin insolubilization in suspension-cultured tomato cells. Phytochemistry, **46**, 1-9.

Bruce, R.J. and West, C.A., (1982). Elicitation of casbene synthetase activity in castor bean. Plant Physiol., **69**, 1181-1188.

Bruce, R.J. and West, C.A., (1989). Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. Plant Physiol., **91**, 889-897.

Campbell, A.D. and Labavich, J.M., (1991). Induction and regulation of ethylene biosynthesis by pectic oligomers in cultured pear cells. Plant Physiol., **97**, 699-705.

Campbell, B.C., (1986). Host-plant oligosaccharins in the honeydew of *Schizaphis graminum* (rondani)(Insecta, Aphidadae). Experientia, 42, 451-452.

Campbell, J.A., Drake, A.M., Lee. V.W.K. and Strother, S., (1995). A putative oligosaccharin growth promoter from *Vitis vinifera* L. primary cell walls. Ann. Bot., **75**, 359-363.

Carpita, N.C. and Gibeaut, D.M., (1993). Structural models of primary cell walls in flowering plants: consistency of the molecular structure with the physical properties of the walls during growth. Plant J., **3**, 1-30.

Carpita, N.C., (1996). Structure and biogenesis of the cell walls of grasses. Annu. Rev. Plant Physiol., **47**, 445-476.

Carter, H.E., Strobach, D.R. and Hawthorne, J.N., (1969). Biochemistry of the shingolipids. XVIII. Complete structure of tetrasaccharide phytoglycolipid. Biochem., **8**, 383-388.

Cervone, F., De Lorenzo, G., Degra, L. and Salvi, G., (1987). Elicitation of necrosis in *Vigna unguiculata* Walp. by homogeneous *Aspergillus niger* endo-polygalacturonase and by α-D-galacturonate oligomers. Plant Physiol., **85**, 626-630.

Cervone. F., Hahn, M.G., De Lorenzo, G., Darvill, A. and Albersheim, P., (1989). Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol., **90**, 542-548.

Chappell, J., Hahlbrock, K. and Boller, T., (1984a). Rapid induction of ethylene biosynthesis in cultured parsley cells by a fungal elicitor and its relationship to the induction of phenylalanine ammonia-lyase. Planta, **161**, 475-480.

Chappell, J. and Hahlbrock, K., (1984b). Transcription of plant defence genes in response to UV light or fungal elicitor. Nature, **311**, 76-78.

Cheong, J-J., Birberg, W., Fugedi, P., Pilotti, A., Garegg, P., Hong, N., Ogawa, T. and Hahn, M.G., (1991a). Structure-activity relationships of oligo-β-glucoside elicitors of phytoalexin accumulation in soybean. Plant Cell, **3**, 127-136.

Cheong, J-J. and Hahn, M.G., (1991b). A specific, high-affinity binding site for the hepta-β-glucoside elicitor exists in soybean membranes. Plant Cell, **3**, 137-147.

Cline, K. and Albersheim, P., (1981). Host-pathogen interactions. XVII. Hydrolysis of biologically active fungal glucans by enzymes isolated from soybean cells. Plant Physiol., **68**, 221-228.

Cosio, E.G., Frey, T. and Ebel, J., (1990). Solubilization of soybean membrane binding sites for fungal β-glucans that elicit phytoalexin synthesis. FEBS Lett., **264,** 235-238.

Cramer, C.L., Ryder, T.B, Bell, J.N. and Lamb, C.J., (1985). Rapid switching of plant gene expression induced by fungal elicitor. Science, **227**, 1240-1243.

Darvill, A.G., and Albersheim, P., (1984). Phytoalexins and their elicitors- a defence against microbial infection in plants. Annu. Rev. Plant Physiol., **35**, 243-275.

Davis, K.R., Lyon, G.D., Darvilo, A.G. and Albersheim, P., (1984). Host-pathogen Interactions. XXV. Endopolygalacturonic acid lyase *from Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. Plant Physiol., **74**, 52-60.

Dean, J.F.D., Gross, K.C. and Anderson, J.D., (1991). Ethylene biosynthesis-inducing xylanse. III. Product characterisation. Plant Physiol., **96**, 571-576.

De Silva, J., Jarman, C.D., Arrowsmith, D.A., Stronach, M.S., Chengappa, S., Sidebottom, C. and Reid, J.S.G., (1993). Molecular characterisation of a xyloglucan-specific endo-(1→4)-β-D-glucanase (xyloglucan endo-transglycosylase) from nasturtium seeds. Plant J., **3**, 701-7011.

