XYLOGLUCAN BIOSYNTHESIS IN *PHASEOLUS VULGARIS*

by

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Preface

I hereby declare that the work represented in this dissertation is my own and is original except where specific reference is made.

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Abbreviations

ADP	Adenosine diphosphate
ara	Arabinose
ATP	Adenosine triphosphate
bd	Blue dextran
Bq	Becquerel
BSA	Bovine serum albumin
cb	Cellobiose
Ch	Chapter
2, 4-D	2, 4-Dichlorophenoxyacetic acid
dm	Decimeter
dnpl	Dinitrophenyllycine
dp/DP	Degree of polymerisation
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
Fru-6-p	Fructose-6-phosphate
Fuc	Fucose
g	Gravity
gal	Galactose
gal a	Galacturonic acid
GDP	Guanosine diphosphate
glc	Glucose
glc 6 p	Glucose-6-phosphate

h	Hour
HEPES	N-2-hydroxyethylpiperazine-N -2-ethane
inc	Incorporated
iso	Isoprimeverose
man	Mannose
MES	2(N-morpholino)ethanesulphonic acid
mg	Milligram
min	Minute
NAA	Naphthyl acetic acid
OD	Optical density
PAL	Phenylalanine ammonia-lyase
PPO	2,5-Diphenyloxazole
raff	Raffinose
rham	Rhamnose
S	Second
suc	Sucrose
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TLE	Thin layer electrophoresis
UDP	Uridine diphosphate
xyl	Xylose

ABSTRACT

A particulate enzyme preparation from suspension-cultured bean (Phaseolus vulgaris) was shown to incorporate xylose from UDP-D-[$U^{14}C$] xylose, fucose from GDP-D-[$U^{14}C$]fucose, and glucose from UDP-D-glucose, into polysaccharide. The xylosyltransferase was dependent upon the presence of UDP-glucose, and was stimulated and apparently protected by GDP-glucose and GDP-mannose, though neither was able to replace UDP-glucose as a glycosyl donor. The product of the reaction was identified as xyloglucan by analysis of the products of enzyme breakdown and acid hydrolysis, and by a cellulose binding study. Molecular weight determination following proteinase K digestion indicated that the nascent xyloglucan is closely attached to protein. Pre-incubation of the enzyme with UDP-glucose stimulated incorporated from UDP-D-[$U^{14}C$] xylose suggesting the simultaneous presence of both nucleotides is not necessary for growth of the polysaccharide. The fucosyltransferase was not dependent upon the presence of other sugar nucleotides, though some stimulation by UDP-galactose occurred. Fucose was transferred from GDP-D-[$U^{14}C$] fucose into a polysaccharide with the characteristics of xyloglucan, as indicated by enzyme and acid hydrolysis. Transfer of glucose from UDP-D-[$U^{14}C$] glucose showed no stimulation by UDP-xylose and was slightly inhibited at higher concentrations of UDP-xylose. The product of the transfer was susceptible to β 1-3 glucanase digestion and did not show enzyme hydrolysis products characteristic of xyloglucan.

The activities of the transferase enzymes were examined in

differentiating bean calluses. Phloem differentiation may be indicated by a peak in β 1-3 glucanase activity 16 days after transfer of a callus onto an "induction" medium.

Activities of xylosyl- and fucosyltransferases showed little variation between non-differentiating and "induced" calluses.

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Chapter 1

INTRODUCTION

General Introduction

The development of a sophisticated plant cell wall has been of profound significance to the evolution and life of most organisms on our planet. In the eukaryotic cell it has allowed the development of complex intracellular organisation with an intricate system of membranous compartments, permitting a division of labour which has played a major role in the refining of photosynthesis, respiration and absorption processes (Bartnicki-Garcia, 1984). The versatile cell wall confers support upon non-woody tissue by allowing the development of high turgor pressure in the cells, and upon woody tissue by the thickening and lignification of the schlerenchyma and tracheary elements. The wall has a protective function, preventing injury to the protoplast by abrasion, desiccation, or microbial attack. It can also function as a food store, and may be involved in recognition phenomena.

Despite the proposal of various models for both the structure and biosynthesis of cell walls, our knowledge of these areas is surprisingly limited, especially in the light of the obvious importance of this structure.

Structure of the Cell Wall

The presence of a cell wall enclosing the protoplast is a distinctive characteristic of plant cells. Although the wall itself

is non-protoplasmic, it is by no means inert, and undergoes many structural changes in the development of the cell. It is situated outside the plasmalemma, and consists of a framework made up of cellulose microfibrils embedded in a complex matrix of polysaccharide, protein and glycoprotein. The reported proportions of wall components vary depending on plant species, tissue age, extraction and analytical methods employed, etc. Roelofson (1959) estimated that a typical primary wall contained one third cellulose, one third hemicellulose and one third pectin and protein. More recently suspension-cultured Douglas Fir cells have been estimated to contain 15% pectic material, 10% xyloglucan, 1% xylan, 23% cellulose, 34% protein and 5% ash (Thomas et al 1987).

Particular attention has been paid to the role of the cell wall in growth and development, and the changes that occur after cell division well illustrate the dynamic nature of the wall. Just after nuclear division a mass of cytoplasmic vesicles fuse to form the cell plate, a thin disk which becomes enlarged by addition of vesicles until the side walls of the dividing cells are reached (Albersheim, 1965). As the cell plate rapidly becomes more substantial, it develops into "primary" cell wall, defined by Wardrop (1962) as that structure which encloses the protoplasts during the period of cell enlargement. Around 20% of the primary cell wall volume is made up of microfibrils - glucan chains packed in aggregates with a definite structure. These have a parallel array, initially perpendicular to the main axis of the plant, but changing to a longitudinal orientation with wall expansion (Albersheim, 1965).

With cessation of growth, the cell wall often becomes thickened by layering of material onto the primary wall, and by this process the "secondary" wall is built up. Secondary walls are much thicker than primary with greatly increased amounts of cellulose and lignin - a complex, cross-linked molecule composed of phenylpropanoid units. Lignification spreads from the middle lamella towards the cell membrane. The microfibrils in secondary walls form layers of parallel bundles, each layer varying by a precise angle from the layers next to it (Albersheim, 1965).

Differentiation into more specialised cell types can involve the development of characteristic wall structures: in tracheids spiral thickenings occur i.e. bundles of cellulose microfibrils which may occur on one or both sides of the middle lamella with the microfibrils parallel to the axis of the spirals.

Thus while the primary wall is sufficiently extensible to allow cell extension and rapid growth, the thickened secondary wall gives the cell its final shape and forms the basis of the plant's mechanical support.

The Components of the Cell Wall

1) The Polysaccharides

The original classification of polysaccharides divided them into three groups: pectic polysaccharides, hemicelluloses and cellulose, where all polysaccharides extracted by hot water, ammonium oxalate, weak acid or chelating agents were pectic polysaccharides, polysaccharides

extracted by relatively strong alkali were hemicelluloses and the residue was mostly cellulose. Although this classification is reasonably accurate, incomplete and overlapping extraction leads to confusion, and currently pectic polysaccharides are considered to be those found in covalent association with galacturonosyl-containing polysaccharides, while hemicelluloses are those polysaccharides found in non-covalent association with cellulose.

The pectins contain galacturonic acid, L-arabinose, D-galactose and L-rhamnose, and exhibit gel-like behaviour. They include rhamnogalacturonans, arabinans and arabinogalactans. Hemicelluloses contain D-xylose, D-glucose, D-mannose and D-glucuronic acid and include xylans, glucomannans, certain glucans and xyloglucans. Cellulose is $\geq$ 90% glucose, with other sugar units often present.

i. Rhamnogalacturonan

This acid polysaccharide consists of an α (1-4) linked galacturonan chain in which 2-linked L-rhamnosyl residues are interspersed conferring a characteristic zig-zag shape on the molecule. In sycamore suspension-culture cells substantial numbers of arabinosyl and galactosyl residues are present in side chains. The rhamnose-containing portions of the molecule are interspersed with a homogalacturonan sequence with 4-linked galacturonic acid (McNeil et al, 1979). Homogalacturonan regions may make up about 5% of the total cell wall in sycamore cells (Talmadge et al, 1973).

Recently two types of rhamnogalacturonan have been defined:

rhamnogalacturonan I has a rhamnose and galacturonic acid backbone and a DP of about 2,000. In sycamore suspension-cultured cells it contains galacturonic acid, arabinose and galactose in the ratio 1:2:1.5:1.5. Rhamnogalacturonan II, also isolated from sycamore suspension-cultured cells, contains rhamnose, galacturonic acid, arabinose and galactose, along with other sugars (Darvill et al, 1978). The molecule is highly branched with no backbone, and the precise structure has yet to be elucidated.

ii. Arabinan.

Primary cell walls contain polymers that are pure arabinans, the dominant constituents being 5-linked α-L-arabinofuranosyl residues. Some of the 5-linked α-L-arabinofuranosyl residues have α-L-arabinofuranosyl residues attached to them at O-2 or O-3, and sometimes at both O-2 and O-3. The point of attachment of the arabinans to other cell wall polysaccharides in situ has not yet been ascertained (McNeil et al, 1984).

iii. Arabinogalactans

These are polysaccharides containing β(1-4) linked D-galactan chains with L-arabinofuranose residues. Arabinogalactan is of two types:- an example of type I is from soybean, and is found in association with a rhamnogalacturonan which contains β-D-galactan units in sidechains. Type II arabinogalactans are highly branched polysaccharides with ramified chains of β-D-galactopyranose residues joined by 1-3 and 1-6 linkages. They are most abundant in gymnosperms, especially larches (Aspinall, 1980).

iv. Xylans

Xylans are all (1 - 4)-β-D-glycans with few branch points in the main chains. They have a DP of 150-200 and have arabinose, glucuronic acid and acetyl groups. In angiosperms glucuronoxylans are the main xylan group, while in monocot primary walls arabinoxylans predominate, and have a two or three-fold screw axis. The secondary wall xylans differ amongst themselves in the nature of side chains glycosidically linked to the xylan backbone. Most frequently a terminal 4-O-methylglucuronosyl residue is linked to one in every 7-10 xylosyl residues (Northcote, 1972).

Two glucoxylans have been isolated from sugar maple sapwood in which 4-linked glucosyl residues are interspersed with 4-linked xylosyl residues in the xylan backbone. A linear unbranched xylan has been isolated from tobacco stalks (Eda et al, 1976), and a primary wall glucuronoxylan has been established as a constituent of suspension-cultured sycamore cells (forming 5% of the wall), which contains terminal, 4-, 2, 4-, and 3, 4-linked xylosyl residues (Darvill et al, 1980). It also contains terminal and 2-linked arabinofuranosyl residues, terminal 4-O-methyl glucuronosyl and terminal glucuronosyl residues.

v. Glucomannans

Glucomannans have a linear chain of β(1-4) linked glucose and mannose which are randomly arranged, and form about 3-5% of the total cell wall material in angiosperms (Timell, 1964). The glucose and mannose are present in the ratio 1:2, with a DP of about 70, and the

chains have a 2-fold screw axis similar to cellulose.

vi. Galactoglucomannans

These are major cell wall components (2-15%) of gymnosperms with D-glucose and D-mannose in the approximate ratio 1:3. In deciduous woods glucomannans devoid of galactose residues with glucose and mannose in the ratio 1:2 occur, making up less than 3% of wall components (Aspinall, 1980).

vii. Non-cellulosic glucans

Chains of β(1 - 3) linked D-glucose or "callose" with 1.9% uronic acid are found in phloem seive tubes and pollen tubes, and the rapid deposition of callose onto the cell wall contributes to cell and tissue sealing as well as being a defence against pathogens (Kauss, 1986).

Mixed β-glucans containing a mixture of 4-linked and 3-linked glucosyl residues have been isolated from rye, oat and barley endosperm (Darvill et al, 1980). A glucan has also been isolated from three day old mung bean hypocotyls containing 3-linked and 4-linked glucopyranosyl residues in the molar ratio 1:1.7, which may have existed in the primary or secondary wall (Buchala & Franz, 1974).

viii. Cellulose

Cellulose is probably the most studied cell wall polymer, and the most abundant in nature. Usually more than 90% is made up of glucose, with other sugars present also. The glucan portion is composed of long, linear chains of β(1 - 4) linked glucopyranose units

with a DP thought to be up to at least 15,000 units in higher plants (Preston, 1974). There are no apparent termination points, and it is not known whether the chains have a parallel orientation as in algal cells (Gardner & Blackwell, 1974), or antiparallel as in chitin (Meyer & Misch, 1937). The relatively rigid linear molecules align closely with their neighbours, and are bound together by extensive hydrogen bonding in orientated aggregates with a definite structure, called microfibrils. Microfibrils are composed of 60-70 chains which are visible under the electron microscope. In secondary walls rope-like aggregates 15-25nm in width are formed. They are probably separated from their nearest neighbours by 50-100nm, at least 2-4 times their own diameter, and the space between them is filled by hydrated encrusting materials of various kinds, including hemicelluloses and lignin (Preston, 1974).

ix. Xyloglucan

Xyloglucan is the best characterised noncellulosic polysaccharide of primary cell walls. It was first described as an "amyloid" component of seeds, as it forms the same coloured complex with iodine as starch (amylose) (Vogel & Schleiden, 1838). It was shown to be composed of glucose, xylose and galactose in the ratio 3:2:1 (Krishna & Ghose, 1942, 1943), and methylation studies by White and Rao (1953) suggested a main chain of 1,4-linked glucose residues with side chains of galactose, and 1,2-linked xylose residues. Kooiman (1960) used a crude commercial enzyme mix called 'Luizym' to hydrolyse Tamarindus indica xyloglucan, and recovered almost all the xylosyl residues as xyl(α1-6) glucose . By methylation analysis he established that the

xylosyl residues were attached to C-6 of the glucose.

The molecular weights recorded for xyloglucans vary considerably depending on the plant source and the method of isolation: sycamore cell culture xyloglucan has been estimated at 7,600, about 50 glucosyl residues (Bauer et al, 1973), while Simmondsia chinensis and Annona muricata xyloglucans have molecular weights of 174,000 and 10,000 respectively (Watanabe et al, 1980; Kooiman, 1967). The major differences between xyloglucans have been highlighted by methylation analysis and result from the presence or absence of fucosyl residues which are attached to C-2 of the majority of otherwise terminal galactosyl residues. Methylation analysis of fragments following partial acid hydrolysis or acetolysis of xyloglucans from various plants has also shown that galactosyl residues are linked through a β-galactosidic bond to C-2 of the xylosyl residues (Darvill et al, 1980).

In recent years, the xyloglucans from several plant sources have been analysed:- Kato and Matsuda (1976) studied xyloglucans from the hemicellulosic fractions of Phaseolus aureus hypocotyl wall preparations. The polysaccharides were homogenous on electrophoresis and ultra-centrifugation, and on acid hydrolysis gave glucose, xylose, galactose and fucose in the ratio 10:7:2.5:1. Results from partial acid hydrolysis suggested a structure in which xylose, galactose and fucose were attached to a sequence of β, 1-4-linked glucose. The xyloglucan accounted for 13.9% of the total non-cellulosic fractions. In a later paper (Kato et al, 1977) xyloglucans from Glycine max, and Vigna sesquipedalis hypocotyls were compared with those from Phaseolus aureus.

Analysis by cellulase fragmentation gave a basic structure based on two repeating, oligosaccharide units, one consisting of glucose and xylose, the other of glucose, xylose, galactose and fucose.

O'Neill and Selvendran (1983) have shown that xyloglucan isolated from Phaseolus coccineus has a 1 - 4-β-D-glucan backbone with L-fucosyl side groups attached to the galactosyl residues. The proportions of sugars in this polymer are 46.4% D-glucose, 9.3% D-galactose, 34.1% D-xylose, 6% L-fucose and 4.2% arabinose. In a later paper, O'Neill and Selvendran (1986) suggested that this xyloglucan does not possess a simple repeating unit, but more probably contains a block-type structure.

While xyloglucans are mainly constituents of primary cell walls of dicots, xyloglucan-type polymers have recently been found as minor constituents of monocot cell walls (Shibuya & Misaki, 1978). They are also found in the role of cotyledonary reserve polysaccharides, present in many nonendospermic leguminous species as massive wall thickenings (Reid, 1985). Most leguminous species containing reserve xyloglucans belong to the sub-family Caesalpinioideae, but a few species in the Faboideae also contain xyloglucan. They are also present in the seeds of many non-leguminous species. In nasturtium seeds the xyloglucan is broken down following germination, by a hydrolytic process involving three enzymes, one of which has a very high degree of specificity (Edwards et al, 1986). Reserve xyloglucans do not generally contain fucose.

2) Other Wall Components

Living primary walls of plants are hydrated and are likely to contain between 60 and 70% of their weight as water. The dry matter consists of the polysaccharides described above, plus smaller amounts of phenols, protein, lipid and other compounds such as tannins. Mineral salts are deposited to varying extents in walls.

i. Lignin

Lignin is an insoluble, aromatic high molecular weight compound, made up of many cross-linked phenylpropanoid units. It is derived by the enzymatic dehydrogenation and subsequent polymerisation of coumaryl, sinapyl and coniferyl alcohols (Neish, 1965). The proportions vary in different plants e.g. coniferyl units are found in both soft and hard woods, while sinapyl units are confined to hard woods. Lignin can form up to 50% of the dry weight in secondary walls (Stace, 1970).

ii. Glycoprotein

The primary walls of some dicotyledenous plants contain a unique hydroxyproline-rich glycoprotein. Around 2-10% of the walls are glycoprotein in which up to 20% of the amino acid is hydroxyproline (Lamport, 1970). The polysaccharide portion of the molecule contains arabinose and galactose (Northcote, 1969). In tomato and carrot cells galactosyl residues are attached to the peptide through o-glycosidic linkages to the amino acid serine (Lamport et al, 1973).

The level of hydroxyproline-rich glycoprotein (HRGP) in plants is increased under stress conditions, particularly pathogenic attack, e.g.

a level of 1% in healthy plants can increase to 10-15% in infected plants (Mazau et al, 1986). Two families of HRGPs have been identified in melon callus and seedlings: HRGPi is an arabinogalactan protein composed of 94% sugars and 6% protein; the sugar moiety contains 66% galactose and 34% arabinose, in the protein moiety hydroxyproline, serine, alanine and glycine are the most abundant amino acid residues, accounting for 45, 12, 10 and 7% respectively; in HRGPii sugars make up 64% and protein 36%; arabinose, galactose and glucose are present at 77, 22 and 1%, and the protein moiety is made up of 39% hydroxyproline, 15% lycine and 7% tyrosine (Mazau et al, 1986).

iii. Protective coverings of the wall

The outer walls of epidermal cells and aerial organs are covered with a protective film of wax and cutin. Wax overlays the cutin and the lipid of cutin penetrates and mingles with the polysaccharides of the wall. Waxes are a complex mixture of long chain alkanes, alcohols, ketones and fatty acids. Alcohols and fatty acids occur as long chain esters or are found uncombined (Martin & Juniper, 1970).

Comparison of Monocot and Dicot Walls

Although compositionally dissimilar, the primary walls of dicots and monocots are probably arranged on a similar architectural plan i.e. cellulose fibres interconnected by hemicelluloses, glycoproteins and pectic polysaccharides. The hemicellulose xyloglucan may account for up to 35% of the polysaccharide in dicot primary walls, but makes

up less than 2% of monocot walls where the dominant hemicellulose is arabinoxylan (Darvill et al, 1980). Dicot primary walls may contain more than 30% pectic polysaccharides (Worth, 1967) but many monocot primary walls contain less than 10% e.g. oat coleoptiles 3% (Ray & Rottenberg, 1964), maize 6% (Darvill, 1976). Levels of hydroxyproline in wall glycoprotein also differ: monocot suspension-cultured cell walls contain 0.13-0.16% whereas dicot suspension-cultured cells contain around 2% (Burke et al, 1974).

Differences are also found between angiosperms and gymnosperms, e.g. xylans form the bulk of hemicellulose in angiosperms while galacto-glucomannans are the most abundant hemicellulose in gymnosperms. It is clear that further differences between plants will emerge as more plant cell walls are analysed.

Changes in Components During Growth

Different tissues can vary markedly in the composition as well as the architecture of their cells. Consistent trends are however evident throughout growth and differentiation:- the main polysaccharide in the cell plate is probably rhamnogalacturonan, though even at this stage some cellulose is incorporated into the walls, which usually show a weak but positive birefringence when viewed under polarizing light (Roelofson, 1959). Rhamnogalacturonans continue to be deposited during primary wall formation, but with increasing deposition of primary wall the ratio of neutral to acidic pectic substances increases, and the less acidic arabinogalactans appear.

At the next developmental stage, deposition of hemicellulose commences, and cellulose microfibrils are laid down in increasing amounts relative to the pectic substances. The kinked, irregular shape of rhamnogalacturonan molecules, and the branched arrangement of the hydrophilic arabinogalactan molecules prevents extensive intermolecular bonding, and allows a large amount of water to enter the interstices of the polymers (Rees, 1972).

As the primary wall approaches maturity and the rate of cell enlargement decreases, the water content and matrix plasticity decrease and extension is arrested. Increasing amounts of hydroxyproline rich protein are incorporated into the wall, which by analogy with collagen, may be a stiffening element (Ramachandran, 1967). With ageing the ratio of glucomannan to xyloglucan in the hemicellulose fraction increases, but the overall proportion of hemicellulose deposited decreases as the proportion of cellulose increases (Northcote, 1963). The cellulose microfibrils have a longitudinal orientation in secondary walls, rather than the transverse orientation they have in primary walls. In some secondary walls, rigidification is completed by the replacement of the water in the matrix by lignin. Subsequent modifications lead to the differentiation of particular cell types: xylem vessel elements for example are elongated cells with secondary side walls in which secondary thickening often only partially covers the primary wall.

A comparison of young and mature tissue of sunflower plants illustrates the change in composition of the components (Fig. 1):-

	Sunflower hypocotyl %	Stem %
Cellulose	38	42
Pectin	46	14
Hemicellulose	8	24
Lignin	8	20

Fig. 1. Comparison of wall components in young (hypocotyl) and mature (stem) sunflower plants (Wareing & Galston, 1962).

The main changes in wall composition during monocot differentiation have been outlined by Carpita (1986) who used maize coleoptiles as a model:- a highly substituted glucorono-arabinoxylan appears during cell elongation, with terminal α-L-arabinofuranosyl and α-D-glucosyluronic acid units, this is followed by cessation of the synthesis of linked and branched arabinosyl-containing units, a large net synthesis of β-glucan, then the gradual loss of arabinosyl and glucuronosyl linkages from most of the glucuronoarabinoxylan of the primary wall.

Bonds and Interconnections of the Cell Wall

The overall strength of the cell wall may be increased through covalent bonds, or non-covalent linkages such as hydrogen, ionic and lectin bonds. These cross-links holding the cell wall matrix together alter many of the properties of the wall, including extensibility, rigidity, cohesion and digestibility (Fry, 1986), however little is known of the actual bonds involved.

i. Covalent bonds

There is evidence for covalent linkages in primary walls between

xyloglucan chains and pectic polyuronides (Bauer et al, 1973, Talmadge et al, 1973), and between xyloglucan and pectic arabinogalactan, since arabinogalactan polymers are released by endopolygalacturonase (Talmadge et al, 1973). Xyloglucan is linked to rhamnogalacturonan through the galactan portion of the arabinogalactan (Albersheim, 1976), and covalent bonds attach neutral pectic polysaccharides arabinan and galactan to acidic ones (Talmadge et al, 1973). Covalent bonds may form between carbohydrates and lignin, ensuring that the components of the secondary wall do not slip with respect to each other (Northcote, 1972).

ii. Hydrogen bonds

Pectic polysaccharides probably interact through non-covalent as well as covalent bonding. They may be connected in some manner to hydroxyproline-rich glycoprotein - in sycamore extracellular polysaccharide rhamnogalacturonan is apparently connected to the hydroxyproline-rich wall protein through a highly branched arabinogalactan (Lamport et al, 1973). It has been proposed that cellulose fibres are held together by hydrogen bonds between glucan chains (Gardner & Blackwell, 1974) and other polysaccharides, such as glucoronoxylans and xyloglucans may hydrogen bond to each other forming aggregates. Such structures lead to gel-formation and may be involved in cross-linking of the primary wall polymers.

A major interconnection of the cell wall is the bonding of xyloglucan to cellulose fibre surfaces through multiple hydrogen bonds (Bauer et al, 1973; Keegstra et al, 1973). When xyloglucan is bound to cellulose, every second glycosidic oxygen in the glucan chain

may hydrogen-bond with the hydrogen of a primary hydroxyl group at position 6 of a glycosyl residue of a given cellulose chain. Of the glycosyl residues in the xyloglucan polymer about one in four have a primary hydroxyl group at position 6, so bonding between xyloglucan and cellulose would be weaker than between two cellulose chains (Frey-Wyssling, 1969). Further lateral associations would be prevented by the fucosyl-galactosyl-xylose side chains resulting in a monolayer of xyloglucan on the cellulose fibre surface (Albersheim, 1976). It is proposed that this would prevent cellulose microfibrils from adhering to one another to form the large aggregates characteristic of secondary walls (Darvill et al, 1980). The xyloglucan chains may also be involved in forming connections between the cellulose fibres and other polymers of the primary cell walls.

iii. Ionic

Calcium has long been known to confer rigidity to cell walls. In the galacturonan 'egg-box' structure of Rees et al (Rees, 1972; Rees & Richardson, 1973) calcium ions between chains of polygalacturonyl residues chelate to the oxygen atoms of four galacturonyl residues distributed between two galacturonan chains. This results in increased rigidity of the galacturonans and cross-linking of the galacturonan chains.

iv. Lectin

Several of the hydroxyproline-rich glycoproteins extracted from plant tissues have carbohydrate binding activity, giving them the characteristics of lectins (Darvill et al, 1980). Two lectin-like

protein fractions extracted from mung bean seedling cell walls bind specifically to galactose residues, though it is not known whether they contain hydroxyproline. This has led to the suggestion that lectins may be involved in establishing a non-covalent protein-glycan network (Kauss & Bowles, 1976).

v. Ester links

It has recently been suggested that pectin molecules may be cross-linked by oxidative coupling of their phenolic substituents through feruloylated sugar residues (Fry, 1986). In grasses, hemicelluloses have been found, in particular a feruloyl-arabino-furanosyl-xylan, which yield feruloyl-arabinose on mild acid hydrolysis. Results also suggest the presence of isodityrosine bridges (an isomer of dityrosine found in the cuticular protein of insects) linking glycoproteins in the wall, though it has still to be shown that the bridges are between, and not within molecules.

Current evidence

Chambat et al (1984) have proposed a tentative model showing possible interactions between polysaccharide consituents of suspension-cultured Rosa glauca cell walls. Two different extraction processes yielded very similar cellulose complex residues and extracts: the cellulose complexes comprised uronic acid containing polysaccharides, and hemicellulose in association with cellulose; graded acid hydrolysis provided evidence for the central role of a homogalacturonan core

interconnecting xyloglucans and arabinogalactans.

A xyloglucan-cellulose complex from pea was examined by Hayashi and Maclachlan (1984) using fluorescence microscopy with a fucose-binding fluorescein lectin as a probe. They found that the macromolecules were present in files of cell shapes with xyloglucan on and between cellulose microfibrils. Since the average chain length of xyloglucan is many times that of the cellulose microfibril diameter, Hayashi and Maclachlan suggest that it could introduce cross-links by binding and contribute rigidity to the wall.

The assembly of β-glucans has been studied in pea protoplasts isolated from the growing regions of etiolated peas (Hayashi et al, 1986). Xyloglucan and cellulose were minor wall components compared with intact cell walls, and bonding between these molecules was weaker. It was found however, that addition of exogenous pea xyloglucan into the culture medium stimulated deposition of new polysaccharide into the protoplast wall, and enhanced the close association of newly formed xyloglucan with cellulose. The presence of xyloglucan outside the protoplasts may lead to a coordination of xyloglucan secretion and cellulose synthesis, so that the two remain in close association as they do in vivo.

Biosynthesis of wall components

i. The carbohydrates

Synthesis of cell wall carbohydrates involves the transfer of

sugars directly or indirectly to the growing polysaccharide chains, a transfer which is accomplished via sugar nucleotides - activated high energy forms of the sugar (Ericson & Elbein, 1980). Glucose, fructose and/or sucrose are translocated from photosynthetic or storage tissues to the sites of wall synthesis and are converted there to UDP- glucose, GDP- glucose etc. (See Fig.2). Sugar nucleotide levels in plants vary: UDP-glucose is the major sugar nucleotide component i.e. 60-70%; UDP-galactose makes up 15-25% of the pool and the other UDP derivitives 1-10%, though the level of nucleotide sugar present does not indicate its rate of participation in a metabolic process (Feingold & Avigad, 1980).

Once a pool of nucleoside diphosphate sugars has been established, at least two stages are involved in polysaccharide biosynthesis: first glycosyl residues are assembled from nucleotide sugars via a sugar transfer to an acceptor substance, a process involving chain initiation, copolymerisation to form heteropolymers, specification of anomeric configuration and linkage position, introduction of branch points, then chain termination (Northcote, 1969). This results in formation of disaccharides, oligosaccharides or polymeric chains. After this stage the glycosyl residues may undergo esterification or etherification. By a different process pre-existing material may be modified to give a branched structure, or transfer of material of a larger molecular weight onto other polysaccharide chains may give new or changed polysaccharide.

Biosynthesis of polysaccharides has generally been investigated

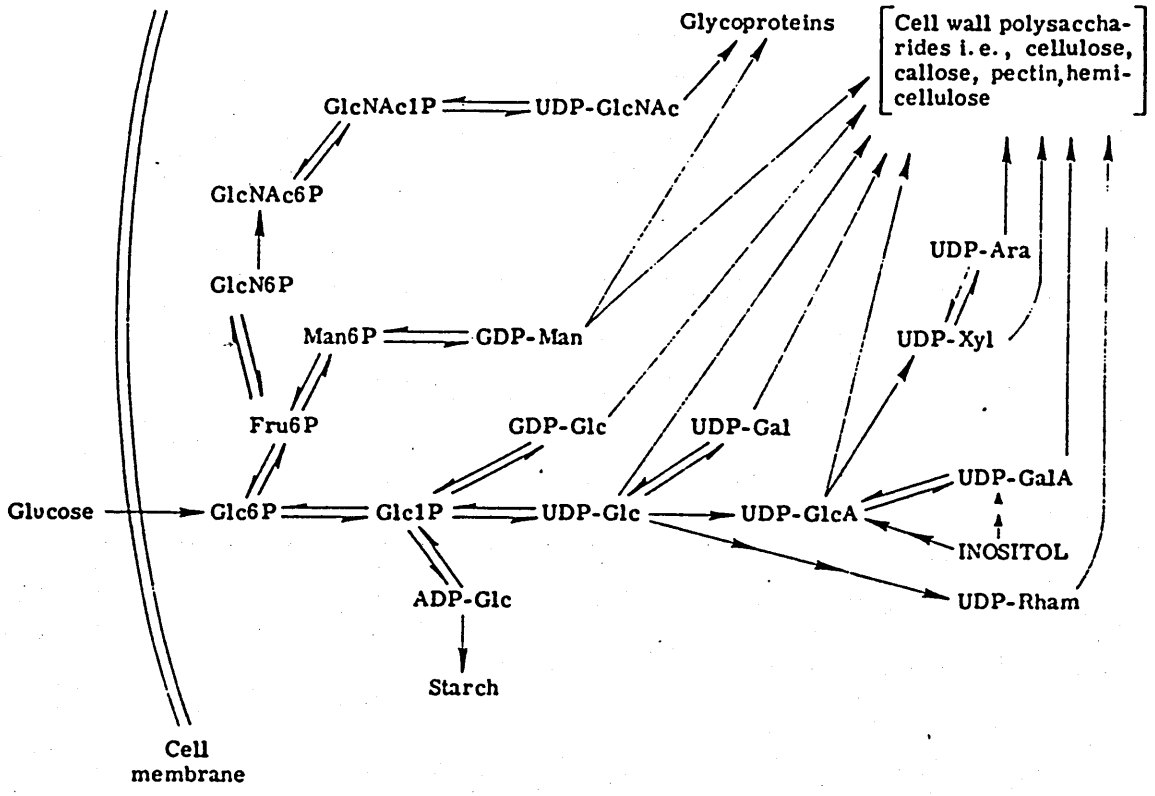


Figure 2

Reactions involved in the synthesis of the various sugar nucleotides that act as precursors of cell wall polysaccharides (From Ericson & Elbein, 1980).

by incubating radioactively-labelled sugar nucelotides with membrane fractions isolated from plant tissues. The products of the incubation are then isolated and characterised. There are two main problems with this sort of study (Kauss, 1974): difficulties arise with product characterisation because most radioactive products are the result of addition of a few sugar molecules to the non-reducing end of an existing polysaccharide chain, rather than de novo polysaccharide synthesis; secondly interconversions of sugar nucleotides may be catalyzed by enzymes in the particulate preparation. A further difficulty is the frequent synthesis of related products, making product identification difficult. These problems can be partially overcome by using short incubations and by adjusting conditions to favour the desired glycosyltransferases, and the biosynthesis of several polysaccharides has been investigated with some degree of success.

Among the first cell-free studies of polysaccharide synthesis were those on β(1-3) glucan: the incorporation of ^{14}C-glucose from UDP-^{14}C-glucose into β(1-3) linked glucan was shown to be catalyzed by a particulate enzyme preparation from mung bean shoots (Feingold et al, 1958). The polysaccharide was soluble in hot dilute alkali, and on partial acid hydrolysis gave rise to a series of oligosaccharides similar to the laminarin series. Some investigators have reported that both β1-3 and β1-4 glucosidic linkages can be produced from UDP-glucose i.e. a low UDP-glucose concentration results in 1-4 linkages, while high UDP-glucose results in 1-3 linkages (Péaud-Lenoël & Axelos, 1970; Clark & Villemez, 1972). It has been shown that at least two enzymes are involved.

The role of β(1-3) glucan formation as a wound response was investigated by Brett (1978). Soybean suspension-culture cells were incubated with UDP-^{14}C-glucose on a linear shaker. Under these conditions low incorporation resulted, but when the cells were re-suspended by stirring with a narrow glass rod, incorporation increased 30-times, apparently due to a wound response. The alkali-insoluble material formed was β(1-3) glucan, and no cellulose synthesis occurred. Similar results have been obtained with cotton fibres (Heiniger & Delmer, 1977).

In vitro cellulose biosynthesis has not been achieved so far, probably reflecting the inherent instability of the synthetase complex, damage to the complex during tissue homogenisation, or failure to provide appropriate substrates or cofactors (Darvill et al, 1980). There is evidence that the direct precursor of cellulose is UDP-glucose (Carpita & Delmer, 1980), but there is no evidence that synthesis of xyloglucan, which has a β1-4 backbone like cellulose, is in any way linked to that of cellulose (Aspinall, 1980).

The biosynthesis of a number of pectic and hemicellulosic polysaccharides has been investigated, for example:- a mung bean particulate enzyme preparation incubated with UDP-galacturonic acid catalyzed the formation of the α1-4 polygalacturonic acid backbone of acidic pectins (Villemez et al, 1965; 1966). The most active glycosyl donor for this reaction was UDP-galacturonic acid, with limited incorporation from TDP- and CDP-galacturonic acid observed in preparations from tomato. Incorporation of arabinose into a pectic fraction was demonstrated

using a preparation from mung bean shoots (Odzuck & Kauss, 1972), and the same preparation catalyzed the transfer of galactose from UDP-^{14}C-galactose into a water soluble galactan, molecular weight 4,600 (McNab et al, 1968).

Glucomannan biosynthesis was investigated by Villemez (1974):- when GDP-^{14}C-glucose was incubated with a mung bean particulate preparation, radioactivity appeared in a β1-4 linked glucan. Addition of GDP-mannose greatly stimulated incorporation, and further research showed that glucose and mannose were incorporated into a β1-4 linked glucomannan. A similar stimulation of incorporation was observed by Waldron & Brett (1983) investigating glucuronoxylan synthesis in pea epicotyls. A pea membrane preparation incorporated glucuronic acid from UDP-^{14}C-glucuronic acid, and this incorporation was stimulated by the presence of UDP-xylose. An enzyme from corncobs will use UDP-xylose to form a xylan-like polysaccharide (Ben-Arie etal, 1973), and a particulate preparation from Zea mays seedlings was capable of producing an alkali-soluble polysaccharide from either UDP-^{14}C-arabinose or UDP-^{14}C-xylose, which on acid hydrolysis yielded xylose and arabinose (Pridham & Hassid, 1966).

Several groups have studied the biosynthesis of xyloglucan, in particular the incorporation of glucose and xylose into the polymer. Using a preparation from pea seedlings, Ray (1980) studied the transfer of glucose and xylose to an unidentified polysaccharide. The transfers appeared to be due to separate enzymes with different requirements e.g. the xylosyl transferase required the presence of a

high concentration of free sugar. On enzymic hydrolysis the products were found to vary with the incubation mixture i.e. in the presence of UDP-glucose alone, hydrolysis with cellulase preparation from Streptomyces or from Trichoderma viride produced cellobiose. With UDP-xylose also present, hydrolysis yielded less cellobiose and more larger oligosaccharides, shown by borohydride reduction to have glucose at the reducing end of the polymer. Trace amounts of ^{14}C-arabinose were found in some samples, suggesting epimerase activity.

Hayashi and Matsuda (1981a) isolated a particulate enzyme preparation from suspension-cultured soybean which catalyzed the transfer of xylose and glucose into xyloglucan. Incorporation from one radiactive sugar nucleotide was dependent on the presence of the other (unlabelled) sugar nucleotide, and in the presence of excess UDP-glucose (optimum 2mmol.dm^{-3}), incorporation from UDP- ^{14}C-xylose was proportional to time. Hydrolysis with Trichoderma viride cellulase yielded nona -, hepta-, penta- and trisaccharides, though the authors suggest that the trisaccharide is from the enzyme hydrolysis of labelled xylan rather than xyloglucan.

Hayashi et al (1981) have suggested an assay for xylosyl transferase based on the product of hydrolysis with cellulase from Aspergillus oryzae. Suspension-cultured soybeans were grown in 2% D-U ^{14}C-glucose to produce labelled xyloglucan which was then hydrolysed with an A. oryzae preparation to produce a disaccharide, identified as ^{14}C-isoprimaverose, along with ^{14}C-monosaccharides. This hydrolysis product is suggested to be diagnostic evidence for xyloglucan xylosyltransferase activity.

It has been proposed (Hayashi & Matsuda, 1981b) that synthesis of xyloglucan proceeds by cooperative transfer of glucose and xylose by xyloglucan synthase, with the polysaccharide composed of a heptasaccharide unit (glc-xyl, 4:3) and a pentasaccharide unit (glc-xyl, 3:2). In a more recent paper (Hayashi et al, 1984), the authors proposed that a basic pentasaccharide unit beoomes a heptasaccharide unit during an elongation process of in vitro synthesis, and that the conversion is dependent upon UDP-xylose concentration. A pulse-chase experiment suggested that the relative amount of heptasaccharide increased with time, as would be expected if the penta- was the immediate precursor of the heptasaccharide unit. A preponderance of UDP-xylose in the reaction mixture resulted in heptasaccharide production, whereas a preponderance of UDP-glucose gave the pentasaccharide.

A recent paper by Camirand and Maclachlan (1986) reports the transfer of ^{14}C-fucose from GDP— ^{14}C-fucose into xyloglucan by pea microsomal membranes. In the presence or absence of UDP-glucose, UDP-xylose or UDP-galactose, ^{14}C-fucose was incorporated into a xyloglucan nonasaccharide: Glc4:Xyl 3:Gal:Fuc. Transfer from UDP-^{14}C-xylose resulted in the formation of an octasaccharide, rather than a nonasaccharide. Cellulose digestion followed by separation on Bio-gel P4 resulted in one peak corresponding to a nonasaccharide with about 85% of the radioactivity. The presence of other sugar nucleotides was not necessary, though incubations with UDP-xylose showed a 7-fold stimulation in incorporation by UDP-glucose. The products however were very similar.

ii. Other wall components: lignin.

The various stages of lignin biosynthesis have been partially elucidated. Early experiments with ^{14}C tracers established that shikimic acid and other aromatic acid precursors were readily converted into lignin (Neish, 1965), and that phenylpyruvic acid, phenolic cinnamic acids and phenylalanine were the most likely precursor compounds of lignin. Phenylalanine probably serves as the main source of cinnamic acid derivatives coniferyl alcohol and sinapyl alcohol, and they probably arise by the stepwise hydroxylation and methylation of cinnamic acid. Formation of lignin probably occurs by a relatively unspecific polymerisation catalysed by a peroxidase which promotes oxidation of the double bonds in the side chain, allowing complex chain-ring and interchain condensation to occur.

Phenylalanine ammonia lyase, the enzyme reponsible for the deamination of phenylalanine, is to be found in high concentration where lignin is being synthesized, but is almost completely absent in tissue where there is no lignin formation (Rubery & Northcote, 1968).

The Role of Primers in Biosynthesis

As attempts were made to achieve polysaccharide biosynthesis in cell-free systems, it became evident that a suitable 'primer' had to be present to initiate proper transfer of sugar to polymer. Although an endogenous acceptor might be present, without an exogenous primer the

amount of monosaccharide that could be incorporated was small and incorporation would only proceed for a short time. In some systems, such as that for glycogen biosynthesis, a partial hydrolysate of the polysaccharide being assembled stimulated synthesis, and a model was devised in which molecules could only arise from the transfer of sugar to an oligosaccharide produced by the enzymatic cleavage of a fragment from a completed molecule. This model is now obsolete.

The current theory is that polysaccharides develop upon protein or lipid cores to which they may remain attached or from which they may be severed after or during synthesis. For some polysaccharides such as keratan sulphate the linkage sequences seem to have the same structures as the oligosaccharides of conventional glycoproteins. Other polysaccharides such as heparin are attached to sequences that have no known equivalent in 'typical' glycoprotein assembly (Stoddart, 1984).

The evidence for primers in β-glucan synthesis has been assessed by Maclachlan (1981). Several factors point to the existence of a primer in plant β-glucan synthesis: firstly, the rates of biosynthesis of isolated plant membrane preparations are much lower than in real plant systems, and are short lived. Secondly, the high molecular weight of insoluble β-glucans (DP up to 10,000) from incubations with tissue slices compared to products from particulate enzyme preparations suggests that intact membranes form larger products (Spencer & Maclachlan, 1972). While certain compounds have been reported to enhance incorporation, no compound has been identified as a primer.

Control of Biosynthesis

During cell development the biosynthesis of cell wall polysaccharides is regulated so that the products differ with successive growth stages. Thus as primary wall formation ceases and secondary growth begins, pectin deposition is discontinued, hemicellulose and cellulose deposition increase and lignin synthesis is initiated. These changes could be brought about by activation or inactivation of certain enzymes, by induction or repression of enzyme synthesis, or by control of transport related to membrane organisation (Northcote, 1985).

There are a number of points at which control may be exerted e.g. the interconversions of nucleoside diphosphate sugars have been considered as potential control points of wall biogenesis during vascular differentiation. Dalessandro and Northcote (1977) studied the epimerases involved during differentiation of xylem cells in pine and fir trees. They showed that the capacity for interconversion of UDP-sugar precursors of pectin and hemicellulose does not change during secondary growth, so a pool of all UDP-sugars is maintained. So for these plants the control must be at sites other than those of epimerisation.

The course of differentiation in bean callus transferred from a maintenance to an induction medium was studied by Haddon et al (1975), using the enzymes involved in lignin and callose biosynthesis as markers for xylem and phloem differentiation respectively. It appeared that changes in the level of callose synthetase activity controlled callose

synthesis during phloem differentiation, and this activity increased in tandem with that of two enzymes involved in lignin synthesis. Thus the rise in PAL (phenylammonia lyase) and caffeate-o-methyl transferase activity (key enzymes in lignin biosynthesis), begins simultaneously with the rise in callose synthetase activity and with the onset of nodule formation. The control mechanism in this experiment was not identified.

Bolwell and Northcote (1983) studied the induction of xylan and arabinan synthetases in bean suspension cultures, enzymes concerned with neutral pectin and hemicellulose formation respectively. Xylan synthetase activity increased on subculture of the bean cells into media which stimulated cell division, while arabinan synthetase activity increased on sub-culture onto media which stimulated differentiation. During the rising phase of this induction, the increases in enzyme activity were inhibited by actinomycin D (an inhibitor of transcription) or by D-2 (4-methyl-2, 6-dinitro anilino)-N-methylpropianamide (an inhibitor of translation). Thus the control for the induction of these enzyme activities involves transcription and possibly also translation.

The possible role of intermediate transfer molecules has been investigated in cell wall polysaccharide biosynthesis. As yet none have been identified, but if a carrier is identified, such as the lipid carriers which are involved in plant glycoprotein synthesis (Bailey et al, 1979, 1980; Hori et al, 1982) this would present another potential site for control of biosynthesis.

The enzyme responsible for the deamination of phenylalanine in the biosynthesis of lignin, i.e. phenylalanine ammonia lyase, has attracted attention because it shows changes in activity in several plant tissues in response to certain stimuli including light, wounding, disease, radiation damage and growth factors such as ethylene. This suggests that the production of lignin precursors and perhaps lignin itself may in part be controlled by a number of environmental and physiological factors.

Interactions of Enzymes in Biosynthesis

The lack of consistency of linkage sequence or monomer composition in the main chain of most heteropolysaccharides suggests that their assembly is not a template-directed process like protein or nucleic acid synthesis, but is more likely to be directed by the specificity of the transferases involved. The systems responsible for the assembly of polymers which contain a variety of linkage types and monomers must involve multiple enzyme-catalyzed reactions requiring delicate coordination. It is not clear with what degree of precision the heteropolysaccharides are synthesized, and research in this area has been impeded by lack of success in complete identification of the polysaccharide products. Recently Waldron and Brett (1985) have proposed two models for control of biosynthesis based on the degree of precision involved:- "imprecise" synthesis involves enzymes specific enough to define residues, but unable to give further regularity to the product. Side chains would be irregularly attached and of

variable length, and the backbone would be synthesized independently of the side chains. "Precise" synthesis would involve synthesis of identical repeating sub-units linked in a precise manner, but of undefined number. It is expected that the backbone of such a precisely synthesized molecule would not be able to grow without concomitant addition of side-chains and non-glycosyl substituents.

The heteropolysaccharide xyloglucan has been relatively well defined with regard to structure and biosynthesis. In terms of the two models proposed by Waldron and Brett, it appears that xyloglucan biosynthesis as described by Ray (1980) for pea epicotyls is 'imprecise' since the backbone can be synthesized independently of the side chains. On the other hand, soybean xyloglucan biosynthesis, as described by Hayashi and Matsuda (1981a & b), appears to be 'precise' since alternate addition of xylosyl and glucosyl residues is proposed.

While knowledge of the mechanisms controlling polysaccharide biosynthesis is limited, even less is understood about the mechanisms governing initiation and termination reactions.

The Site of Polysaccharide Biosynthesis

Heteropolysaccharides are thought to be synthesized within the lumen of the endomembrane system, principally in the Golgi apparatus, and secreted into the cell wall as high molecular weight polymers (Hassid, 1967). The synthesis of homo-polysaccharides cellulose and

callose on the other hand probably takes place on the cell surface (Darvill et al, 1980).

In eukaryotic cells there is a continuous transport of membranes and material contained in membrane-bound vesicles outwards to the plasma membrane and cell wall from the Golgi apparatus, and an inward movement from plasma membrane to endosomes and vacuoles. Following synthesis the heteropolysaccharides are packaged in vesicles, probably in discrete compartments of the Golgi cisternae, and moved under controlled conditions to the plasma membrane where a calcium dependant membrane fusion takes place (Morris & Northcote, 1977). The vesicles are partly directed by microtubules at the plasma membrane. It is believed that the Golgi cisternal membranes arise from the rough endoplasmic reticulum, which changes to smooth endoplasmic reticulum then to Golgi cisternae (Northcote, 1974). The endoplasmic reticulum may also be involved in heteropolysaccharide biosynthesis where heteropolysaccharide is synthesized as a glycoprotein, as in mucilage synthesis in maize root tips (Pickett-Heaps & Northcote, 1966).

Some cell wall enzymes are present at the cell surface of intact cells, for example cellulase, β(1-3) glucanase, peroxidase etc., and cellulose synthesis is believed to take place here. Multi-subunit particles found on the interior face or the plasmamembrane in oocystis freeze-fracture studies appear to be associated with the ends of cellulose fibrils (Brown & Montezinos, 1976; Montezinos & Brown, 1976). Since each cellulose fibril consists of a large number of glucan chains, a multi-subunit enzyme complex would be expected to participate in cellulose synthesis. Microtubules are implicated in the process as

the orientation of the cellulose fibrils often correlates with microtubule position (Robinson, 1977).

A tentative proposal was advanced by Preston (1964) for a mechanism in which an enzyme complex on the outside of the plasma-membrane attached glycosyl residues in a coordinated manner to the ends of (1 - 4)-β-D-glucans at the tips of microfibrils. This form of end growth would require the translation of either the microfibril or the enzyme complex or both. A second theory attributes microfibril formation simply to the operation of physico-chemical forces. Current research involves reassembly of dissociated components.

Hayashi and Matsuda (1981c) have postulated that xyloglucan may be synthesized in the membrane system prior to cellulose synthesis, which begins as soon as the vesicles fuse with the plasmalemma. At the moment of fusion, cellulose synthesis starts and allows continuous formation of a microfibril network. The presence of cellulose microfibrils, the authors argue, may be essential for the deposition of matrix polysaccharides, and these may be necessary for fibre elongation in order to form normal microfibrils. Thus the direction of micro-fibril orientation is probably regulated by the xyloglucan coating round the cellulose microfibrils. The authors believe that a β 1-4 glucan 4-β-D-glucosyltransferase which has been isolated from soybeans is a latent pro-enzyme set aside for polysaccharide assembly in the plant cell wall.

Xyloglucan and Cell Wall Growth

The 'acid secretion' theory of auxin action proposed by Hager et al (1971), and Cleland (1971), attempts to explain how cell wall extension occurs, and several workers have implicated xyloglucan metabolism in this process. According to the theory, auxin acts by stimulating an outwardly directed hydrogen pump in the plasmamembrane, and this acidifies the cell wall. The lower pH somehow breaks bonds in the wall to allow extension, and therefore elongation.

Labavitch and Ray (1974) found that an increase in soluble xyloglucan from pea epicotyls began 15 min after exposure to 1AA. Metabolic inhibitors and calcium ions inhibited auxin promotion of elongation and of xyloglucan metabolism in parallel, thus the authors concluded that changes in xyloglucan may represent the means by which auxin modifies the cell wall to cause elongation. In a later paper (Jacobs & Ray, 1975) neutral buffer was reported to inhibit the 1AA-induced increase in soluble xyloglucan.

Nishitani and Matsuda (1983) found that treating Vigna angularis epicotyl segments with 0.1 $moldm^{-3}$ 1AA caused a change in xyloglucan composition but not quantity, i.e. there was a decrease in the amount of high molecular weight xyloglucan (106 x 10^4 Daltons) and an increase in the amount of low molecular weight xyloglucan (10 x 10^4 Daltons). Hayashi and Maclachlan (1984) suggested that xyloglucan is important in the maintenance of wall integrity, and inhibition of its synthesis, or acceleration of breakdown contributes to wall loosening and swelling.

An interesting effect of a xyloglucan oligosaccharide has recently been reported by York et al (1984). It was found that 2, 4-D-stimulated elongation of etiolated pea segments was inhibited by the application of nonasaccharide-rich Bio-gel P-2 fractions of endo-β-glucanase digested sycamore xyloglucan at 10^{-8} moldm^{-3}. Also an endo-β-glucanase was isolated, the activity of which was stimulated 50 times by auxin, and which could convert pea xyloglucan to hepta- and nonasaccharide fragments. The nonasaccharide-rich fraction inhibited 2, 4-D-stimulated growth at 1 $\mu g ml^{-1}$, whereas a heptasaccharide-rich fraction was inactive (York et al, 1984).

The nonasaccharide may be affecting protein synthesis, and could play an important part in plant development. It may act as a feedback inhibitor of auxin-stimulated growth, or may inhibit growth of axillary buds following its transportation down the stem, thus functioning to regulate apical dominance (Fry et al, 1983).

The Technique of Plant Tissue Culture

The term 'tissue culture' is used as a blanket term to cover the cultivation of all plant parts, whether a single cell, a group of cells or an organ. Tissue culture methods vary, and may involve a liquid medium made semi-solid with agar, in which case the living cells are placed on the surface or embedded in the medium, or a liquid medium with the plant material wholly or partially immersed. Liquid suspension cultures must be agitated or subjected to forced aeration,

and an orbital shaker is employed for this purpose in most laboratories. Movement of the liquid medium maintains even cell and cell aggregate distribution, and promotes gaseous exchange.

During incubation of suspension cultures the amount of cell material increases until a point of maximum yield is reached. If at this point the culture is diluted by sub-culture to the initial cellular content, it will grow with a similar growth pattern to the initial culture, yielding a similar amount of material. The culture can thus be continuously propagated by successive batch cultures of appropriate duration. Cells will only grow in culture when supplied with suitable nutrients. Certain macro-nutrients are essential for growth, including carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur, potassium, calcium, sodium, magnesium and chlorine. Micro-nutrients required include iodine, boron, cobalt, manganese, copper, zinc and iron. Carbon is supplied usually as sucrose, nitrogen as nitrate in the form of ammonia or organic urea. Vitamins may be added to supply organic factors. Most cultures require growth hormones in the form of an auxin, a cytokinin or both. The auxins, which stimulate cell expansion, are usually 1AA, NAA or 2, 4-D. Cytokinins, which stimulate cell division, include zeatin and kinetin. Hormones are physiologically active in very small amounts, usually in the range 10^{-3} to 10^{-5} $mol.dm^{-3}$.

In suspension culture techniques all media, instruments and culture vessels are sterilised to kill vegetative spores, cells or

other reproductive structures of micro-organisms liable to cause culture-contamination. Apparatus and instruments are thus handled using aseptic technique.

The initiation of a cell suspension culture requires a relatively large amount of callus to serve as the inoculum e.g. approx. 2-3 g for 100 cm^3. When the plant material is first placed in the medium, there is an initial lag period prior to any sign of cell division (Fig. 3), followed by an exponential rise in cell number and a linear increase in the cell population. Following a gradual deceleration in the division rate, the cells enter a stationary/non-dividing phase. Maximum cell density is often reached within 18-25 days, though it may be as short as 6-9 days. A 'critical cell density' may be determined below which the culture will not grow e.g. for a clone of sycamore cells this value is $9\text{-}15 \times 10^3$ cells/cm^3.

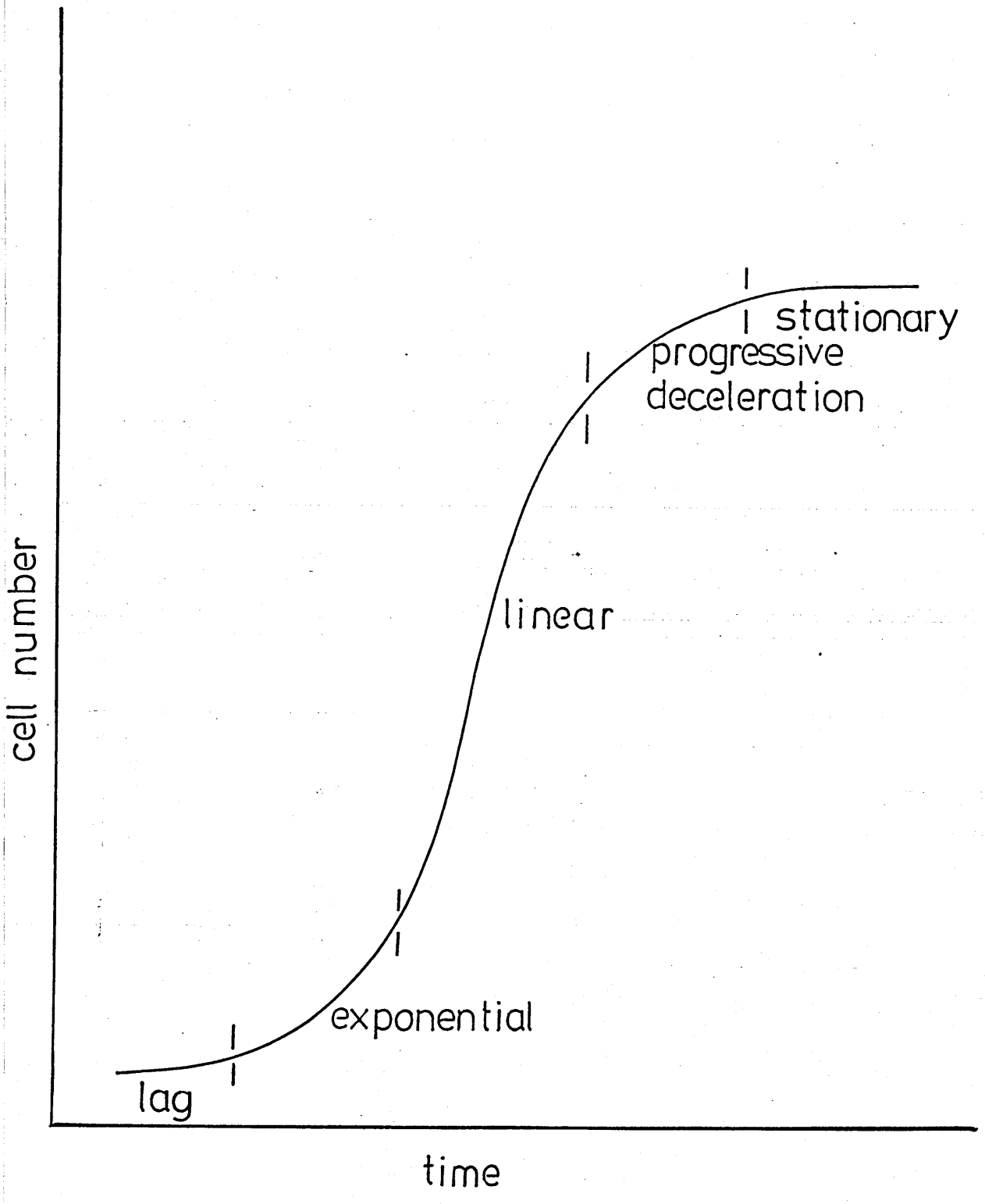
The Use of Plant Tissue Culture in Studies of Cell Wall Biosynthesis and Differentiation

Solid callus and suspension cultures have been used as a source of experimental material for several years. There are obvious advantages of using cultured tissue:- tissue derived from one original plant source provides uniform cells yielding reproducible results, and a large degree of control can be exerted on the system. Potential disadvantages are the requirement for continuous maintenance of the system, and the possibility that cultures may change over long periods

Figure 3

Growth curve of a cell suspension grown under batch conditions, relating total cell number per unit volume to time.

Fig.3


cell number
time
lag
exponential
linear
progressive
deceleration
stationary

of time (Dixon, 1985).

Cultured cells have been widely used in studies of wall biogenesis and structure as they offer an easily obtained source of homogenous primary cell walls, which are apparently very similar to those obtained from plant tissues, for example the xyloglucan of bean plant suspension culture primary cell walls is structurally similar to that of suspension-cultured sycamore cells with only minor differences (Wilder & Albersheim, 1973). Further evidence is found on comparing the structurally complex rhamnogalacturonan II in the walls of suspension-cultured sycamore with that of intact pea, bean and tomato cell walls (Talmadge et al, 1973).

Tissue culture has also been used in studies of differentiation, in studying the process by which an undifferentiated cell becomes, for example, a xylem cell. The xylem cell is often selected for studies of cell differentiation because of the relative ease of inducing xylem differentiation, and the ease of identification of vessels, due to the unusual pattern of secondary wall thickening. The nutritional requirements for xylem differentiation are relatively simple, minimum requirements being an auxin and a cytokinin. Wetmore and Rier (1963) showed that cellular differentiation could be induced in calluses by application of auxin (IAA or NAA) and sucrose, reflecting the pattern of growth exhibited by cells in the intact plant.

Xylem vessel formation has also been achieved from single cells in culture. Torrey (1975) achieved up to 7% differentiated cells in

a suspension-culture of Centaurea cyanus in suitable media, and Kohlenbach (1975) observed up to 30% differentiation of isolated mesophyll cells to tracheids using mechanically isolated cells of Zinnia elegans.

Dudley and Northcote (1978) found differentiation of xylem and phloem cells in Phaseolus vulgaris suspension cells, when transferred from a 'maintenance' to an 'induction' medium containing hormones to induce differentiation.

Activity of the enzyme phenylalanine ammonia lyase (PAL) has been used as a marker for xylem differentiation due to its involvement in the phenylpropanoid pathway which provides the monomers for lignin synthesis. Bevan and Northcote (1979a) found a correlation between increased PAL activity and increased numbers of xylem vessels on transfer of tissue from a maintenance to an induction medium. In a second paper (Bevan & Northcote, 1979b), the interaction of auxin and cytokinin in the induction of PAL activity in suspension cultures was investigated. The presence of NAA was found to be necessary two days prior to the increase of PAL activity, while kinetin caused an immediate rapid increase.

Bolwell and Northcote (1983) correlated the effect of growth hormones on bean cell suspension-cultures with the production of polysaccharide synthases, and found that sub-culture of cells into induction medium brought about an increase in xylan synthase.

More recently, Falconer and Seagull (1985) have studied changes

in wall deposition and microtubule organisation during xylogenesis in suspension-cultured Zinnia elegans. Differentiation occurred in two bursts, at 4-6 days and 10-13 days after culturing. Percentage and timing of differentiation were extremely variable, but the system closely paralled intact shoots and roots with regard to changes in microtubule assays assessed during cell division, expansion and differentiation.

These results again highlight the value of suspension-culture techniques and the possibility of making meaningful extrapolations from suspension-cultured tissue to whole plants.

Introduction to Experimental Work

Xyloglucans are complex, hemicellulosic heteropolysaccharides which have a β1-4 linked glucan backbone with α-xylosyl residues attached to the 6-position of β-glucosyl residues. Additional fucosyl-galactosyl residues are present attached to xylose in some species. The structures of many xyloglucans have been elucidated (Kooiman, 1960; Bauer et al, 1973; Watanabe et al, 1980; Darvill et al, 1980; Kato & Matsuda, 1976, 1977; O'Neill & Selvendran, 1983, 1986), and the biosynthesis of the molecule has also been studied by a number of groups (Ray, 1980; Hayashi & Matsuda (1981a & b); Camirand & Maclachlan, 1986).

It is likely that several enzymes, or an enzyme complex, is involved in the synthesis of such a complex molecule, but studies of xyloglucan biosynthesis tend to concentrate on the actions of one transferase, or on the interactions of not more than two transferase enzymes. Ray (1980) examined a UDP-xylose:xyloglucan xylosyltransferase associated with pea Golgi membranes and found its action was stimulated by UDP-glucose. No stimulation of transfer of glucose from UDP-glucose by UDP-xylose was found. Hayashi and Matsuda (1981a) investigated a similar xylosyltransferase enzyme in suspension-cultured soybean cells and found its action was dependent upon the presence of UDP-xylose in incubations, contrasting with the findings of Ray. Hayashi and Matsuda (1981a) proposed that the xyloglucan synthase complex transfers glucose and xylose alternately, whereas Ray proposed that xylose can be

transferred to a pre-formed glucan backbone. These contrasting mechanisms for transfer provide examples for the two models of wall synthesis proposed by Waldron and Brett (1985), which define synthesis by degree of precision. "Imprecise synthesis", involving enzymes specific enough to define residues and linkages with side chains irregularly attached and of variable length, would permit synthesis of the backbone independently of the side chains - i.e. xyloglucan biosynthesis as visualised by Ray (1980). "Precise" synthesis, involving synthesis of identical, repeating sub-units linked in a precise manner, but of undefined number, would not permit synthesis of the backbone without concomitant addition of side chains and non-glycosyl substituents - i.e. xyloglucan biosynthesis as visualised by Hayashi and Matsuda (1981a). A recent study of the transfer of fucose from GDP-fucose to xyloglucan by Camirand and Maclachlan (1986) suggests this also occurs by an "imprecise" mechanism, i.e. by addition of fucose to a preformed backbone. Clarification of the mode of synthesis of this complex polysaccharide should increase our understanding of cell wall polysaccharide synthesis in general.

The present thesis reports the results of experiments <u>in vitro</u> concerning the incorporation of the four sugars which comprise bean xyloglucan from their corresponding sugar nucleotides, by particulate enzyme preparations from dark-grown bean seedlings, and bean suspension cultures. Characterisation of enzyme products is attempted as far as possible, and related transferase systems such as UDP-glucose: β1-3

glucantransferase and UDP-xylose:xylantransferase are also investigated. Interactions of the transferases are studied in order to clarify the degree of precision of synthesis. Finally, an attempt is made to relate enzyme transferase activities in immature (undifferentiated) tissue to those in differentiating material.

Chapter 2

MATERIALS AND METHODS

Chemicals and Sundries

UDP-D-[$U^{14}C$]xylose (258.7 $mCi.mmol^{-1}$) was purchased from New England Nuclear, Stevenage, Herts, U.K. UDP-D-[$U^{14}C$]glucose (12GBq. $mmol^{-1}$), UDP-D-[$U^{14}C$]galactose (11.04 $GBq.mmol^{-1}$), GDP-D-[$U^{14}C$]fucose (7.4 $GBq.mmol^{-1}$), D-[$U^{14}C$]glucose (10 $GBq.mmol^{-1}$), D-[$U^{14}C$]xylose (2.81 $GBq.mmol^{-1}$) and L-[$U^{14}C$]phenyalanine (19.4 $GBq.mmol^{-1}$) were purchased from Amersham International, Amersham, U.K. Non-radioactively-labelled sugar nucleotides were purchased from Sigma.

All chemicals were of analar or laboratory grades, and were purchased as follows:- Agar, HEPES, BSA, EDTA, Tris, $NaBH_4$, tannic acid, DNP-lycine, blue dextran, larch xylan, laminarin, guaiacol, phloroglucinol, all commercial enzyme preparations and hormones (with the exception of 2, 4-D) from Sigma ; DTT, Tween 80, Amberlite IR45 and IRA 120, cadmium oxide, ethylene diamine, 2, 4-D and gum acacia from BDH Chemicals Ltd., Poole, England; Sephadex G25 and G100, Sepharose CL-2B and CL-6B from Pharmacia Ltd., Midsummer Boulevard, Milton Keynes, Bucks; PPO from International Enzymes, Windsor, Berkshire, England; cellulose powder from Machery-Nagel Ltd., Duren, FRG; Bio-gel P2 from Bio-Rad Laboratories Ltd., Caxton Way, Watford, Hertfordshire; trans-cinnamic acid and 2, 6-dichloroquinonechloroimide from Koch-Light Laboratories, Colnbrook, Bucks., and TLE plates pre-coated with silica gel G from Camlab, Cambridge, U.K.

[1-^{3}H]xylol - 6 glucose (isoprimeverose) was a generous gift

from Dr. S.C. Fry, Department of Botany, Edinburgh University, and purified tamarind xyloglucan was kindly given by Dr. J.S.G. Reid, Department of Biological Science, Stirling University.

Visking tubing was purchased from Griffin and George, Frederick Street, Birmingham B1 3HT.

Plant Material

Beans (*Phaseolus vulgaris*) var Canadian Wonder were supplied by Sinclair McGill (Ayr, Scotland) and arrived pre-treated with "Lindare Thiram". Peas (*Pisum sativum*) var Alaska used for cellulase preparation were also supplied by Sinclair McGill. Beans for hypocotyl preparations were soaked for between 6 and 8 h in tap water, then subsequently planted in trays (38 x 30 cm) which contained moist vermiculite, and grown in darkness at 22°C for approx. 7 days. Peas for cellulase preparation were treated as described (p.56).

Media

Callus and suspension cultures were grown on the B5 medium of Gamborg *et al* (1968), supplemented with 2% sucrose, 2 $mgdm^{-3}$ 2, 4-D and 20% deproteinised coconut milk, adjusted to pH 5.5 before autoclaving at 120°C for 20 min. Solid media contained 1% agar, added before autoclaving. Inductior media were as described (p.208).

Tissue Culture

Seeds of *Phaseolus vulgaris* were surface-sterilised in a buffered

solution of sodium hypochloride (1%) (Milton, Richardson-Vicks Ltd., Egham, Surrey) for 6 h. After washing in distilled water they were placed on sterile water-agar at 25°C in the dark to germinate. The callus which formed after 3-4 weeks growth was removed from the explant and transferred to fresh medium. Thereafter portions of approx. 1 g were transferred to fresh medium every 4-6 weeks. To set up suspension-cultures, friable callus was transferred after about 4 sub-cultures into liquid medium in 500 cm^3 Erlenmeyer suspension-culture flasks, rotating at 100 rpm at 25°C. Sub-culturing of suspension was initially carried out after 3-4 weeks, after which the interval decreased to every 5-7 days.

Particulate Enzyme Preparation:- 1. Hypocotyls

Bean hypocotyls (25 g) were excised using scissors into 75 cm^3 buffer containing HEPES (100 $mmol dm^{-3}$) brought to pH 6.8 with 1 $mol dm^{-3}$ KOH, dithiothreitol (1 $mmol dm^{-3}$), EDTA (1 $mmol dm^{-3}$), sucrose (0.4 $mol dm^{-3}$), 0.1% BSA and $MnCl_2$ (50 $mmol dm^{-3}$). Homogenisation was performed using 2 passes of a Polytron Kinematica at setting 5 for 10 secs. The homogenate was passed through four layers of muslin, and the filtrate was centrifuged at 97,000 x g (average) for 30 min. Pellets were resuspended in homogenisation buffer (0.5 cm^3). All procedures were carried out at 0-4°C.

Particulate Enzyme Preparation - 2. Suspension-Culture

Suspension-culture cells were filtered through four layers of

muslin, and filtered-tissue was ground with acid-washed sand with a pestle and mortar, in an equal volume of Tris (100 $mmol.dm^{-3}$) adjusted to pH 7.5 with HCl, or in the case of the xylan-xylosyltransferase preparation in potassium phosphate (0.1 $mol.dm^{-3}$) pH 7.2, $MnCl_2$ (1 $mmol.dm^{-3}$), DTT (1 $mmol.dm^{-3}$), sucrose (0.4 $mol.dm^{-3}$) and 1% BSA. After filtering through muslin for a second time, the filtrate was centrifuged for 30 min at 97,000 xg. or for 60 min in the case of the xylan-xylosyltransferase preparation. The pellet was resuspended in buffer (0.5 cm^3) in a glass homogeniser. All procedures were carried out at 0-4°C.

Crude Enzyme Preparation for Solid Callus and Suspension-Cultures

Callus tissue or filtered suspension-cultured tissue from differentiation experiments was ground with acid-washed sand with a pestle and mortar in an equal volume of Tris/HCl, or potassium phosphate buffer for investigation of the xylan-xylosyltransferase. The homogenate was filtered through four layers of muslin and the filtrate was added to incubations, without centrifugation, as required.

Enzyme Assays

Xyloglucan-xylosyltransferase

The standard incubation mixture consisted of particulate enzyme preparation (50 mm^3), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-glucose (2 $mmol.dm^{-3}$) and UDP-D-[$U^{14}C$]xylose (21 $\mu mol.dm^{-3}$, 740 Bq). This was used for hypocotyl and suspension-culture particulate enzyme preparations.

For crude preparations from suspension cultures and calluses incubation mixtures consisted of enzyme preparations (500 mm^3), $MnCl_2$ (1.82 $mmol.dm^{-3}$), UDP-glucose (0.36 $mmol.dm^{-3}$) and UDP-D-[$U^{14}C$] xylose (3.82 $\mu mol.dm^{-3}$, 740 Bq).

Xyloglucan-glucosyltransferase

The standard incubation mixture for hypocotyl preparations consisted of enzyme preparation (50 mm^3), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-D-[$U^{14}C$]glucose (200.77 $\mu mol.dm^{-3}$, 925 Bq) and UDP-xylose (200 $\mu mol.dm^{-3}$). For particulate preparations from suspension-culture preparations incubations consisted of enzyme preparation (50 mm^3), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-glucose (2 $mmol.dm^{-3}$) and UDP-D-[$U^{14}C$]glucose (200.77 $\mu mol.dm^{-3}$, 925 Bq). The incubation mixture used for investigation of β(1-3) glucan biosynthesis in differentiation experiments using solid callus consisted of enzyme preparation (500 mm^3), UDP-D-[$U^{14}C$]glucose (36.5 $\mu mol.dm^{-3}$, 925 Bq) and $MnCl_2$ (1.82 $mmol.dm^{-3}$).

Xyloglucan-galactosyltransferase

The standard incubation mixture for hypocotyl preparations consisted of enzyme preparation (50 mm^3), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-glucose (20 $\mu mol.dm^{-3}$) UDP-xylose (20 $\mu mol.dm^{-3}$) and UDP-D-[$U^{14}C$] galactose (0.841 $\mu mol.dm^{-3}$, 925 Bq). For suspension-culture preparations a variety of mixtures was used as described in the text.

Xyloglucan-fucosyltransferase

The standard incubation mixture for hypocotyl enzyme preparations consisted of enzyme preparation (50 mm^3), UDP-glucose (20 $\mu mol.dm^{-3}$), UDP-xylose (20 $\mu mol.dm^{-3}$), UDP-galactose (20 $\mu mol.dm^{-3}$), $MnCl_2$ (10 $mmol.dm^{-3}$) and GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq). For suspension-cultures this was changed to enzyme preparation (50 mm^3), UDP-glucose (2 $mmol.dm^{-3}$), UDP-xylose (2 $mmol.dm^{-3}$), UDP-galactose (2 $mmol.dm^{-3}$), $MnCl_2$ (10 $mmol.dm^{-3}$) and GDP-D-[$U^{14}C$] fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq). For solid callus culture preparation and crude preparations from suspension cultures incubations consisted of enzyme preparation (500 mm^3), UDP-glucose (0.36 $mmol.dm^{-3}$), UDP-xylose (0.36 $mmol.dm^{-3}$), UDP-galactose (0.36 $mmol.dm^{-3}$), $MnCl_2$ (1.82 $mmol.dm^{-3}$) and GDP-D-[$U^{14}C$]fucose (0.23 $\mu mol.dm^{-3}$, 925 Bq).

Xylan-xylosyltransferase

The standard incubation mixture consisted of enzyme preparation (50 mm^3), $MnCl_2$ (10 $mmol.dm^{-3}$) and UDP-D-[$U^{14}C$]xylose (0.77 $\mu mmol.dm^{-3}$, 740 Bq). For solid callus cultures and crude preparations from suspension-cultures incubations comprised enzyme preparation (500 mm^3), $MnCl_2$ (1.82 $mmol.dm^{-3}$) and UDP-D-[$U^{14}C$]xylose (0.14 $\mu mol.dm^{-3}$, 740 Bq).

Incubations were in a total volume of 100 or 550 mm^3, made up to volume with H_2O. Incubations were carried out at 25°C and were terminated by addition of 70% (v/v) ethanol (1 cm^3). In samples retained for product analysis, radioactivity in incubations was doubled. Incubations were performed in duplicate.

Extraction of [^{14}C]labelled Polysaccharides after Incubation:-

1. Hemicellulose Extraction

Terminated incubations were centrifuged at 10,000 x g for 1 min. The pellets were extracted twice in water (1 cm^3), and then twice in 70% ethanol (v/v) (1 cm^3). The pellet was then extracted twice with 24% (w/v) KOH (1 cm^3) for 15 min at 100°C. Pellets were centrifuged at approx. 700 x g for 5 min. each time, and the supernatants were retained and neutralised with glacial acetic acid (1 cm^3) after the second extraction. Pure ethanol was added to give a 70% solution. The solutions were then left for 48 h at 4°C with approx. 50 mg cellulose powder added to facilitate precipitation. After precipitation, the solutions were centrifuged for 5 min. at approx. 700 x g. The pellets were resuspended in pure ethanol (0.5 cm^3) and this was added to water (0.5 cm^3) before measuring radioactivity incorporated by counting on a scintillation spectrometer.

Extraction of [^{14}C]labelled Polysaccharides After Incubation:-

2. Total Polysaccharide

Terminated incubation mixtures were centrifuged at 10,000 x g for 1 min. The pellets were washed five times in 70% (v/v) ethanol (1 cm^3), and then four times in water to remove the majority of the radioactivity and any low molecular weight products. Measurement of incorporation of radioactivity into total polysaccharide was achieved by counting on a scintillation spectrometer.

Centrifugation

Sedimentation of membranous material at 97,000 x g (average) involved the use of a Sorval OTD 65-B ultra-centrifuge and AH627 rotor.

Sedimentation of muslin filtrate for the pea cellulase preparation at 13,000 x g involved the use of an MSE-40 centrifuge.

Centrifugation at 10,000 x g during treatment of terminated incubations was achieved using an Eppendorf microfuge and 1.5 cm^3 Eppendorf polythene vials.

Sedimentation of precipitated hemicellulose was carried out at 100 x g using an MSE-minor S centrifuge and 10 cm^3 conical tubes.

Preparation of Samples for Enzyme/Acid Hydrolysis

Pellets to be retained for analysis were counted in toluene scintillant. After counting samples were centrifuged at 10,000 x g for 1 min, and then extracted twice in pure ethanol. After a further centrifugation, ethanol was removed and the pellets were dried in air at reduced pressure overnight.

Total Acid Hydrolysis of Polysaccharide

Total acid hydrolysis was carried out in trifluoroacetic acid (2 $moldm^{-3}$) in sealed Reacti-vials (Pierce & Warriner (U.K.) Ltd., Chester). The samples were autoclaved at 120°C for 1 h or more,

after which the non-hydrolysed material was removed by centrifugation at 10,000 x g for 5 min, and the hydrolysate was evaporated to dryness under reduced pressure.

Preparation of Pea Cellulase for Enzyme Hydrolysis

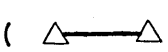
Pea cellulase was prepared using a modification of the method of Byrne et al (1975). Pea seeds were soaked and grown on moist vermiculite in darkness at room temperature for five days. The seedlings were then sprayed with 0.1% 2, 4-D, 0.1% Tween 80, and 0.1 mol.dm^{-3} NaCl, adjusted to pH 7. Approx 10 cm^3 spray were used per 500 seedlings, which were then left for a further 5 days. Seedling apices (20 g) were then excised and homogenised in 2 volumes of buffer containing sodium phosphate (20 mmol.dm^{-3}) pH 6.2, 1% glucose and 5% glycerol, using two passes of 10 seconds at speed 5 of a Polytron Kinematica. The homogenate was passed through four layers of muslin, and then the filtrate was centrifuged for 10 min at 13,000 x g. The supernatant was retained. All procedures were carried out at 2-4°C.

Assessment of Pea Cellulase Activity

The activity of extracted pea cellulase was assayed viscometrically using a Volac semimicroviscometer. To 2.7 cm^3 of a 1% solution of carboxymethylcellulose was added 0.3 cm^3 of pea cellulase preparation, and the resulting drop in viscosity was measured at intervals over an hour. As a control, 0.3 cm^3 phosphate buffer was added to 2.7 cm^3 carboxymethylcellulose. The resulting change in viscosity was recorded (Fig.1). With fresh enzyme or enzyme frozen for 48 h, a

Figure 1

Reduction in viscosity of carboxymethylcellulose by pea cellulase enzyme preparation:-


)control: carboxymethylcellulose + buffer


): carboxymethylcellulose + fresh enzyme

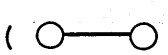
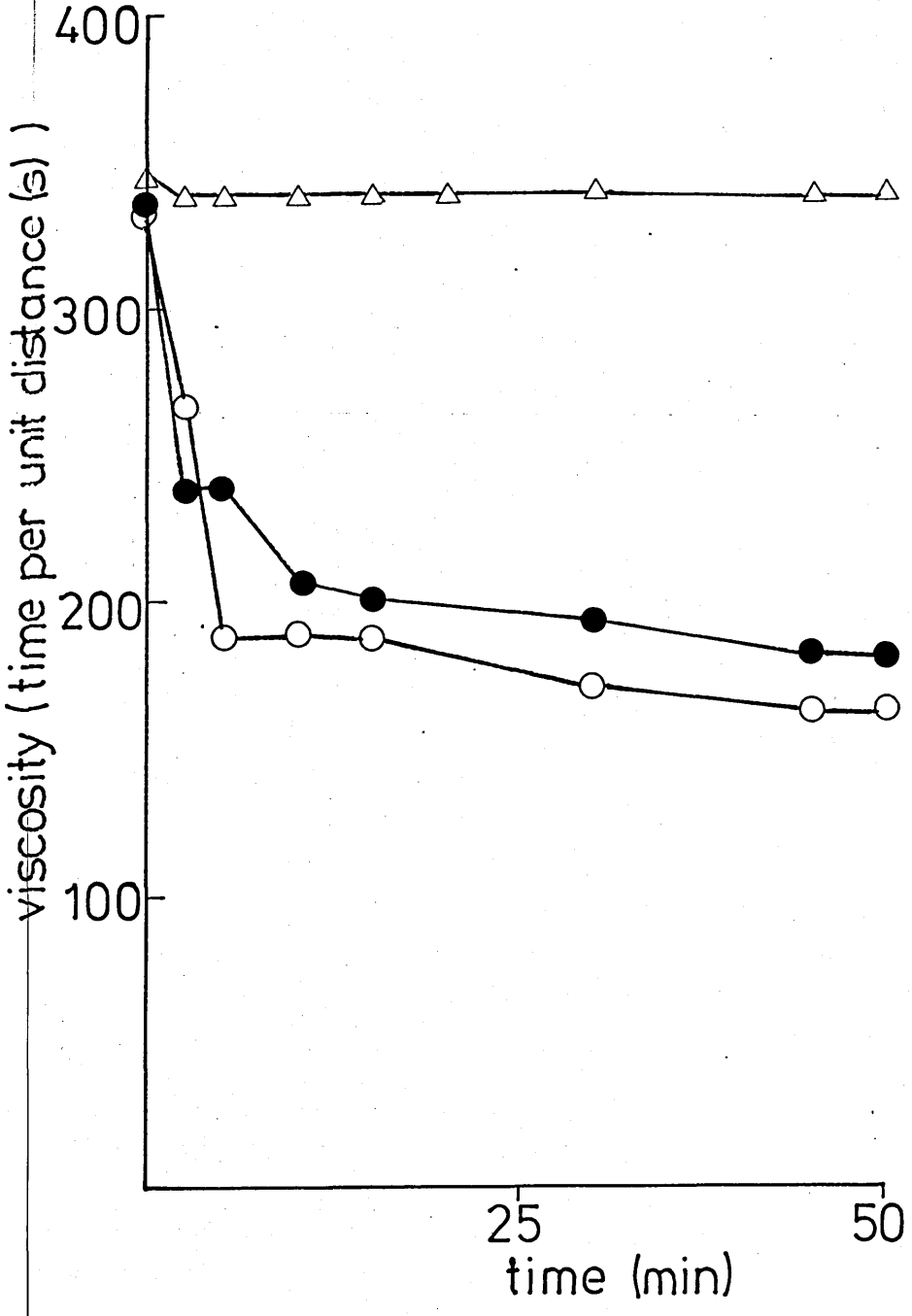

): carboxymethylcellulose + fresh enzyme

Fig.1


400
300
200
100
viscosity (time per unit distance (s))
25
50
time (min)

rapid drop in viscosity took place over the first 5 min. of incubation, followed by a slower decrease over the next 40 min. The initial drop was more marked with fresh enzyme, but overall there was little difference. Samples incubated with buffer showed no reduction in viscosity over 40 min.

Dialysis of Pea Cellulase Preparation

Where pea-cellulase-incubated polysaccharide was to be analysed by paper chromatography, glucose and glycerol were removed from the cellulase preparation before incubation. This was done by dialysis. Pea cellulase preparation was pipetted into Visking tubing and dialysed against sodium phosphate pH 6.2 (20 $mmol dm^{-3}$) for 3 days, with constant stirring. The sodium phosphate was changed twice a day. Enzyme activity was assayed viscometrically and found to be unaffected by dialysis.

Enzyme Hydrolysis of [^{14}C]labelled Polysaccharide

Enzymic hydrolysis with driselase (Sigma) was carried out with 50 mg driselase powder in 50 $mmol dm^{-3}$ acetate buffer (1 cm^3) adjusted to pH 4.5 with NaOH. The powder was dissolved in buffer, left to settle at room temperature for 10 min, then centrifuged at 10,000 x g for 1 min to remove carrier material. The supernatant was removed and added to the polysaccharide pellet. Incubation was generally carried out overnight, at 25°C, then boiled to terminate.

Enzymic hydrolysis with laminarinase (Sigma) was carried out with 10 mg laminarinase in acetate buffer (50 mmol.dm^{-3}), pH 4.5. In general 1 cm^3 enzyme solution was added to polysaccharide pellets. Where non-radioactively-labelled samples of purified tamarind xyloglucan, cellulose powder or laminarin were hydrolysed, 10 mg of each was incubated with 1 cm^3 enzyme. Incubations were terminated by boiling for 5 min.

Enzymic hydrolysis with Trichoderma viride (Sigma) was carried out with 5 mg enzyme powder in 50 mmol.dm^{-3} (1 cm^3) buffer pH 3.5 (1 cm^3) for 24 h at 60°C. Incubations were terminated by boiling for 5 min.

Enzymic hydrolysis with Proteinase K (Sigma) was carried out with 0.5 mg Proteinase K in 10 mmol.dm^{-3} Tri/HCl, pH 7.5 (1 cm^3). Proteinase k digestion was performed at 25°C for 2 h, or overnight. Incubations were terminated by boiling for 5 min, or by addition of 100% cadoxen (0.5 cm^3) to incubations if the sample was to be analysed by gel-filtration.

Separation of Oligosaccharides by Descending Paper Chromatography

Oligosaccharides produced by enzymic hydrolysis where a wide range of product sizes was anticipated were separated for 48 h in Solvent A - butan -1-ol/pyridine/water, 4:3:4 by volume (Leloir et al, 1971). Where mono- and disaccharide products were to be separated, Solvent B was used - butan-1-ol/pyridine/water, 6:4:3 by volume

(Leloir et al, 1971) for 24 h. Separation of the xylan-hydrolysate series was achieved by running for 24 h in Solvent C - ethylacetate/pyridine/water, 10:4:3 by volume (Dalessandro & Northcote, 1981).

Separation of Monosaccharides by Descending Paper Chromatography

Monosaccharides produced by total acid hydrolysis were separated for 24 h in Solvent D - ethylacetate/pyridine/water, 8:2:1. Sugars were separated from sugar alcohols for 8 h in Solvent E - ethyl methyl ketone/acetic acid/sat. boric acid, 9:1:1 (Rees & Reynolds, 1958).

Preparation of Malto-oligosaccharide Markers for Descending Paper Chromatography

To prepare malto-oligosaccharide markers for molecular weight estimation, 3% H_2SO_4 (1 cm^3) was added to four conical glass tubes containing 100 mg starch powder. The tubes were left at room temperature for 15, 30, 60 min and overnight. The reaction was terminated by neutralisation with Amberlite 1R 45 ion-exchange resin in the bicarbonate form. The solution (5 mm^3) was then used as required.

Detection of Sugar Markers

Marker sugars, 10 mm^3, were used at a concentration of 10 $mg.cm^{-3}$, and along with enzyme hydrolysate markers were detected by the method of Trevelyan et al (1950). Chromatograph sheets were passed through a bath of acetone saturated with silver nitrate and allowed to dry. They were then passed through a bath containing 0.5% NaOH. On drying the sugars showed up as grey/brown spots.

Elution of Radioactivity from Paper Chromatograms

Strips of chromatography paper which had been counted in toluene/PPO scintillant were washed three times in sulphur-free toluene to remove the scintillant. The remaining toluene was then allowed to evaporate. The strips were agitated in water (3 cm^3 per strip) for 5 min, three times. The washings were retained and centrifuged at 7,000 x g for 5 min. to remove paper particles. The washings were dried by rotary evaporation at 40°C.

Estimation of Radioactivity

Pellets of water-insoluble material produced in incubations from suspension-cultured tissue, or of precipitated hemicellulose, were vortex-mixed with 5.5 cm^3 of scintillation fluid/water (10:1, v/v). The scintillation fluid used was Koch-Light xylene-based scintillant.

Samples which were to be retained for analysis after counting were washed twice in pure ethanol and then dissolved in 1 cm^3 scintillation fluid, consisting of PPO in toluene (Sulphur -free) ($4g.dm^{-3}$) (Harris & Northcote, 1970). Strips of chromatography paper or thin layer electrophoresis plate strips were likewise counted in 1 cm^3 toluene scintillant.

All samples were counted for radioactivity in a Packard liquid-scintillation spectrometer, model 3380.

Cadoxen Solubilisation of Hemicellulose

Cadoxen was prepared by the method of Wood and McCrae (1978). To make approx. 1 dm^3 of cadoxen, ethylene diamine (280 g), cadmium oxide (100 g) and water (720 cm^3) were stirred at room temperature for 3 h, and then at 4^oC for 18 h. The mixture was allowed to settle and the supernatant (100% cadoxen) decanted and stored at 4^oC.

To solubilise polysaccharide, the dried pellet was mixed with 0.25 cm^3 cadoxen and sonicated for 3-4 days at room temperature.

Gel Filtration

Analysis of pea cellulase hydrolysed products involved the use of Sephadex G-25 gel media, using a column 1 x 50 cm with a flow rate of approx. 0.5 $cm^3 min^{-1}$ at room temperature. The running buffer was that used for the cellulase preparation.

Estimation of the molecular weight of the (^{14}C) xylose-labelled cadoxen-solubilised product was performed using Sepharose CL-6B and CL-2B gel filtration media. The use of the cross-linked form of sepharose prevented solubilisation of the gel-filtration media by the cadoxen. Columns measured 25 x 1 cm. Proteinase K digested product was analysed using sepharose CL-6B and CL-2B in 48 x 1 cm columns. For both analyses samples were run in 50% cadoxen with blue dextran and dinitrophenyllycine were used as markers. The flow rate of columns was approx 0.5 $cm^3 min^{-1}$.

Analysis of GDP-D-[U^{14}C]fucose incubation product involved the use of Sephadex G100 gel media, using a column 1 x 15 cm with a flow rate of approx. 0.5 $cm^3 min^{-1}$, eluting with water.

Analysis of (^{14}C) fucose-labelled samples which had been digested with cellulase was achieved using Bio-gel P2 gel filtration media using a column 150 x 1 cm. Distilled water was used for elution, and the column had an elution rate of approximately 0.4 $cm^3 hr^{-1}$.

Ion Exchange

To prepare Amberlite IR45 in the bicarbonate form for neutralising solutions, the resin was washed with 96% ethanol and then washed twice with water. It was soaked overnight in $NaHCO_3$ (0.1 $mol.dm^{-3}$) and then washed ten times with distilled water. Resin was added to the solution to be neutralised until it was detected as neutral with litmus paper.

Deionisation of solutions was achieved using Amberlite 1R120 for removal of cations, and Amberlite 1RA 400 for removal of anions. The resins were packed into gel-filtration columns. Amberlite 1R 120 was treated for 30 min with HCl (0.1 $mol.dm^{-3}$) and then washed with two or more column volumes of water, until the eluant was neutral. Samples were passed through the resin in a total volume of 10 cm^3 and two further column volumes of water were then passed through to elute the deionised material. Amberlite 1RA 400 was treated with HCl (0.1 $mol.dm^{-3}$) for 30 min, washed with water till the eluant was neutral, then treated with NaOH (0.1 $mol.dm^{-3}$) for 30 min. After washing with

water till neutral, the sample was passed through (10 cm^3) with two column volumes of water.

Phenol Method for Sugar Estimation

Sugars and oligosaccharides in fractions from gel filtration columns were detected by the phenol method of Dubois et al (1956). A solution of 5% phenol (w/v) (1 cm^3) was added to the test solution. Concentrated H_2SO_4 (5 cm^3) was added rapidly onto the surface to facilitate mixing. This was allowed to cool for 10 min at room temperature, and then placed in a water bath at 25-30°C for 10 min. The optical density was read at 490 nm on a Pye Unicam SP8-500.

Borohydride Reduction of Radioactively-labelled Xylose Marker

A marker was produced from [^{14}C]-labelled xylose (2.6 $mmol.dm^{-3}$, 7.4 KBq), by first incubating for 1 hour at room temperature with 1 $mol.dm^{-3}$ aqueous NH_3 (0.5 cm^3) and $NaBH_4$ (10 mg). After incubation, glacial acetic acid was added dropwise to neutralise the solution, which was then rotary evaporated to dryness four times with redistilled methanol (10 cm^3).

Borohydride Reduction of Radioactively-labelled Disaccharides

Disaccharide samples were reduced for 1 hour at room temperature with 1 $mol.dm^{-3}$ aqeous NH_3(0.5 cm^3) and $NaBH_4$ (10 mg). The solution was neutralised with glacial acetic acid, then rotary evaporated to dryness four times with redistilled methanol (10 cm^3). To this was added 3% H_2SO_4 (1 cm^3), and the solution was autoclaved for 1 hour.

Neutralisation was carried out with Amberlite 1R45 resin. The eluate was rotary evarporated to dryness.

Thin-layer Electrophoresis

Electrophoresis was carried out on plastic sheets pre-coated with silica-gel G (Camlab, Cambridge, U.K.) at 300 V for 4 hours. The buffer consisted of pyridine/acetic acid/water (1:10:90 by vol.). Markers (10 µl of 10 $mg cm^{-3}$ xylose and UDP-xylose) were located by spraying the plates with methanolic 0.5% α-napthol, allowing to dry, spraying with 10% (v/v) H_2SO_4 in methanol, and drying at between 70 and 110°C.

Production of Cellulose Oligomers

A range of cellulose oligomers of varying size was produced by hydrolysing cellulose powder (1 g) with 3% H_2SO_4 (1 cm^3) at room temperature, for varying lengths of time up to 60 min. Hydrolysis was terminated by addition of Amberlite 1R 45 in the bicarbonate form.

Pre-incubation of Particulate Enzyme Preparation

Particulate enzyme preparations were made from suspension-cultured tissue as described (p. 50) and were resuspended in 0.5 cm^3 buffer. To each was added the relevant pre-incubation components, i.e. 10 $mmol.dm^{-3}$ $MnCl_2$, 2 $mmol.dm^{-3}$ UDP-glucose and 2 $mmol.dm^{-3}$ GDP-glucose, depending on the treatment. Pre-incubation was carried

out at 25°C or at 0-4°C in polypropylene centrifuge tubes. At the end of the pre-incubation (20-50 min), centrifuge tubes were topped up with ice-cold buffer and centrifuged for a second time, at 97,000 x g, for 30 min. Pellets were resuspended in 0.5 cm^3 buffer, and added to incubations as required.

Measurement of Protein

To measure protein in enzyme preparations, the Tannin assay method of Mejbaum-Katzenellenbogen and Dobryszycka (1959) was employed. Tannin reagent was made by dissolving tannic acid (20 g) and phenol (4 g) in 196 cm^3 HCl (1N), heating to 80°C and filtering. Protein to be measured was dissolved in a total of 1 cm^3 buffer or H_2O, and brought to 30°C in a waterbath. To this was added 1 cm^3 of the tannin reagent, also at 30°C, and the solution was incubated for 10 min. To terminate the incubation, 1 cm^3 of gum acacia (0.2% w/v in H_2O) was added. The turbidity of the solution was then measured at 500 nm in a Pye Unicam Spectrophotometer, SP8-500. Samples were expressed as µg BSA equivalents by comparison with a calibration curve (Fig.2).

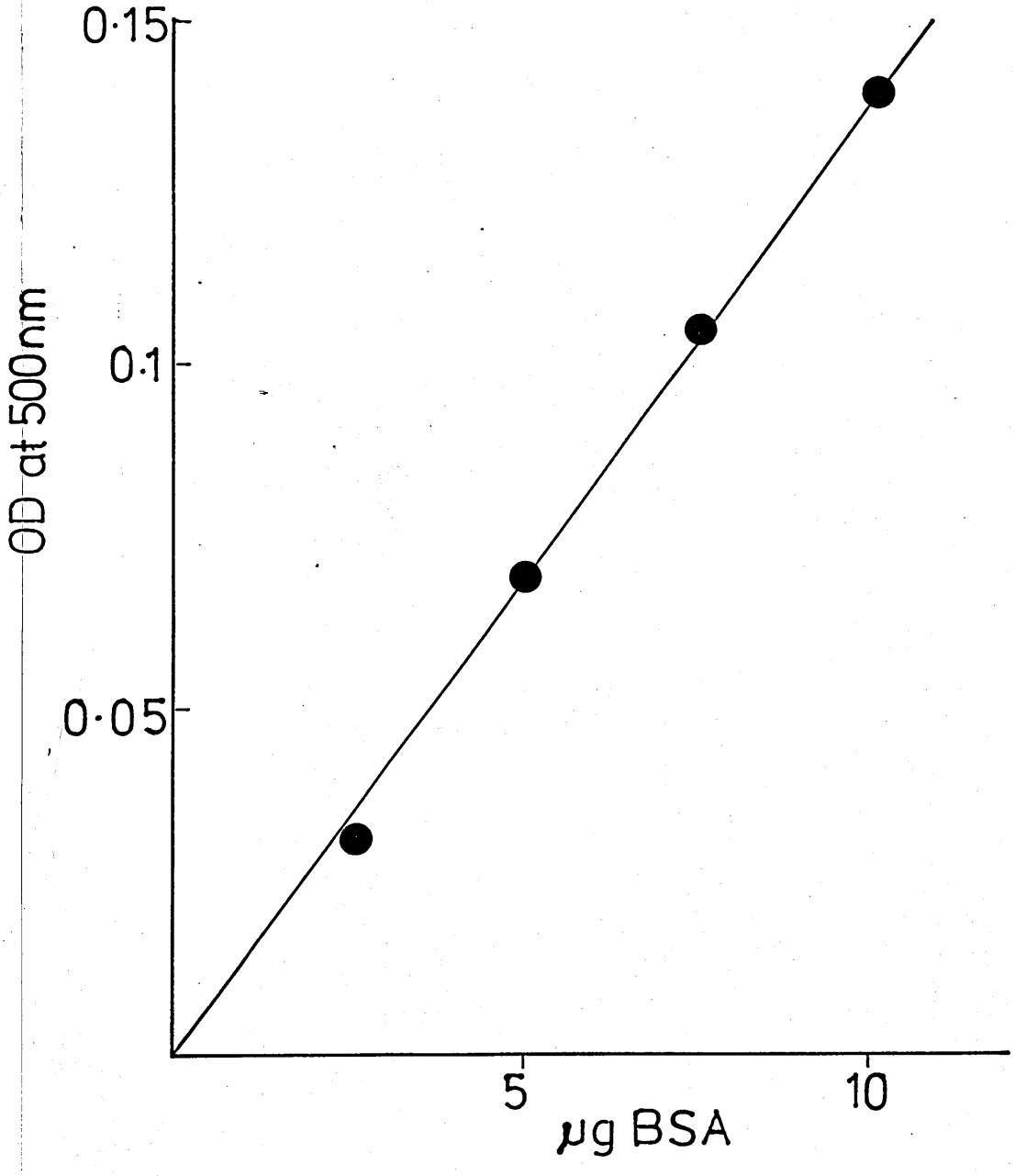
Detection of Lignin by Phloroglucinol/HCl Staining

Phloroglucinol (5 drops) was added to suspension-culture tissue cells on a microscope slide. This was followed by 5 drops of 50% HCl, then a further 5 drops of phloroglucinol. Tissue was observed for characteristic pink staining under a Zeiss Photomicroscope II on phase contrast at x 192.