De Silva, J., Arrowsmith, D., Hellyer, A., Whiteman, S. and Robinson, S., (1994). Xyloglucan endotransglycosylase and plant growth. J. Exp. Bot., **45**, 1693-1701.

De Wit, P.J.G.M., Toma, I.M.J. and Joosten, H.A.J., (1988). Race-specific elicitors and pathogenicity factors in the *Cladosporium fulvum-tomato* interaction. Physiology and Biochemistry of Plant-Microbial Interactions. (Noel T. Keen, Tsune Kosuge, and Linda L. Walling, Eds.). The American Society of Plant Physiologists.

Dietrich, A., Mayer, J.R. and Hahlbrock., (1990). Fungal elicitor triggers rapid, transient, and specific protein phosphorylation in parsley cell suspension culture. J. Biol. Chem., **265**, 6360-6368.

Drøbak, B.K., Ferguson, I.B., Dawson, A.P. and Irvine, R.F., (1988). Inositol-containing lipids in suspension-culture plant cells. Plant Physiol., **87**, 217-222.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F., (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem., **28**, 350-356.

Ebel, J., Ayers, A.R. and Albersheim, P., (1976). Host-pathogen interactions XII. Response of suspension-cultured soybean cells to the

elicitor isolated from *Phytophthora megasperma* var sojae, a fungal pathogen of soybeans. Plant Physiol., **57**, 775-779.

Eberhart, S., Doubrava, N., Marfa, V., Mohnen, D., Soutwick, A., Darvill, A. and Albersheim, P., (1989). Pectic cell wall fragments regulate tobacco thin-cell-layer explant morphogenesis. Plant Cell, **1**, 747-755.

Emmerling, M. and Seitz, H.U., (1990). Influence of a specific xyloglucan-nonasaccharide derived from cell walls of suspension cultured cells of *Daucus carota* L. on regenerating carrot protoplasts. Planta, **182**, 174-180.

Farmer, E.E., Pearce, G. and Ryan, C.A., (1989). *In vitro* phosphorylation of plant plasma membrane proteins in response to the proteinase inhibitor inducing factor. Proc. Nat. Acad. Sci. USA, **86**, 1539-1542.

Farmer, E.E., Moloshok, T.D., Saxton, M.J. and Ryan C.A., (1991). Oligosaccharide signalling in plants. Specificity of oligouronide-enhanced plasma membrane protein phosphorylation. J. Biol. Chem., **266**, 3140-3145.

Felix, G., Regenass, M. and Boller, T., (1993). Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation and establishment of a refractory state. Plant J., **4**, 307-316.

Freshour, G., Clay, R.P., Fuller, M.S., Albersheim, P., Darvill, A.G. and Hahn, M., (1996). Developmental and tissue-specific structural

alterations of the cell-wall polysaccharides of *Arabidopsis thaliana* roots. Plant Physiol., **110**, 1413-1429.

Fry, S.C. and Street, H.E., (1980). Gibberelin-sensitive suspension culture. Plant Physiol., **65**, 472-477.

Fry, S.C., (1982). Isodityrosine, a new cross-linking amino acid from plant cell-wall glycoprotein. Biochem., J., **204**, 449-455.

Fry, S.C., (1986a). In-vivo formation of xyloglucan nonasaccharide: A possible biologically active cell-wall fragment. Planta, **169**, 443-453.

Fry, S.C., (1986b). Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu. Rev. Plant. Physiol., **37**, 165-186.

Fry, S.C., (1988). The Growing Plant Cell Wall: Chemical and Metabolic Analysis. Longman Scientific and Technical, Harlow, UK.

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K. and Matthews, K.J., (1992). Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochem. J., **282**, 821-828.

Fry, S.C., Aldington, S., Hetherington, P.R. and Aitkin, J., (1993). Oligosaccharides as signals and substrates in the plant cell wall. Plant Physiol., **103**, 1-5.

Fry, S.C., York, W.S., Albersheim, P., Darvill, A.G., Hayashi, T., Joseleau, J., Kato, Y., Lorences, E.P., Maclachlan, G.A., McNeil, M., Mort, A.J., Reid, J.S.G., Seitz, H.U., Selvendran, R.R., Voragen, A.G.J.

and White, A.R., (1993). An unambiguous nomenclature for xyloglucan-derived oligosaccharides. Physiol. Plant., **89**, 1-3.