Figure 2

Calibration curve for estimation of protein in samples determined by tannin assay.

Fig.2



Determination of Phenol by Extraction in Sodium Hydroxide Solution

Phenol analyses of suspension-cultured tissue were performed using a modification by Stafford (1960) of the method of Geirer (1954) for native lignin preparations. Samples of tissue were dried overnight at 60°C and then 50 mg of dried tissue was extracted three times with water. The residue was extracted for 16 h with 0.5N NaOH (2 cm^3). Samples of the extract (1 cm^3) were added to 0.5 $mol.dm^{-3}$ NaOH (1 cm^3). Samples of the extract (1 cm^3) and freshly prepared ethanolic solution (26 $\mu g cm^{-3}$) of 2, 6-dichloroquinonechloroimide (1 cm^3) and were incubated for 1 h at room temperature. The extinctions at 610 nm were measured on a Pye Unicam Spectrophotometer, SP8-500. The quantities of phenols present in the solutions were expressed in terms of μg of guiacol by comparison with a standard curve (Fig.3).

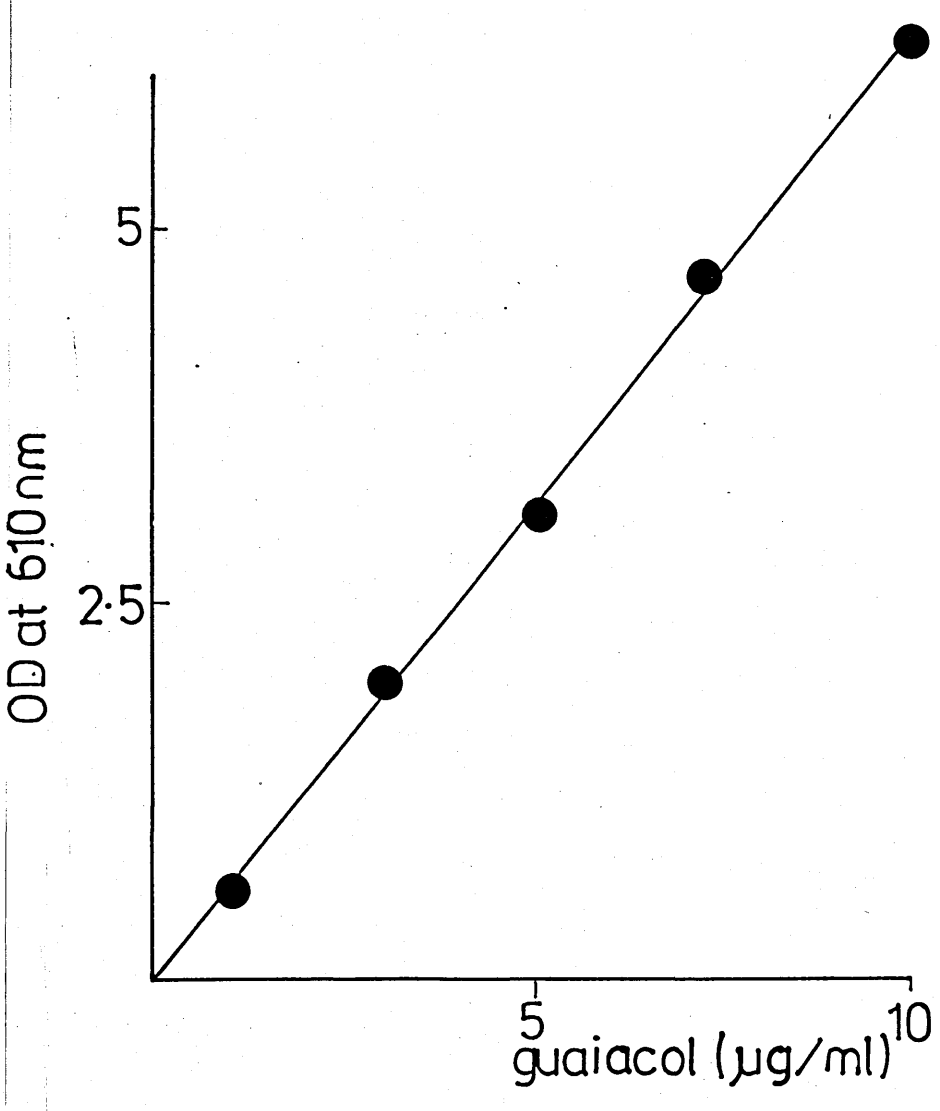
Assay for Phenylalanine Ammonia-Lyase Activity

The assay for phenylalanine ammonia-lyase activity was adopted from Bevan and Northcote (1979). Filtered suspension cultured tissue (1 g fresh weight) were ground with a little acid-washed sand in 1 cm^3 100 $mmol.dm^{-3}$ Tris/HCl containing 1% mercaptoethanol pH 8.8 at 4°C. The homogenate was centrifuged at 10,000 x g for 1 min and the supernatant was used for the enzyme assay. The assay contained 38.5 $mmol.dm^{-3}$ Tris/HCl and 3.9 $mmol.dm^{-3}$ L-phenylalanine, containing 4.27 KBq, in a total volume of 60 μl. The assay was started by incubation at 38°C stopped after 2 h by drying the entire assay mixture onto a silica gel thin-layer plate. A solution of 1% cinnamic acid was streaked through the origin, and the chromatogram was developed in toluene: ethyl formate: formic

Figure 3

Guaiacol calibration curve for estimation of phenol content of samples

Fig.3



acid, 70:20:10 v/v/v. Cinnamic acid has an Rf of 0.6 in this solvent while phenylalanine remains at the origin. The cinnamic acid was visualised under ultraviolet light, then TLC plates were cut into 0.5 cm strips and counted in toluene scintillant (0.3 cm^3).

PAL activity was expressed as percentage conversion rate to cinnamic acid, or more accurately as nmol of cinnamic acid produced mg^{-1} protein.

Chapter 3

XYLOGLUCAN BIOSYNTHESIS BY A PARTICULATE ENZYME PREPARATION FROM PHASEOLUS VULGARIS:- PRELIMINARY INVESTIGATION USING HYPOCOTYLS

I. Incorporation of Radioactivity from Four Radioactively-Labelled Sugar Nucleotides by a Particulate Enzyme Preparation from Phaseolus vulgaris

The biosynthesis of xyloglucan from UDP-glucose and UDP-xylose has previously been studied by Hayashi and Matsuda (1981a) in suspension-cultured soybean cells. A modified version of their method was used to investigate incorporation of glucose, xylose, galactose and fucose from UDP-D-[U^{14}C]glucose, UDP-D-[U^{14}C]xylose, UDP-D-[U^{14}C]galactose and GDP-D-[U^{14}C]fucose by a bean hypocotyl particulate enzyme preparation. Thus HEPES buffer (100 mmol.dm^{-3}) was used at pH 6.8 for homogenisation and resuspension of membrane pellets. Incubation contents were as described (Ch.2 p.52) and incubations were terminated at 0, 30, 60 and 120 min. Radioactivity incorporated was recorded (Fig. 1a - d) and pmol incorporation at 60 min. calculated to facilitate comparison of incorporation levels (Table 1).

Incorporation generally increased with time up to 30 min. then levelled out. Incorporation of radioactivity from UDP-D-[U^{14}C] glucose and UDP-D-[U^{14}C]galactose was at a much higher level than from UDP-D-[U^{14}C] xylose and GDP-D-[U^{14}C] fucose.

Effect of UDP-xylose on incorporation of radioactivity from UDP-D-[U^{14}C] glucose

A particulate enzyme preparation from bean hypocotyls was incubated with two incubation mixtures for 0, 5, 15 or 30 min.

Figure 1

Time course of incorporation of radioactivity into hemicellulose by particulate enzyme preparation from bean hypocotyls, from four incubation mixtures:-

a) UDP-D-[$U^{14}C$]glucose (202 $\mu mol.dm^{-3}$, 1,850 Bq), $MnCl_2$ (10 $mmol.dm^{-3}$) and UDP-xylose (200 $\mu mol.dm^{-3}$)

b) UDP-D-[$U^{14}C$]xylose (201 $\mu mol.dm^{-3}$, 740 Bq) $MnCl_2$ (10 $mmol.dm^{-3}$) and UDP-glucose (2 $mmol.dm^{-3}$)

c) UDP-D-[$U^{14}C$]galactose (1.7 $\mu mol.dm^{-3}$, 1,850 Bq), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-glucose (20 $\mu mol.dm^{-3}$) and UDP-xylose (20 $\mu mol.dm^{-3}$)

d) GDP-D-[$U^{14}C$]fucose (2.5 $\mu mol.dm^{-3}$, 1,850 Bq), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-glucose (20 $\mu mol.dm^{-3}$), UDP-xylose (20 $\mu mol.dm^{-3}$) and UDP-galactose (20 $\mu mol.dm^{-3}$).

Fig.1a

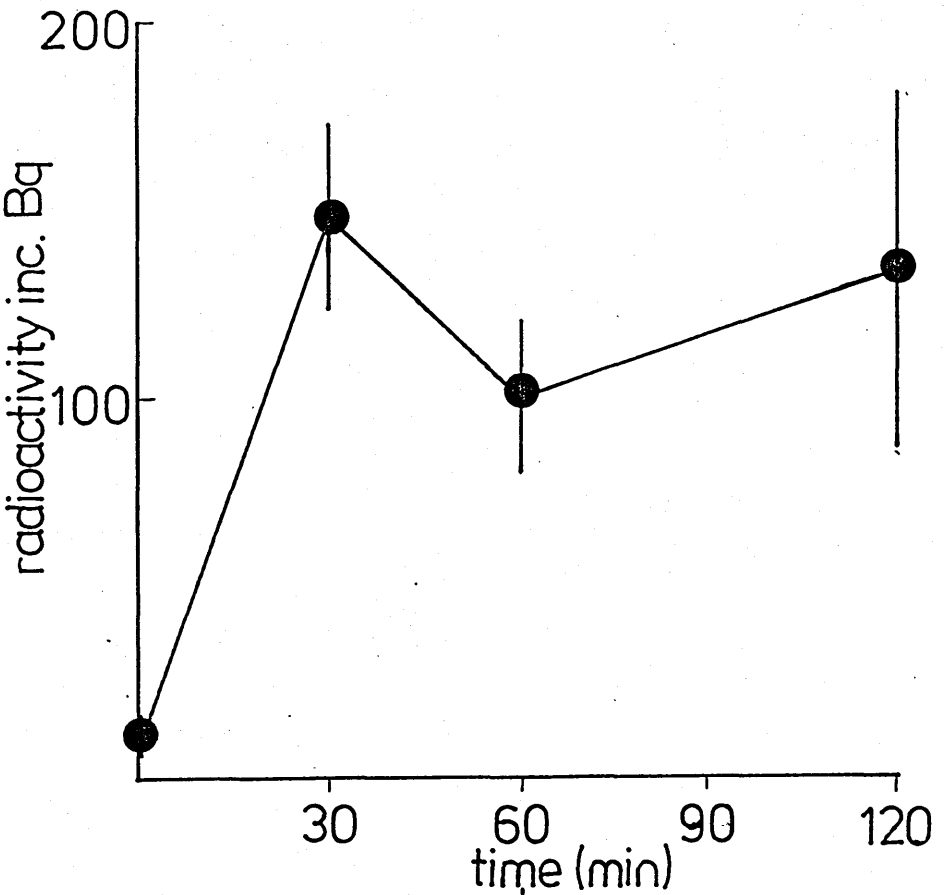

200
100
radioactivity inc. Bq
30
60
90
120
time (min)

Fig.1b

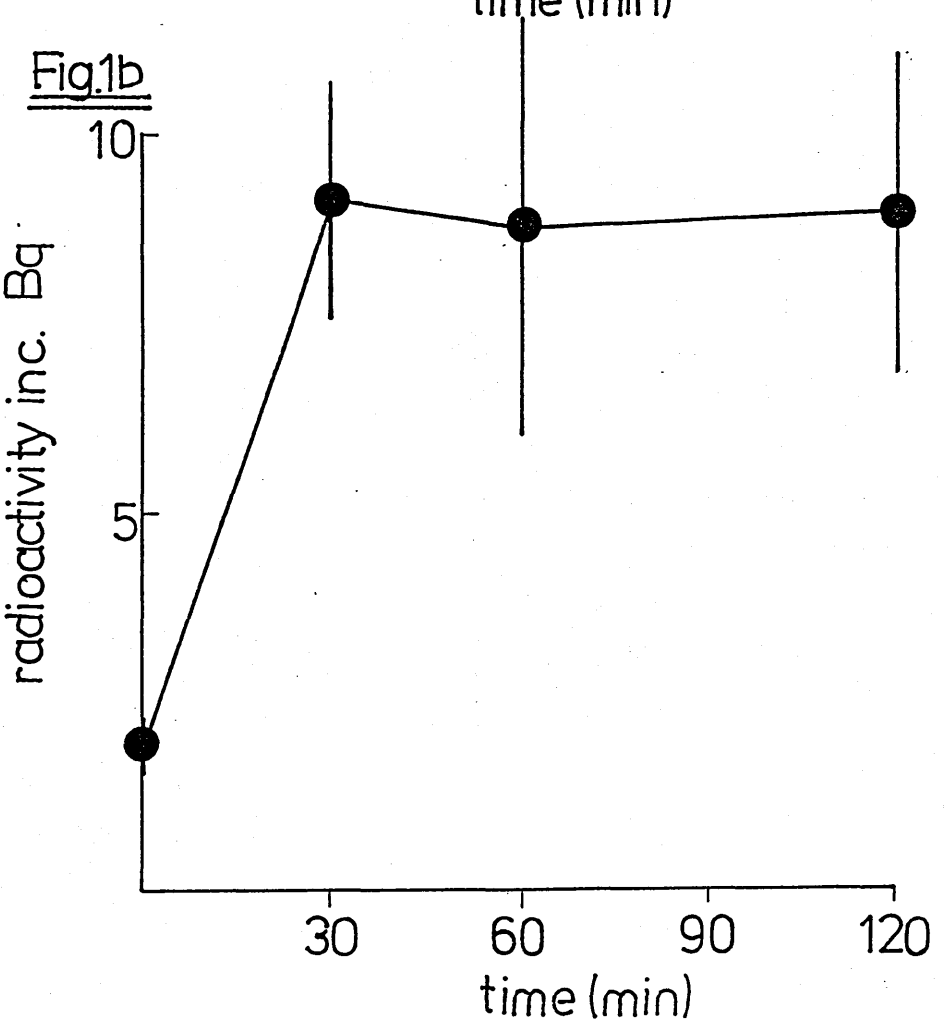

10
5
radioactivity inc. Bq
30
60
90
120
time (min)

Fig.1c

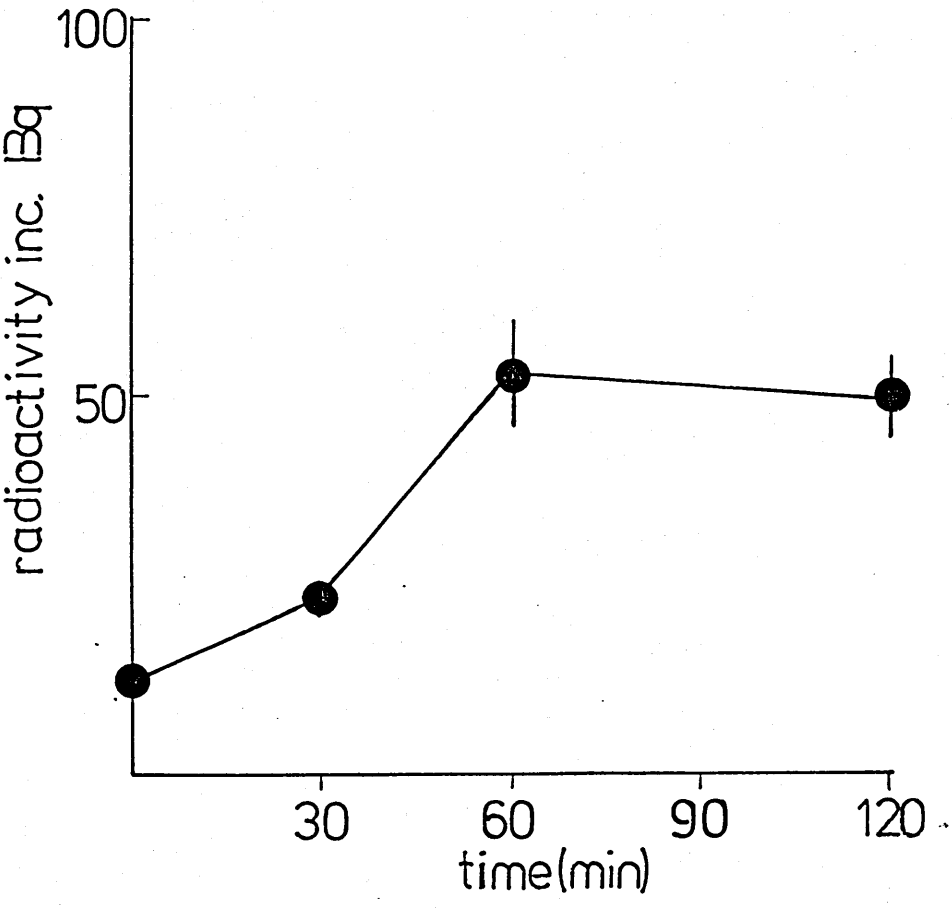

100
50
radioactivity inc. Bq
30
60
90
120
time(min)

Fig.1d

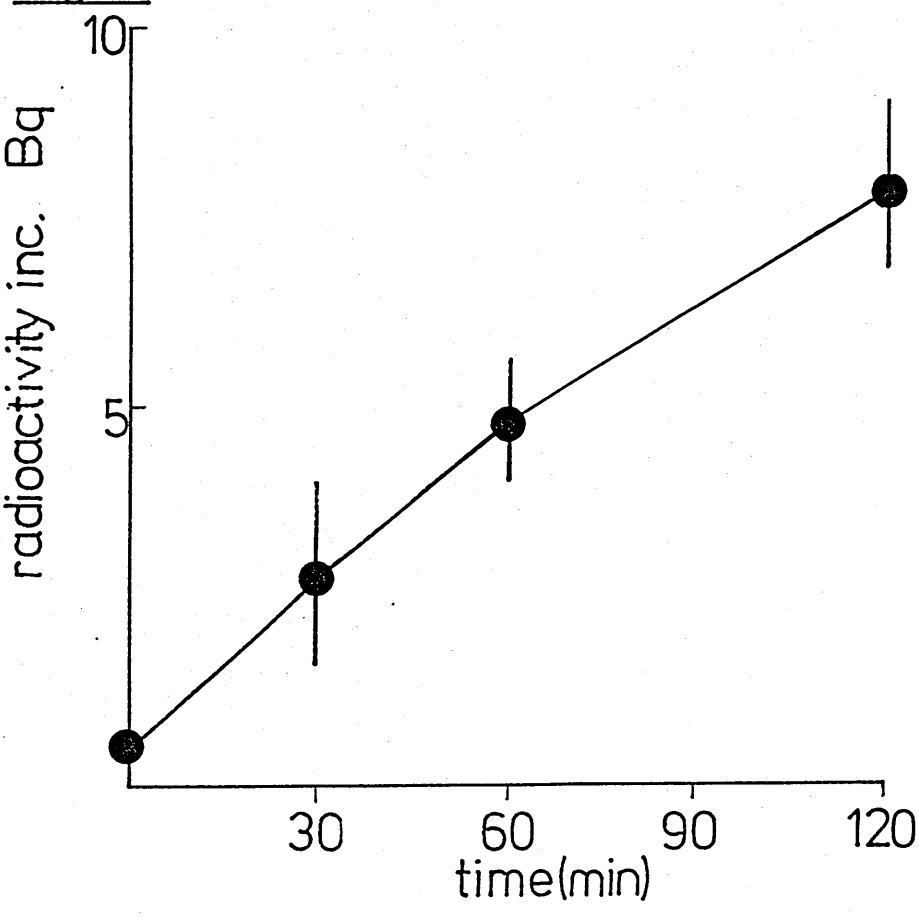

10
5
radioactivity inc. Bq
30
60
90
120
time(min)

Table 1

Incorporation at 60 min in pmol of monosaccharide from four radioactively-labelled sugar nucleotides by particulate enzyme preparation from bean hypocotyls (incubation mixture a-d as in Fig. 1).

Sugar nucleotide	pmol incorporated
UDP-D-[U^{14}C]glucose	1090 ± 266
UDP-D-[U^{14}C]xylose	240 ± 110
UDP-D-[U^{14}C]galactose	4.9 ± 0.56
GDP-D-[U^{14}C]fucose	0.66 ± 0.11

Incubations comprised:- UDP-D-[U^{14}C]glucose (0.77 μmol.dm^{-3}, 925 Bq), and $MnCl_2$ (10 mmol.dm^{-3}), or UDP-D-[U^{14}C]glucose (0.77 μmol.dm^{-3}, 925 Bq), UDP-xylose (20 μmol.dm^{-3} and $MnCl_2$ (10 mmol.dm^{-3}).

The presence of UDP-xylose in incubations seemed to reduce overall incorporation from UDP-D-[U^{14}C]glucose (Fig. 2a & b), and this may be due to competitive inhibition. However, as only one combination of nucleotide concentrations was used, the possibility of stimulation of incorporation from UDP-D-[U^{14}C]glucose by UDP-xylose cannot be excluded.

Optimisation of incubation conditions for maximum incorporation of radioactivity

Attempts were made to increase incorporation and to decrease zero time values:- cations and cation concentrations were varied, sugar nucleotide concentrations and buffer pH were altered, and a variety of buffers including HEPES, MES, MOPS and TES, each at 0.1 mol.dm^{-3}, was tried. HEPES buffer consistently gave maximum incorporation from UDP-D-[U^{14}C]glucose, and GDP-D-[U^{14}C]fucose, but there was little difference between buffers for incorporation at 30 min from UDP-D-[U^{14}C]xylose (Fig. 3a-c). Incorporation levels from the standard incubation mixtures were not improved by varying sugar nucleotide concentrations, and varying cations and cation concentrations gave extremely variable and inconsistent results, with no two enzyme preparations exhibiting the same properties: Fig. 4(a & b) for example contrasts the effects of combinations of cations on

Figure 2

Time course of incorporation of radioactivity from UDP-D-[U^{14}C] glucose into hemicellulose by a particulate enzyme preparation from bean hypocotyls in the presence (●—●) and absence (○—○) of UDP-xylose (20 μmol.dm^{-3}).

Fig. 2

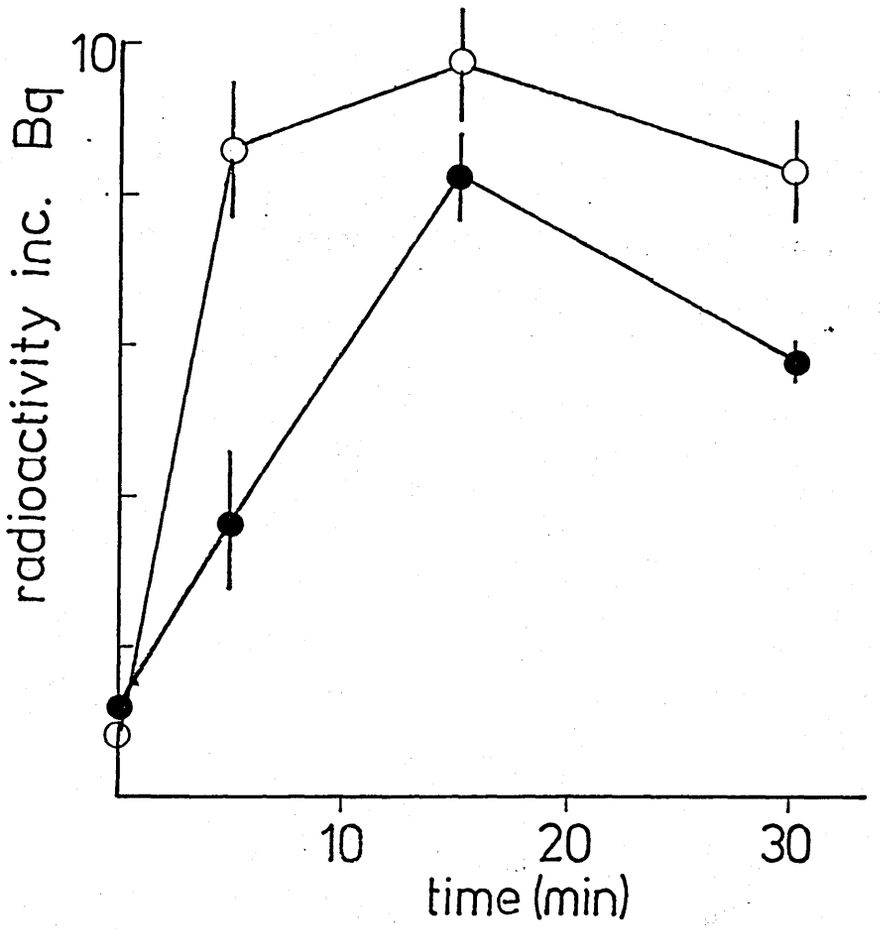


Figure 3

Incorporation of radioactivity into hemicellulose by a particulate enzyme preparation from bean hypocotyls from 60 min standard incubations with:-

a) UDP-D-[U^{14}C]glucose

b) GDP-D-[U^{14}C]fucose

c) UDP-D-[U^{14}C]xylose

using a. HEPES, b. MES c. MOPS or d. TES as resuspension and homogenisation buffer.

Fig.3a

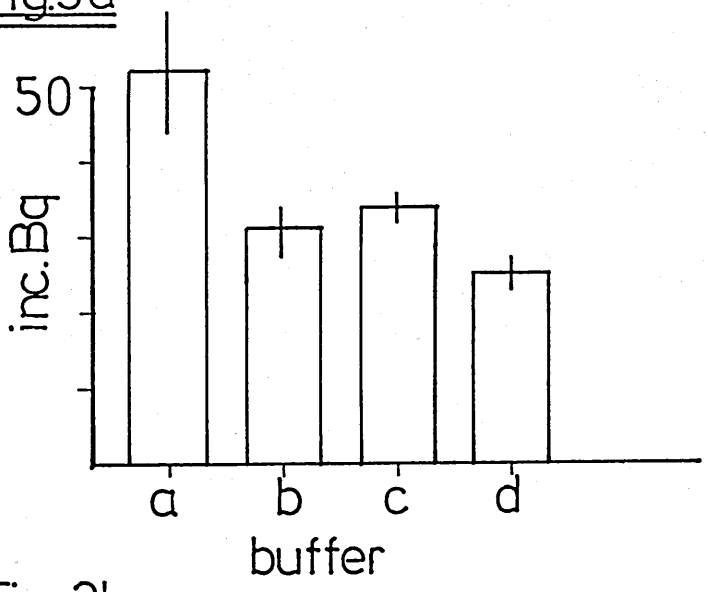


Fig.3b

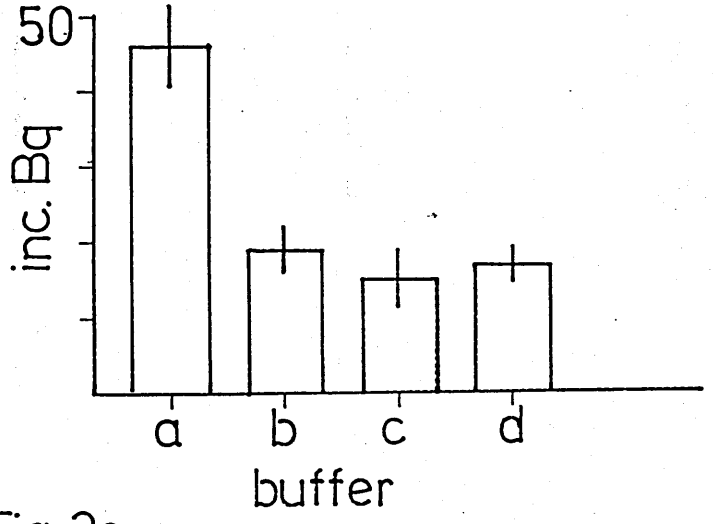
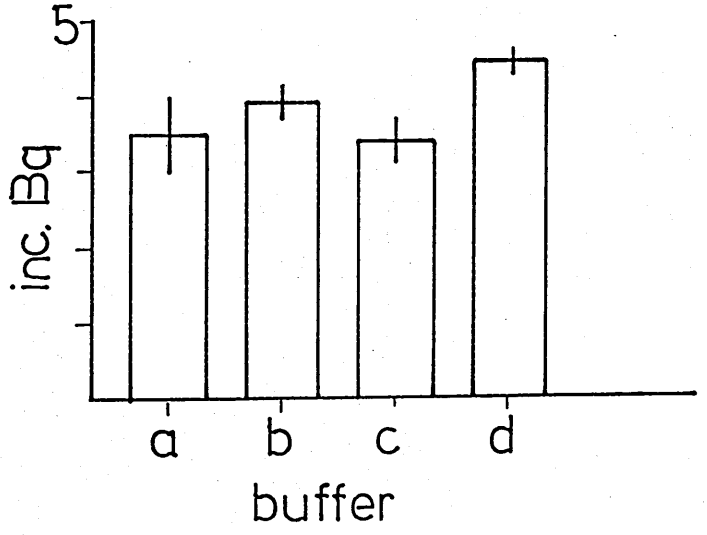


Fig.3c



incorporation at 60 min. from three sugar nucleotides, using two different enzyme preparations. The only case where a similar pattern of incorporation was observed for the two enzyme preparations was where incorporation from UDP-D-[U^{14}C]xylose was measured (Fig. 4c), and in this case overall incorporation was too low for any conclusions to be drawn. Results for incorporation from UDP-D-[U^{14}C]glucose (Fig. 4a) and GDP-D-[U^{14}C]fucose (Fig. 4b) were extremely variable.

The effect of freezing the particulate enzyme preparation at -15°C for 48 hours was studied. Incorporation from UDP-D-[U^{14}C] glucose was reduced by between 15 and 40%, and it was concluded that enzyme preparations should be freshly prepared for maximum activity.

II. Characterisation of Enzyme Incubation Products

Estimation of molecular weights of UDP-D-[U^{14}C]glucose and UDP-D-[U^{14}C] galactose incubation products

Pellets retained for analysis from the previous time course experiments were hydrolysed with a pea cellulase preparation, obtained by a modification of the method of Byrne et al (1975) (Ch.2 p.56). Radioactively-labelled pellets from 60 min. incubations with UDP-D-[U^{14}C]glucose and UDP-D-[U^{14}C]galactose were incubated as described, (Ch.2 p.58) with enzyme preparation or with buffer alone as a control. Enzyme hydrolysates and controls were run down a 1 x 50 cm Sephadex G-25 gel-filtration column. Samples of 0.5 cm^3 were collected and counted for radioactivity.

Figure 4

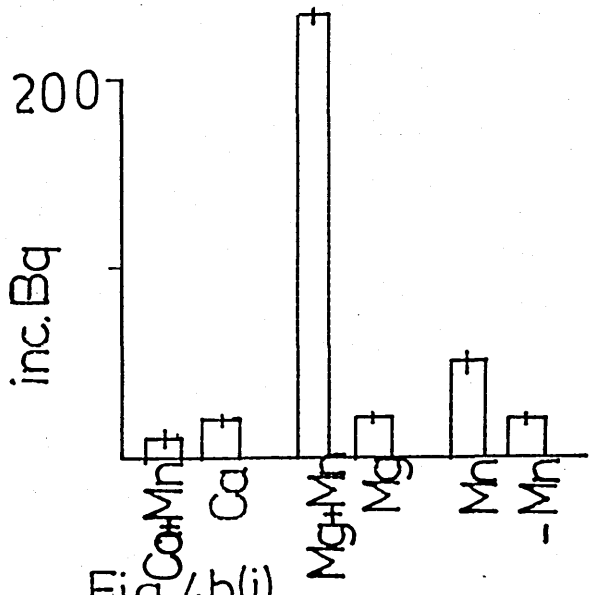
Incorporation of radioactivity into hemicellulose by two particulate enzyme preparation (i and ii) from bean hypocotyls using 60 min standard incubations with varying concentrations with:-

a) GDP-D-[U^{14}C]fucose

b) UDP-D-[U^{14}C]glucose

c) UDP-D-[U^{14}C]xylose

Fig.4a(i)



4a(ii)

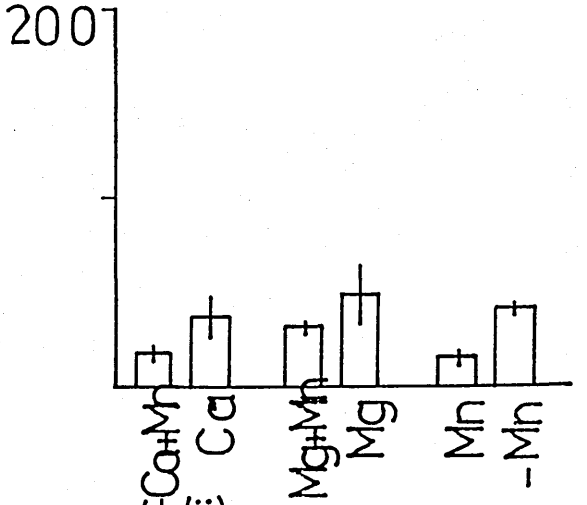
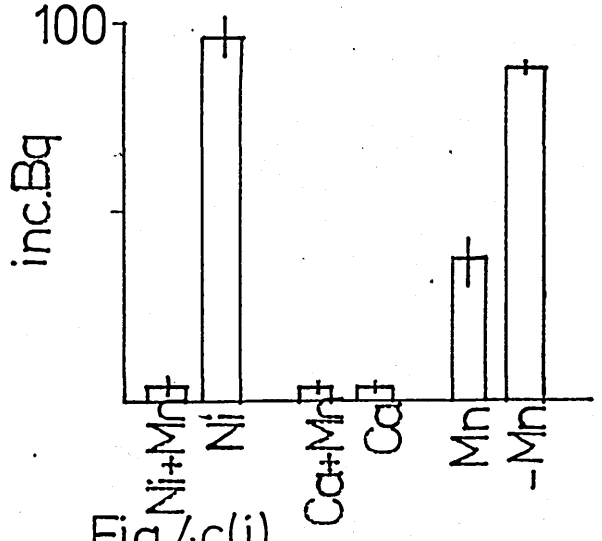


Fig.4b(i)



4b(ii)

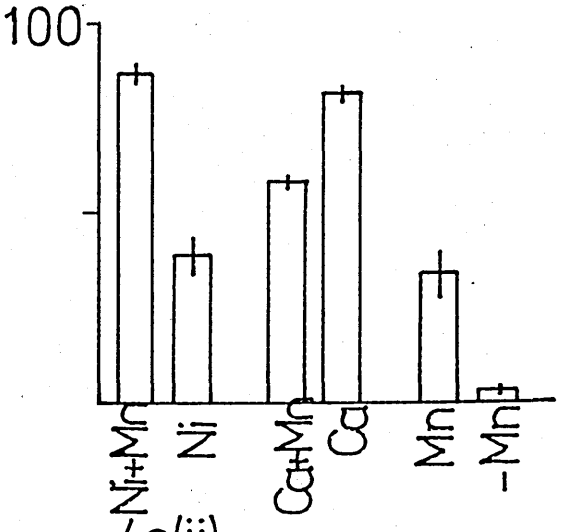
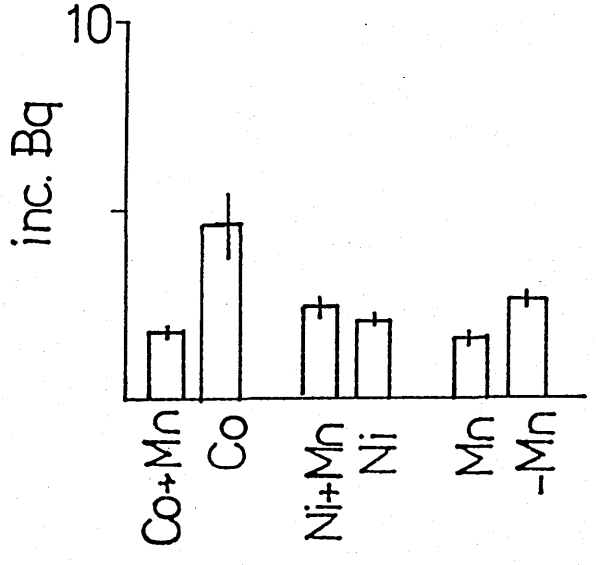
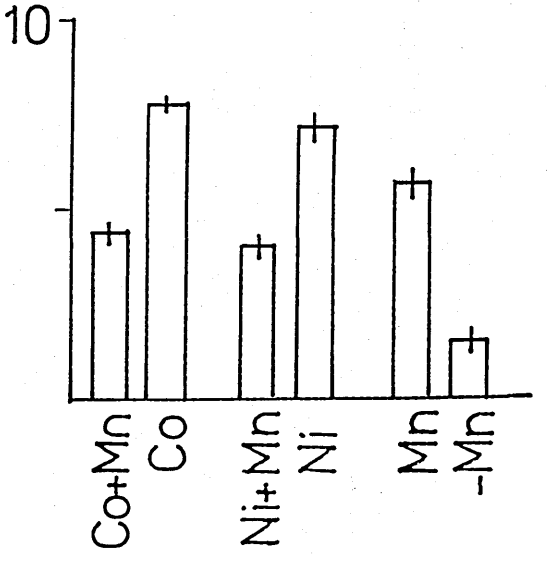


Fig.4c(i)



4c(ii)



Results (Fig.5a & b) indicated that a high molecular weight product is being broken down into a range of smaller molecular weight products in the presence of the cellulase enzyme. Hayashi et al (1981) have indicated that on digestion with Aspergillus oryzae enzymes, xyloglucan polymers synthesized in a cell-free system from suspension-cultured soybean cells gave a [^{14}C]disaccharide with a chromatographic mobility identical to isoprimeverose (6 - O -α- D - xylopyranosyl-D-glucopyranose). The gel-filtration column however did not give clear enough resolution to allow identification of enzymic breakdown products. Paper chromatography was therefore employed as a means of separation.

Descending paper chromatography of enzyme-hydrolysed [^{14}C]glucose - and [^{14}C]galactose-labelled incubation products.

Radioactively-labelled pellets from time course incubations of 60 min. duration and containing approx. 25 Bq, were incubated with pea cellulase preparation which had first been dialysed to remove glycerol and glucose (Ch.2 p. 58). Following incubation and deionisation (Ch.2 p. 63) samples were run for 36 hours in solvent A with glucose and malto-oligosaccharide starch hydrolysates as markers (Ch.2 p.60). Resulting radioactive peaks (Fig. 6a & b) suggest most of the radioactivity was in a monosaccharide peak in both samples, with a smaller, higher molecular weight peak in the [^{14}C]glucose-labelled sample.

In a further experiment, samples from incubations of 15, 30 and

Figure 5a

Sephadex G-25 profile of buffer (●——●) and pea cellulase (○——○) incubated hemicellulose (labelled with radioactivity from UDP-D-[U^{14}C]glucose during 60 min standard incubations

Fig.5a

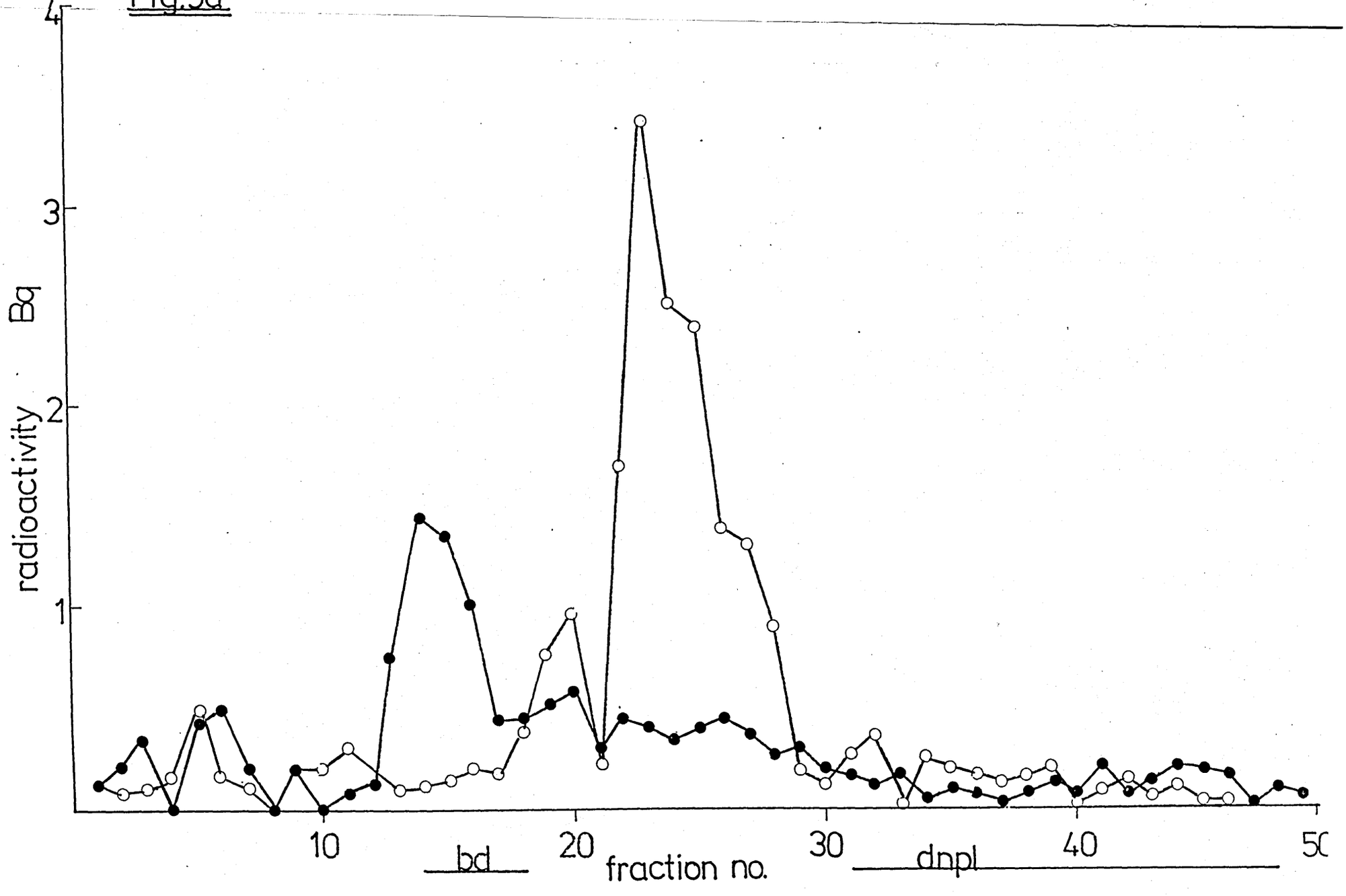


Figure 5b

Sephadex G-25 profile of buffer (●——●) and pea cellulase (○——○) incubated hemicellulose (labelled with radioactivity from UDP-D-[$U^{14}C$]galactose during 60 min standard incubations.