Fry, S.C., (1995). Polysaccharide-modifying enzymes in the plant cell wall. Annu. Rev. Plant Physiol. Plant Mol. Biol., **46**, 497-520.

Fry, S.C., (1996). Oligosaccharin mutants. Trend in Plant Science, 1, 326-328.

Garcia-Romera, I. and Fry, S.C., (1997). Upper limits for endogenous oligogalacturonides and free galacturonic acid in rose cell-suspension culture: implications for the action of exo- and endo-polygalacturonases *in vivo*. J. Plant Physiol., **150**, 241-246.

Gollin, D.J., Darvill, A.G. and Albersheim, P., (1984). Plant cell wall fragments inhibit flowering and promote vegetative growth in *Lemna gibba* G3. Biol. Cell., **51**, 275-280.

Gibeaut, D.M. and Carpita, N.C., (1994). Biosynthesis of plant cell wall polysaccharides. FASEB J., **8**, 904-915.

Grab, D., Feger, M. and Ebel, J., (1989). An endogenous factor from soybean (*Glycine max L.)* cell cultures activates phosphorylation of a protein which is dephosphorylated in vivo in elicitor-challenged cell. Planta, **179**, 340-348.

Gorshkova, T.A., Wyatt, S.E., Salnikov, V.V., Gibeaut, D.M., Ibragimov, M.R., Lozovaya, V.V. and Carpita, N.C., (1996). Cell-wall polysaccharides of developing flax plants. Plant Physiol., **110**, 721-729.

Green, T.R. and Ryan, C.A., (1972). Wound-induced proteinase inhibitor in plant leaves: a possible defence mechanism against insects. Science, **175**, 776-777.

Hahn, M,G., Darvill, A.G. and Albersheim, P., (1981). Host-pathogen interactions. XIV. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. Plant Physiol., **68**, 1161-1169.

Ham, K., Albersheim, P. and Darvill, A.G., (1995). Generation of β-glucan elicitors by plant enzymes and inhibition of the enzymes by a fungal protein. Can. J. Bot., **73** (sup. 1), S1100-1103.

Hayashi, T., Marsden, M.P.F. and Delmer, D., (1987). Pea xyloglucan and cellulose. V. Xyloglucan-cellulose interactions *in vitro* and *in vivo*. Plant Physiol., **83**, 384-389.

Hoson, T. and Masuda, Y., (1991). Effect of xyloglucan nonasaccharide on cell elongation induced by 2,4-dichlorophenoxyacetic acid and indole-3-acetic acid. Plant Cell Physiol., **33**, 777-782.

Ishii, T. and Saka, H., (1992). Inhibition of auxin-stimulated elongation of cell in rice lamina joints by a feruloylated arabinoxylan trisaccharide. Plant Physiol., **33**, 321-324.

Ishii, T. and Nishijima, T., (1995). Inhibition of gibberellin-induced elongation growth of rice by feruloyl oligosaccharides. Plant Cell Physiol., **36**, 1447-1451.

Ishii, T., (1997). Structure and functions of feruloylated polysaccharides. Plant Science, **127**, 111-127.

Ishii, K. and Teasdale, R.D., (1997). Effects of xylooligosaccharides on suspension cultured cells and protoplasts in *Pinus radiata.* Plant Cell Tissue and Organ Culture, **49**, 189-193.

Jarvis, M.C., (1984). Structure and properties of pectin gels in plant cell walls. Plant, Cell, Env., **7**, 153-164.

Joseleau, J.P., Cartier, N., Chambat, G., Faik, A. and Ruel, K., (1992). Structural features and biological-activity of xyloglucans from suspension cultured plant-cells. Biochimie, **74**, 81-88.

Kobayashi, A., Tai, A. and Kawasu, K., (1995). Structural elucidation of an elicitor-active oligosaccharide, LN-3, prepared from algal laminaran. J. Carbohydr. Chem., **14**, 819-832.

Kogel, G., Beissmann, B., Reisener, H.J. and Kogel, K., (1991). Specific binding of a hypersensitive lignification elicitor from *Puccinia graminis* f. sp. *tritici* to the plasma membrane from wheat (*Triticum aestivum* L.). Planta, **183**, 164-169.

Laine, R.A. and Hsieh, T.C-Y., (1987). Inositol containing shingolipids. Met. Enzymol., **138**, 186-195.

Loewus, F.A. and Loewus, M.W., (1980). *Myo*-inositol: biosynthesis and metabolism. In: The Biochemistry of Plants. A Comprehensive Treatise. Vol. 3. Carbohydrates: Structure and Function. (P.K. Stumpf and E.E. Conn eds.). Academic Press, London, UK.

Lorences, E.P., McDougall, G.J. and Fry, S.C., (1990). Xyloglucan- and cello-oligosaccharides: antagonists of the growth-promoting effect of H^+. Physiol. Plant., **80**, 109-113.

Marfa, V., Gollin, D.J., Eberhard, S., Mohnen, D., Darvill, A.G. and Albersheim, P., (1991). Oligogalacturonides are able to induce flowers to form on tobacco explants. Plant J., **1**, 217-225.

Masuta, C., Van den Bulcke, M., Bauw, G., Van Montagu, M. and Caplan, A.B., (1991). Differential effect of elicitors on the viability of rice suspension cells. Plant Physiol., **97**, 619-629.

McDougall, G.J. and Fry, S.C., (1988). Inhibition of auxin-stimulated growth of pea stem segments by a specific nonasaccharide of xyloglucan. Planta, **175**, 412-416.

McDougall, G.J. and Fry, S.C., (1989). Anti-auxin activity of xyloglucan oligosaccharides: the role of groups other than the terminal α-L-fucose residue. J. Exp. Bot., **40**, 233-238.

McDougall, G.J. and Fry, S.C., (1990). Xyloglucan oligosaccharides promote growth and activate cellulase: evidence for a role of cellulase in cell expansion. Plant. Physiol., **93**, 1042-1048.

McDougall, G.J. and Fry, S.C., (1991). Xyloglucan nonasaccharide, a naturally-occurring oligosaccharin, arises *in vivo* by polysaccharide breakdown. J. Plant Physiol., **137**, 332-336.

McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P., (1984). Structure and function of the primary cell wall of plants. Annu. Rev. Biochem., **53**, 625-63.

Melotto, E., Greve, L.C. and Labavitch, J.M., (1994). Cell wall metabolism in ripening fruit. VII Biologically active pectin oligomers in ripening tomato (*Lycopersicon esculentum* Mill.) fruits. Plant Physiol., **106**, 575-581.

Messiaen, J., Read, N.D., Cutsem, P.V. and Trewavas, A.J., (1993). Cell wall oligogalacturonides increase cystolic free calcium in carrot protoplasts. J. Cell Science., **104**, 365-371.

Moerschbacher, B.M., Flott, B.E., Noll, U. and Reisener, H-J., (1989). On the specificity of an elicitor preparation from stem rust which induces lignification in wheat leaves. Plant Physiol. Biochem., **27**, 305-314.

Moloshok, T. and Ryan, C.A., (1989). Di- and trigalacturonic acid and $\Delta^{4,5}$–di- and trigalacturonic acids: inducers of proteinase inhibitor genes in plants. Met. Enzymol., **47**, 566-570.

Mutaftschiev, S., Macaya, A., Prat, R., Devillers, P. and Golberg, R., (1993). Early effects of plant cell wall fragments on plant cell growth. Plant Physiol. Biochem., **31**, 459-467.

Nothnagel, E.A., McNeil, M., Albersheim, P. and Dell, A., (1983). Host-pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. Plant Physiol., **71**, 916-926.

Pelissier, B., Thibaud, J.B., Grignon, C. and Esquerre-Tugaye, M.T., (1986). Cell surfaces in plant-microorganism interactions. VII. Elicitor preparations from two fungal pathogens depolarize plant membranes. Plant Sci., **46**, 103-109.

Percy, A.E., O'Brien, I.E.W., Jameson, P.E., Melton, L.D., MacRae, E.A. and Redgwell, R.J., (1996). Xyloglucan endotransglycosylase activity during fruit development and ripening in apple and kiwifruit. Physiol. Plant., **96**, 43-50.

Perotto, S., Donovan, N., Drobak, B.K. and Brewin, N.J., (1995). Differential expression of a glycosyl inositol phospholipid antigen on the peribacteroid membrane during pea nodule development. Mol. Plant-Microbe Interactions, **8**, 560-568.

Philips, N., Imoto, S., Ohata, Y. and Nakanishi, K., (1992). Parital purification of an endogenous elicitor from suspension cultured cells of red bean, *Vigna angularis*. Experientia, **48**, 683-687.