Fig.5b

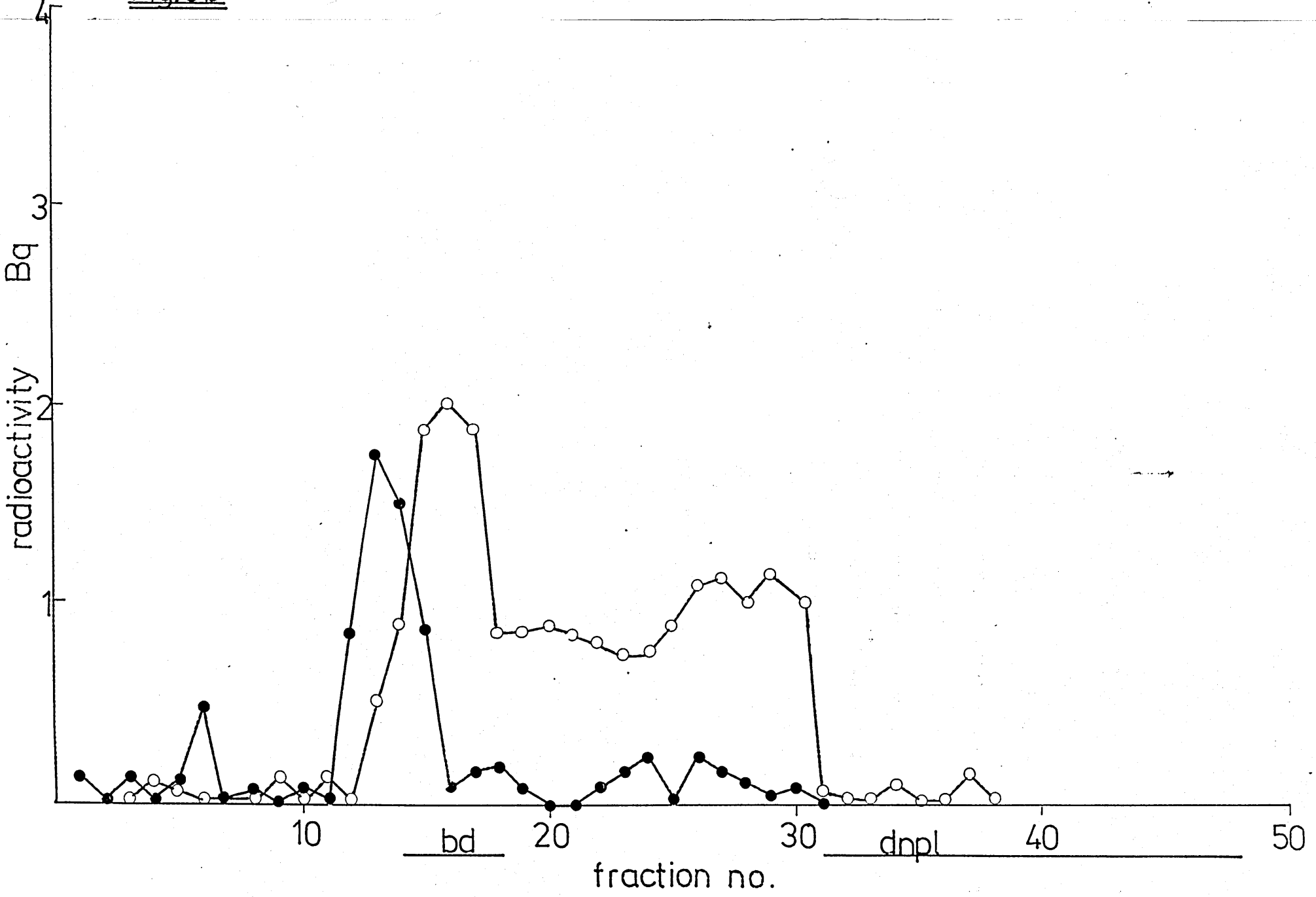


Figure 6

Paper chromatographic analysis of 36 h pea cellulase hydrolysates of hemicellulose labelled with radioactivity incorporated from:-

a) UDP-D-[U^{14}C] glucose

b) UDP-D-[U^{14}C] galactose

Fig.6a

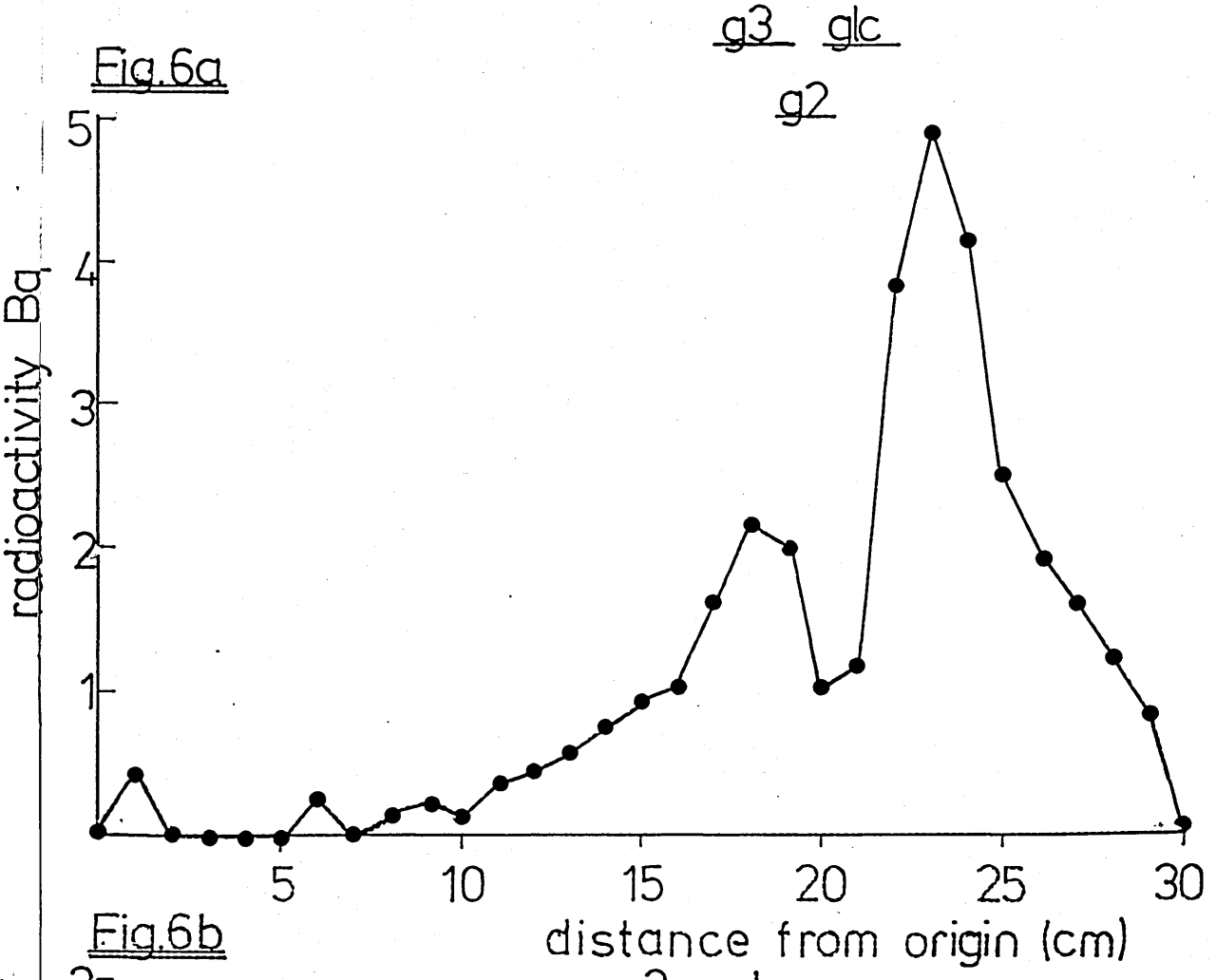
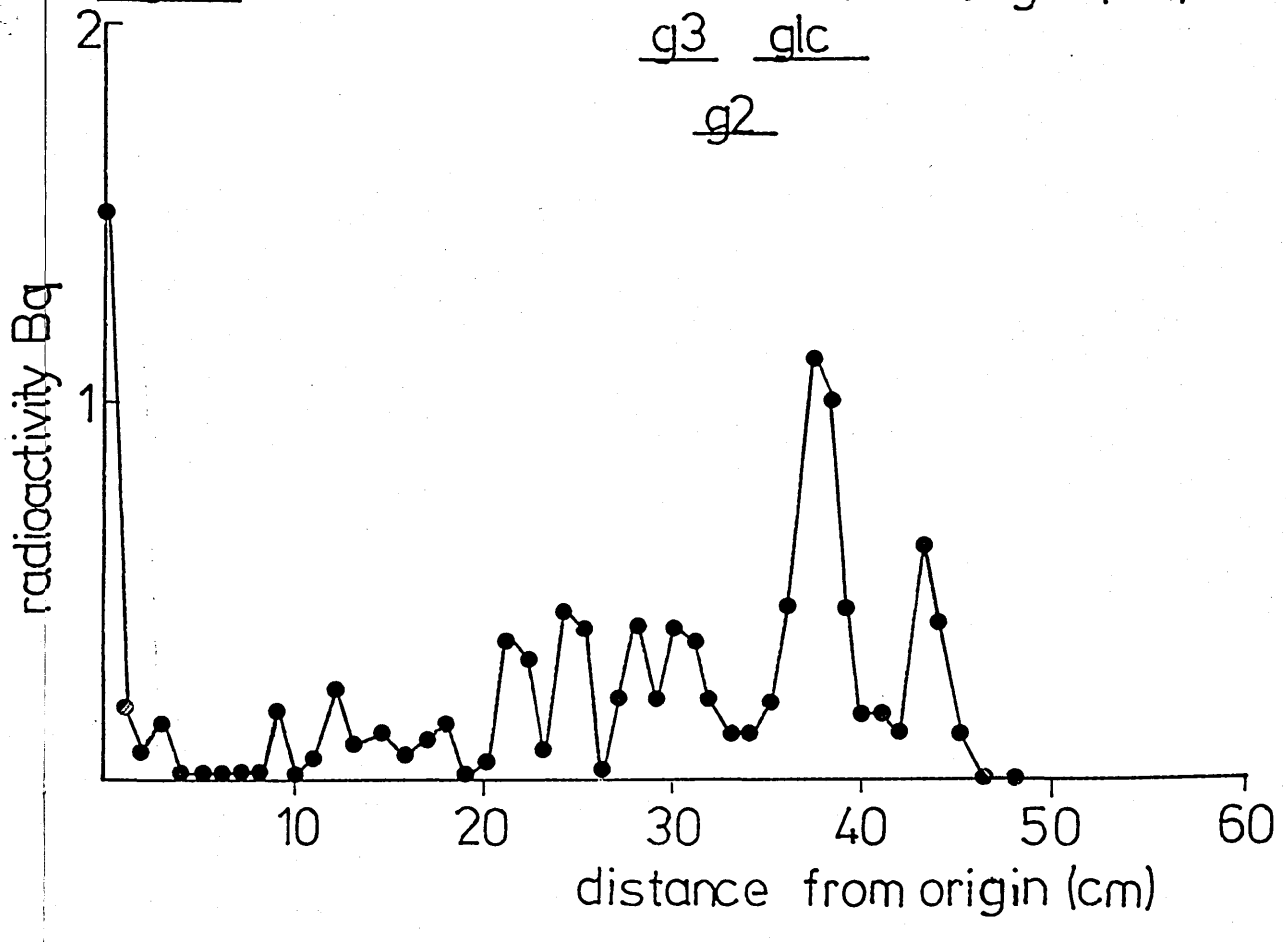

g3 glc
g2
radioactivity Bq
1
2
3
4
5
5
10
15
20
25
30
distance from origin (cm)

Fig.6b


g3 glc
g2
radioactivity Bq
1
2
10
20
30
40
50
60
distance from origin (cm)

60 minutes duration were hydrolysed and analysed by paper chromatography as in the previous experiment. A disaccharide co-chromatographing with a cellobiose marker was present at higher levels in 15 minute incubation products than in 30 and 60, where more monosaccharides were observed. Resolution achieved using this chromatography system however was probably inadequate for accurate product identification.

Estimation of GDP-D-[U^{14}C]fucose incubation product molecular weight

Unlike galactose and glucose which are present in a variety of plant polysaccharides, fucose has been reported only as a constituent of some plant xyloglucans (Aspinall et al, 1977, Bauer et al, 1980), and of glycoprotein (Hori et al, 1985). Characterisation of the ^{14}C fucose-labelled product was therefore attempted by molecular weight estimation and then by proteinase digestion. The product was partially soluble in water, and on running down a 15 x 1 cm sephadex G100 column it eluted with the blue dextran marker (Fig.7) indicating a molecular weight of around 100,000 Daltons.

Protease digestion of [^{14}C]fucose-labelled incubation product

Two [^{14}C]fucose-labelled samples from 60 minute incubations, containing approx. 30 Bq were incubated with Proteinase K or buffer as described (Ch.2 p. 59). Following centrifugation radioactivity in pellets and supernatants was counted (Table 2).

The majority of radioactivity was present in the pellet in both buffer- and enzyme-treated samples, indicating little or no breakdown of the product.

Figure 7

Saphadex G-100 profile of [^{14}C]fucose-labelled hemicellulose product from 60 min standard incubation of GDP-D-[U^{14}C] fucose with particulate enzyme preparation from bean hypocotyl.

Fig.7

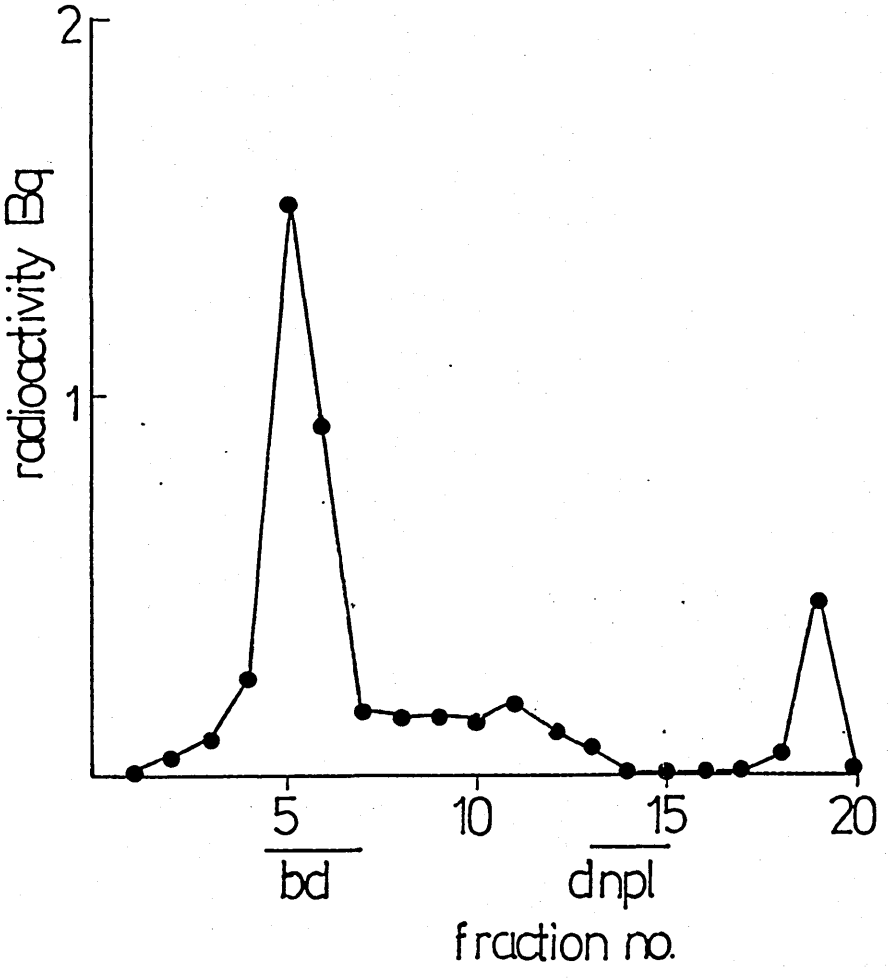


Table 2

Protease digestion of [^{14}C]fucose-labelled hemicellulose product from a 60 min standard incubation of GDP-D-[U^{14}C]fucose with particulate enzyme preparation from bean hypocotyl, incubated with a. enzyme or b. buffer.

	Solution	Radioactivity Bq	% breakdown by enzyme
a.	Supernatant (enz. inc.)	3.8	16.4%
	Pellet (enz. inc.)	23.2	
b.	Supernatant (buf. inc.)	2.4	10.5%
	Pellet (Buf. inc.)	22.7	

III. Preliminary Differentiation Experiment:- Enzyme Activity in Different Parts of Bean Hypocotyls

Enzyme activity in actively growing and mature tissue was compared. Particulate enzyme preparations were made using the top or bottom 2 cm of 7-8 day old dark-grown hypocotyls (total length approx. 8 cm). Preparations were made with 25 g tissue in 75 ml buffer as in the standard preparation. Incubations, which were of 15 minutes duration, were performed using the standard procedure, with UDP-D-[$U^{14}C$]glucose, UDP-D-[$U^{14}C$]galactose, UDP-D-[$U^{14}C$]xylose or GDP-D-[$U^{14}C$]fucose.

Clear differences in enzyme activities are apparent (Fig. 8a-d):- incorporation from UDP-D-[$U^{14}C$]glucose was about doubled in bottom sections compared with top sections; that from UDP-D-[$U^{14}C$]galactose was roughly similar in top and bottom sections; incorporation from both UDP-D-[$U^{14}C$]xylose and GDP-D-[$U^{14}C$]fucose was much higher in top sections, being almost negligible in bottom sections.

Discussion

The time courses of incorporation from four sugar nucleotides (Fig. 1a-d) suggest that in bean hypocotyls far more glucose and galactose are incorporated into hemicellulose than xylose and fucose. This may reflect the wide variety of cell wall polysaccharides into which these sugars are incorporated. It may also be that the particulate enzyme preparation and incubation contents are more

Figure 8

Time course of incorporation of radioactivity from bean particulate enzyme preparations from top (■—■) and bottom (□—□) sections of hypocotyls, from incubations with:-

a) UDP-D-[U^{14}C]glucose

b) UDP-D-[U^{14}C]galactose

c) UDP-D-[U^{14}C]xylose

d) GDP-D-[U^{14}C]fucose

Fig.8a

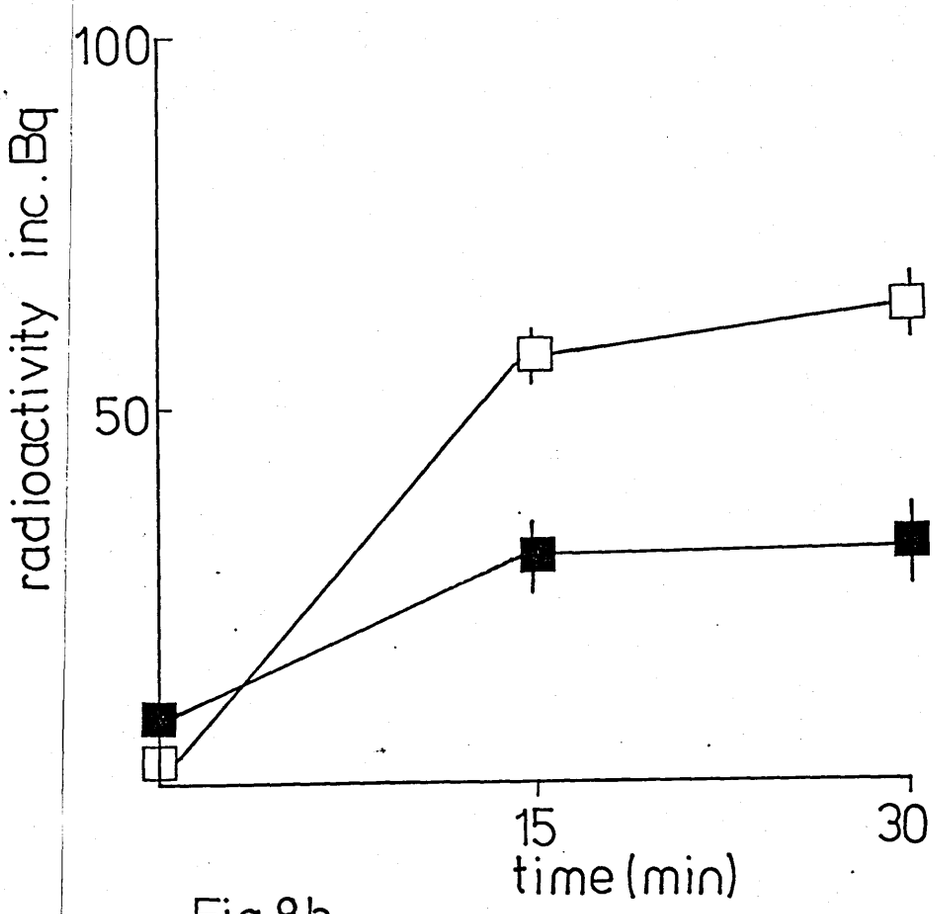

100
50
radioactivity inc.Bq
15
30
time (min)

Fig.8b

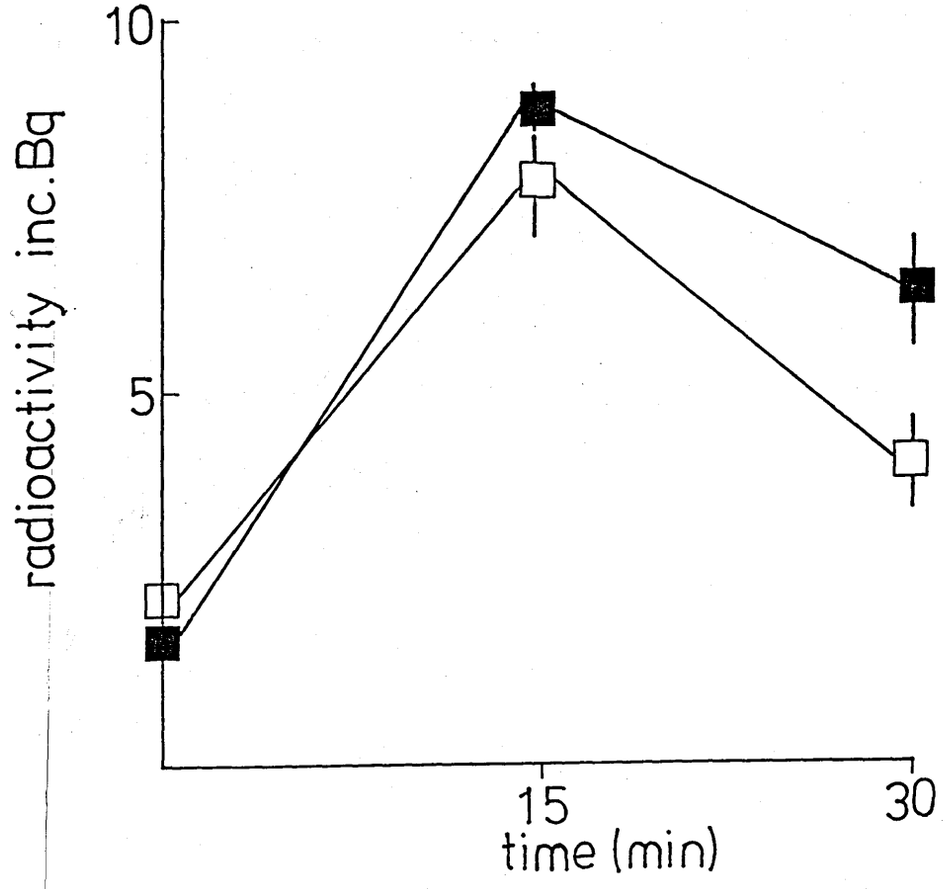

10
5
radioactivity inc.Bq
15
30
time (min)

Fig.8c

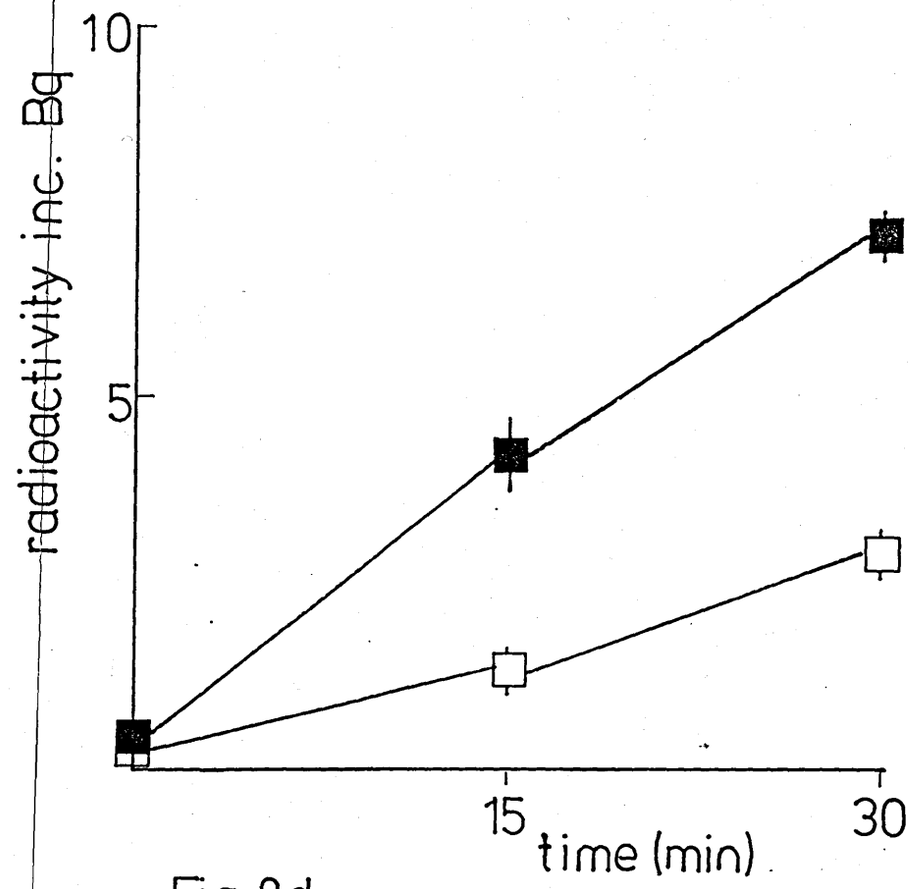
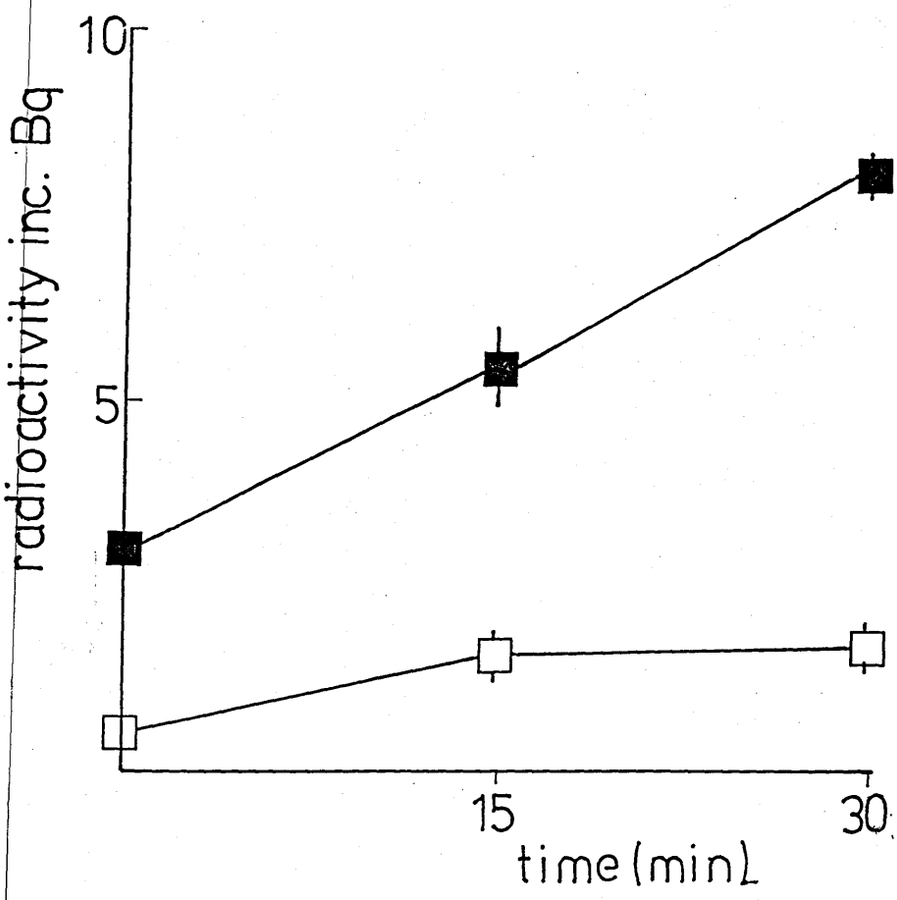

radioactivity inc. Bq
10
5
15
30
time (min)

Fig.8d


radioactivity inc. Bq
10
5
15
30
time (min)

favourable for the galactosyl- and glucosyl-transferases than for the fucosyl- and xylosyltransferases. A further possibility is that glucose and galactose are transferred from the sugar nucleotides into the same product/products due to epimerase activity causing inter-conversion of sugars.

Stimulation of incorporation from UDP-D-[$U^{14}C$]glucose by UDP-xylose was reported by Ray (1980), who studied xylosyl and glucosyl transfer by Golgi membranes from pea epicotyls. A UDP-xylose concentration of 0.15 $\mu mol dm^{-3}$ stimulated incorporation from UDP-D-[$U^{14}C$]glucose by a factor of up to five. No such stimulation was observed for the bean hypocotyl system, suggesting a different product is being made. This is probably due to the differences between the experimental systems i.e. Ray used a more specific area of plant, namely apices or first node, rather than whole epicotyls; secondly, the enzymes involved were localised on Golgi membranes, extracted by use of differential centrifugation using sucrose gradients. A further factor is that the properties of the pea and bean enzymes may differ.

Variation between enzyme activities in preparations from different batches of hypocotyls was highlighted during attempts to optimise conditions for maximal incorporation. The vast variation in results from different preparations made it impossible to optimise any incubation conditions, other than buffer used, despite using hypocotyls of identical maturity.

Characterisation of the products of incubations with bean hypocotyl particulate preparations is obviously complicated by variation in activities of different preparations. Products of incubations with UDP-D-[$U^{14}C$]glucose and UDP-D-[$U^{14}C$]galactose are apparently of high molecular weight, and can be hydrolysed by cellulase preparations (Figs. 5 & 6) to yield mainly oligo- and monosaccharides. The product of incubation with GDP-D-[$U^{14}C$]fucose is evidently not a glycoprotein and is of high molecular weight (Fig. 7), suggesting it may be xyloglucan. Low or variable incorporation rates made it impossible to identify the products more fully.

A preliminary experiment to investigate changes occurring during differentiation showed clear differences in incorporation from different parts of the hypocotyl. Thus fucose and xylose showed higher incorporation levels in top sections than bottom sections (Fig. 8c-d), galactose showed little difference, and glucose showed higher incorporation in bottom sections. These results probably reflect the different compositions of cell walls in these different hypocotyl regions. The top sections are composed of a higher percentage of primary walls whereas the more mature bottom sections are composed of a higher percentage of secondary wall with a different polysaccharide composition. Xyloglucan, for example, is regarded as a component of primary walls, and the higher levels of incorporation from UDP-xylose and GDP-fucose in top sections may represent incorporation into xyloglucan.

The results of the differentiation experiment and preceding

experiments indicate the potential advantages of using tissue in which primary walls predominate for investigating xyloglucan bio-synthesis. Such tissue can be provided by suspension-culture, which also has the advantage of using one cell line only, eliminating much of the variation exhibited by the hypocotyl preparations. The techniques and experiments in this chapter were adapted for use with suspension-cultured bean tissue in subsequent investigations, using calluses derived from bean hypocotyl, and were developed to allow a more thorough investigation of the enzymes involved in the synthesis of xyloglucan, in particular the xylosyltransferase.

Chapter 4

INVESTIGATION OF THE XYLOGLUCAN XYLOSYL-TRANSFERASE USING A PARTICULATE ENZYME PREPARATION FROM SUSPENSION-CULTURED BEAN CELLS

The general principles of cell suspension-culture were described in Chapter 1 (p.37), and potential advantages in using such tissue for studying xyloglucan biosynthesis were outlined at the end of the preceding chapter. Before using such material it was necessary to determine optimum experimental conditions, so extraction procedure, buffer, effect of freezing and of culture age on enzyme activity were examined. At least one, and sometimes all four transferases were considered, and the results were found to be relatively consistent for all sugar nucleotides.

As reported in Methods (Ch.2 p.51), mechanical homogenisation was found to be unsuitable for suspension-cultured tissue, so material was hand ground with sand in a mortar.

i. Investigation of extraction procedure: time course of incorporation of radioactivity from UDP-D-[U^{14}C]glucose, UDP-D-[U^{14}C]galactose, UDP-D-[U^{14}C]xylose and GDP-D-[U^{14}C]fucose: a. hemicellulose extraction.

Incubations were performed with a particulate enzyme preparation from suspension-cultured bean cells, using the enzyme preparation and assays outlined in Ch. 2 (p.51), i.e.tissue was ground with a mortar and pestle, and Tris/HCl (pH 7.5, 100 mmol.dm^{-3}) was used instead of HEPES (buffers were compared in a later experiment). Incubations were terminated at 0, 15 and 30 min. and hemicellulose extraction was performed as in previous experiments.

Incorporation for all nucleotides increased with time (Fig. 1),

Figure 1

Time course of incorporation of radioactivity into hemicellulose by particulate enzyme preparation from suspension-cultured bean, from four incubation mixtures:-

(■——■) UDP-D-[U^{14}C]glucose (2.002 mmol.dm^{-3}, 925 Bq), $MnCl_2$ (10 mmol.dm^{-3}) and UDP-xylose (20 μmol.dm^{-3})

(▲——▲) UDP-D-[U^{14}C]xylose (21 μmol.dm^{-3}, 740 Bq), $MnCl_2$ (10 mmol.dm^{-3}) and UDP-glucose (2 mmol.dm^{-3})

(□——□) UDP-D-[U^{14}C]galactose (21 μmol.dm^{-3}, 925 Bq), $MnCl_2$ (10 mmol.dm^{-3}), UDP-glucose (20 μmol.dm^{-3}) and UDP-xylose (20 μmol.dm^{-3})

(●——●) GDP-D-[U^{14}C]fucose (1.25 μmol.dm^{-3}, 925 Bq), $MnCl_2$ (10 mmol.dm^{-3}), UDP-glucose (2 mmol.dm^{-3}), UDP-xylose (2 mmol.dm^{-3}) and UDP-galactose (2 mmol.dm^{-3})

Figure 2

Time course of incorporation of radioactivity into polysaccharide by particulate enzyme preparation from suspension-cultured bean from four incubation mixtures with:-

(■——■) UDP-D-[U^{14}C]glucose

(▲——▲) UDP-D-[U^{14}C]xylose

(□——□) UDP-D-[U^{14}C]galactose

(●——●) UDP-D-[U^{14}C]fucose

(Incubation mixtures as detailed in Fig. 1)

Fig.1

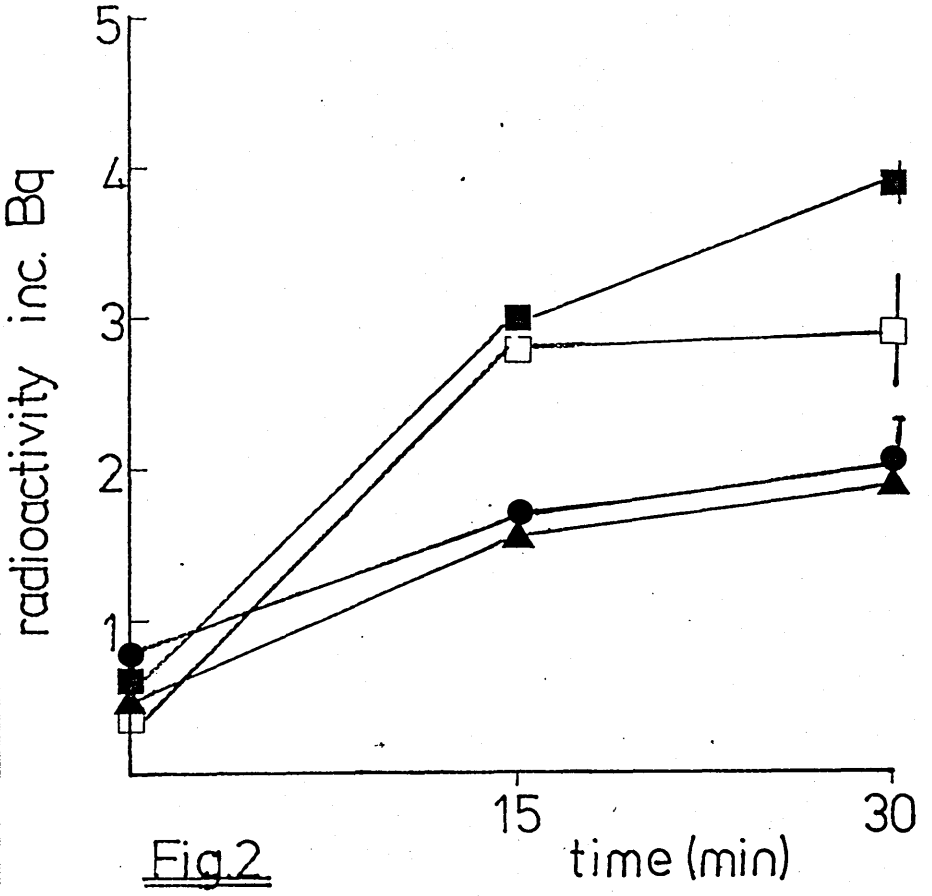
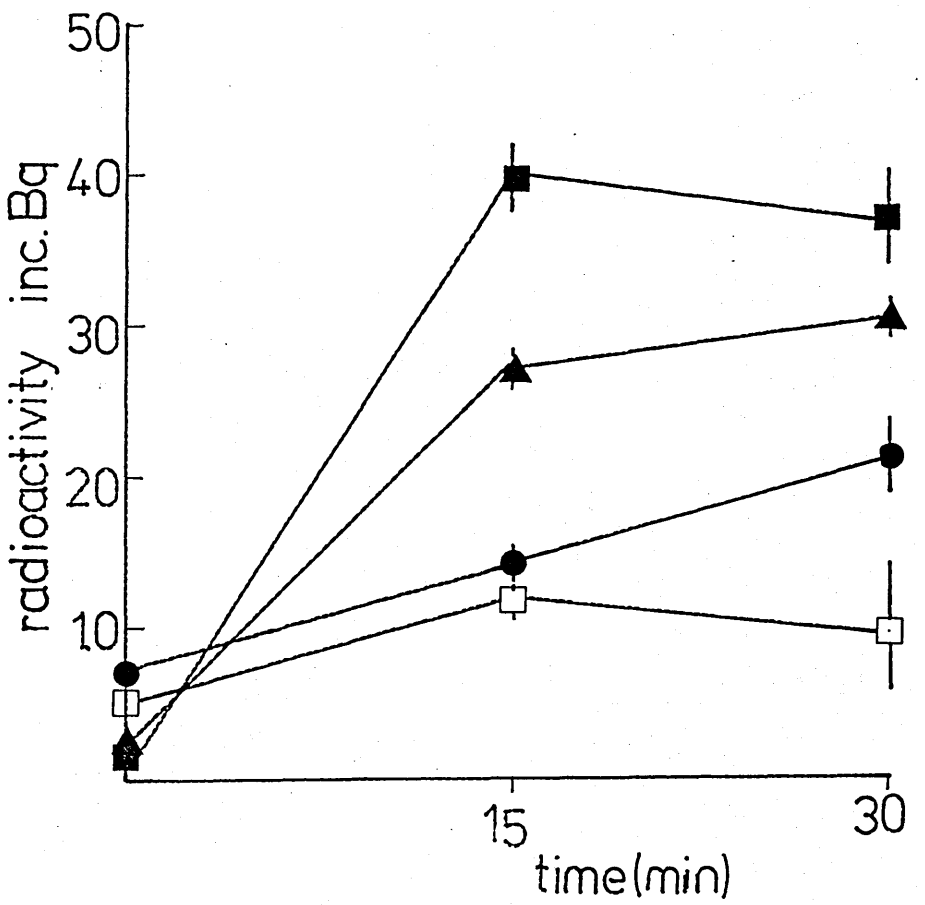

radioactivity inc. Bq
5
4
3
2
1
15
30
time (min)

Fig.2


radioactivity inc. Bq
50
40
30
20
10
15
30
time (min)

zero time values were low and replicates good. Overall incorporation however was very low (Table 1), suggesting the extraction procedure is too rigorous for suspension-cultured tissue, and may be extracting some polysaccharide product.

ii. Investigation of extraction procedure: time course of incorporation of radioactivity from four sugar nucleotides:

b. whole polysaccharide extraction

Incubations were performed as in the previous experiment. Following termination, five extractions with 70% ethanol (1 cm^3) were performed, followed by four with H_2O (1 cm^3).

Time courses of incorporation were similar for all sugar nucleotides (Fig.2) and replicates generally good. Incorporation levels were more than ten times those recorded following hemicellulose extraction (Table 2), except for UDP-galactose where levels were four times higher. Zero values were generally low. Incorporation from UDP-D-[$U^{14}C$]glucose and UDP-D-[$U^{14}C$]galactose dipped at 30 min, but a more detailed time course would be required to conclude anything from this.

iii. Comparison of incorporation of radioactivity from UDP-D-[$U^{14}C$] glucose and UDP-D-[$U^{14}C$]xylose using HEPES or Tris/HCl

Particulate enzyme preparations were made from suspension-cultured material, using either Tris/HCl or HEPES as homogenisation and resuspension buffer. Incubations were terminated at 0, 15 and 30 min.

Table 1

Incorporation at 30 min in pmol of monosaccharide from four radioactively-labelled sugar nucleotides into hemicellulose.

Sugar nucleotide	pmol incorporated
UDP-D-[U^{14}C]glucose	865 ± 123
UDP-D-[U^{14}C]xylose	5.6 ± 0
UDP-D-[U^{14}C]galactose	6.8 ± 2.72
GDP-D-[U^{14}C]fucose	0.24 ± 0.2

Table 2

Incorporation at 15 min in pmol of monosaccharide from radioactively-labelled sugar nucleotides into polysaccharide. Incubation mixtures as in Fig. 1.

Sugar nucleotide	pmol incorporated
UDP-D-[U^{14}C]glucose	3.5 ± 0.5
UDP-D-[U^{14}C]xylose	0.8 ± 0.2
UDP-D-[U^{14}C]galactose	1.1 ± 0.1
UDP-D-[U^{14}C]fucose	2.0 ± 0.5

While incorporation levels from UDP-D-[U^{14}C]glucose were similar for Tris/HCl and HEPES (Fig. 3a), incorporation from UDP-D-[U^{14}C] xylose was considerably greater where Tris/HCl was used (Fig. 3b). Tris/HCl was therefore chosen as the standard buffer for incubations with suspension-cultured tissue.

iv. Effect of suspension-culture age on incorporation from UDP-D-[U^{14}C] xylose and GDP-D-[U^{14}C]fucose

Bean suspension-cultures were routinely sub-cultured every 7-10 days, and could be maintained by sub-culture for several months. Enzyme activity was assumed to vary with time after sub-culture, so to determine on which day after sub-culture activity was maximal, enzyme preparations were made from flasks of different aged tissue. Flasks were set up with approx. equal amounts of tissue, and enzyme preparations were made at two day intervals up to 14 days. Incubations with UDP-D-[U^{14}C]xylose and GDP-D-[U^{14}C]fucose were performed (0 & 15 min duration) and incorporation was compared (Fig. 4a& b). For both sugar nucleotides incoporation was at a high level on day 2, then peaked again on day 8. As there is generally insufficient material for an enzyme preparation on day 2, it was decided to use tissue around 6-8 days after sub-culturing to give good enzyme activity and cell bulk.

v. Effect of freezing enzyme on incorporation of radioactivity from UDP-D-[U^{14}C]xylose

An enzyme preparation was made and incubations performed with the

Figure 3

Incorporation of radioactivity into hemicellulose by bean suspension particulate enzyme preparation using Tris/HCl (●—●) or HEPES (○—○) as homogenisation and resuspension buffer:-

incorporation from

a) UDP-D-[U^{14}C]glucose

b) UDP-D-[U^{14}C]xylose

using standard incubation conditions

Fig.3a

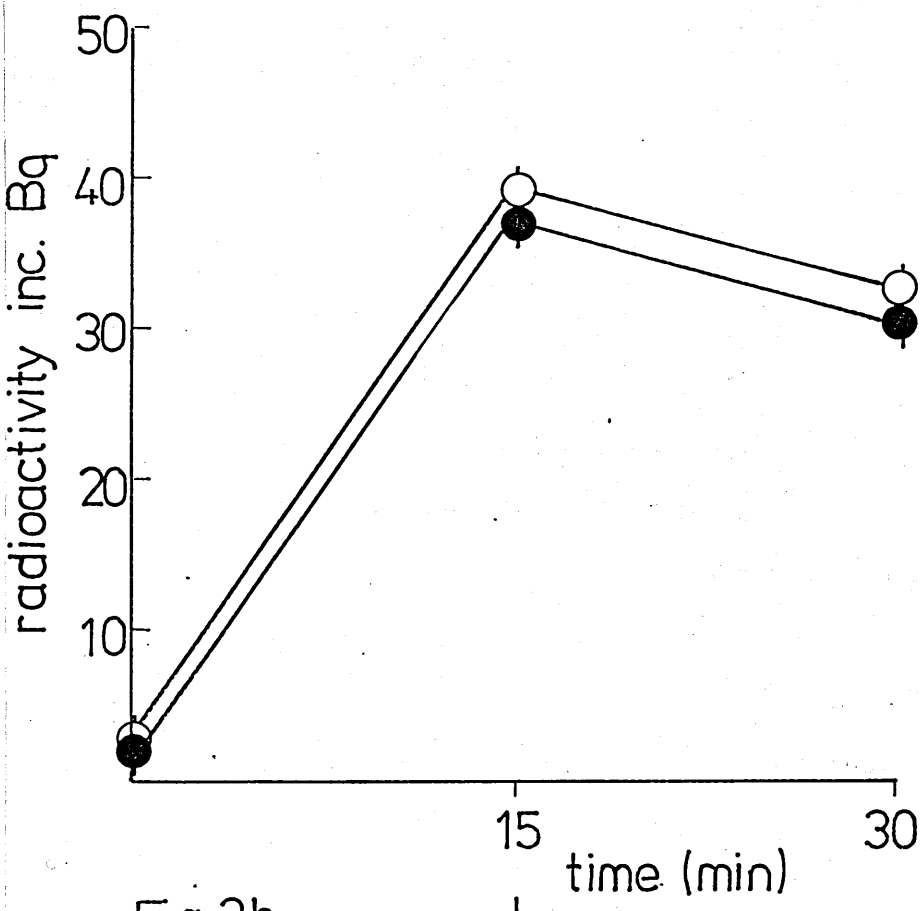

50
40
30
20
10
radioactivity inc. Bq
15
30
time (min)

Fig.3b

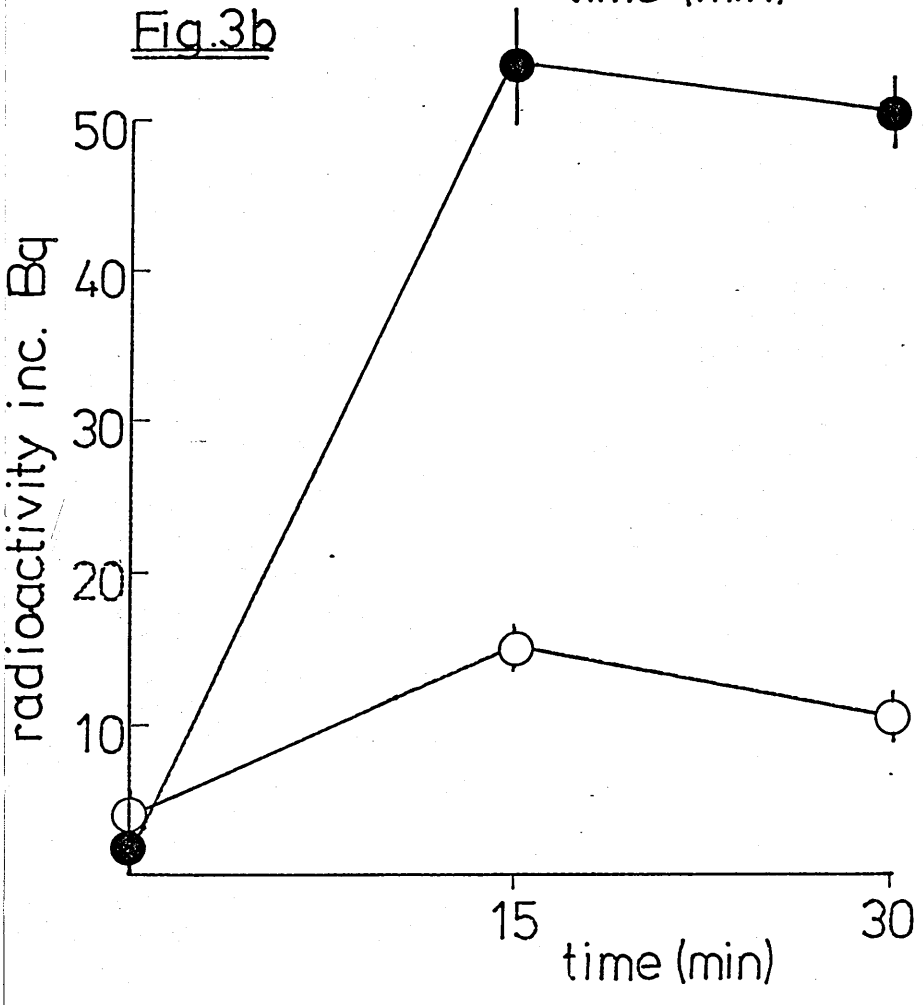

50
40
30
20
10
radioactivity inc. Bq
15
30
time (min)

Figure 4

Incorporation of radioactivity into hemicellulose by bean-suspension particulate enzyme preparation using material of increasing maturity: incorporation at 15 min, using standard incubation conditions from:-

a) UDP-D-[U ^{14}C] xylose

b) GDP-D-[U ^{14}C]fucose

Each incubation corresponded to approx. 3.5 g wet weight of tissue.

Fig. 4a

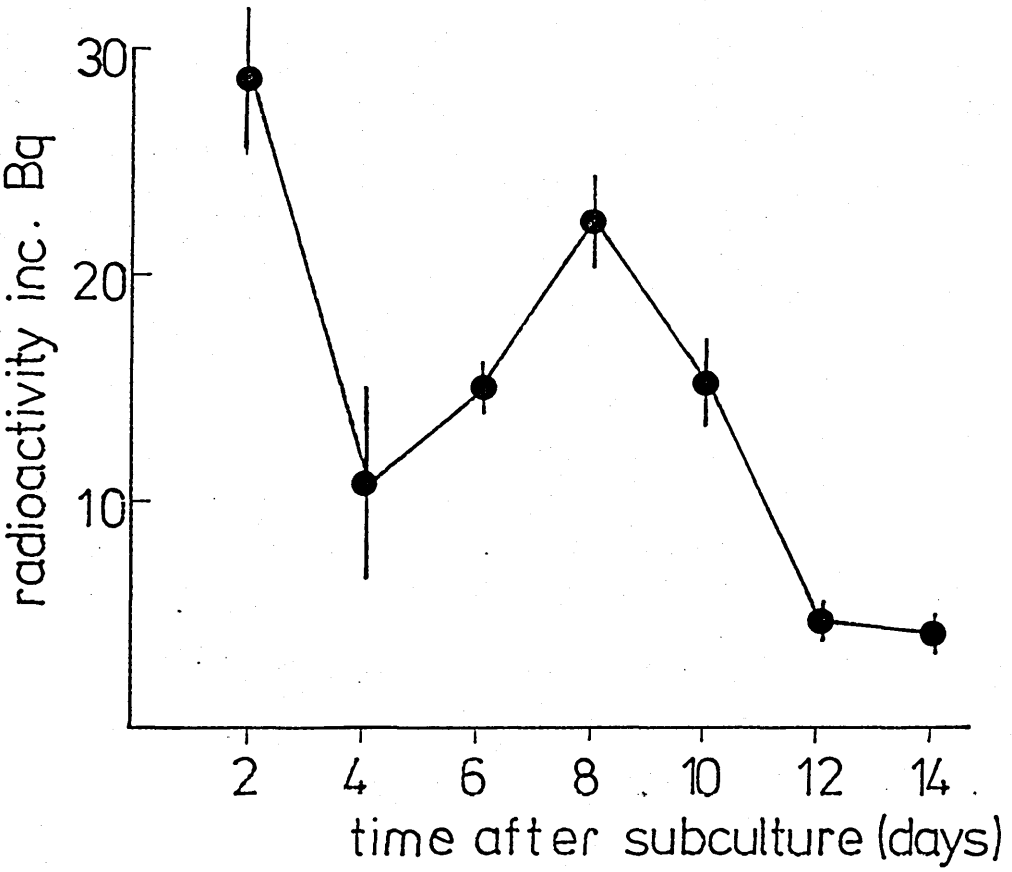
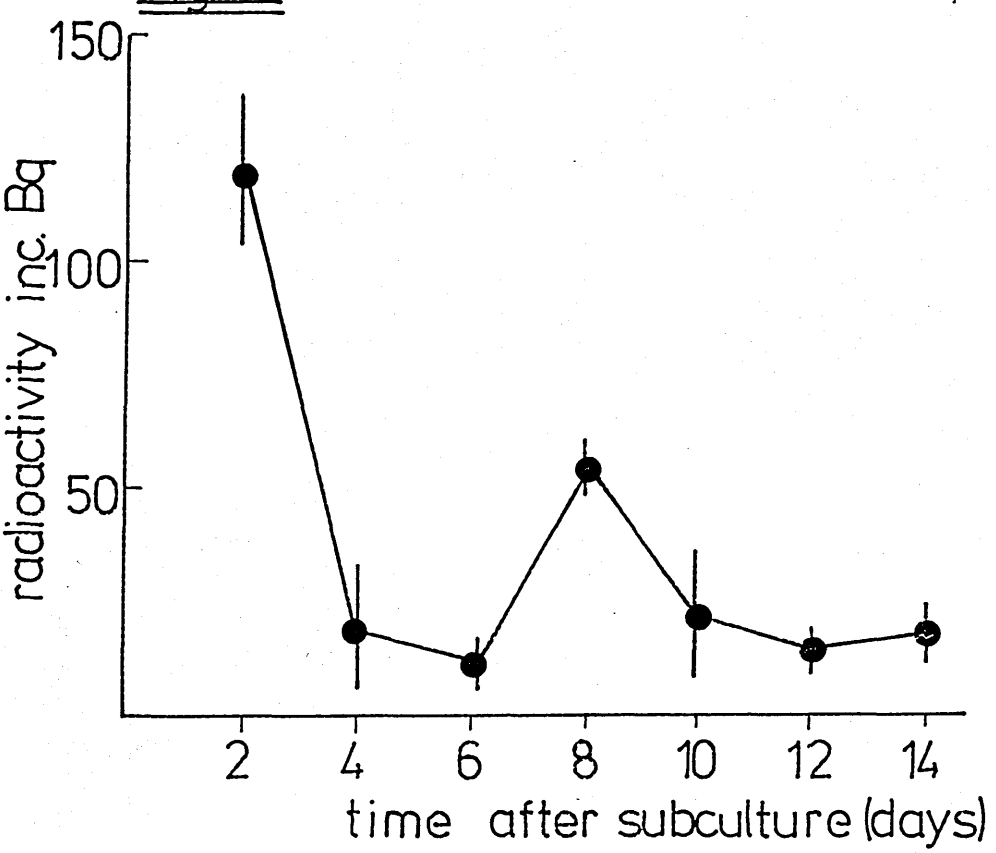

radioactivity inc. Bq
30
20
10
2
4
6
8
10
12
14
time after subculture (days)

Fig. 4b


radioactivity inc. Bq
150
100
50
2
4
6
8
10
12
14
time after subculture (days)

standard incubation mixture, with incubations terminated at 0, 15 and 60 min. Three sets of incubations were performed with the same particulate enzyme preparation, which was kept at -15°C between experiments. One set of incubations was performed with fresh enzyme, a second set 24 h later and the third 7 days later.

Incorporation decreased considerably after 24 h of freezing (Fig. 5) - a 69% loss in activity. It was therefore clearly not feasible to use frozen preparations for experiments.

Having established the basic experimental conditions, it was then possible to study the four transferase enzymes in more detail. First the effects of sugar-nucleotide mixture and cations on the xylosyltransferase were investigated.

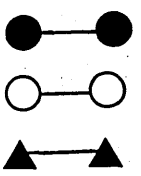
Effect of varying UDP-xylose concentration on incorporation of radioactivity from UDP-D-[U^{14}C]xylose

Incubations were performed with varying UDP-xylose concentrations up to 200.77 μmol.dm^{-3}, with UDP-glucose present in one set of incubations. Incubations comprised UDP-D-[U^{14}C]xylose (200.77 μmol.dm^{-3}, 740 Bq), $MnCl_2$ (10 mmol.dm^{-3}) and UDP-glucose, where present, at 2 mmol.dm^{-3}. Incubations were terminated at 0 and 15 min.

Incorporation was generally low and zero time values high unless UDP-glucose was present (Fig.6). Zero time and 15 min values decreased with increasing UDP-xylose concentration, which suggests

Figure 5

Effect of freezing bean suspension particulate enzyme preparation on incorporation of radioactivity from UDP-D-[U^{14}C]xylose:-



(●—●) fresh preparation

(○—○) preparation frozen for 24 h

(▲—▲) preparation frozen for 7 days

Fig.5

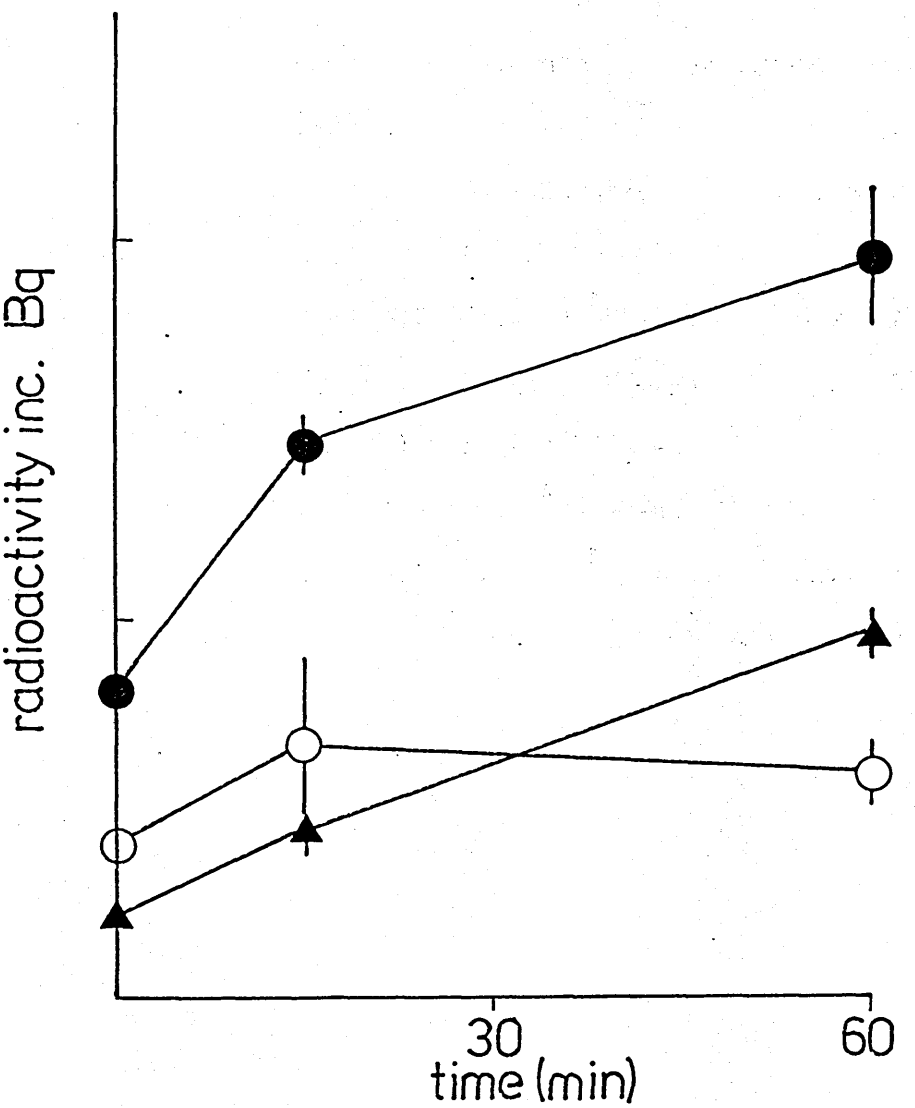


Figure 6

Effect of varying UDP-xylose concentration on incorporation of radioactivity from UDP-D-[$U^{14}C$]xylose by particulate enzyme preparation from bean-suspension culture, using incubations comprising $MnCl_2$ (10 mmoldm^{-3}) with:-

(■—■) 1 μmoldm^{-3} UDP-D-[$U^{14}C$]xylose (740 Bq)

(▲—▲) 3 μmoldm^{-3} UDP-D-[$U^{14}C$]xylose (740 Bq)

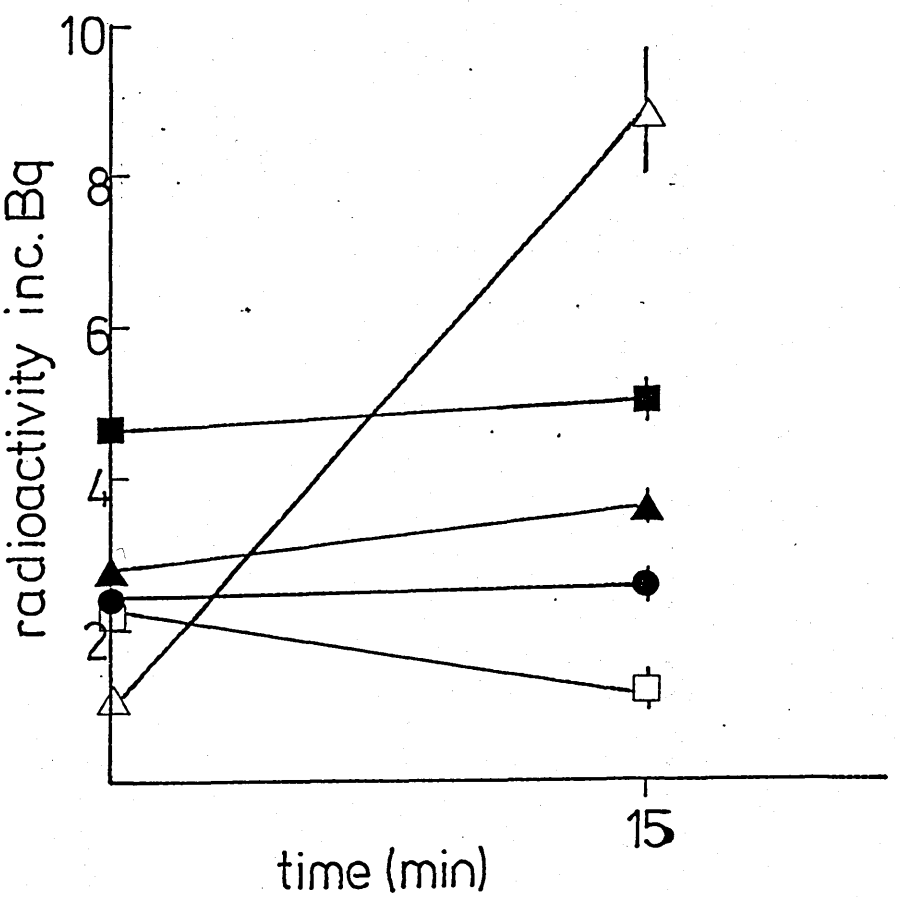
(●—●) 21 μmoldm^{-3} UDP-D-[$U^{14}C$]xylose (740 Bq)

(□—□) 201 μmoldm^{-3} UDP-D-[$U^{14}C$]xylose (740 Bq)

(△—△) 3 μmoldm^{-3} UDP-D-[$U^{14}C$]xylose (740 Bq)

plus UDP-glucose (2 mmoldm^{-3})

Fig.6



that the radioactivity is being non-covalently bound to the polysaccharide rather than incorporated, an effect which decreases with increasing UDP-xylose concentration as the specific activity decreases.

Effect of varying UDP-glucose concetration on incorporation of radioactivity from UDP-D-[U^{14}C]xylose

Incubations were performed with varying UDP-glucose concentrations and comprised UDP-D-[U^{14}C]xylose (21 μmol.dm^{-3}, 740 Bq) UDP-glucose (0-5 mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3}). Incubations were terminated at 0 and 15 min.

There was a clear stimulation of incorporation from UDP-D-[U^{14}C] xylose by UDP-glucose (Fig.7) and zero time values were low, suggesting non-covalent binding is unlikely to be occurring. Incorporation increased steadily with increasing UDP-glucose concentration up to 2 mmol.dm^{-3}, then decreased markedly at 5 mmol.dm^{-3}. Of the concentrations used, 2 mmol.dm^{-3} appears to be optimal.

Effect of varying UDP-xylose concentration at constant UDP-glucose concentration on incorporation of radioactivity from UDP-D-[U^{14}C] xylose

The optimum UDP-glucose concentration (determined in the previous experiment) was used to establish the optimum UDP-xylose concentration for incorporation with UDP-glucose present in incubations. Incubations comprised UDP-xylose (1-201 μmol.dm^{-3}, 740 Bq), UDP-glucose (2 mmol.dm^{-3}), $MnCl_2$ (10 mmol.dm^{-3}) and were terminated at 0 and 15 min. Incorporation was calculated in pmol, and this showed that maximum incorporation was

Figure 7

Effect of varying UDP-glucose concentration on incorporation from UDP-D-[U ^{14}C]xylose at 15 min by enzyme preparation from bean suspension-cultures (using standard incubations with varying UDP-glucose concentration).

Figure 8

Effect of varying UPD-xylose concentration at constant UDP-glucose concentration on incorporation from UDP-D-[U ^{14}C] xylose at 15 min by bean suspension-culture enzyme preparation (using standard incubations with varying UDP-xylose concentration).

Fig.7

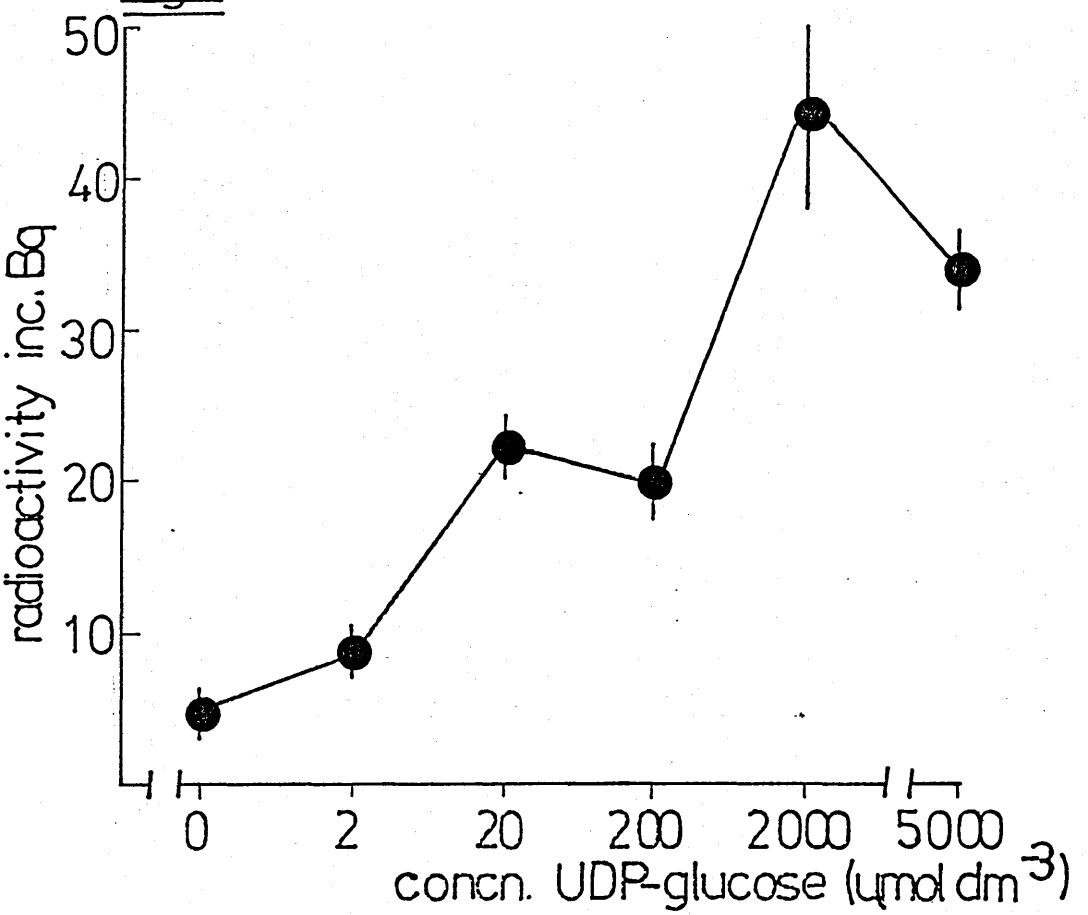

radioactivity inc. Bq
50
40
30
20
10
0
2
20
200
2000
5000
concn. UDP-glucose (μmol dm^{-3})

Fig.8

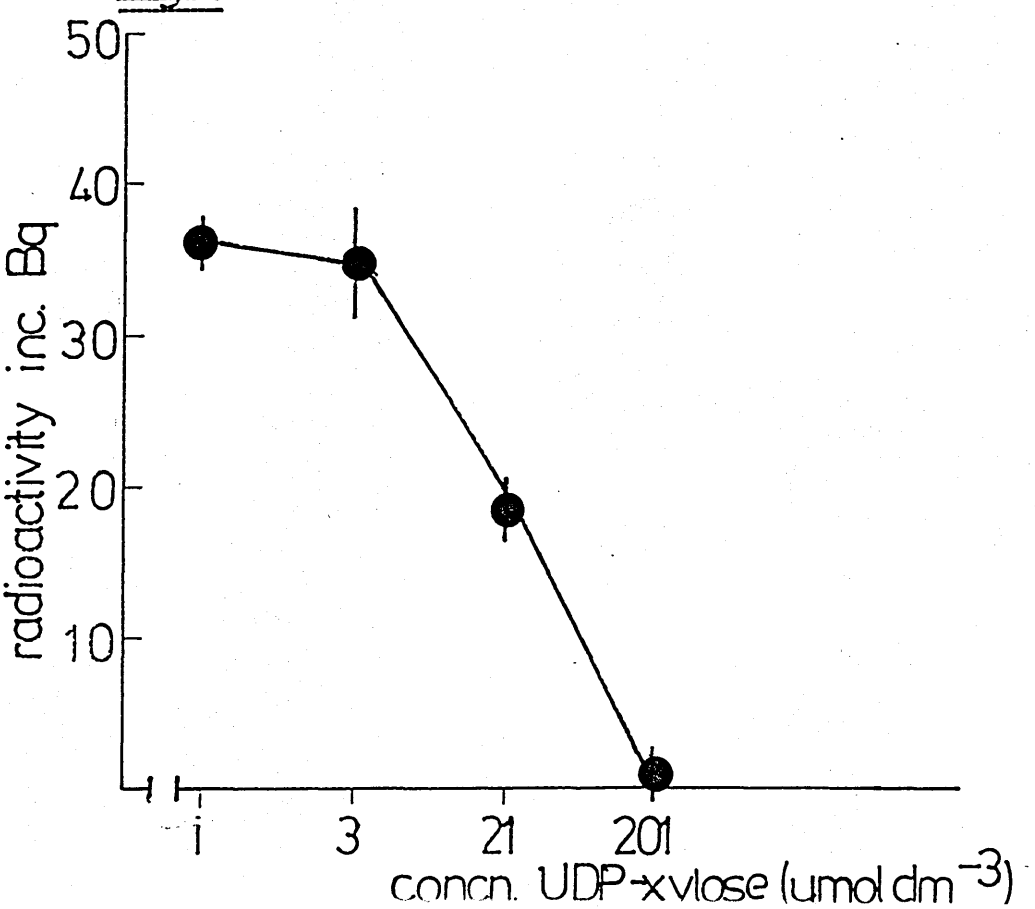

radioactivity inc. Bq
50
40
30
20
10
i
3
21
201
concn. UDP-xylose (μmol dm^{-3})

Table 3

Effect of varying UDP-xylose concentration on relative incorporation of radioactivity from UDP-^{14}C-xylose by particulate enzyme preparation from bean suspension culture.

Conc. UDP-D-[U^{14}C]xylose (μmoldm^{-3})	Relative incorporation of radioactivity from UDP-D-[^{14}C] xylose (%)
1	5
3	14
21	51
201	27

achieved with 21 $\mu mol.dm^{-3}$ UDP-xylose in incubations (Fig.8, Table 3).

Effect of divalent cations on incorporation of radioactivity from UDP-D-[U ^{14}C]xylose

The effect of a range of divalent cations on incorporation from UDP-D-[U ^{14}C]xylose was determined. The chloride salts of Ca^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+} were used at 10 $mmol.dm^{-3}$, otherwise standard incubation conditions were used.

Incorporation was maximal in the presence of Mn^{2+}, though Co^{2+} and Mg^{2+} also stimulated incorporation (Table 4).

Optimisation of Mn^{2+} concentration for incorporation of radioactivity from UDP-D-[U ^{14}C]xylose

The optimum Mn^{2+} concentration was determined using a range of $MnCl_2$ concentrations from 0 to 100 $mmol.dm^{-3}$. Standard incubation contents were used, with incubations terminated at 0 and 15 min.

Incorporation increased steadily with increasing Mn^{2+} concentration up to 10 $mmol.dm^{-3}$, then decreased sharply (Fig. 9). A further set of identical incubations was then performed using Mn^{2+} concentrations from 10 to 100 $mmol.dm^{-3}$. Again 10 $mmol.dm^{-3}$ gave maximum incorporation (Fig.10).

From these experiments the standard incubation mixture was established, i.e. UDP-glucose (2 $mmol.dm^{-3}$), UDP-D-[U ^{14}C]xylose (21 $\mu mol.dm^{-3}$, 740 Bq) and $MnCl_2$ (10 $mmol.dm^{-3}$). The xylosyl transferase

Figure 9

Effect of varying Mn^{2+} concentration on incorporation at 15 min from UDP-D-[U ^{14}C]xylose by suspension-cultured bean enzyme preparation using standard incubations.

Figure 10

Optimisation of Mn^{2+} concentration for incorporation from UDP-D-[U ^{14}C]xylose by bean-suspension culture particulate enzyme preparation: incorporation from standard incubation mixture at 15 min.

Fig.9

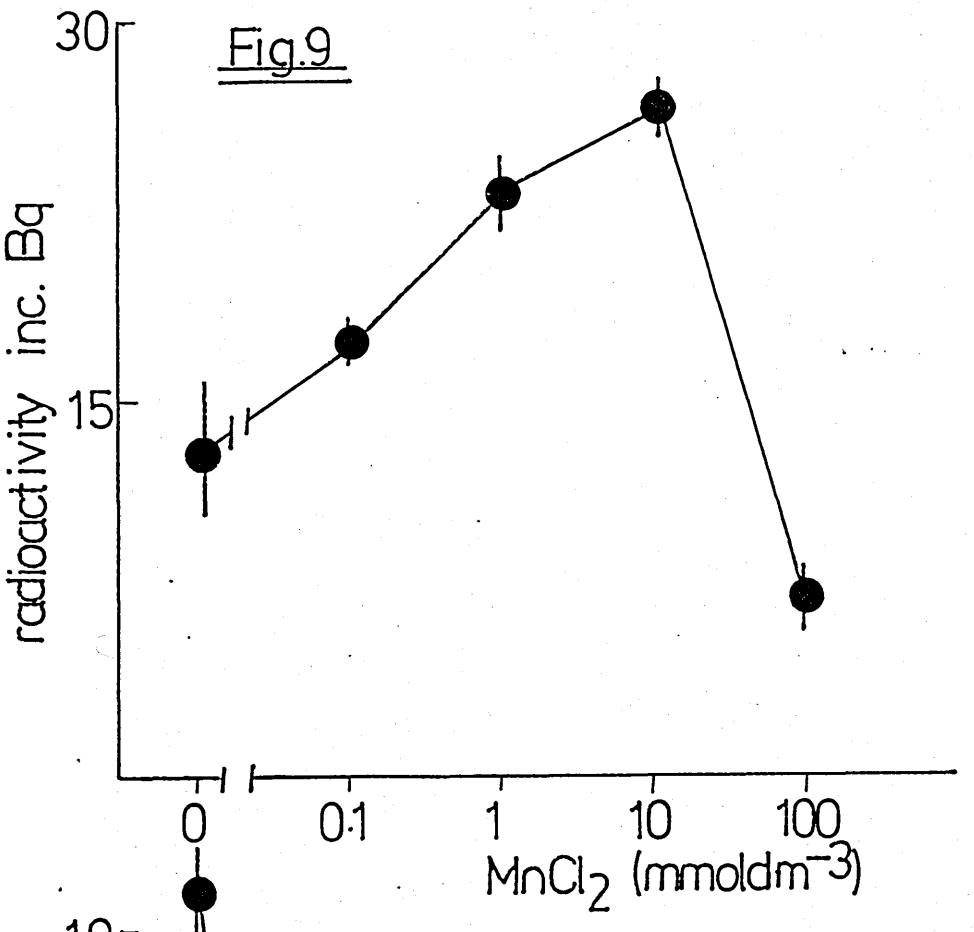
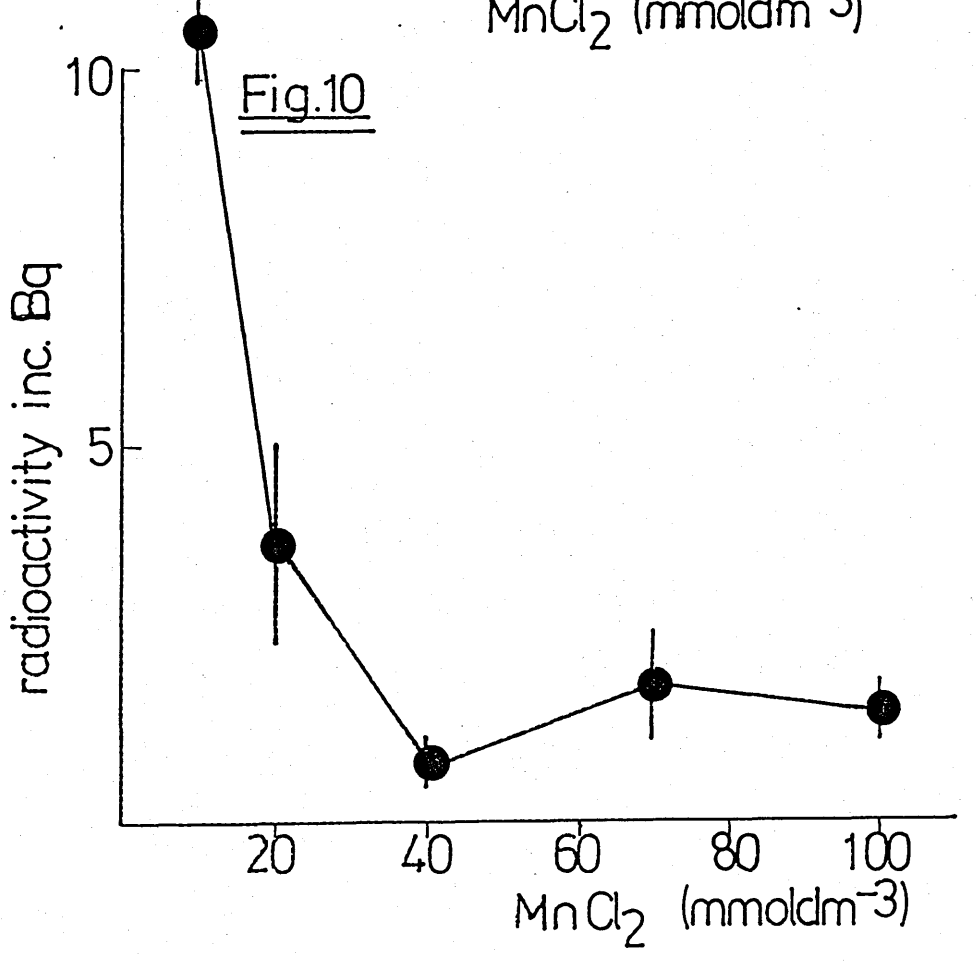

30
15
radioactivity inc. Bq
0
0·1
1
10
100
MnCl2 (mmoldm−3)

Fig.10


10
5
radioactivity inc. Bq
20
40
60
80
100
MnCl2 (mmoldm−3)

was next investigated by performing a series of time-courses.

Time-course of incorporation of radioactivity from UDP-D-[U^{14}C]xylose

The incorporation of radioactivity from UDP-D-[U^{14}C]xylose was investigated using the previously established incubation conditions. Incubations were terminated at 0, 5, 10, 15, 30 and 60 min.

Incorporation increased in an almost linear fashion (Fig. 11) and began to tail off by 60 min.

Extended time course of incorporation of radioactivity with and without UDP-glucose

The time course of incorporation from UDP-D-[U^{14}C]xylose was extended to 120 min and incubations without UDP-glucose were included to study the stimulatory effect of UDP-glucose on incorporation. Standard incubation conditions were used, with incubations terminated at 0, 15, 60 and 120 min.

Incorporation in the presence of UDP-glucose was approximately linear up to 60 min (as in the previous experiment), then levelled off (Fig.12). With no UDP-glucose present incorporation was very low, and the high time zero value suggests non-covalent binding is occurring. The amount of radioactivity incorporated, or more likely the amount of radioactivity non-covalently bound in these incubations decreased with time.

Figure 11

Time course of incorporation of radioactivity from UDP-D-[U ^{14}C] xylose by particulate enzyme preparation from suspension-cultured bean (using standard incubation conditions).

Figure 12

Time course of incorporation from UDP-D-[U ^{14}C]xylose by bean particulate enzyme preparation from suspension-cultured bean in the presence (●—●) and absence (○—○) of UDP-glucose (using standard conditions).

Fig.11

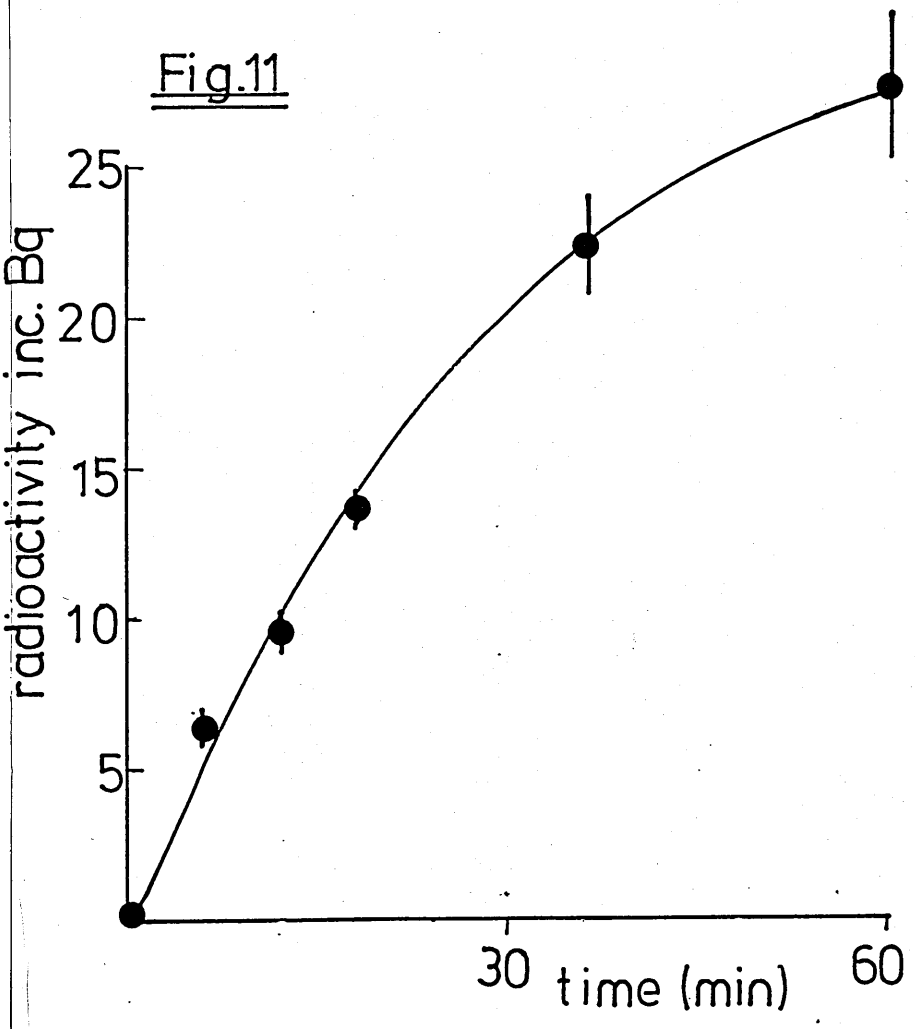
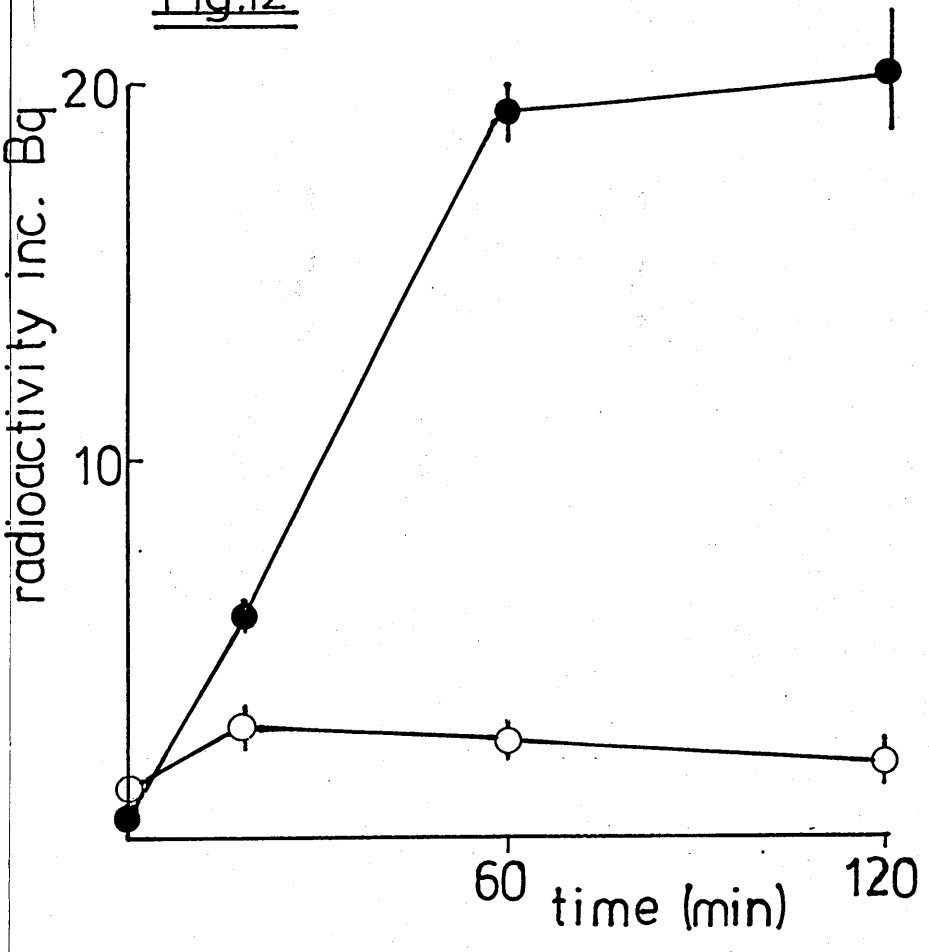

radioactivity inc. Bq
25
20
15
10
5
30
time (min)
60

Fig.12


radioactivity inc. Bq
20
10
60
time (min)
120

It is evident that UDP-glucose is required for incorporation from UDP-D-[U^{14}C]xylose, and this suggests that glucose and xylose may form part of the same polysaccharide product, possibly a xyloglucan. There may however be other explanations for the apparent stimulation: UDP-glucose may be serving as a protectant of UDP-xylose by providing an alternative substrate for degradive enzymes (e.g. pyrophosphorylases), and may not be incorporated into the polysaccharide at all.

Several variations of the original time course were carried out to investigate the role of UDP-glucose in the incorporation.

Effect of UDP-glucose addition after commencement of time course

The time course of incorporation from UDP-D-[U^{14}C]xylose was further investigated by adding UDP-glucose (2 mmoldm^{-3}) to incubations after a 50 min time lapse. Three sets of incubations were used: in one set UDP-glucose was present from time zero with incubations terminated at 0, 15, 80 and 120 min; a second set had no UDP-glucose present and the third set had UDP-glucose added to incubations after 50 min. In the latter two sets incubations were terminated at 0, 80, 95 and 120 min.

No stimulation of incorporation occurred on addition of UDP-glucose after 50 min (Fig.13), with incorporation at a similar level to incubations with no UDP-glucose present at any time during incubation. Earlier addition of UDP-glucose to incubations (20 min after commencement of incubation) made no difference to the time course.

Lack of stimulation by UDP-glucose could be due to a number of factors: the radioactive substrate may be subject to enzyme degradation in the absence of the protectant UDP-glucose as suggested previously, or the transferase enzyme may be losing activity rapidly in the absence of the UDP-glucose substrate. To test this latter theory a time course was performed, in which a fresh enzyme preparation was added to incubations along with UDP-glucose after a 30 min time lapse.

Effect of UDP-glucose and fresh enzyme addition after commencement of time course

Three sets of incubations were performed, one set of standard incubations and two sets with UDP-D-[U^{14}C]xylose and $MnCl_2$ only present initially. To one of these latter two sets UDP-glucose and fresh enzyme preparation (50 µl) were added 20 min after commencement of incubation. Incubations were terminated at 0, 15 and 60 min.

No stimulation of incorporation was observed on addition of UDP-glucose and fresh enzyme (Fig. 14). Zero time values were higher where UDP-glucose was absent. This implies that lack of stimulation by late-added UDP-glucose is not due to loss of enzyme activity. However, if activity is lost due to UDP-D-[U^{14}C]xylose breakdown in the absence of UDP-glucose, addition of fresh enzyme would not be effective, so this result still does not explain the loss of stimulation by UDP-glucose.

Figure 13

Effect of delayed addition of UDP-glucose to incubations on incorporation from UDP-D-[U^{14}C]xylose by particulate enzyme preparation:-

(●——●) standard incubation contents

(○——○) standard incubation contents but with UDP-glucose added 50 min after commencement of incubation

(▲——▲) standard incubation minus UDP-glucose

Figure 14

Effect of delayed addition of UDP-glucose with fresh enzyme to incorporation from UDP-D-[U^{14}C]xylose by particulate enzyme preparation from suspension-cultured bean:-

(●——●) standard incubation conditions

(○——○) standard conditions with UDP-glucose plus 50 mm^3 added 20 min after commencement of incubation

(▲——▲) standard incubation minus UDP-glucose

Fig.13

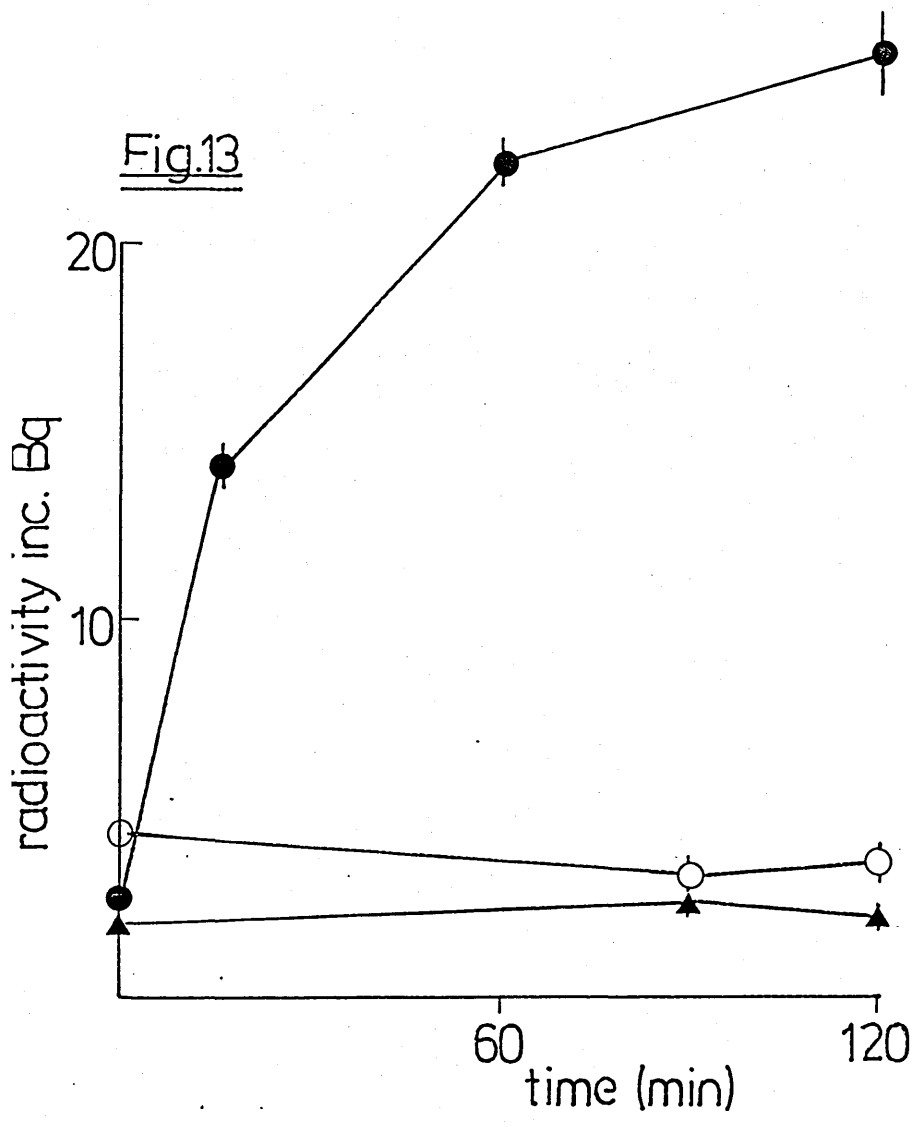
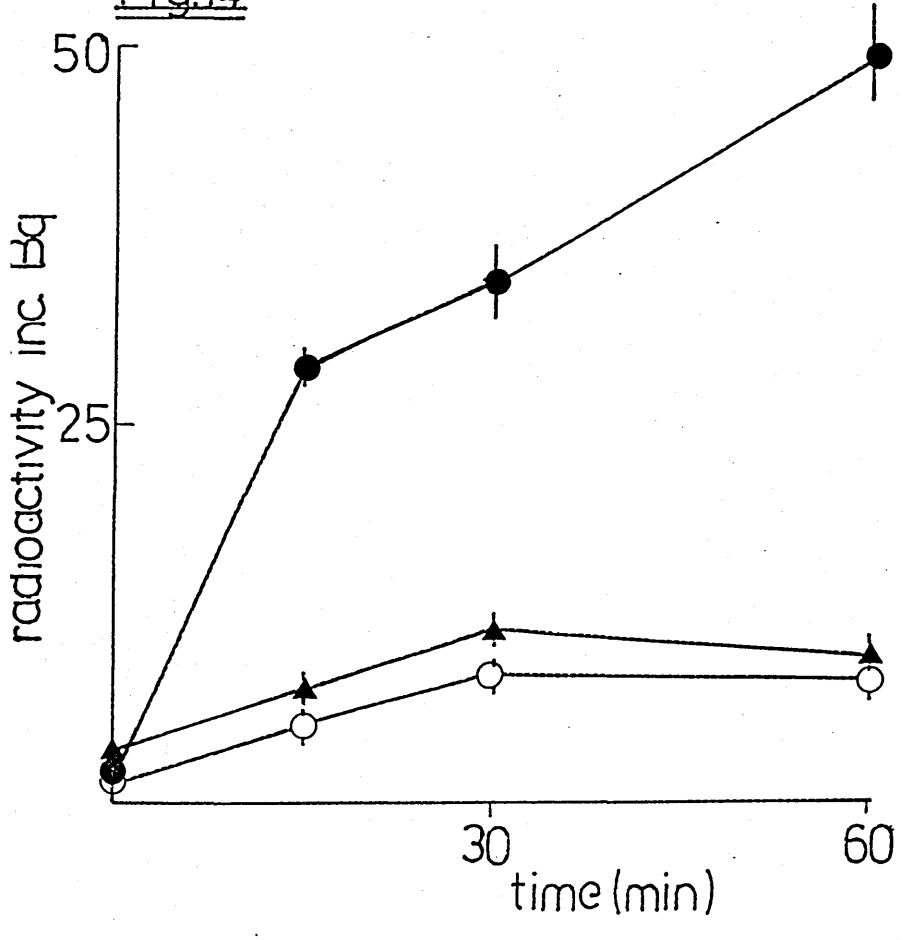

20
10
radioactivity inc. Bq
60
120
time (min)

Fig.14


50
25
radioactivity inc Bq
30
60
time (min)

Effect of addition of UDP-D-[U^{14}C]xylose to UDP-glucose containing incubations

The apparent loss of enzyme activity in the absence of substrate was further investigated by adding UDP-D-[U^{14}C]xylose after commencement of incubation, to incubations containing UDP-glucose and $MnCl_2$ only. Two sets of incubations were performed: standard incubations with all sugar nucleotides present from the start, and incubations where UDP-D-[U^{14}C]xylose was added 20 minutes after commencement of incubation. Incubations were terminated at 0, 15 and 60 min.

High incorporation rates were found where all sugar nucleotides were present from time zero (Fig. 15). Incorporation at 60 min was reasonably high where UDP-D-[U^{14}C]xylose was added after a 20 min delay, indicating that the enzyme did not lose activity in the absence of UDP-xylose. This result however still does not determine whether UDP-glucose protects the UDP-xylose, the enzyme, or both. Nor does it determine whether UDP-glucose is being incorporated into polysaccharide. Its role as a protectant was therefore further investigated by examining possible substitute protectants.

Effect of ATP and GDP as protectants in UDP-D-[U^{14}C]xylose incubations

The potential of ATP and GDP protectants was examined in an experiment where sugar nucleotides were added to incubations after commencement of incubation. Incubations were performed with either ATP or GDP present in all cases, with UDP-D-[U^{14}C]xylose and UDP-glucose

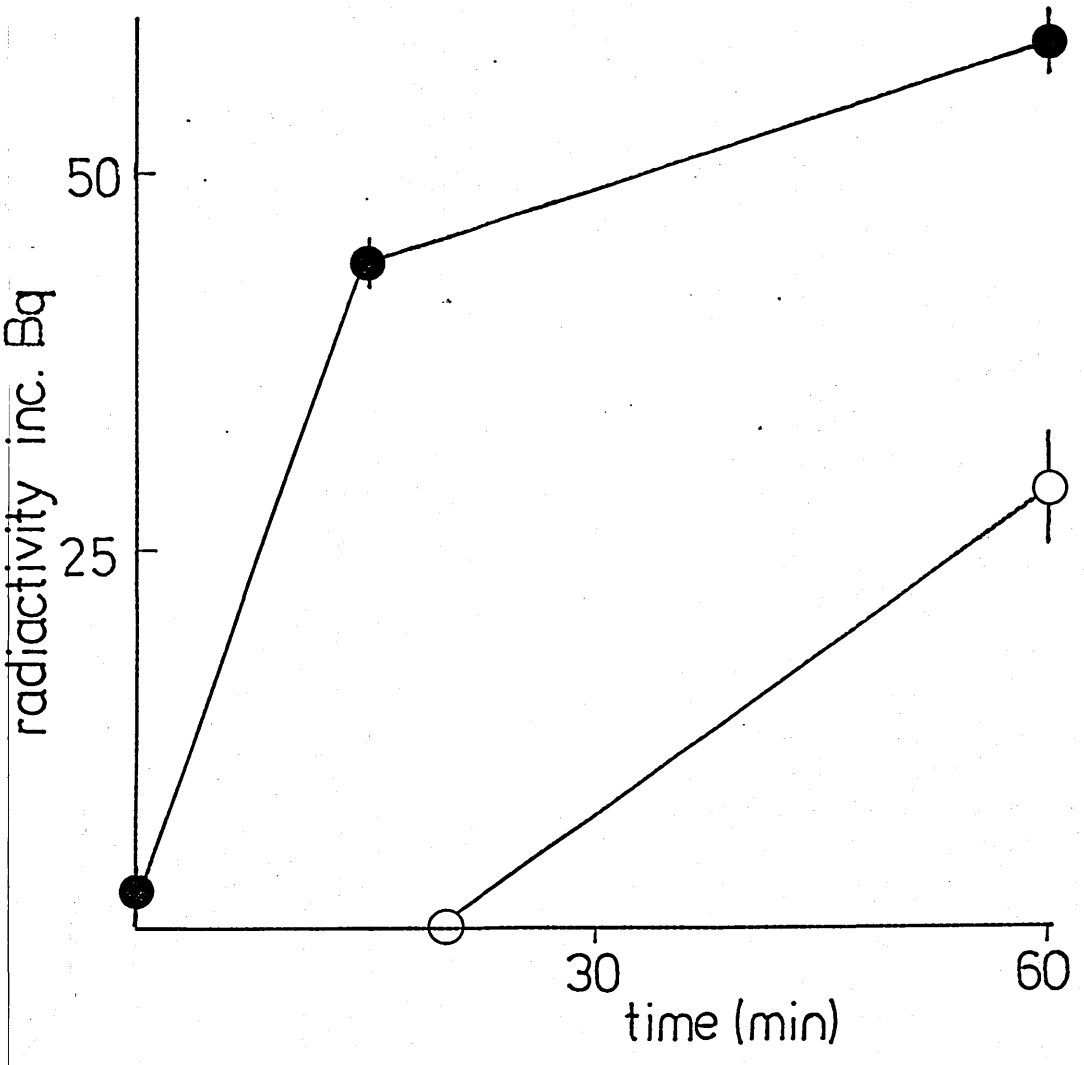
Figure 15

Effect of delayed addition of UDP-D-[U^{14}C]xylose to incubations containing UDP-glucose on incorporation of radioactivity by particulate enzyme preparation from suspension-cultured bean:-

(●—●) standard incubation conditions

(○—○) standard incubation conditions with UDP-D-[U^{14}C] xylose added 20 min after commencement of incubation.