Reiter, W-D., Chapple, C. and Somerville, C.R., (1993). Altered growth and cell walls in a fucose-deficient mutant of *Arabidopsis*. Science, **261**, 1032-1035.

Reiter, W-D., Chapple, C. and Somerville, C.R., (1997). Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. Plant J., **12**, 335-345.

Roberts, A.W., Donican, S.G. and Haigler, C.H., (1997). A secreted factor induces cell expansion and formation of metaxylem-like tracheary

elements in xylogenic suspension cultures of Zinnia. Plant Physiol., **115**, 683-692.

Roby, D., Broglie, K., Gaynor, J. and Broglie, R., (1991). Regulation of a chitinase gene promotor by ethylene and elicitors in bean protoplasts. Plant Physiol., **97**, 433-439.

Rocco, A., Castaneda, P. and Perez, L.M., (1993). Oligosaccharides released by pectinase treatment of Citrus limon seedlings are elicitors of the plant response. Phytochemistry, **33**, 1301-1306.

Rouet-Mayer, M., Matieu, Y., Cazale, A., Guern, J. and Lauriere, C., (1997). Extracellular alkalinisation and oxidative burst induced by fungal pectin lyase in tobacco cells are not due to the perception of oligogalacturonide fragments. Plant Physiol. Biochem., **35**, 321-330.

Ryan, C.A., (1974). Assay and biochemical properties of the proteinase inhibitor-inducing factor, a wound hormone. Plant Physiol., **54**, 328-332.

Ryan, C.A., Bishop, P. and Pearce, G., (1981). A sycamore cell wall polysaccharide and a chemically related tomato leaf polysaccharide possess similar proteinase inhibitor-inducing activities. Plant Physiol., **68**, 616-618.

Ryan, C.A. and Farmer, E.E., (1991). Oligosaccharide signals in plants: a current assesment. Annu. Rev. Plant Physiol. Plant Mol. Biol., **41**, 651-674.

Sakurai, N. and Nevins, D.J., (1997). Relationship between fruit softening and wall polysaccharides in avocado (*Persea americana* Mill) mesocarp tissues. Plant Cell Physiol., **38**, 603-610.

Schmidt, W.E. and Ebel, J., (1987). Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions form soybean *Glycine max*. Proc. Nat. Acad. Sci. USA, **84**, 4117-4121.

Schroder, R. and Knoop, B., (1995). An oligosaccharide growth factor in plant suspension cultures: a proposed structure. J. Plant Physiol., **146**, 139-147.

Sharon, A., Fuchs, Y. and Anderson, J.D., (1993). The elicitation of ethylene biosynthesis by a *Trichoderma* xylanase is not related to the cell wall degradation activity of the enzyme. Plant Physiol., **102**, 1325-1329.

Sharp, J.K., Valent, B. and Albersheim, P., (1984a). Purification and partial characterisation of a β-glucan fragment that elicits phytoalexin accumulation in soybean. J. Biol. Chem., **259**, 11312-11320.

Sharp, J.K., McNeil, M. and Albersheim, P., (1984b). The primary structure of one elicitor-active and seven elicitor-inactive hexa(β-D-glucopyranosyl)-D-glucitols isolated from the mycelial wall of *Phytophthora megasperma*. J. Biol. Chem., **259,** 11321-11336.

Showalter, A.M., (1993). Structure and function of plant cell wall proteins. Plant Cell, **5**, 9-23.

Sims, I.M. and Bacic, A., (1995). Extracellular polysaccharides from suspension cultures of *Nicotiana plumbaginifolia*. Phytochemistry., **38**, 1397-1405.

Skare, N.H., Paus, F. and Raa. J., (1975). Production of pectinase and cellulase by Cladosporium cucumerinum with dissolved carbohydrates and isolated cell walls of cucumber as carbon source. Physiol. Plant., **33**, 229-233.

Smith, R.C. and Fry, S.C., (1991). Endotransglycosylation of xyloglucans in plant cell suspension cultures. Biochem. J., **279**, 529-535.

Sowden, J.C., (1952). 6-C^{14}-D-glucose and 6-C^{14}-D-glucoronolactone. J. Am. Chem. Soc., **74**, 4377-4379.

Subikova, V., Slovakova, L, and Farkas, V., (1994). Inhibition of tobacco necrosis virus infection by xyloglucan fragments. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz, J. of Plant Diseases and Protection, **101**, 128-131.

Tani, M., Fukui, H., Shimomura, M. and Tabata, M., (1992). Structures of endogenous oligogalacturonides inducing shikonin biosynthesis in *Lithospermum* cell cultures. Phytochemistry., **31**, 2719-2723.