Fig.15



present from the start or added 30 min after commencement of incubation. Incubations were standard plus 2 mmol.dm^{-3} ATP or GDP, or $MnCl_2$ (10 mmol.dm^{-3}) plus 2 mmol.dm^{-3} ATP or GDP with UDP-D-[U^{14}C] xylose and UDP-glucose added after 30 min. Incubations were terminated at 0, 15 and 60 min (See Figure legend for incubation details).

The presence of ATP or GDP in standard incubations did not appear to affect the time course of incorporation. Delayed addition of UDP-D-[U^{14}C]xylose and UDP-glucose resulted in a low level of incorporation with GDP, and practically none with ATP (Fig. 16a & b). Thus under conditions investigated, ATP was not effective as an enzyme protectant and GDP had little effect.

ATP and GDP were selected as potential protectants due to the unlikelihood of their becoming incorporated into polysaccharide product. Due to their inability to act as protectants however, two sugar nucleotides with a structure closely resembling that of UDP-glucose were examined.

Effect of GDP-mannose and GDP-glucose as protectants in UDP-D-[U^{14}C] xylose incubations

Incubations were performed with and without GDP-mannose and GDP-glucose, in the presence and absence of UDP-glucose. Three standard incubation time courses were set up, one with 2 mmol.dm^{-3} GDP-glucose and one with 2 mmol.dm^{-3} GDP-mannose added, and two sets

Figure 16

Effect of ATP and GDP on incorporation from UDP-D-[U^{14}C] xylose by particulate enzyme preparation from bean suspension-culture tissue using incubations:-

a) With GTP present (2 mmol.dm^{-3}), in (●—●) standard incubation mixture or (○—○) incubations with UDP-glucose (2 mmol.dm^{-3}) and UDP-D-[U^{14}C]xylose added 30 min after commencement of incubation

b) With ATP present (2 mmol.dm^{-3}), in (●—●) standard incubation mixture or (○—○) incubations with UDP-glucose (2 mmol.dm^{-3}) and UDP-D-[U^{14}C]xylose added 30 min after commencement of incubation.

Fig.16a

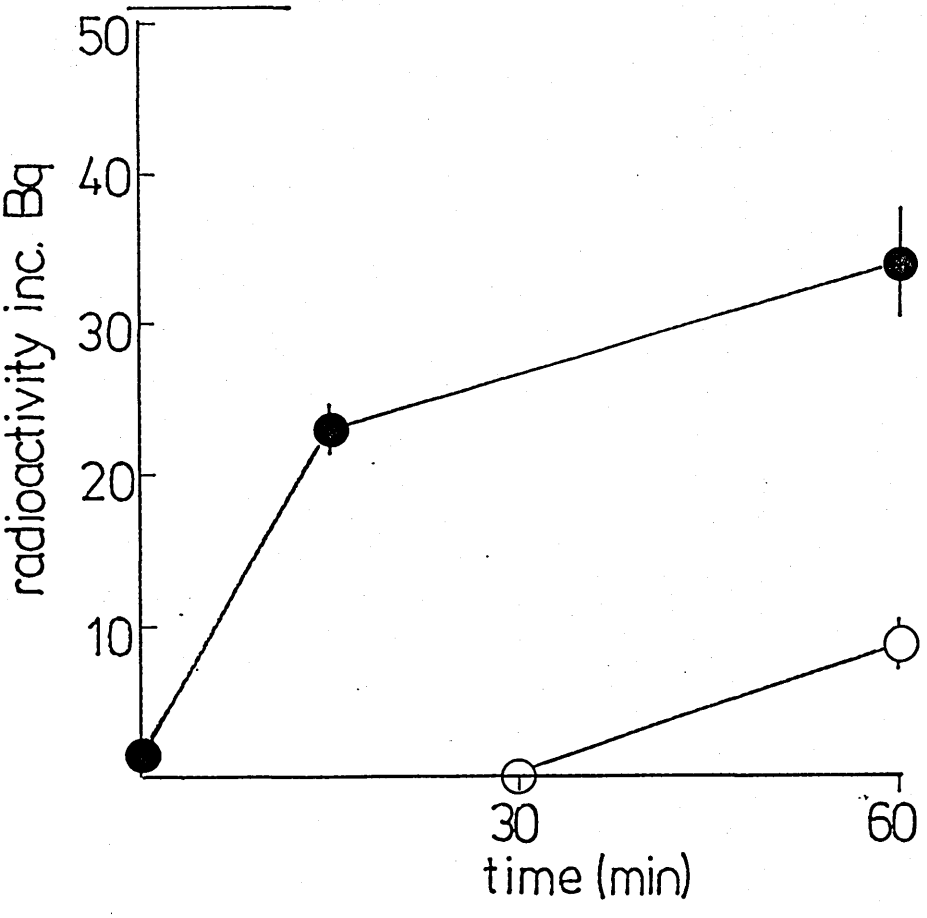
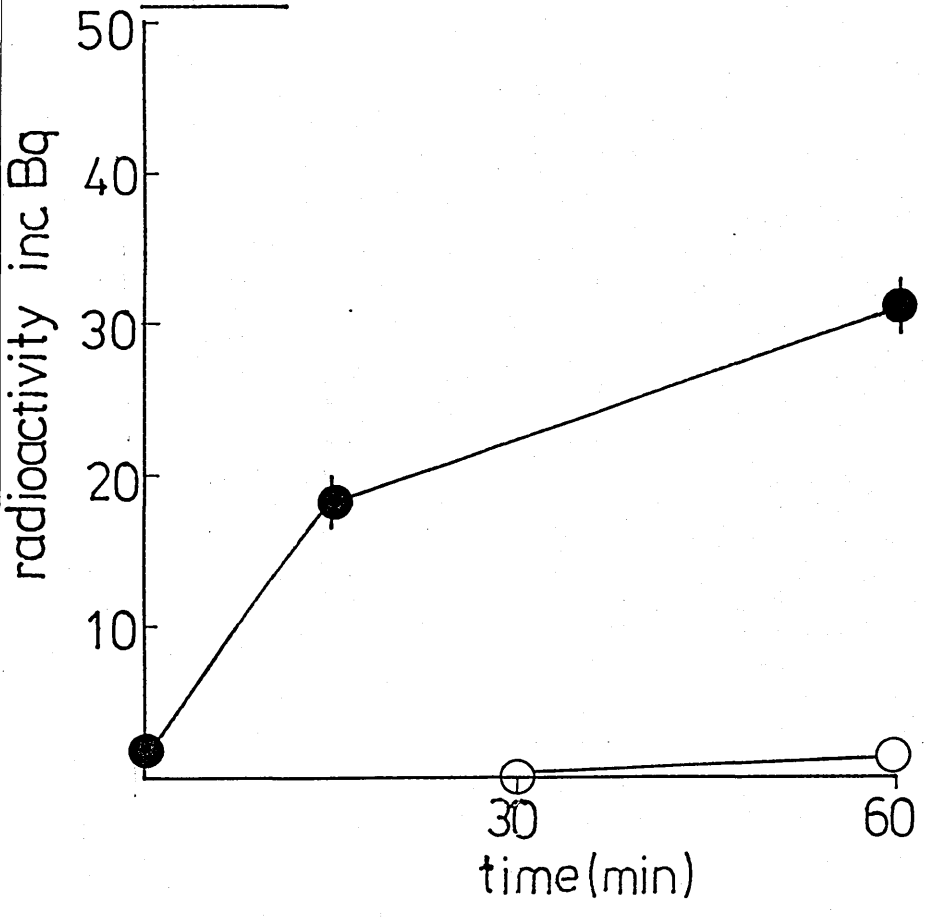


Fig.16b



of incubations with the standard assay contents, minus UDP-glucose but plus either GDP-glucose (2mmoldm^{-3}) or GDP-mannose (2 mmoldm^{-3}). Incubations were terminated at 0, 15, 30 and 60 min.

There was little incorporation in the absence of UDP-glucose in incubations containing either GDP-glucose or GDP-mannose (Fig.17a-c) i.e. they did not provide a substitute for UDP-glucose as stimulators of xylosyltransferase activity. However, where UDP-glucose was present incorporation was considerable (up to 28 Bq) and was apparently stimulated by the presence of GDP-glucose and GDP-mannose in incubations (by 38 and 23% respectively) compared to UDP-glucose only.

As GDP-glucose appeared to have the greater stimulatory effect, its protective role was further investigated.

Effect of delayed UDP-glucose addition to GDP-glucose containing incubations

In a modification of an earlier time course experiment (p.114), UDP-glucose was added to incubations 20 min after commencement, with 2 mmoldm^{-3} GDP-glucose present in incubations. Incorporation was compared to incubations with GDP-glucose but no UDP-glucose present, and to incubations with all nucleotides present from time zero. Incubations were terminated at 0, 15, 30, 60 and 180 min.

Where UDP-glucose was either present or absent from time zero,

Figure 17

Effect of GDP-mannose and GDP-glucose on incorporation of radioactivity from UDP-D-[U^{14}C]xylose by particulate enzyme preparation from suspension-cultured bean:-

a) Incubations with GDP-glucose (2 mmol.dm^{-3}) containing

(●—●) standard incubation mixture, or

(○—○) standard incubation mixture minus UDP-glucose

b) Incubations with GDP-mannose (2 mmol.dm^{-3}) containing

(●—●) standard incubation mixture, or

(○—○) standard incubation mixture minus UDP-glucose

c) Incubations with standard incubation mixture

Fig.17a

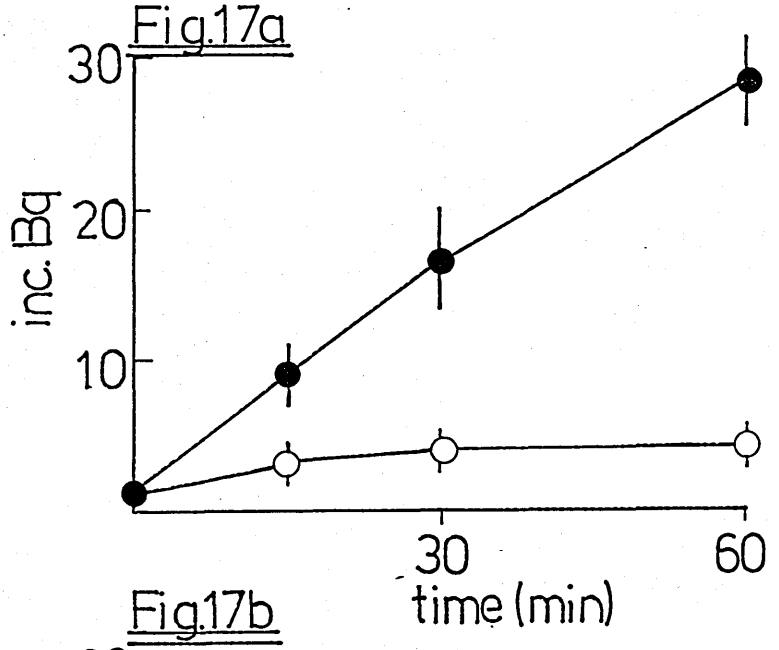


Fig.17b

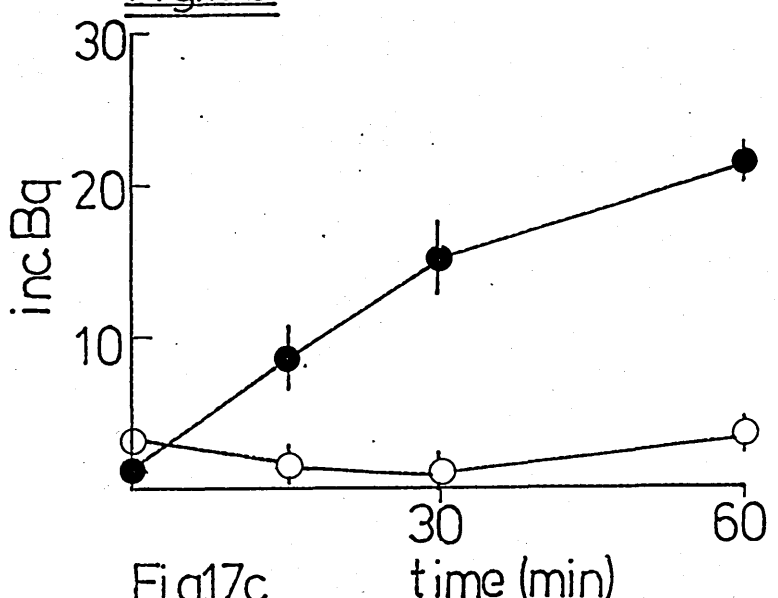
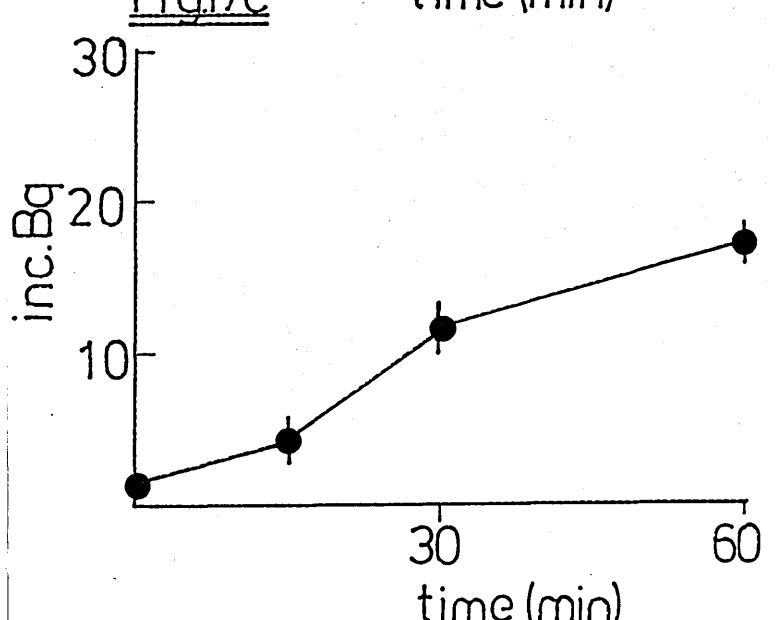


Fig.17c



results were similar to those of the original experiment i.e. stimulation of incorporation by UDP-glucose. Where UDP-glucose was added belatedly, incorporation commenced at a rate similar to that of incubations where it was present from the start (Fig. 18). This stimulation did not occur in the absence of GDP-glucose (Fig. 18), suggesting GDP-glucose has a protective role, protecting either the transferase enzyme or the sugar nucleotide substrate in the absence of UDP-glucose.

Protection of the xylosyltransferase by GDP-mannose and GDP-glucose in the absence of other nucleotides

Activity of the xylosyltransferase enzyme is generally lost by holding at room temperature for any length of time in the absence of sugar nucleotide substrates. The effectiveness of the protectants GDP-glucose and GDP-mannose was therefore tested by incubating each with enzyme preparation for 20 min before addition of any other sugar nucleotides. Two sets of incubations were performed for each protectant: the protectants were present at 2 $mmol.dm^{-3}$ in each set from time zero, and the standard incubation contents were either also present from time zero or added after 20 min. Incubations were terminated at 0, 15 and 60 min.

Incorporation of radioactivity where all sugar nucleotides were present from time zero was similar for GDP-glucose and GDP-mannose-containing incubations (Fig.19) with incorporation increasing up to 60 min. Incorporation still occurred in both sets of incubations where sugar nucleotides were added after a 15 min delay, and this incorporation was greater where GDP-mannose was the protectant

Figure 18

Effect of GDP-glucose on incorporation of radioactivity from UDP-D-[U ^{14}C]xylose by particulate enzyme preparation from suspension-cultured bean using standard incubation conditions:-

a) With GDP-glucose (2 mmol.dm^{-3}) ●—●

b) With GDP-glucose (2 mmol.dm^{-3}) and UDP-glc (2 mmol.dm^{-3}) added 20 min from time zero ○—○

c) With GDP-glucose (2 mmol.dm^{-3}) and no UDP-glucose present ▲—▲

Fig.18

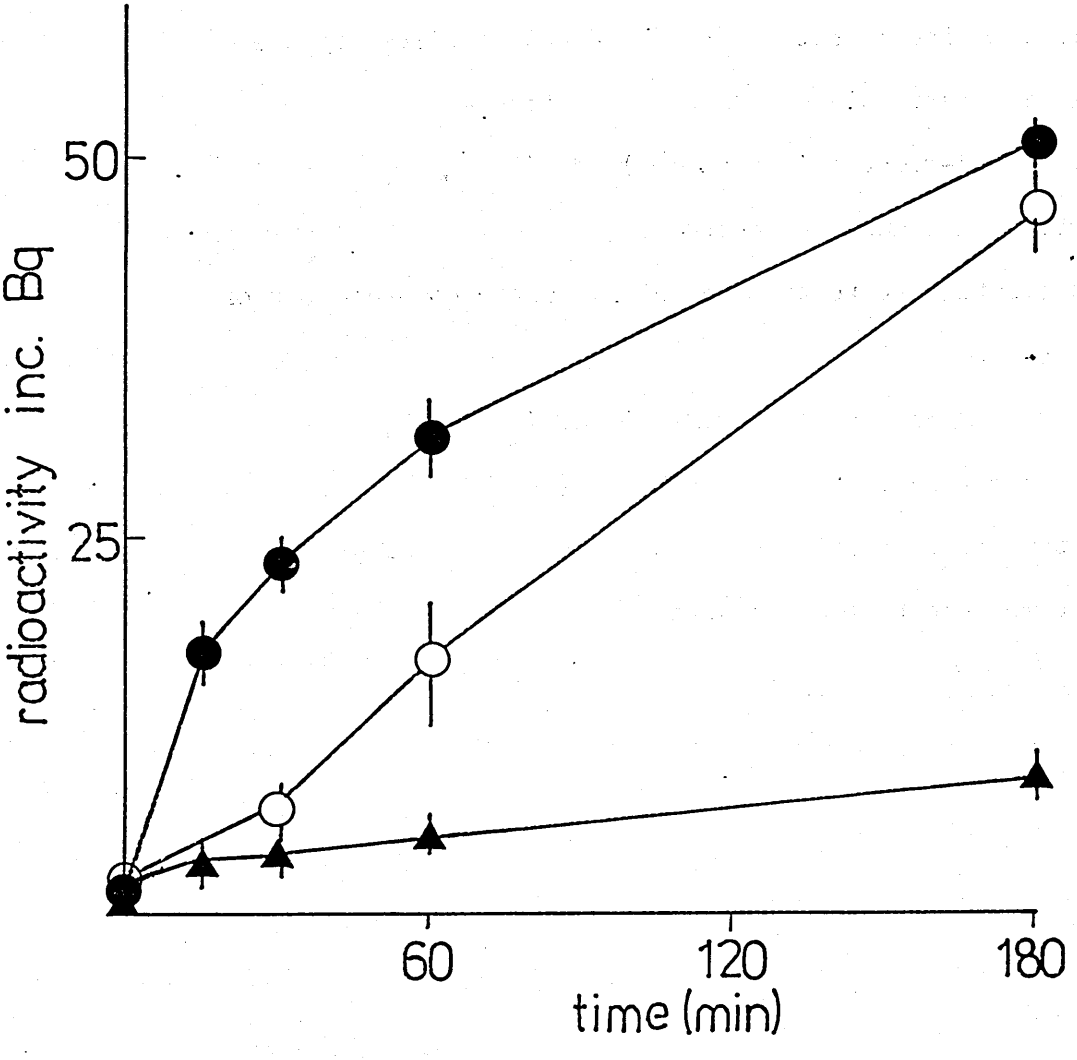


Figure 19

Effect of addition of sugar nucleotides, 20 min after commencement of incubation, to incubations containing GDP-glucose or GDP-mannose, on incorporation from UDP-D-[$U^{14}C$]xylose by bean suspension particulate enzyme preparation:-

a) With GDP-glucose (2 mmol.dm^{-3}) and (●—●) standard incubation contents or (○—○) standard incubation contents added 20 min after commencement of incubation

b) With GDP-mannose (2 mmol.dm^{-3}) and (●—●) standard incubation contents or (○—○) standard incubation contents added 20 min after commencement of incubation

Fig.19a

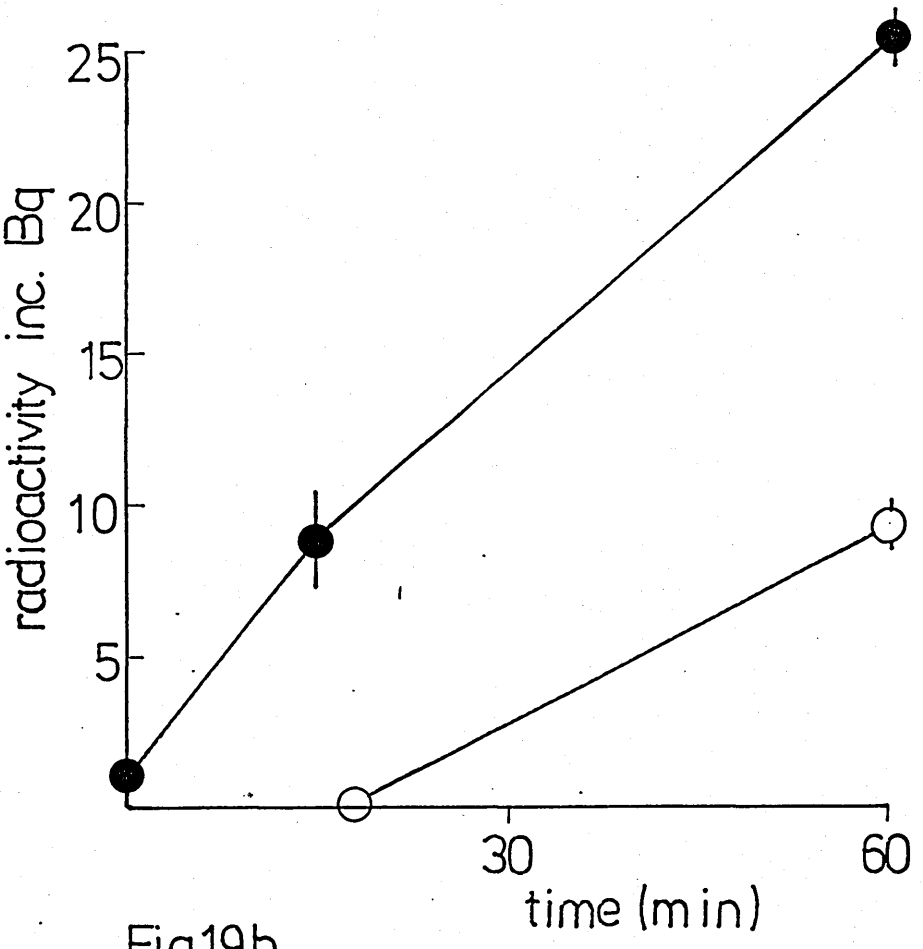
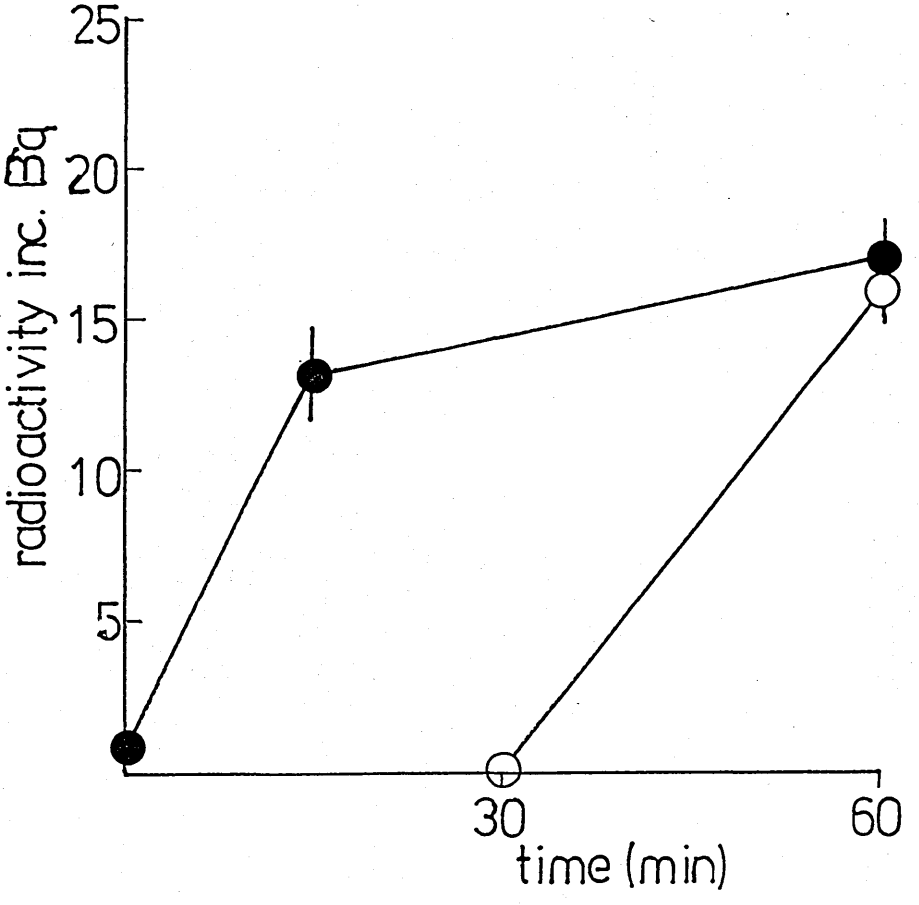


Fig.19b



used. Both protectants can therefore effectively protect the xylosyltransferase in the absence of substrates, indicating that this is at least one of the ways in which the protectants act.

The protection of UDP-xylose by GDP-glucose was examined by analysing incubation washings to determine the fate of UDP-xylose in the presence and absence of protectants.

TLE analysis of incubations performed with or without GDP-glucose

To determine whether UDP-D-[U^{14}C]xylose was broken down in incubations without protectants, TLE analysis of the first ethanol extraction was carried out. Incubations were performed and the first 70% ethanol extraction supernatant, which generally contains about 70% of the total experimental radioactivity, was retained for analysis. Incubations, which were of 60 min duration, had four basic nucleotide combinations:- the standard incubation mixture was used, standard incubation plus GDP-glucose (2 mmoldm^{-3}), standard incubation plus GDP-glucose minus UDP-glucose, or standard incubation minus UDP-glucose. The extraction supernatants were dried down under nitrogen and analysed by TLE as described (Ch.2 p.65), using UDP-xylose and xylose as markers.

Comparison of radioactive traces (Fig. 20 a-d) showed firstly that radioactivity levels are lower when UDP-glucose is present in incubations, presumably due to greater incorporation of radioactivity. Peaks of radioactivity co-chromatographing with UDP-xylose and xylose

Figure 20

Thin-layer-electrophoretic analysis of first ethanol wash from incubations of bean suspension particulate enzyme preparation with UDP-D-[U^{14}C]xylose:-

a) Standard incubation plus GDP-glucose (2 mmoldm^{-3})

b) Standard incubation

c) Standard incubation plus GDP-glucose minus UDP-glucose

d) Standard incubation minus UDP-glucose

Fig. 20a

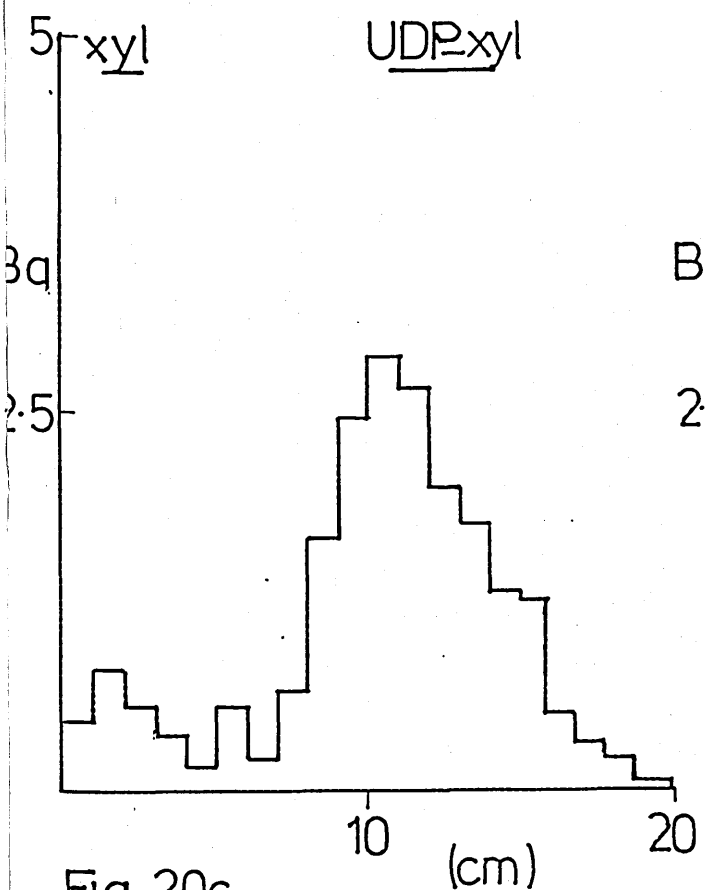


Fig. 20b

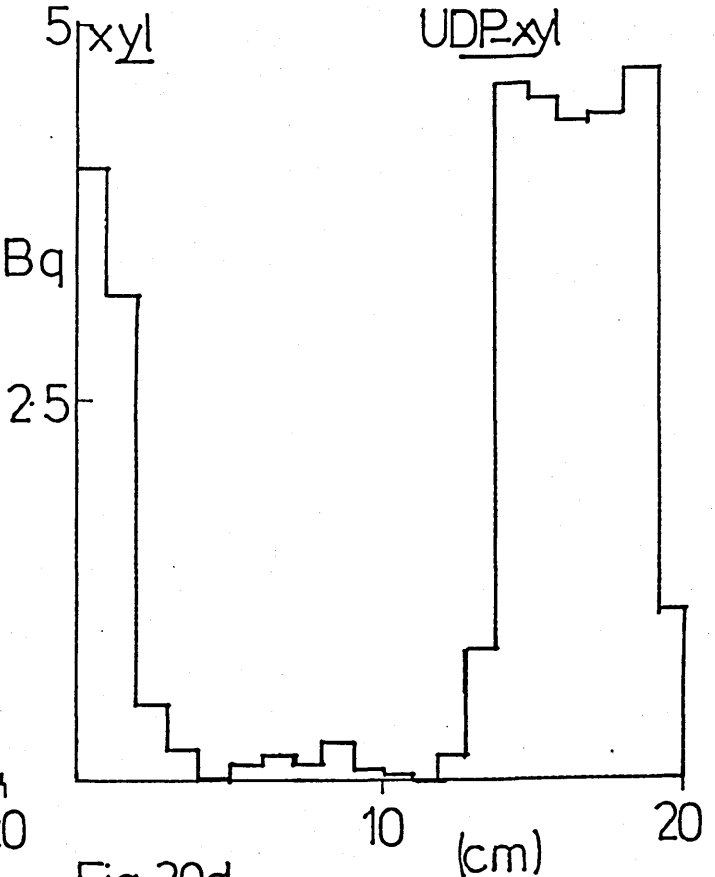


Fig. 20c

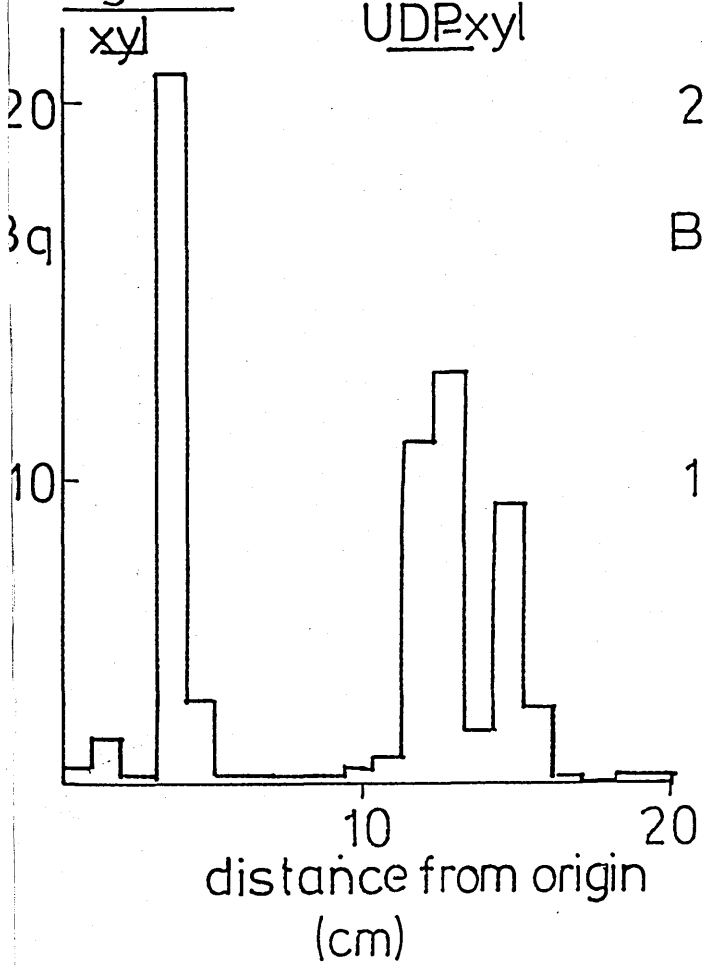
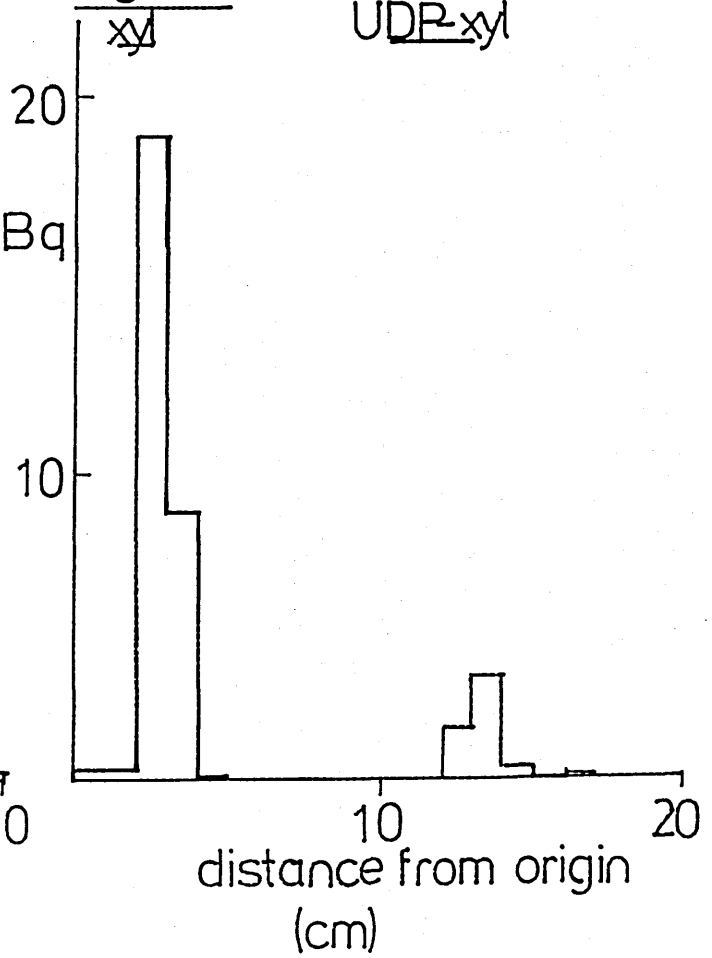


Fig. 20d



were detected in each analysis (Fig. 20 a-d), and the relative quantities of each varied with the incubation mixture used: presence of GDP-glucose in incubations from which UDP-glucose was absent (Fig.20c) resulted in much more UDP-D-[U^{14}C]xylose in washes than where GDP-glucose was absent (Fig. 20d). This was also true for incubations in which UDP-glucose was present i.e. GDP-glucose presence in addition to that of UDP-glucose (Fig.20a) resulted in nearly all the radioactivity being present as UDP-xylose rather than xylose.

This experiment confirms the role of GDP-glucose in protecting UDP-xylose from breakdown but does not rule out the possibility of protection of the glycosyltransferase by the GDP-glucose.

GDP-glucose and GDP-mannose appear to have a similar protective quality to UDP-glucose, but they do not demonstrate the same stimulatory effect on incorporation from UDP-D-[U^{14}C]xylose. It therefore seems likely that UDP-xylose and UDP-glucose contribute to the same product. In order to test this theory a pre-incubation experiment was performed.

Effect of pre-incubation with UDP-glucose on incorporation of radioactivity from UDP-D-[U^{14}C]glucose

Ray (1980) found that a pre-incubation of Golgi membranes from pea epicotyls with UDP-glucose resulted in increased incorporation from UDP-D-[U^{14}C]xylose in the absence of UDP-glucose i.e. it appeared that glucan chains were pre-formed before addition of xylose from UDP-xylose.

This was investigated for the particulate enzyme preparation from suspension-cultured bean.

The pre-incubation was performed as described (Ch.2 p.65). It involved a 20 min pre-incubation of the particulate enzyme preparation followed by a 30 min centrifugation to remove unreacted incubation mixture, then resuspension and incubations up to 60 min. Enzyme activity was monitored by performing a time course with pre-incubation. Treatments were as follows:- 1. standard incubation with no pre-incubation, with or without UDP-glucose in incubations; 2. pre-incubation with $MnCl_2$ and UDP-glucose (2 $mmol dm^{-3}$) followed by incubations, two sets either with or without UDP-glucose; 3. pre-incubation with $MnCl_2$ only followed by incubations, two sets with or without UDP-glucose, and 4. pre-incubation with $MnCl_2$ only at 0-4°C followed by incubations, with or without UDP-glucose. Incubations were terminated at 0, 15 or 60 min.

Pre-incubation at 0-4°C reduced incorporation from UDP-D-[$U^{14}C$] xylose in the presence of UDP-glucose by over 50% compared with the standard time course where no pre-incubation was performed (Fig.21 a & b). In other treatments very little incorporation occurred, and zero time values were particularly high in UDP-glucose-free incubations (Fig.21c & d). If a 50% loss in activity occurs with a pre-incubation at 0-4°C, it seems likely that an even greater loss will occur during a 25°C pre-incubation. Having already established the protective role of GDP-glucose in a previous experiment (p.119) it was decided to include

Figure 21

Effect of pre-incubation with UDP-glucose on incorporation of radioactivity from UDP-D-[U^{14}C]xylose by particulate enzyme preparation from suspension-cultured bean following four treatments:-

a) No pre-incubation

b) Pre-incubation for 30 min at 1-4^{o}C with $MnCl_2$ (10 mmoldm^{-3})

c) Pre-incubation for 30 min at 25^{o}C with $MnCl_2$ (10 mmoldm^{-3})

d) Pre-incubation for 30 min at 25^{o}C with UDP-glucose (2 mmoldm^{-3}) and $MnCl_2$ (10 mmoldm^{-3})

All incubations were performed as standard (●—●) or standard minus UDP-glucose (○—○).

Fig. 21a

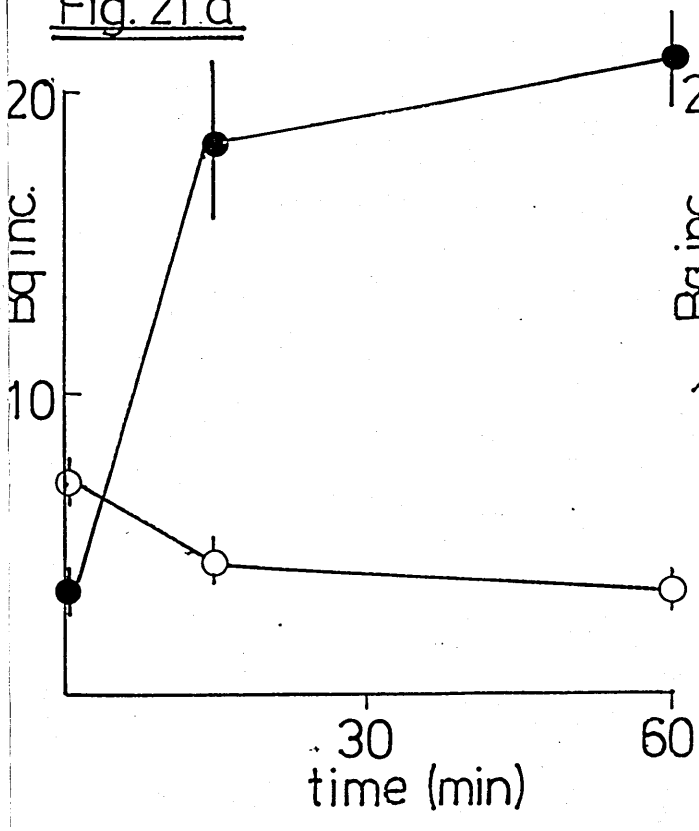


Fig. 21b

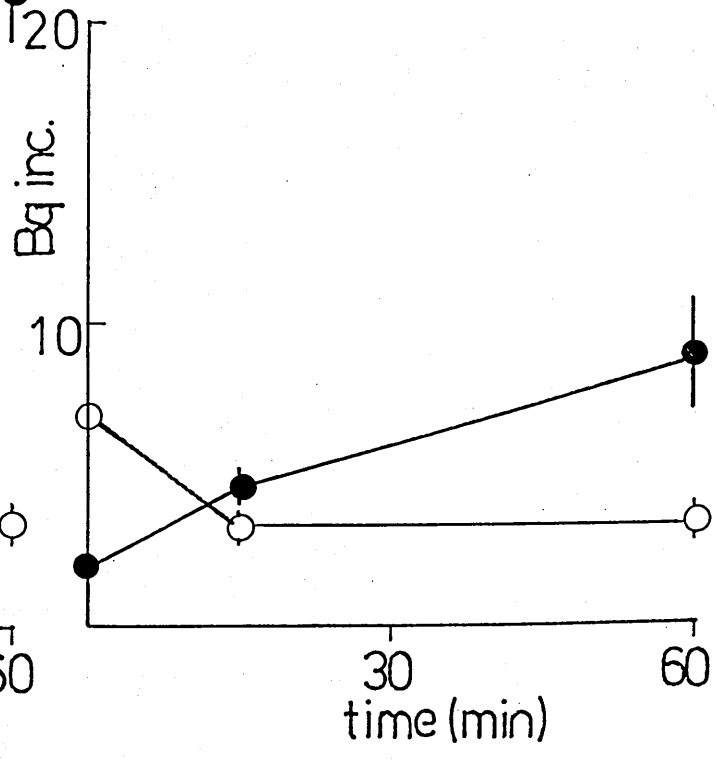


Fig. 21c

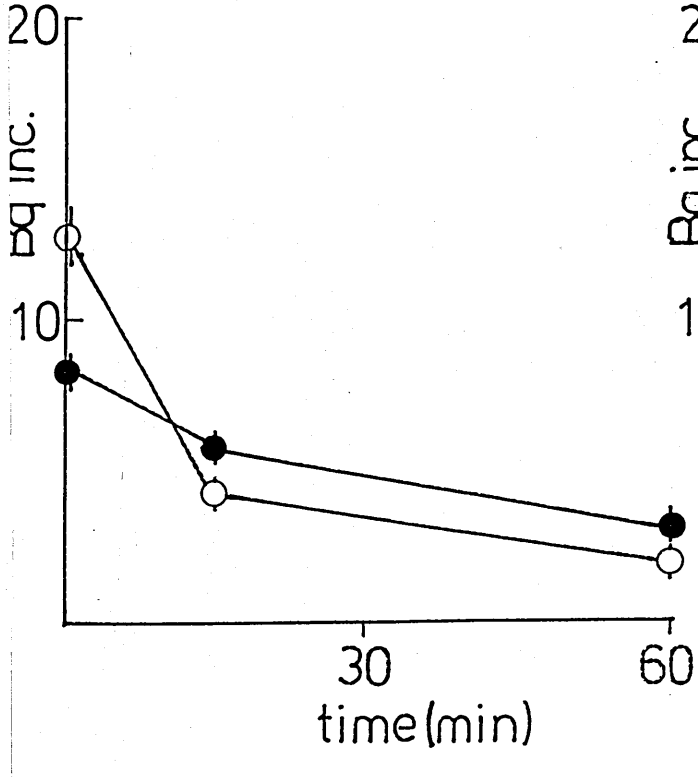
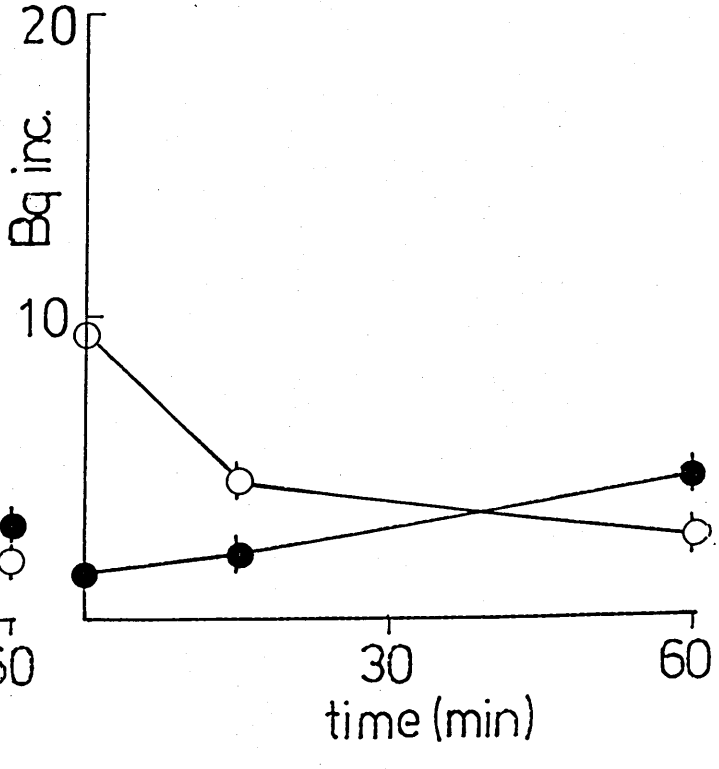


Fig. 21d



GDP-glucose in the 20 min pre-incubation.

The general scheme of the previous pre-incubation experiment was followed, but several modifications were made for the sake of simplicity. No pre-incubation on ice was performed, nor standard time-course without pre-incubation. Pre-incubations comprised GDP-glucose (2 $mmol.dm^{-3}$) and $MnCl_2$, with or without UDP-glucose. Following a 20 min pre-incubation, samples were centrifuged and then incubated for 0, 15 or 60 min.

Incorporation this time was higher with more typical time courses (Fig. 22). Incorporation up to 15 Bq occurred when UDP-glucose was present only in the pre-incubation, and incorporation where UDP-glucose was present in pre- and time course incubations was enhanced over incubations where it was only present in time course incubation. These results suggest preformation of the glucan chain during the pre-incubation.

As overall incorporation was still low in each case, the experiment was repeated with GDP-glucose present in the pre-incubation and also in the time course incubations. This improved incorporation further, illustrating the enhanced incorporation after pre-incubation with UDP-glucose still more clearly (Fig. 23 a & b).

Discussion

The transfer of xylose from UDP-xylose into a polysaccharide has

Figure 22

Effect of pre-incubation with UDP-glucose and GDP-glucose on incorporation of radioactivity from UDP-D-[U ^{14}C]xylose by particulate enzyme preparation from suspension-cultured bean following two treatments:-

a) Pre-incubation for 30 min with GDP-glucose (2 mmol.dm^{-3}), UDP-glucose (2 mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3})

b) Pre-incubation for 30 min with GDP-glucose (2 mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3})

All incubations were performed as standard (●—●) or standard minus UDP-glucose (○—○).