Thain, J.F., Doherty, H.M., Bowles, D.J. and Wildon, D.C., (1990). Oligosaccharides that induce proteinase inhibitor activity in tomato plants cause depolarisation of tomato leaf cells. Plant, Cell, Environ., **13**, 569-574.

Thompson, J.E., Smith, R.C. and Fry, S.C., (1997). Xyloglucan undergoes interpolymeric transglycosylation during binding to the plant cell wall

in vivo: evidence from $^{13}C/^{3}H$ dual labelling and isopycnic centrifugation in caecium trifluoroacetate. Biochem. J., **327**, 699-708.

Tran Thanh Van, M., Toubart, P., Cousson, A., Darvill, A.G., Gollin, D, J., Chelf, P. and Albersheim, P., (1985). Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins. Nature, **314**, 615-617.

Varner, J.E. and Lin, L-S., (1989). Plant cell architecture. Cell, **56**, 231-239.

Wakabayashi, K., Hoson, T. and Kamisaka, S., (1997). Changes in amounts and molecular mass distribution of cell-wall polysaccharides of wheat (Triticum aestivum, L.) coleoptiles under water stress. J. Plant Physiol., **151**, 33-40.

Warneck, H. and Seitz, H.U., (1993). Inhibition of gibberellic acid-induced elongation-growth of pea epicotyls by xyloglucan oligosaccharides. J. Exp. Bot., **44**, 1105-1109.

Warneck, H.M., Haug, T. and Seitz, H.U., (1996). Activation of cell wall-associated peroxidase isoenzymes in pea epicotyls by a xyloglucan-derived nonasaccharide. J. Exp. Bot. **47**, 1897-1904.

Warneck, H.M., Fulton, D.C., Seitz, H.U. and Fry, S.C., (1998). Transport, degradation and cell wall-integration of XXFGol, a growth-regulating nonasaccharide of xyloglucan, in pea stems. Planta, **204**, 78-85.

Walker-Simmons, M., Jin, D., West, C.A., Hadwiger, L. and Ryan, C.A., (1984). Comparison of proteinase inhibitor-inducing activities and phytoalexin elicitor activities of a pure fungal endopolygalacturonase, pectic fragments and chitin. Plant Physiol, **76**, 833-836.

Walker-Simmons, M. and Ryan, C., (1986). Proteinase inhibitor I accumulation in tomato suspension cultures. Plant Physiol, **80**, 68-71.

Wright, K. and Northcote, D.H., (1975). An acidic oligosaccharide from maize slime. Phytochemistry., **14**, 1793-1798.

Yamazaki, N., Fry, S.C., Darvill, A.G. and Albersheim, P., (1983). Host-pathogen interactions. XXIV. Fragments isolated from suspension-cultured sycamore cell walls inhibit the ability of the cells to incorporate [^{14}C]leucine into proteins. Plant Physiol., **72**, 864-869.

York, W.S., Darvill, A.G. and Albersheim, P., (1984). Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. Plant Physiol., **75**, 295-297.

Yoshikawa, M., Keen, N.T. and Wang, M-C., (1983). A receptor on soybean membranes for a fungal elicitor of phytoalexin accumulation. Plant Physiol, **73**, 497-506.

Zablackis, E., Huang, J., Muller, B., Darvill, A.G. and Albersheim, P., (1995). Characterisation of the cell-wall polysaccharides of *Arabidopsis thaliana* leaves. Plant Physiol., **107**, 1129-1138.

Zablackis, E., York, W.S., Pauly, M., Hantus, S., Reiter, W-D., Chapple, C.C.S., Albersheim, P. and Darvill, A.G., (1996). Substitution of L-

fucose by L-galactose in cell walls of *Arabidopsis mur*1. Science, **272**, 1808-1810.

Zabotina, O.A., Gur'yanov, O.P., Malikov, R.G., Ayupova, D.A., Beldman, G., Voragen, A.J.G. and Lozovaya, V.V., (1995). The isolation and biological activity of oligosaccharins from the pea shoots. Rus. J. Plant Physiol., **42**, 366-371.

Zabotina, O.A., Gur'yanov, O.P., Ayupova, D.A., Beldman, G., Voragen, A.J.G. and Lozovaya, V.V., (1996). Biologically-active soluble oligosaccharides from the pea stem tissues. Plant Cell Rep., **15**, 954-957.