Fig. 22a

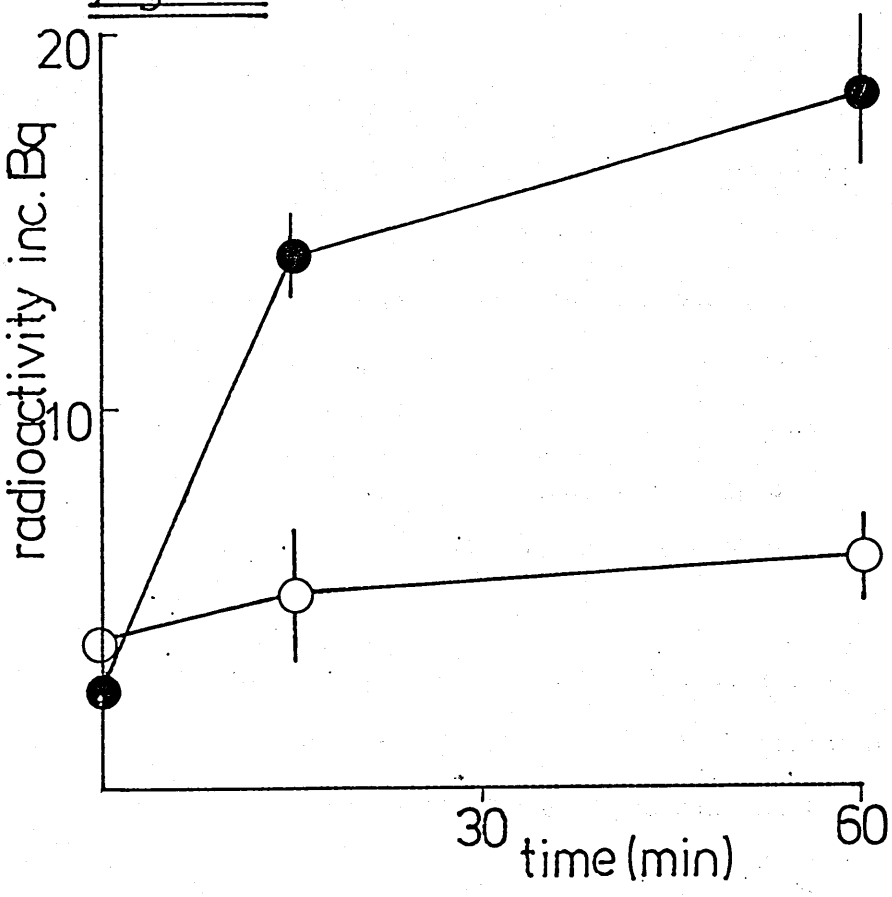


Fig. 22b

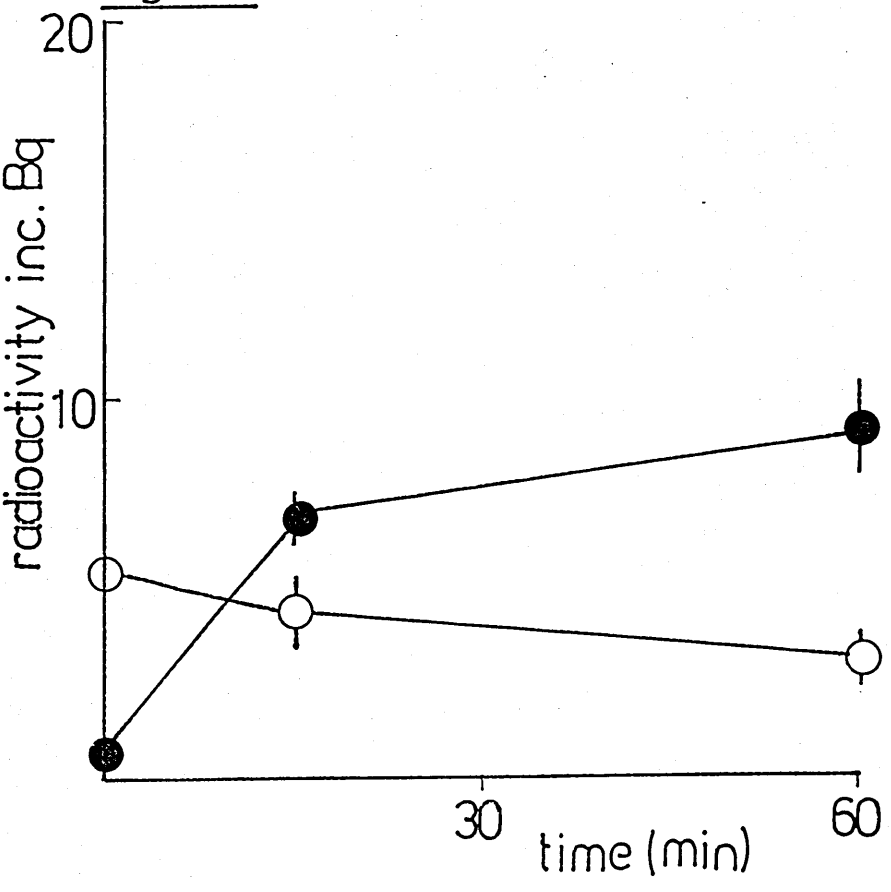


Figure 23

Effect of GDP-glucose presence in pre-incubation and incubation on incorporation of radioactivity from UDP-D-[U^{14}C]xylose by particulate enzyme preparation from bean suspension culture following two treatments:-

a) Pre-incubation with GDP-glucose (2 mmol.dm^{-3}), UDP-glucose (2 mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3})

b) Pre-incubation with GDP-glucose and $MnCl_2$ (10 mmol.dm^{-3})

Incubations were performed with GDP-glucose (2 mmol.dm^{-3}) and standard incubation (●——●), or with GDP-glucose (2 mmol.dm^{-3}) and standard incubation minus GDP-glucose (○—○).

Fig.23a

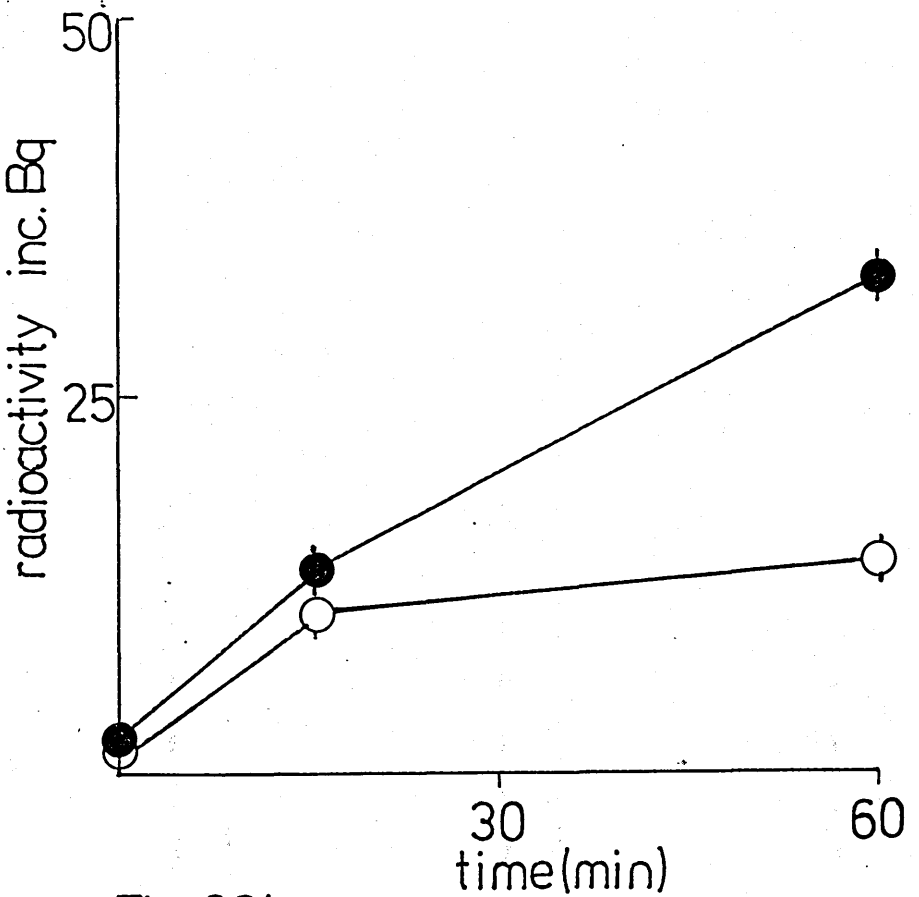
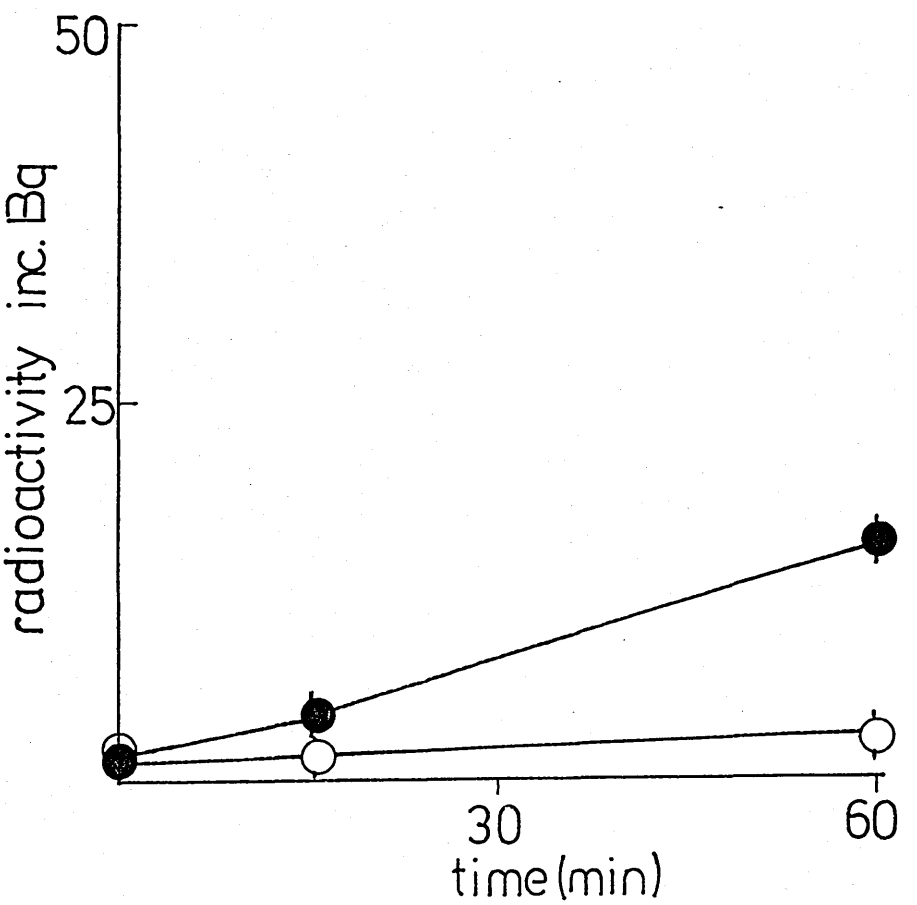


Fig.23b



previously been investigated by Ray (1980) in Golgi membranes of pea seedling, and by Hayashi and Matsuda (1981a) in particulate enzyme preparation from suspension-cultured soybean cells. There are marked similarities and differences between these systems, and the bean suspension-culture xylosyltransferase investigated in this study. Comparing the basic experimental conditions, Tris/HCl was a satisfactory buffer for the bean suspension-culture enzyme preparation, but was found by Hayashi and Matsuda to inhibit the transferase reaction. Both bean preparation and the soybean preparation of Hayashi and Matsuda lost activity on freezing, and in both cases enzyme activity was maximal on the sixth day after sub-culturing.

Ray (1980) found that incorporation from UDP-D-[U^{14}C]xylose into polymeric products was stimulated by UDP-glucose and by Mn^{2+}, though a divalent cation was not necessary for incorporation. Hayashi and Matsuda (1981a) also found that incorporation from UDP-D-[U^{14}C]xylose depended on UDP-glucose, the optimum concentration being around 2 mmol.dm^{-3}, and on Mn^{2+} which had an optimum concentration of 10 mmol.dm^{-3}. These conditions are identical to those found optimal for [^{14}C]xylose transfer by the particulate enzyme preparation from bean suspension-cultures, but stimulation of the enzyme by other sugar nucleotides was found to vary between systems. The pea Golgi-membrane transferase system was stimulated by GDP-glucose at 5 or 50 μmol.dm^{-3}, while the soybean suspension-culture system was stimulated equally by TDP or UDP-glucose, and at 50% by GDP-glucose. No such stimulation occurred for the bean suspension-culture xylosyltransferase. The

stimulation of the xylosyltransferase by GDP-glucose or GDP-mannose in the presence of UDP-glucose was apparently not investigated for the pea or soybean systems.

Pre-incubation of the xylosyltransferase system was performed for all three plant systems. Hayashi and Matsuda (1981) found that enzyme activity was lost on pre-incubation with UDP-glucose, and concluded that this fact, combined with the structure they propose for the xyloglucan product, suggests that the xylosyltransferase acts co-operatively with a glucosyltransferase, developing the xyloglucan chain by alternate transfer of glucose and xylose. Ray (1980) however found that pre-treatment of the pea Golgi membranes could stimulate incorporation from UDP-xylose even when the membranes were removed from the UDP-glucose solution before incubation with UDP-xylose. The stimulation obtained by pre-treatment was smaller than the stimulation obtained on addition of UDP-glucose during the transferase reaction. A similar result was found for the bean particulate enzyme preparation, though the stimulation in this case was much enhanced by the presence in the pre-incubation and incubation of GDP-glucose.

These results suggest that at least some of the xylose from UDP-xylose is transferred into xyloglucan by the particulate-enzyme preparation from bean suspension-cultured tissue, and further evidence was sought by analysing the [^{14}C]xylose-labelled product.

Chapter 5

CHARACTERISATION OF THE [^{14}C]XYLOSE-LABELLED PRODUCT FROM BEAN SUSPENSION-CULTURE ENZYME PREPARATION

The first step in the characterisation of the [^{14}C]xylose-labelled product was to establish that the radioactivity was still in the form of [^{14}C]xylose, and that no epimerisation to [^{14}C]arabinose or other modification had taken place.

Total acid hydrolysis of [^{14}C]labelled product

A [^{14}C]xylose-labelled pellet from a 60 min standard incubation, containing 60 Bq, was hydrolysed with 2N TFA for 1 hour (Ch.2 p.55). After rotary evaporating to dryness, the hydrolysate was spotted onto Whatman No.1 paper in 300 mm^3 H_2O then run in solvent D for 24 h. The sugars arabinose, xylose and glucose were applied as markers.

The radioactivity mostly co-chromatographed with xylose (Fig.1) with a small peak of [^{14}C]arabinose indicating a degree of epimerase activity (less than 6%).

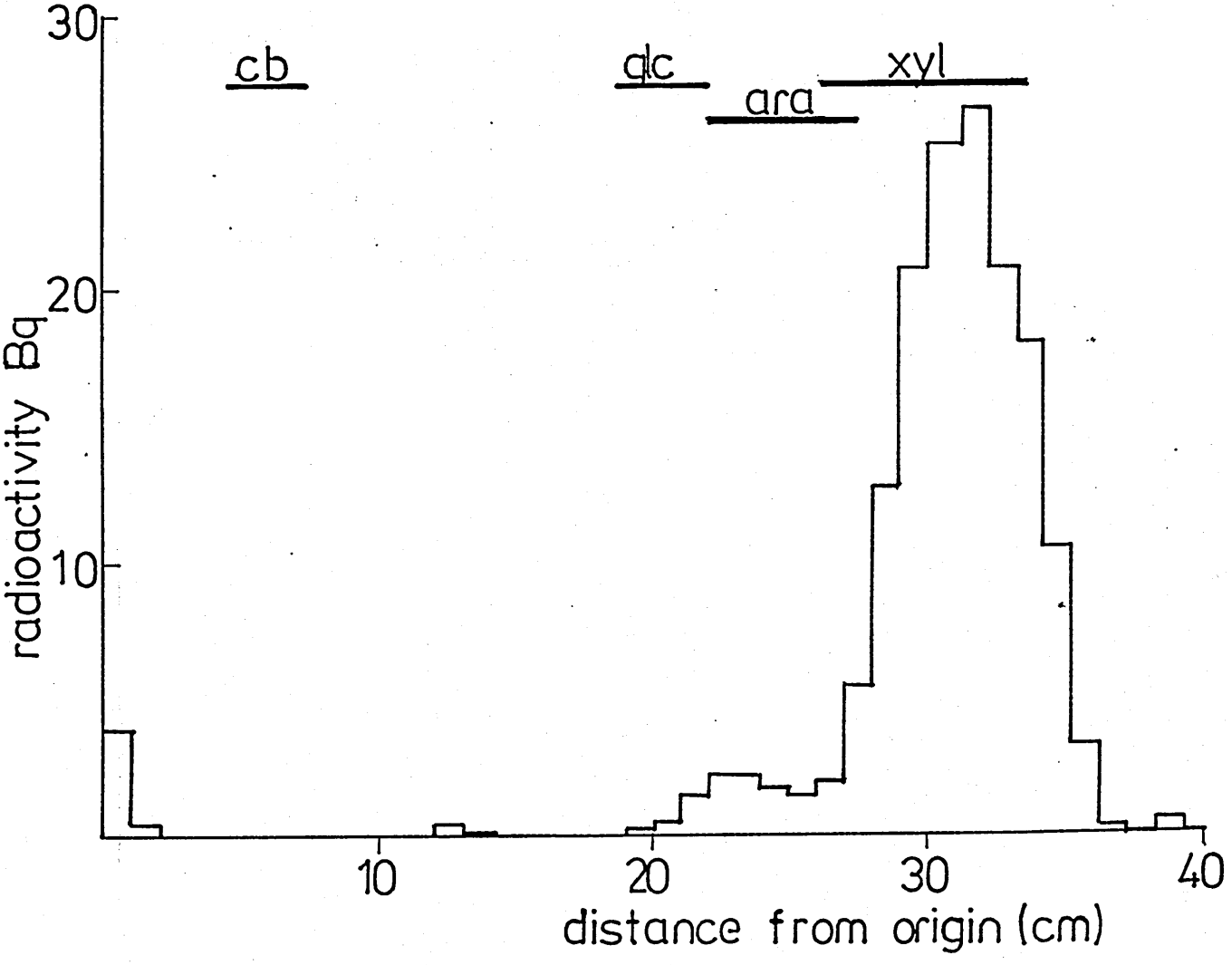
Estimation of [^{14}C]xylose-labelled product molecular weight

The molecular weight of the [^{14}C]xylose-labelled product was assessed by gel-filtration chromatography, initially using a Sepharose CL 6B column. A 60 min standard incubation product containing 25 Bq was first solubilised with cadoxen (Woods & McCrae, 1978; Ch.2 p.62). The solubilised product was run down a 25 x 1cm gel-filtration column in 50% cadoxen with blue dextran and dinitrophenyllycine as markers. Fractions of 0.5 cm^3 were collected, and the radioactive product was detected by scintillation counting

Figure 1

Paper chromatographic analysis of total acid hydrolysates of polysaccharide labelled with radioactivity incorporated from UDP-D-[U ^{14}C]xylose during 60 min incubations with standard incubation mixture.

Fig.1


30
20
10
radioactivity Bq
cb
glc
ara
xyl
10
20
30
40
distance from origin (cm)

using xylene scintillant.

The product eluted with the blue dextran marker, beyond the exclusion limit of the column, i.e. its molecular weight was greater than 1,000,000 (Fig.2a).

The experiment was repeated using a Sepharose CL 2B column for more accurate molecular weight estimation. The same procedure was followed and a radioactive peak was detected between blue dextran and dinitrophenyllycine markers (Fig. 2b). The product molecular weight is therefore in the range 1×10^6 - 20×10^6 Daltons.

Hayashi and Matsuda (1981b) have suggested that the activity of xyloglucan xylosyltransferase can be distinguished from other polysaccharide syntheases by analysis of the products using certain cellulase preparations. Hydrolysis of [^{14}C]xylose-labelled product with Aspergillus oryzae resulted in all of the xylosyl residues being recovered in [^{14}C]isoprimeverose (6-O-α-D-xylopyranosyl-D-glucopyranose), and based on this finding an assay method for the activity of xyloglucan-xylosyltransferase was developed by Hayashi and Matsuda. The enzyme preparation, driselase, which contains xylanase and glucanase activity, can also be used as it does not cleave the bond between the glucose in the glucan backbone and the xylose side chain, yielding the characteristic disaccharide.

Before performing driselase hydrolysis of the [^{14}C]xylose product, the chromatographic mobility of isoprimeverose was investigated.

Figure 2

Sepharose profile of cadoxen-solubilised polysaccharide (labelled with radioactivity from UDP-D-[$U^{14}C$]xylose during 60 min incubations with the standard incubation mixtures):-

a) Sepharose CL 6B profile

b) Sepharose CL 2B profile

Fig. 2a

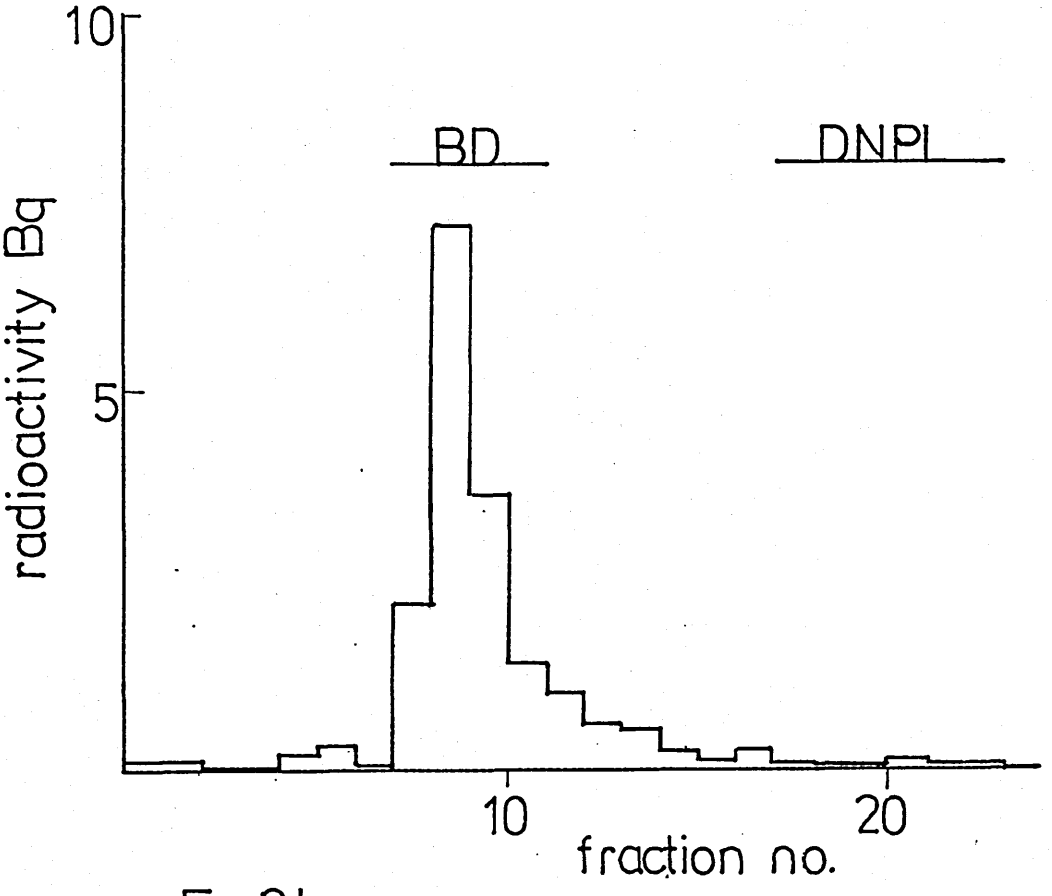
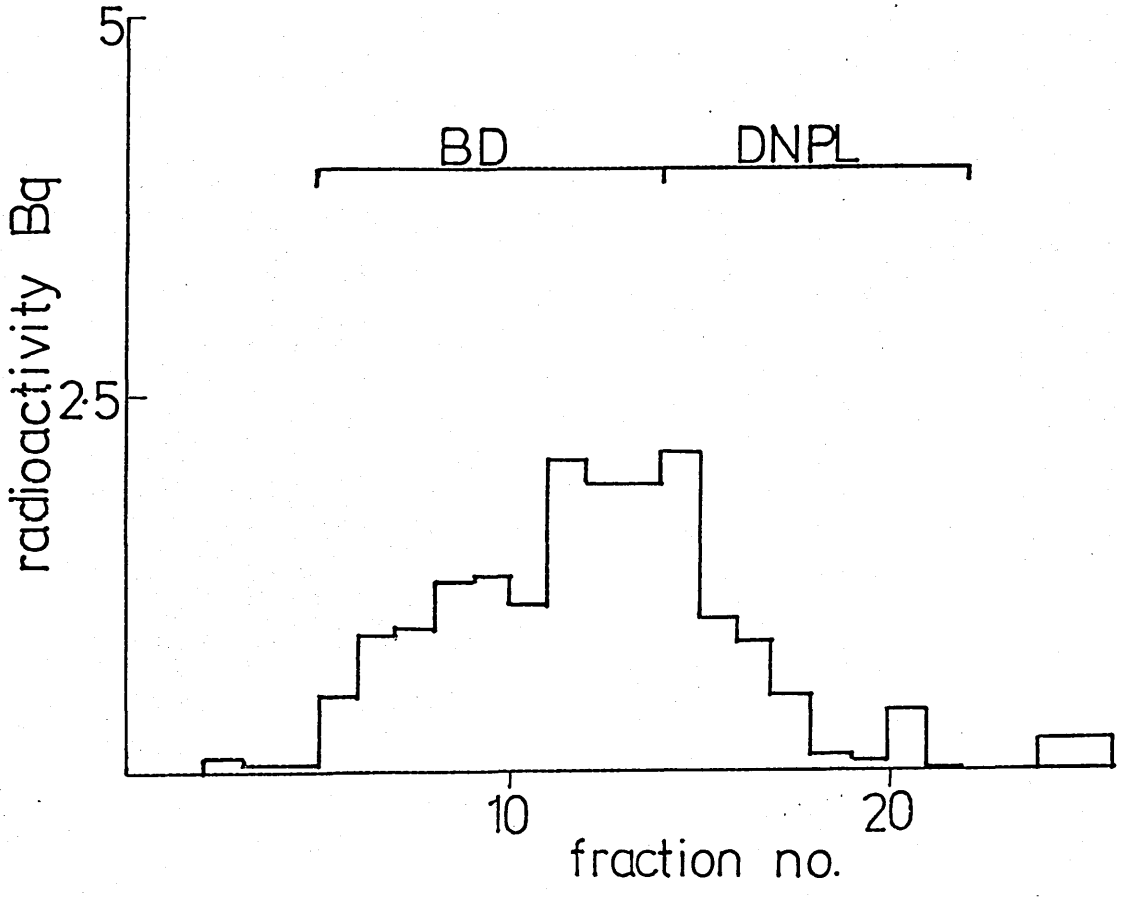

10
5
radioactivity Bq
BD
DNPl
10
20
fraction no.

Fig. 2b


5
2·5
radioactivity Bq
BD
DNPL
10
20
fraction no.

Descending paper chromatography of [^{3}H]isoprimeverose and mono- and disaccharide markers

Descending paper chromatography of a [^{3}H]isoprimeverose sample (containing 23.5 Bq) was performed for 36 h in solvent B. Cellobiose, glucose and galactose were used as markers.

The isoprimeverose showed a clear peak between cellobiose and galactose (Fig.3). Glucose ran slightly more quickly than galactose on this solvent system. Isoprimeverose could therefore be distinguished from the monosaccharides glucose and galactose, and from the disaccharide cellobiose.

The chromatographic mobility of isoprimeverose was compared with that of purified tamarind xyloglucan hydrolysates.

Driselase hydrolysis of purified tamarind xyloglucan

A sample of purified tamarind xyloglucan was hydrolysed with driselase overnight (Ch.2 p.58). The supernatant was rotary evaporated to dryness, dissolved in 300 mm^3 H_2O and spotted onto Whatman No.1 paper. Descending paper chromatography was carried out in solvent A for 30 h. Glucose and cellobiose were used as markers.

The hydrolysed xyloglucan gave three slightly overlapping peaks, the fastest moving co-chromatographing with glucose, the slowest

Figure 3

Paper chromatography of [^{3}H]isoprimeverose with mono- and disaccharide markers

Figure 4

Paper chromatographic separation of sugar markers, and driselase hydrolysates of purified tamarind xyloglucan (peaks 1, 2 & 3)

Fig.3

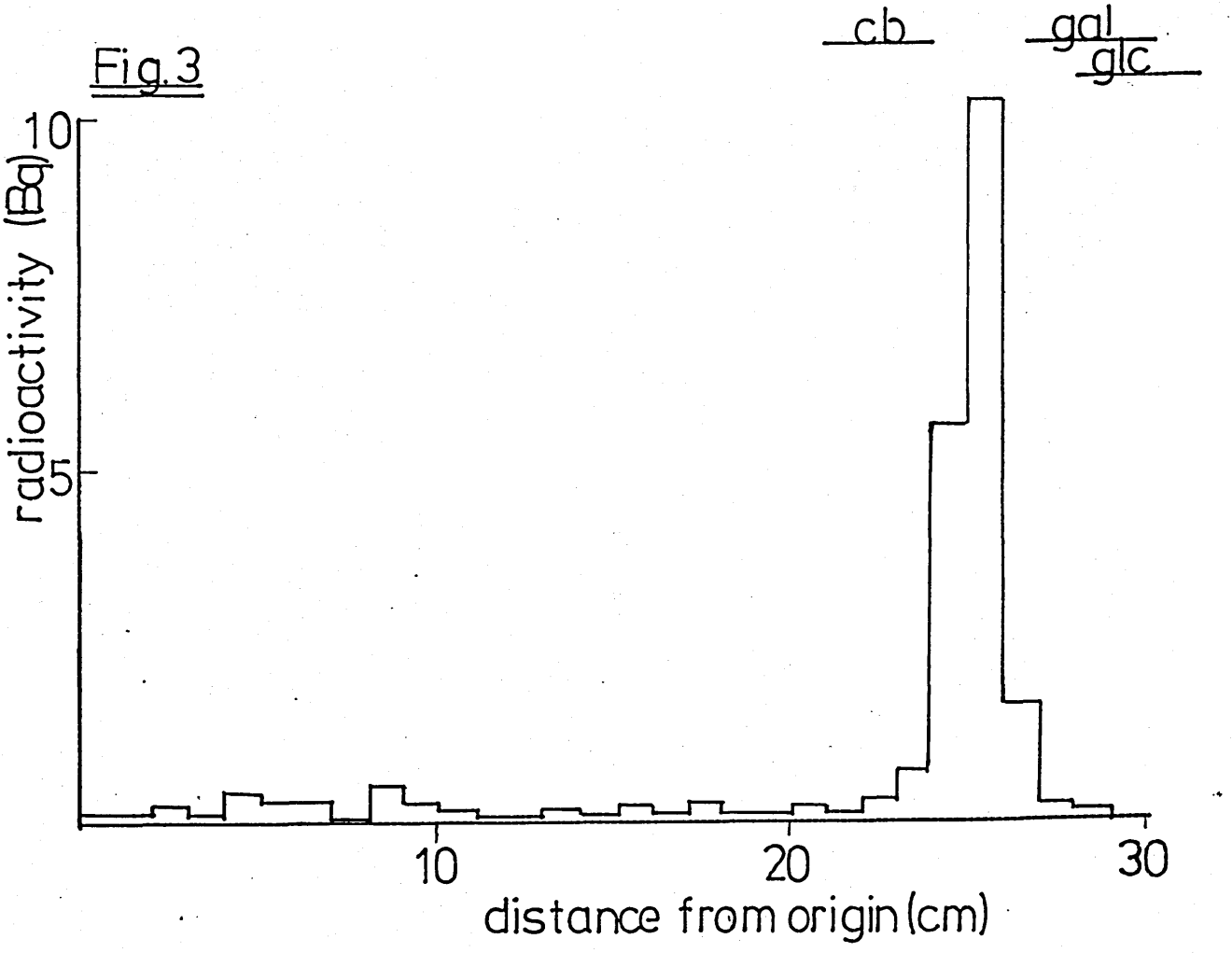
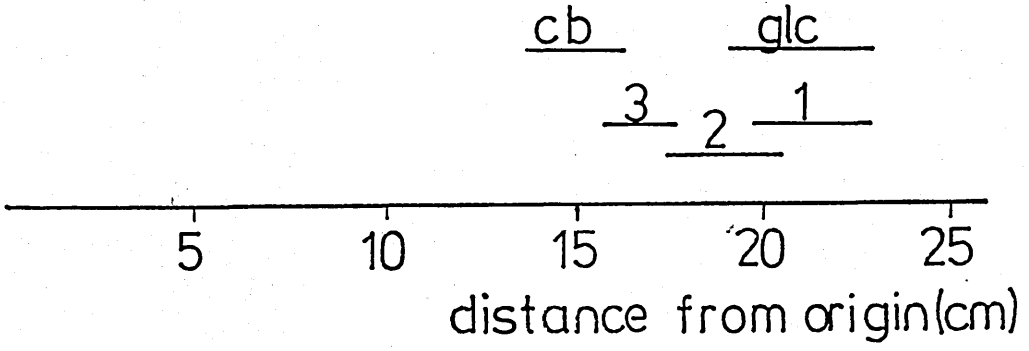

cb
gal
glc
radioactivity (Bq)
10
5
10
20
30
distance from origin (cm)

Fig.4


cb
glc
3
2
1
5
10
15
20
25
distance from origin (cm)

slightly ahead of cellobiose, with the middle peak between the two (Fig.4). The middle peak thus appeared to have the same chromatographic mobility as the [^{3}H]isoprimeverose in the previous experiment, with a monosaccharide peak, apparently of glucose.

Driselase hydrolysis of [^{14}C]xylose-labelled product - 1.

A [^{14}C]xylose-labelled 60 min incubation product containing 30 Bq was hydrolysed overnight with driselase (Ch.2 p. 58). Following hydrolysis, the supernatant was run in solvent A for 30 h with glucose and xylose as markers.

The radioactive peak ran between cellobiose and glucose (Fig.5), overlapping slightly with cellobiose, i.e. it was probably isoprimeverose.

The peak was further characterised by eluting and subjecting to complete acid hydrolysis.

Complete acid hydrolysis of [^{14}C]xylose-labelled disaccharide peak

The radioactively-labelled peak from the previous experiment was eluted as described (Ch.2 p. 61) and rotary evaporated to dryness. The residue was hydrolysed with TFA, then run in solvent D for 24 h. Xylose, glucose and arabinose were used as markers.

The resulting radioactive peak co-chromatographed with xylose with little suggestion of arabinose, so the disaccharide peak is

Figure 5

Paper chromatographic analysis of driselase hydrolysates of polysaccharide labelled with radioactivity incorporated from UDP-D-[U^{14}C]xylose during 60 min standard inbucations.

Figure 6

Paper-chromatographic separation of total acid hydrolysate of eluted disaccharide peak from Fig.5.

Fig.5

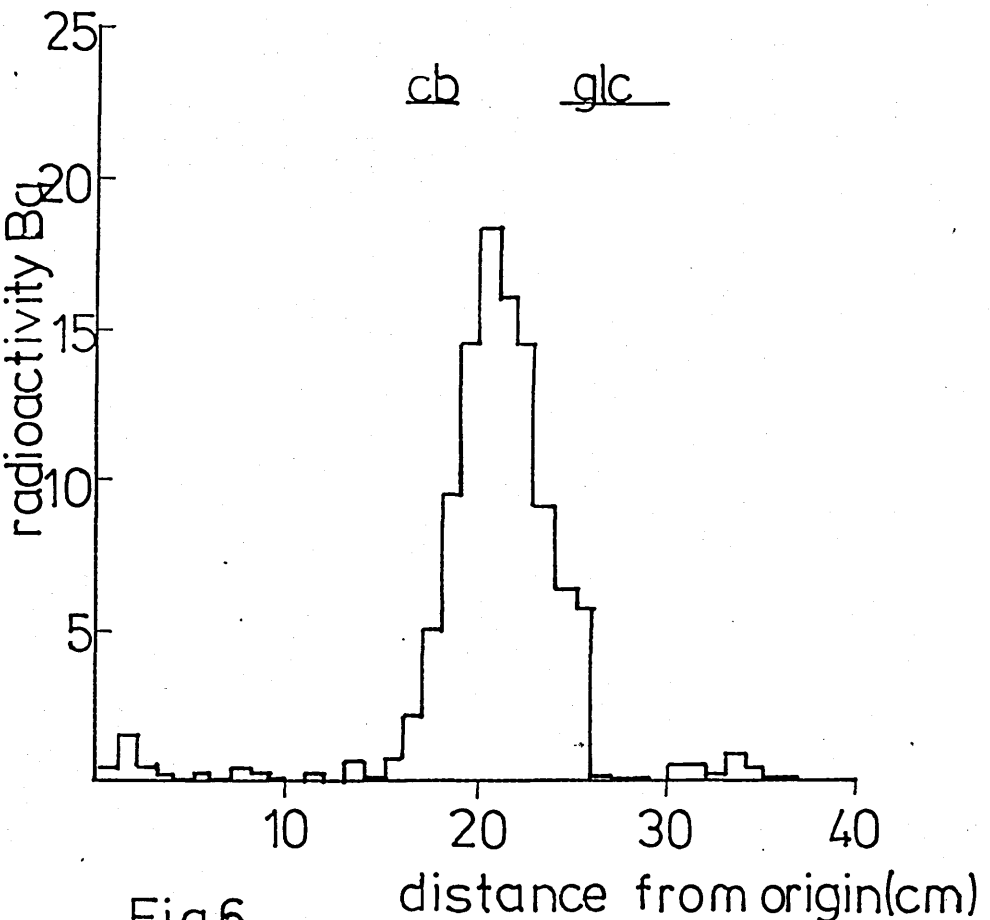
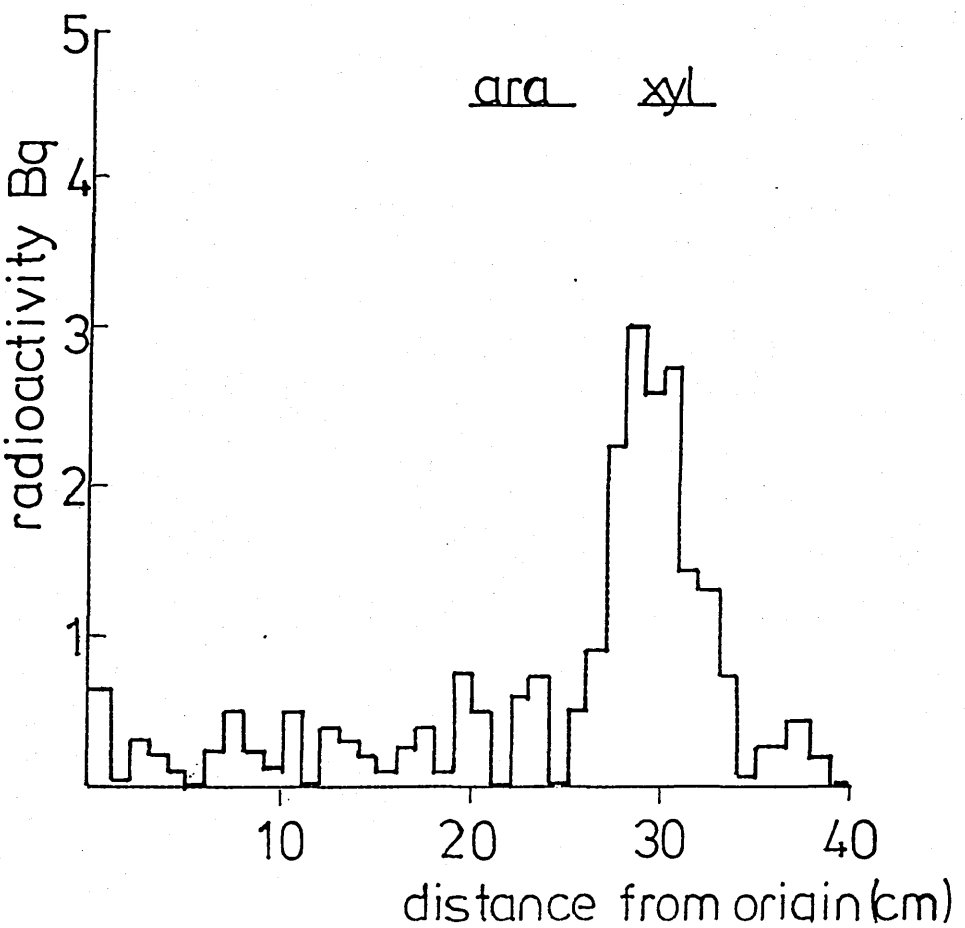

cb
glc
radioactivity Bq
25
20
15
10
5
10
20
30
40
distance from origin(cm)

Fig.6


ara
xyl
radioactivity Bq
5
4
3
2
1
10
20
30
40
distance from origin(cm)

[^{14}C]xylose-labelled (Fig.6).

Driselase hydrolysis of [^{14}C]xylose-labelled product - 2.

The [^{14}C]xylose-labelled driselase-hydrolysate products were again separated by descending paper chromatography in Solvent A. A 60 min incubation sample containing approx. 100 Bq was hydrolysed then the radioactive peaks resulting from paper chromatography were eluted and re-chromatographed three times to isolate the disaccharide peak. This final peak (Fig.7) co-chromatographed exactly with [^{3}H] isoprimeverose.

More information about the [^{14}C]xylose disaccharide was next sought by performing borohydride reduction. Borohydride reduces the sugar in the reducing position in a molecule to a sugar alcohol, and following reduction the disaccharide can be hydrolysed to yield the sugar and sugar alcohol. If in this case the disaccharide is in fact isoprimeverose, i.e. with glucose in the reducing position, the reaction will yield [^{14}C]xylose and non-radioactive glucitol, whereas a xylose backbone to the molecule would result in xylose being in the reducing position, and [^{14}C]xylitol would be produced.

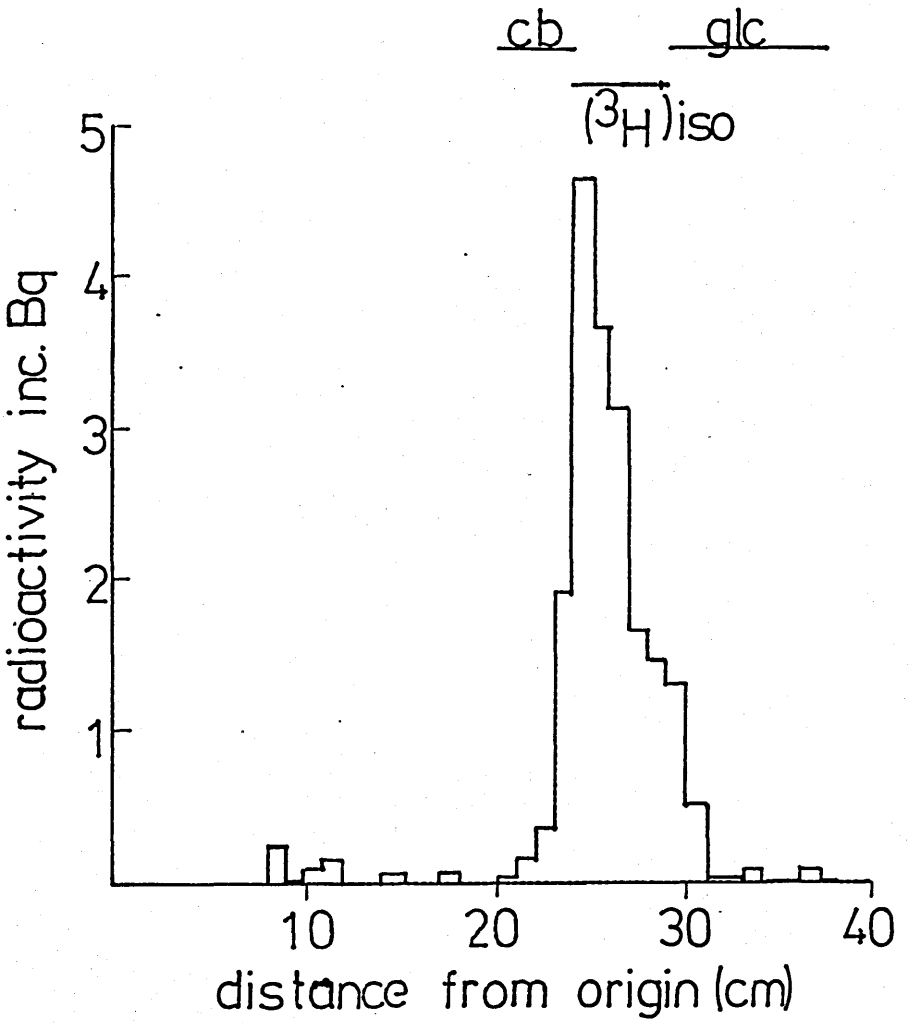
Analysis of [^{14}C]xylose-labelled disaccharide by borohydride reduction and hydrolysis

Radioactively-labelled xylitol was first produced for use as a marker to assist in identification of the product of borohydride reduction. The [^{14}C]xylitol was produced by borohydride reduction

Figure 7

Paper chromatographic analysis of driselase hydrolysates of polysaccharide labelled with radioactivity incorporated from UDP-D-[U^{14}C] xylose during 60 min standard incubations-disaccharide peak resulting from three successive elutions.

Fig.7


cb
glc
(3H)iso
radioactivity inc. Bq
5
4
3
2
1
10
20
30
40
distance from origin (cm)

of [^{14}C]xylose (see Ch.2 p.64) and this was then run against glucose and xylose on paper overnight in Solvent E (Rees & Reynolds 1978; Ch.2 p. 60).

The xylitol peak ran well beyond glucose and xylose on this solvent system (Fig.8), providing a suitable means of separating [^{14}C]xylose from [^{14}C] xylitol.

Borohydride reduction of the [^{14}C]xylose-labelled disaccharide was then carried out, using the eluted disaccharide peak identified in an earlier experiment (p.146 , Fig.7). The peak, which contained about 50Bq, was reduced with borohydride and hydrolysed as described (Ch.2 p. 64), then run in Solvent E with xylose and glucose as markers.

The [^{14}C]xylose peak co-chromatographed with xylose (Fig. 9). A small peak of presumably non-hydrolysed radioactively-labelled material ran 3-5 cm from the origin, and a small peak around 20 cm from the origin may correspond to a small quantity of [^{14}C]xylitol. The majority of the radioactive label however is in xylose, supporting the conclusion that the disaccharide product is isoprimeverose.

The glucan backbone of xyloglucan is made up of β(1-4)-linked glucose molecules. However, UDP-glucose is a precursor of β(1-3) glucan as well as β(1-4) glucan chains, and it is therefore conceivable that xylose might be added as a side-chain to β(1-3) glucan, rather than to β(1-4) glucan. To test this, the [^{14}C]xylose-labelled product

Figure 8

Chromatographic separation of glucose, xylose, and [^{14}C] xylitol produced by borohydride reduction of [^{14}C] xylose.

Figure 9

Paper chromatographic analysis of borohydride-reduced [^{14}C]xylose-labelled disaccharide peak (identified in Fig.7)

Fig.8

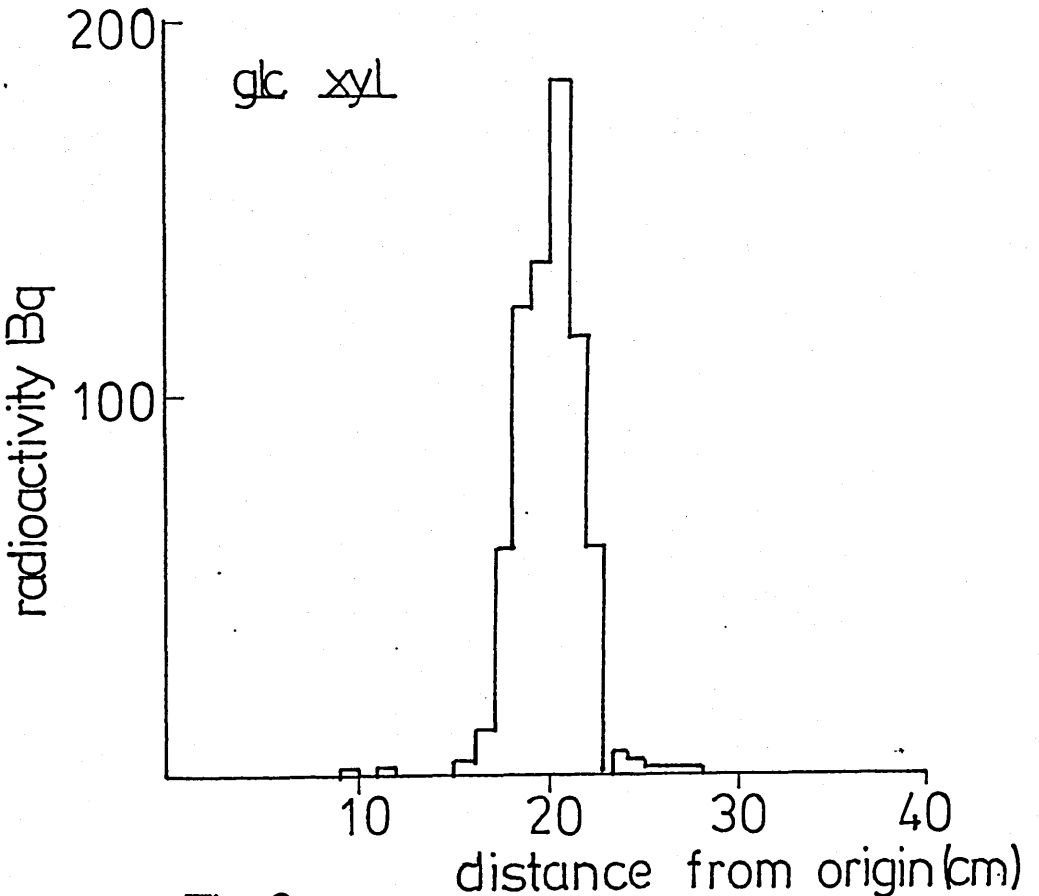
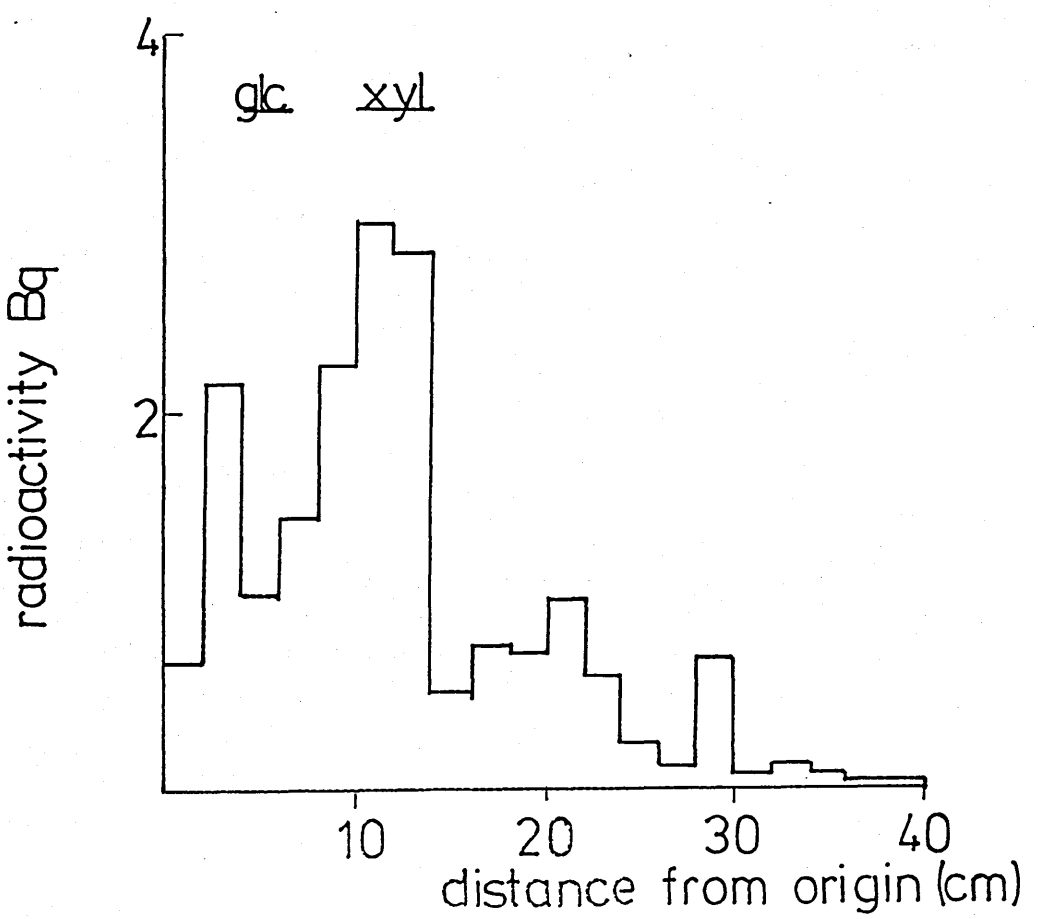

200
100
glc. xyl.
radioactivity Bq
10
20
30
40
distance from origin (cm)

Fig.9


4
2
glc. xyl.
radioactivity Bq
10
20
30
40
distance from origin (cm)

was subjected to laminarinase (β(1-3) glucanase) digestion.

Hydrolysis of the [^{14}C]xylose-labelled product with β(1-3) glucanase

Laminarinase is an enzyme preparation with β(1-3) glucanase, and possibly some β(1-4) glucanase activity. It breaks the β(1-3) glucan laminarin down to give a series of products including laminaritetrose, laminaritriose, laminaribiose and glucose.

To assess the activity of laminarinase, the hydrolysates of incubations of laminarinase with cellulose, xyloglucan and laminarin were compared.

Samples (10 mg) were suspended in either 1 cm^3 50 $mmol.dm^{-3}$ acetate buffer pH 4.5, or in buffer plus 10 mg laminarinase (1 cm^{-3}). Incubations were carried out at 25°C overnight. Samples of each hydrolysate were run in solvent D with glucose as a marker.

The glucose marker moved about 22 cm from the origin. The laminarin was hydrolysed to give a smear of lower molecular weight products with a clear peak at glucose. Xyloglucan and cellulose hydrolysates both showed a faint peak at glucose, but this occurred in enzyme- and buffer-incubated samples, and was probably due to the presence of a small quantity of glucose in the samples, rather than enzymic breakdown.

The laminarinase showed little activity against the β(1-4) glucans and in particular against xyloglucan, so it was suitable for

distinguishing between β(1-3) and β(1-4) glucan backbones.

Laminarinase hydrolysis of [^{14}C]xylose-labelled product

Two [^{14}C]xylose-labelled pellets froma standard incubation, and containing 38 Bq, were incubated with either buffer, or with buffer plus laminarinase, for 5 h. The supernatants were run in solvent D with glucose as a marker.

The glucose marker ran about 25 cm from the origin, but the radioactively-labelled buffer and enzyme incubated samples moved only 2 cm from the origin, with no evidence of any breakdown. The experiment was repeated with a 24 h enzyme incubation, but there was still no evidence of enzyme activity, indicating that the [^{14}C]xylose-labelled product does not have a β(1-3) glucan backbone.

Investigation of [^{14}C]xylose-labelled product binding to cellulose

Bauer et al (1973) have presented evidence in support of the hypothesis that hemicelluloses bind strongly to cellulose, and that this attachment is mediated through hydrogen bonds. In their experiments, 36-84% of a sycamore extracellular xyloglucan bound to Whatman cellulose in less than 5 min, and approximately 15-20% of the cellulose-bound xyloglucan was released by overnight incubation with Trichoderma viride endoglucanase. Valent and Albersheim (1974) found that the percentage binding of nine sugar xyloglucan fragments was increased by the addition of organic solvents, by reducing the

tendency of the fragments to hydrogen bond to the solvent. The percentage binding of xyloglucan fragments to cellulose at 2°C varied from 33% in 60% acetone to 38% in 65% acetone. At 25°C in 70% acetone 45% binding occurred.

A further property of xyloglucan and its digestion products reported by Aspinall et al (1969) is the desorption of cellulose-bound xyloglucan by 24% KOH, which brought about desorption of up to $\frac{2}{3}$ of the adsorbed radioactivity.

These properties were investigated for the [^{14}C] xylose-labelled product following the method of Camirand and Maclachlan (1986), who investigated the biosynthesis of a fucose-containg xyloglucan non-asaccharide by pea microsomal membranes. As investigations of cellulose binding are generally carried out with partially solubilised xyloglucan, the first step was the partial digestion of [^{14}C]xylose-labelled product. Pellets from 60 min UDP-D-[^{14}C]xylose incubations using the standard incubation mixture were pooled to give an 830 Bq sample. This was hydrolysed at 25°C with 0.5 ml 50 mg.cm^{-3} driselase in 50 mmol.dm^{-3} acetate buffer, pH 4.5. The supernatant was removed at 30 min intervals up to 90 min and fresh enzyme solution (0.5 cm^3) was added. The supernatant was dried onto a strip of Whatman No.1 paper and counted in toluene scintillant at each time interval. From the 30, 60 and 90 min samples, 116 Bq, 94 Bq and 32 Bq respectively were released into the supernatant. After elution from the paper and pooling of samples, a 228 Bq sample resulted. This sample was rotary

evaporated to dryness, dissolved in 400 mm^3 H_2O and then added with acetone to 100 mg cellulose powder to give an 83% acetone solution. This was stirred for 30 min and then left to settle for 2 h. After centrifuging at 700 x g for 5 min the supernatant was counted, by drying onto a chromatography paper strip and counting in tolvene scintillant. The supernatant contained 93.6 Bq i.e. 41% of the radioactivity. Thus 59% of the radioactivity was bound to the cellulose powder. This figure agrees well with that of Valent and Albersheim who found 45% binding in 70% acetone at 25°C.

The cellulose powder was then extracted for 30 min with 0.5cm^3 24% KOH. It was centrifuged as above, the supernatant was neutralised with two drops of acetic acid then counted in xylene scintaillant. This contained 56.8 Bq, i.e. 30.6% of the bound radioactivity was released from the cellulose by KOH.

These figures agree well with those recorded, and provide further evidence that the product is xyloglucan.

It is thought that some polysaccharides may be attached to protein during and/or following biosynthesis (Waldron & Brett, 1985). This was investigated for the [^{14}C]xylose-labelled product by analysing the products of proteinase digestion by gel-filtration. Firstly a proteinase digestion was performed to confirm that the product was not a glycoprotein containing short, [^{14}C]labelled oligosaccharide side chains.

Proteinase K digestion of [^{14}C]xylose-labelled polysaccharide product

Two [^{14}C]xylose-labelled pellets from a 60 minute standard incubation were incubated with Proteinase K or buffer as described (Ch.2 p.59). The samples contained 87.9 and 55.4 Bq respectively. After centrifuging, the supernatant and pellet from each incubation were counted in xylene scintillant.

There was no appreciable difference between enzyme-and buffer-incubated products (Table 1) i.e. no solubilisation by Proteinase K.

A more detailed examination of the products of Proteinase K digestion was carried out to identify any changes to the product brought about by hydrolysis. Before proceeding with this examination, the activity of Proteinase K against purified tamarind xyloglucan was examined as a control. An enzyme-incubated tamarind xyloglucan sample was solubilised in cadoxen (Ch.2 p.62) and then run down a sepharose CL6B column (48 x 1 cm) in 50% cadoxen with dinitrophenyllycine and blue dextran as markers. Samples of 0.5 cm^3 were collected and carbohydrate detected using phenol and suphuric acid (Ch.2 p.64). All carbohydrate was detected as one high molcular weight peak with no evidence of breakdown to smaller products.

Gel-filtration of Proteinase-K digested [^{14}C]xylose-labelled product

Samples labelled with [^{14}C]xylose from 60 min standard incubations were pooled to give two samples containing approx. 90 Bq each. Samples were incubated with Proteinase K or with buffer as described (Ch.2 p.59), and the incubation was terminated by addition of 100%

Table 1

Proteinase-K hydrolysis of [^{14}C]xylose-labelled polysaccharide product from 60 min standard incubations with UDP-D-[U^{14}C]xylose, incubated with a. enzyme or b. buffer

	Solution	Radioactivity Bq	% breakdown by enzyme
a.	Supernatant (enz. inc.)	23.2	16.4%
	Pellet (enz. inc.)	3.8	
b.	Supernatant (buf. inc.)	22.7	10.5%
	Pellet (Buf. inc.)	2.4	

cadoxen. Radioactivity was detected by counting in xylene scintillant, and blue dextran and dinitrophenyllycine were used as markers.

There was a marked difference in elution patterns of incubation products from the two incubations (Fig. 10a & b). The buffer incubation resulted in one single peak of relatively high molecular weight material, while the enzyme incubation gave two peaks, corresponding to the high molecular weight material of the buffer incubation, and a much lower molecular weight product, with an approximate molecular weight of 100,000. This suggests product breakdown by Proteinase K, and indicates that the product is attached to a protein. Estimations of molecular weights of products are likely to be inaccurate, as the column contracted by about 4 cm while samples were being run, presumably due to changes in its property brought about by cadoxen.

Effect of cellulose oligomer addition to standard UDP-D-[U^{14}C]xylose incubations

The existence of a 'primer' or 'acceptor' for cell wall polysaccharide biosynthesis has been postulated (Maclachlan, 1981; Stoddard, 1984). If such a molecule exists for the UDP-xylose xylosyltransferase system, its presence in incubations should increase incorporation of radioactivity markedly.

The effect of cellulose fragments of varying size on incorporation from UDP-D-[U^{14}C]xylose was measured by performing 60 min standard

Figure 10

Sepharose CL-2B profile of Proteinase-K-hydrolysed, cadoxen-solubilised polysaccharide (labelled with radioactivity from UDP-D-[U^{14}C]xylose during 60 min standard incubations:-

a) incubated with buffer

b) incubated with Proteinase-K

Fig.10a

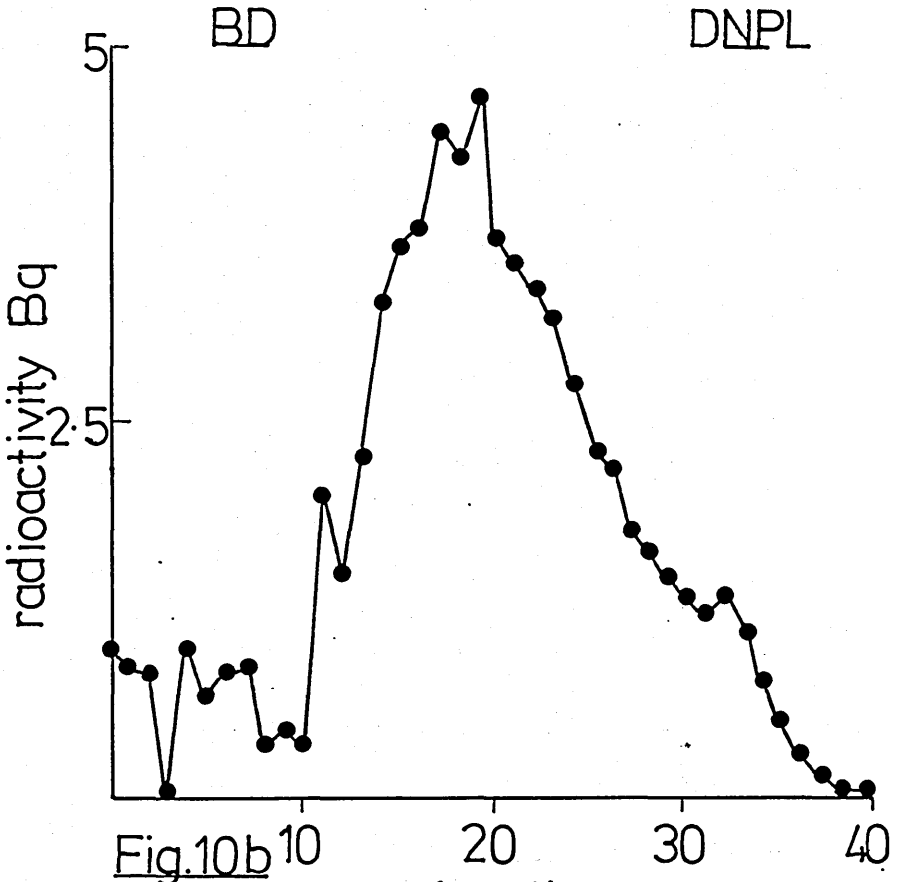


Fig.10b

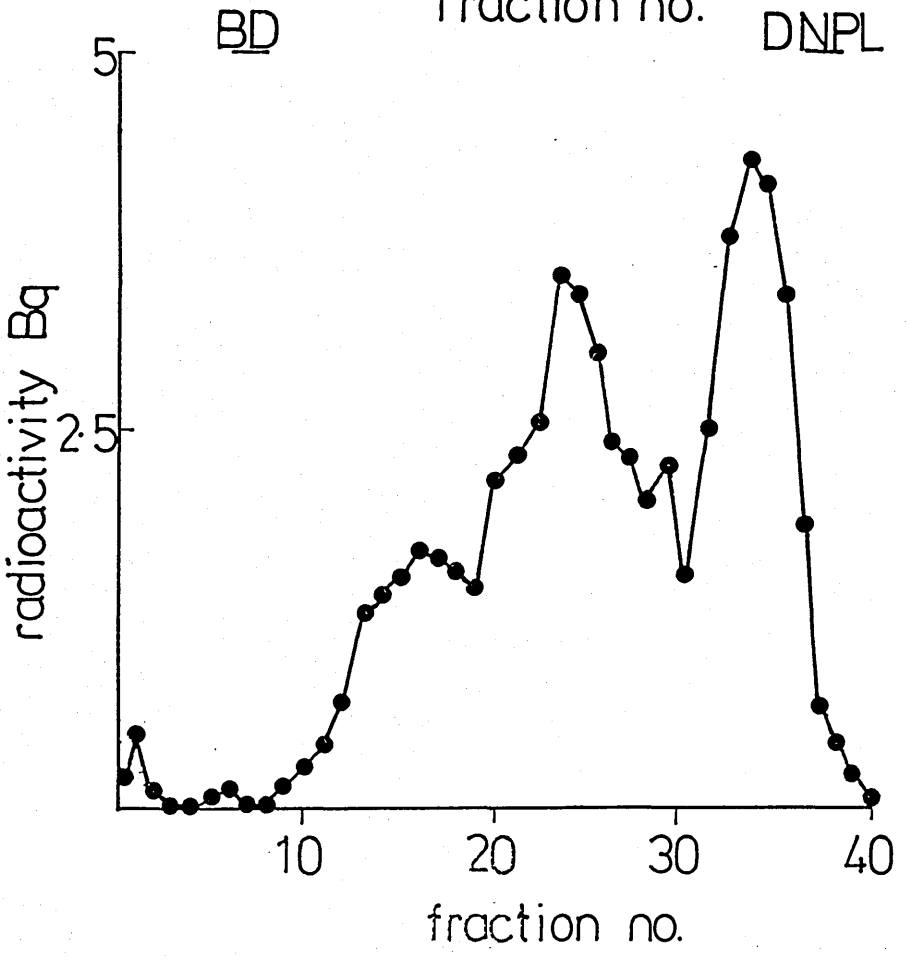
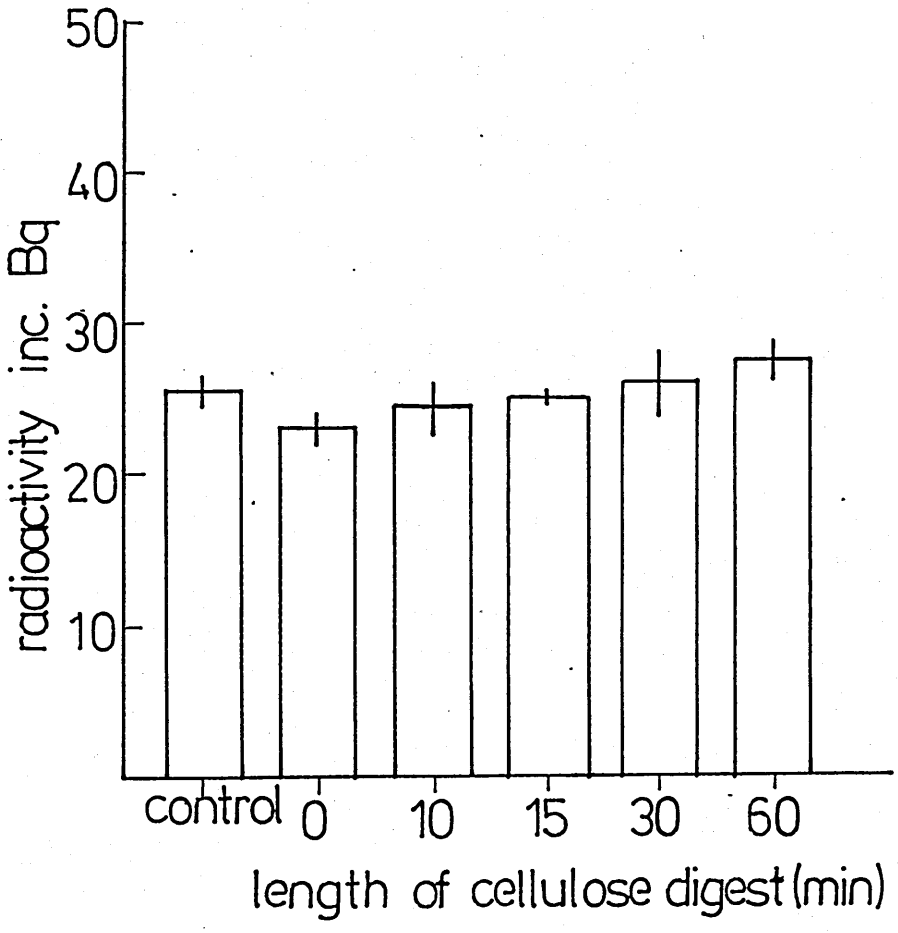


Figure 11

Effect of cellulose oligomer addition on incorporation from UDP-D-[$U^{14}C$]xylose by particulate enzyme preparation from bean suspension cultures: incorporation at 60 min standard incubations.

Fig.11


50
40
30
20
10
radioactivity inc. Bq
control
0
10
15
30
60
length of cellulose digest (min)

incubations with cellulose oligomers present in incubations. Cellulose oligomers were produced as described (Ch.2 p. 65). Standard incubations containing 740 Bq were used with 10 mm^3 of cellulose oligomer solution added to each. A control with no added cellulose was performed, and extracted pellets were counted in xylene scintillant.

There was no evidence of stimulation of incorporation of radioactivity by cellulose oligomer addition (Fig.11). There was however a slight inhibition of incorporation which was maximal where oligomer length was greatest (i.e. minimum hydrolysis time). Maximum inhibition however only totalled 16%, so the addition of cellulose oligomers seems to have little effect on the xylosyltransferase system.

Discussion

The product of incubation of UDP-D-[U^{14}C]xylose with bean particulate enzyme preparation is a [^{14}C]xylose-labelled polysaccharide with a molecular weight of between 1 x 10^6 and 20 x 10^6. There may also be a small proportion of a [^{14}C]arabinose-labelled product formed by epimerase action. At least some of the [^{14}C]xylose-labelled product is broken down by driselase to a characteristic disaccharide which co-chromatographs with isoprimeverose, a feature characteristic only of xyloglucan, according to Hayashi & Matsuda (1981c). This disaccharide was further characterised by borohydride reduction and found to have xylose at the non-reducing end, presumably with glucose

in the reducing position derived from the backbone of the polysaccharide product. The polysaccharide was further confirmed as a β(1-4) glucan by its resistance to enzymic breakdown by laminarinase, a β(1-3) glucanase.

Further evidence that the polysaccharide is xyloglucan was provided by a cellulose binding study. Of a [^{14}C]xylose-labelled sample, partially hydrolysed by driselase, 59% of the radioactivity was found to bind to cellulose powder in 83% acetone, and of this 30.6% was released from the cellulose by KOH.

The participation of a protein intermediate in heteropolysaccharide biosynthesis in plants has been postulated (Maclachlan, 1985) and a glycoprotein-dependant mechanism has been proposed (Robyt, 1979), as a result of studies on the biosynthesis of bacterial α-dextrans and fructans. There is however no direct evidence for involvement of protein intermediates in heteropolysaccharide biosynthesis. Incubation of the [^{14}C]xylose-labelled product from bean suspension cultures with proteinase K did not result in any release of radioactivity into the supernatant (Table 1), however analysis of cadoxen- solubilised [^{14}C] xylose-labelled product which had been incubated with Proteinase K resulted in a modification of the product (Fig. 10). This resulted in two peaks of radioactivity from the enzyme-incubation where only one resulted from the buffer incubation. This suggests that the molecular weight of the [^{14}C]labelled polysaccharide product is increased by its attachment to a protein. No attempt was made to characterise or identify

this protein as it was considered outwith the scope of this study, but this finding, as suggested by Spencer and Maclachlan (1972), does help to explain the relatively high molecular weight of polysaccharide products formed *in vitro* compared to those *in vivo*.

A second difference between *in vivo* and *in vitro* products highlighted by Maclachlan (1981) is the rapidity of synthesis in whole tissue compared with enzyme preparations, suggesting a missing element in these circumstances. Many groups have experimented with potential 'primers' adding e.g. cellodextrins, laminaridextrans and cellobiose to incubations to no effect. In this study cellulose powder and cellulose oligomers of varying length were added to incubations with UDP-D-[U^{14}C]xylose but no effect upon incorporation was observed (Fig.11).

As a final stage in the identification of the [^{14}C]xylose-labelled product, comparison was made with the product of a xylan-xylosyltransferase (Chapter 6).

Chapter 6

INVESTIGATION OF INCORPORATION OF XYLOSE FROM UDP-D-[U ^{14}C] XYLOSE INTO XYLAN BY BEAN SUSPENSION CULTURE

In the cell wall the main xylose-containing polysaccharides are the xyloglucans and the xylans. The xylans include arabinoxylans, which predominate in monocot primary walls, and glucuronoxylans, which form the principal xylan group in angiosperms. Xylose incorporation from UDP-xylose into xyloglucan was investigated by Dalessandro and Northcote (1981) in sycamore tissue, which was fractionated into cambial cells, differentiating xylem cells and differentiated xylem cells. Particulate enzyme preparations obtained from homogenates of these cells catalyzed the transfer of D-[U^{14}C]xylose from UDP-D-[U^{14}C]xylose into an alkali-soluble β(1 - 4)-linked xylan. The enzyme preparation was performed with potassium phosphate buffer and was centrifuged at 100,000 x g for 60 min (details in Chapter 2, p.53). The method of Dalessandro and Northcote (1981) was used in this study to investigate the biosynthesis of xylan by a particulate enzyme preparation from bean suspension culture to allow comparison of xylan and xyloglucan incubation products.

Incorporation of radioactivity from UDP-D-[U^{14}C]xylose into xylan by a particulate enzyme preparation from bean suspension culture.

Xylan biosynthesis in bean suspension-cultures was first investigated by modifying the incubation contents used by Dalessandro and Northcote (1981). The enzyme was prepared as outlined in Chapter 2 (p. 53) and incubations comprised either UDP-D-[U^{14}C] xylose (201 μmol.dm^{-3}, 370 Bq) and $MnCl_2$ (10 mmol.dm^{-3}), or UDP-D- U^{14}C xylose (0.38 μmol.dm^{-3}, 370 Bq) and $MnCl_2$ (10 mmol.dm^{-3}).

Incorporation of radioactivity into polysaccharide occurred in

Table 1

Incorporation of radioactivity from UDP-D-[U^{14}C]xylose into polysaccharide by particulate enzyme preparation from suspension-cultured bean from two incubation mixtures:

a) UDP-D-[U^{14}C]xylose (201.4 μmol.dm^{-3}, 370 Bq) and $MnCl_2$ (10 mmol.dm^{-3})

b) UDP-D-[U^{14}C]xylose (0.38 μmol.dm^{-3}, 370 Bq)

Incubation mixture concentration of UDP-D-[U^{14}C]xylose		Incubation time (min) 0	60
a)	200.4 μmol.dm^{-3}	1.7 ± 0 Bq	26.6 ± 0.3 Bq
b)	0.38 μmol.dm^{-3}	1.7 ± 0.2 Bq	61.9 ± 1.5 Bq

both incubation mixtures (Table 1), but was greatest where UDP-D-[U^{14}C]xylose was present at 0.38 μmol.dm^{-3} i.e. higher specific activity. Zero time values were low, so non-covalent binding did not appear to be a problem.

Further investigation of this system was carried out by looking at incorporation from UDP-D-[U^{14}C]xylose into xylan using the buffer used in previous investigations of xyloglucan biosynthesis i.e. Tri/HCl pH 7.5, 100 mmol.dm^{-3}.

Comparison of incorporation from UDP-D-[U^{14}C]xylose using potassium phosphate and Tris/HCl buffers

Enzyme preparations were made as previously (Ch.2, p.51), using either potassium phosphate or Tris/HCl for homogenisation and resuspension.

The standard incubation mixture was used and incubations were terminated at 0, 15 and 60 min.

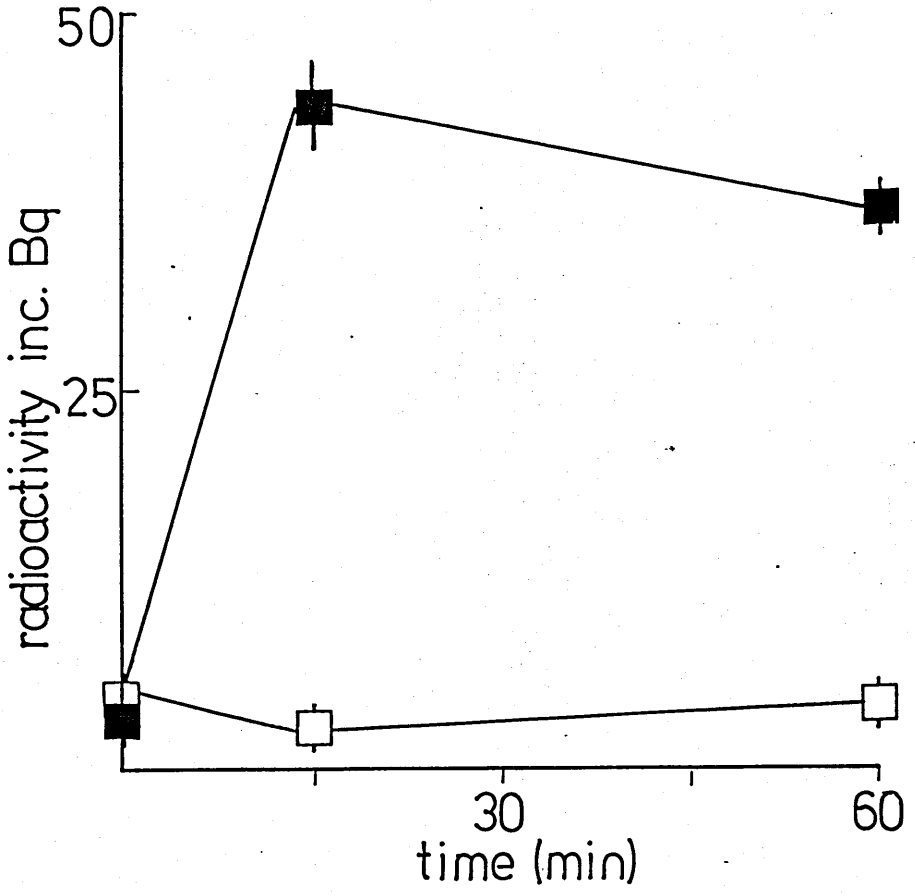
Incorporation was considerably greater where potassium phosphate was used as the buffer (Fig. 1). Where Tris/HCl was used zero time values were relatively high, and incorporation increased only marginally over 60 min. Thus the enzyme complex which is active when potassium phosphate is used does not seem to be active when Tris/HCl is used. This suggests the product is not xyloglucan, the synthesis of which appears to require different conditions.

This was further investigated by analysing the products of the two systems.

Figure 1

Incorporation of radioactivity from UDP-D-[U ^{14}C]xylose by particulate enzyme preparation from suspension-cultured bean using (■—■) potassium phosphate or (□—□) Tris/HCl as homogenisation and resuspension buffers (standard incubations).

Fig.1



Analysis of [^{14}C]xylose-labelled xylan product by enzyme hydrolysis.

A [^{14}C]-xylose-labelled product from a xylan preparation, containing approx. 75 Bq was incubated for 6 hours with driselase in acetate buffer (Ch.2 p. 58). A sample of commercial xylan was similarly hydrolysed for use as a marker along with xylose. Samples were run on paper chromatography in solvent C for 24 hours.

The hydrolysed xylan marker gave a series of peaks corresponding to a small amount of partially-hydrolysed high molecular-weight material at the origin, clear penta-, tetra-, and tri-saccharide peaks and less distinct di- and mono-saccharide peaks. The mono-saccharide peak cochromatographed with the xylose marker. The radio actively-labelled sample (Fig. 2) gave a very similar pattern of peaks, with the clearest peaks corresponding to penta- and tetra-saccharides, and smaller, less distinct peaks of tri-, di- and monosaccharides.

The driselase hydrolysis products from the [^{14}C] xylose-labelled xylan incubation product thus appeared to be identical to those from the xylan hydrolysis, suggesting the incubation product is a xylan. This was checked by comparing the driselase hydrolysis products of a xylan preparation with those from a xyloglucan preparation.

Driselase hydrolysis of [^{14}C] xylose-labelled xyloglucan product

A [^{14}C]xylose-labelled xyloglucan preparation sample containing approximately 95 Bq was hydrolysed for 6 hours with driselase (Ch.2 p.58) and run on the same solvent system as above. Cellobiose and glucose

Figure 2

Paper-chromatographic separation of driselase-hydrolysed products of incubation of UDP-D[U^{14}C]xylose with bean particulate enzyme preparation (using xylan preparation standard incubations); run with driselase-hydrolysed xylan markers, x 2- x 5.

Figure 3

Paper-chromatographic separation of driselase-hydrolysed products of incubation of UDP-D-[U^{14}C]xylose with bean particulate enzyme preparation (using xyloglucan preparation standard incubations); run with sugar markers.

Fig.2

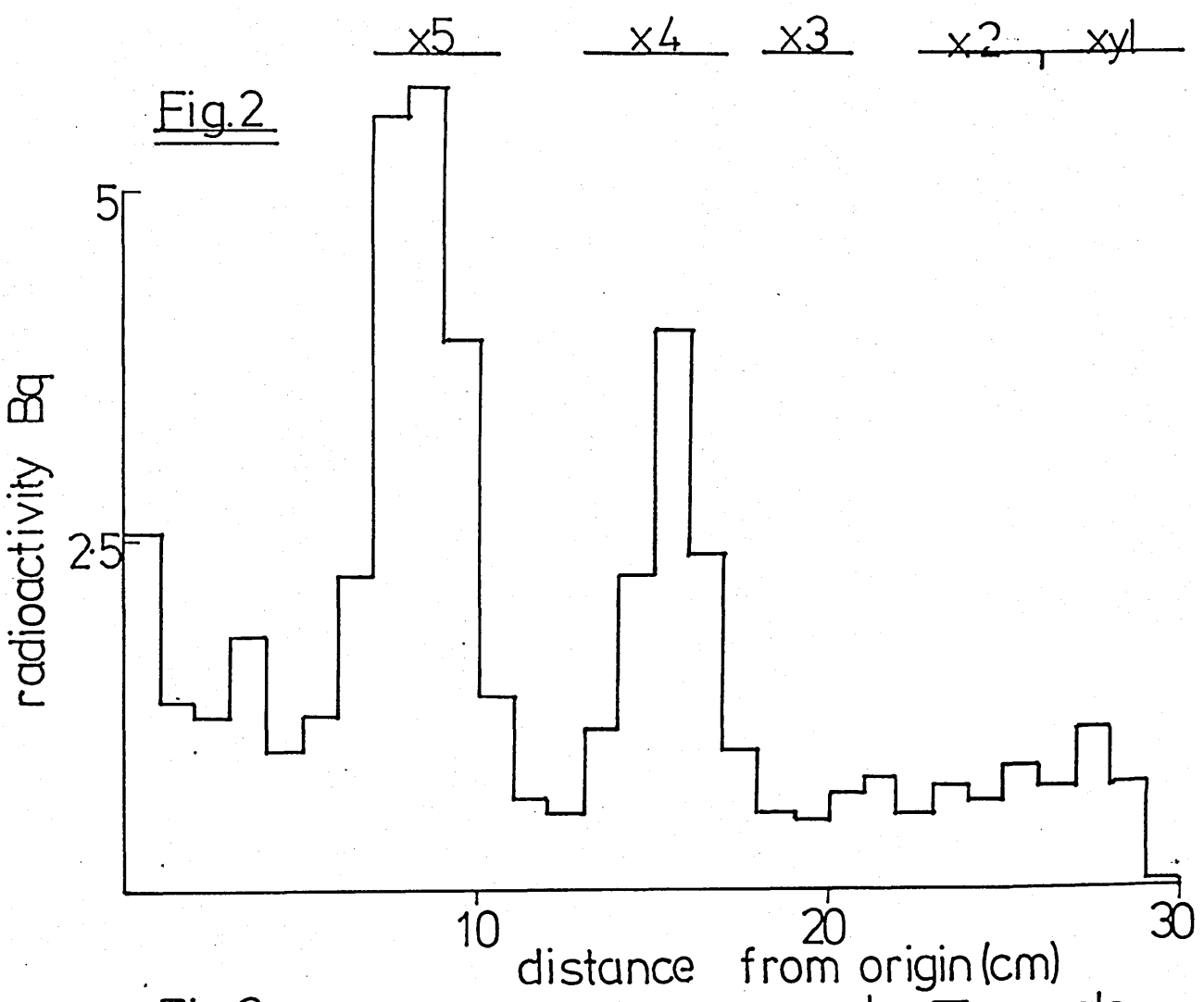
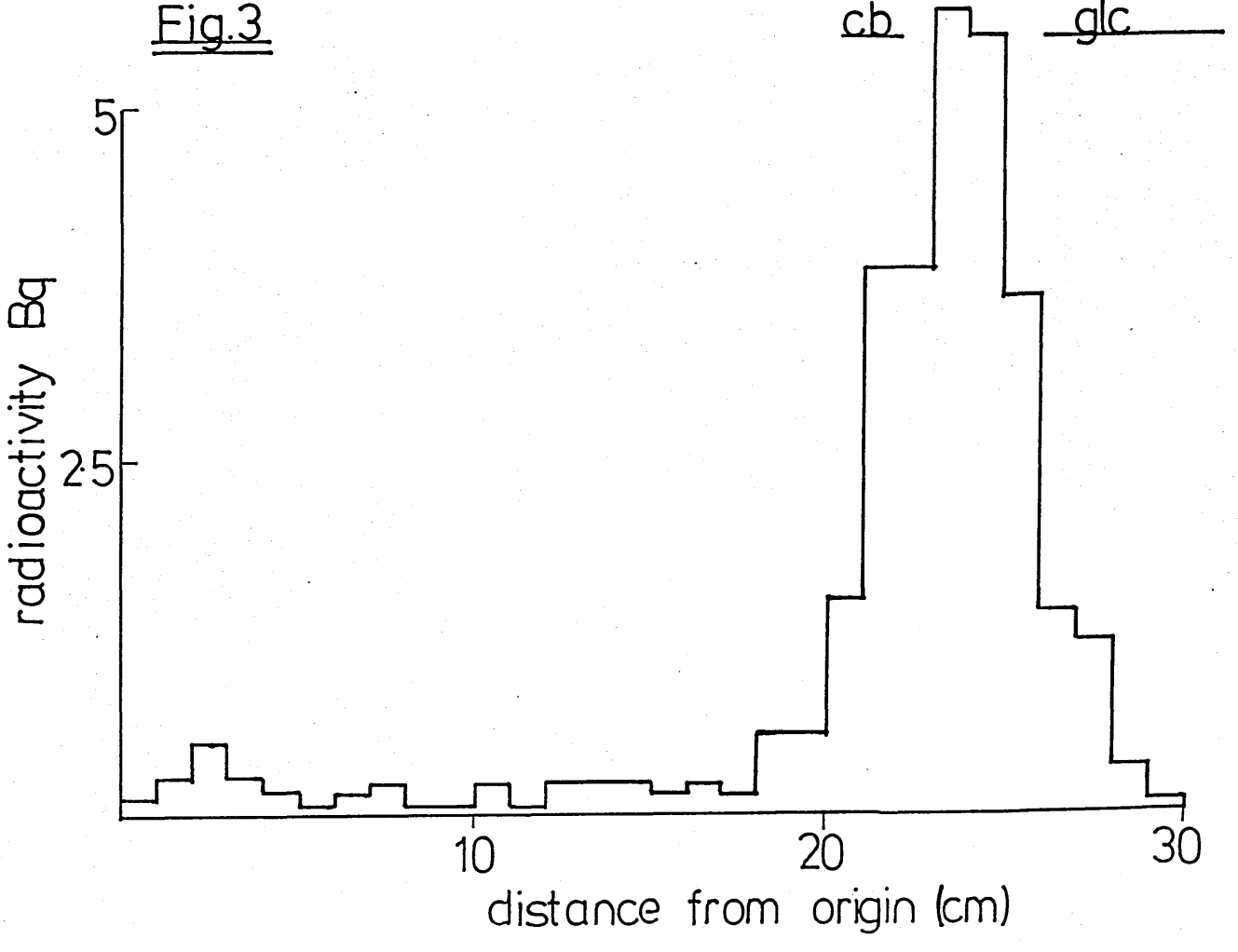

x5
x4
x3
x2
xyl
5
2·5
radioactivity Bq
10
20
30
distance from origin (cm)

Fig.3


cb
glc
5
2·5
radioactivity Bq
10
20
30
distance from origin (cm)

were used as markers.

A single peak of radioactivity was found, running between the cellobiose and glucose markers, i.e. probably a disaccharide, and a small peak at the origin corresponding to non-hydrolysed material (Fig. 3). The peak was spread over several centimeters, so may correspond to a mixture of different sized products. Given that the enzyme hydrolysis and chromatography systems used for xylan and xyloglucan products were identical, it is evident that the initial incubation products are not the same.

Hydrolysis of [^{14}C]xylose-labelled xylan product by incubation with laminarinase

Although no xyloglucan with a B(1-3) linked glucan backbone has been reported amongst higher plant cell wall polysaccharides, as a final characterisation step the xylan-incubation product was hydrolysed with a B(1-3) glucanase, laminarinase. This enzyme had little activity against (1-4) linked glucan derivatives such as xyloglucan (see earlier experiment, Ch.5 p.149).

Laminarinase hydrolysis was carried out as described (Ch.2 p.59) and the hydrolysate was run on the same chromatography system as in the previous experiment. Laminarinase-hydrolysed laminarin and glucose were used as markers.

The sample did not move from the origin and no peaks were detected. The laminarinase-hydrolysed laminarin gave a series of peaks on development with silver nitrate, the clearest being tri- and disaccharides,

and a monosaccharide which co-chromatographed with the glucose marker. The radioactively-labelled sample did not therefore appear to contain β(1-3) glucan linkages.

Particulate enzyme preparations from suspension-cultured bean appear to be capable of incorporating xylose from UDP-D-[U^{14}C] xylose into xyloglucan or into xylan, depending upon the experimental conditions used. The two systems seem to be distinct with their own specific requirements.

Discussion

The incorporation of [^{14}C]xylose from UDP-D-[U^{14}C]xylose into xylan was investigated by Dalessandro and Northcote (1981), and the same system was used to investigate xylan biosynthesis in suspension-cultured bean cells. Although Dalessandro and Northcote used enzyme preparations from differentiating or differentiated xylem cells where secondary walls would be expected to predominate, similar results were found for the bean suspension-culture tissue, suggesting similar products may result from the incubations.

The products of the xylan preparation and the xyloglucan preparation from chapter 4 were compared. The major differences between the preparations were the buffer used and the duration of centrifugation. Using the xyloglucan preparation, incorporation of

radioactivity from UDP-D-[U^{14}C] xylose only occurred when UDP-glucose was present (chapter 4), suggesting the products of these systems differed, despite the same tissue being used for both.

Driselase hydrolysis of the radioactively-labelled product of the xylan preparation gave a series of peaks, co-chromatographing with certain products resulting from the driselase hydrolysis of larch-wood xylan, mainly four and five carbon hydrolysates. Driselase hydrolysis of the radioactively-labelled product of the xyloglucan preparation resulted in one peak only, a disaccharide which co-chromatographed with isoprimeverose. This confirmed that the products of the two enzyme systems differ.

Further characterisation of the [^{14}C]-labelled product of the xylan system by hydrolysis with laminarinase showed that it was unlikely to have β(1-3) glucan linkages as no breakdown of product occurred with this enzyme.

If the product of the enzyme system with particulate preparation from bean investigated here is a xylan, then it is most unlikely that the product of the glucose-stimulated incorporation investigated in the previous two chapters is also a xylan, as the two systems clearly have different requirements and properties. This provides further evidence that the product is xyloglucan.

Chapter 7

INVESTIGATION OF INCORPORATION OF GLUCOSE FROM UDP-D-[$U^{14}C$] GLUCOSE BY BEAN SUSPENSION CULTURE

There exists a wide range of glucose-containing cell wall polysaccharides. This fact, coupled with the possibility of epimerase or other modification of the UDP-glucose precursor, is likely to make isolation and identification of specific synthetase enzymes and their products difficult. Glucosyl transferases involved in the biosynthesis of xyloglucan have however been studied by two groups. Hayashi and Matsuda (1984) studied a xyloglucan-4-B-D-glucosyltransferase in suspension-cultured soybean cells. They found that incorporation of [^{14}C]glucose into xyloglucan depended on the presence of UDP-xylose in the incubation mixture, the optimum concentration being 10 - 30 μmol.dm^{-3}. Ray (1980) studied a glucosyltransferase of pea Golgi membranes, and found no stimulation by UDP-xylose, though at concentrations above 50 μmol.dm^{-3} inhibition occurred.

The incorporation of glucose from UDP-D-[U^{14}C]glucose was investigated for the bean suspension culture by first studying the effect of varying sugar nucleotide composition of incubation.

Effect of varying UDP-xylose concentration on incorporation of radioactivity from UDP-D-[U^{14}C]glucose - 1.

Incubations were performed for 0 and 15 min. and contained UDP-D-[U^{14}C]glucose (0.8 μmol.dm^{-3}, 925 Bq) $MnCl_2$ (10 mmol.dm^{-3}) and a range of UDP-xylose concentrations from 0 to 200 μmol.dm^{-3}. Incorporation of radioactivity from incubations was negligible at all concentrations of UDP-xylose.

The experiment was repeated with non-radioactive UDP-glucose and

UDP-xylose present in incubations.

Effect of UDP-xylose concentration on incorporation of radioactivity from UDP-D-[U^{14}C]glucose - 2.

Incubations were performed with a constant UDP-glucose concentration, and varying UDP-xylose concentrations. Incubations contained 0-200 μmol.dm^{-3} UDP-xylose, UDP-D-[U^{14}C]glucose (200.77 μmol.dm^{-3}, 925 Bq) and $MnCl_2$ (10 mmol.dm^{-3}), and were terminated at 0 and 15 min.

Incorporation was generally high but with wide variation between replicates (Table 1). Maximum incorporation occurred in the absence of UDP-xylose, and incorporation decreased with increasing UDP-xylose, concentration. There was no evidence of stimulation of incorporation by UDP-xylose.

The lack of stimulation of incorporation by UDP-xylose, wide variation between replicates and lack of incorporation in the absence of unlabelled UDP-glucose suggests that much of the incorporation may be into β(1-3) glucan rather than xyloglucan. To investigate this further, analysis of the product of incorporation from UDP-D-[U^{14}C] glucose was attempted.

Total acid hydrolysis of [^{14}C]glucose-labelled product

To determine whether epimerisation or any other modification of the substrate was affecting incubations with UDP-D-[U^{14}C]glucose, complete acid hydrolysis of the labelled product was carried out.

Table 1

Incorporation of radioactivity from UDP-D-[U^{14}C]glucose by particulate enzyme from bean suspension culture: incubations were terminated at 15 min and contained $MnCl_2$ (10 mmoldm^{-3}), UDP-D-[U^{14}C]glucose (200.77 μmoldm^{-3}, 925 Bq) and UDP-xylose (0-200 μmoldm^{-3}).

Concentration UDP-xylose (μmoldm^{-3})	Incorporation of radioactivity at 15 min (Bq)
0	155.9 ± 24.3
2	140.9 ± 8.9
20	108.3 ± 57.1
200	101.5 ± 35.5

This was followed by descending paper chromatography to separate, and allow identification of, the products of hydrolysis. Fifteen minute incubations were carried out, using the standard preparation. Following extraction, a pellet which contained approx. 40 Bq was hydrolysed with TFA as described (ch.2 p.55), and then rotary evaporated to dryness. It was spotted onto Whatman No.1 paper and run in Solvent D (ch.2 p.60) for 30 min.

The radioactive peak appeared to co-chromatograph with glucose. There was little evidence of epimerisation to [^{14}C]galactose (Fig. 1)

Enzymic hydrolysis of [^{14}C]glucose-labelled product using driselase

Driselase is an enzyme mixture which contains xylanase and glucanase activity. It will not break the glucose-xylose bond between the glucose of the xyloglucan backbone and the xylosyl side chains, so hydrolysis of xyloglucan with driselase yields a distinctive disaccharide, isoprimeverose, which can be used to confirm the presence of xyloglucan (as previously described, ch.5 p.139).

A [^{14}C]glucose-labelled pellet containing 150 Bq was obtained from a 15 minutes incubation using standard conditions. It was hydrolysed with driselase for 6 h as described (Ch. 2 p.58) and the resulting hydrolysates were separated on Whatman No.1 paper in solvent B. Peaks of radioactivity initially occurred as smears, and it was necessary to elute peaks three times to isolate di- and monosaccharides. Cellobiose, glucose and [^{3}H]isoprimeverose were used as markers.

Figure 1

Paper chromatographic analysis of total acid hydrolysates of [^{14}C]glucose-labelled product of incubation of UDP-D-[U^{14}C] glucose with particulate enzyme preparation from suspension-cultured bean.

Figure 2

Paper chromatographic analysis of driselase hydrolysates of [^{14}C] glucose-labelled product from incubation of UDP-D-[U^{14}C] glucose with particulate enzyme preparation from suspension-cultured bean.

Fig.1

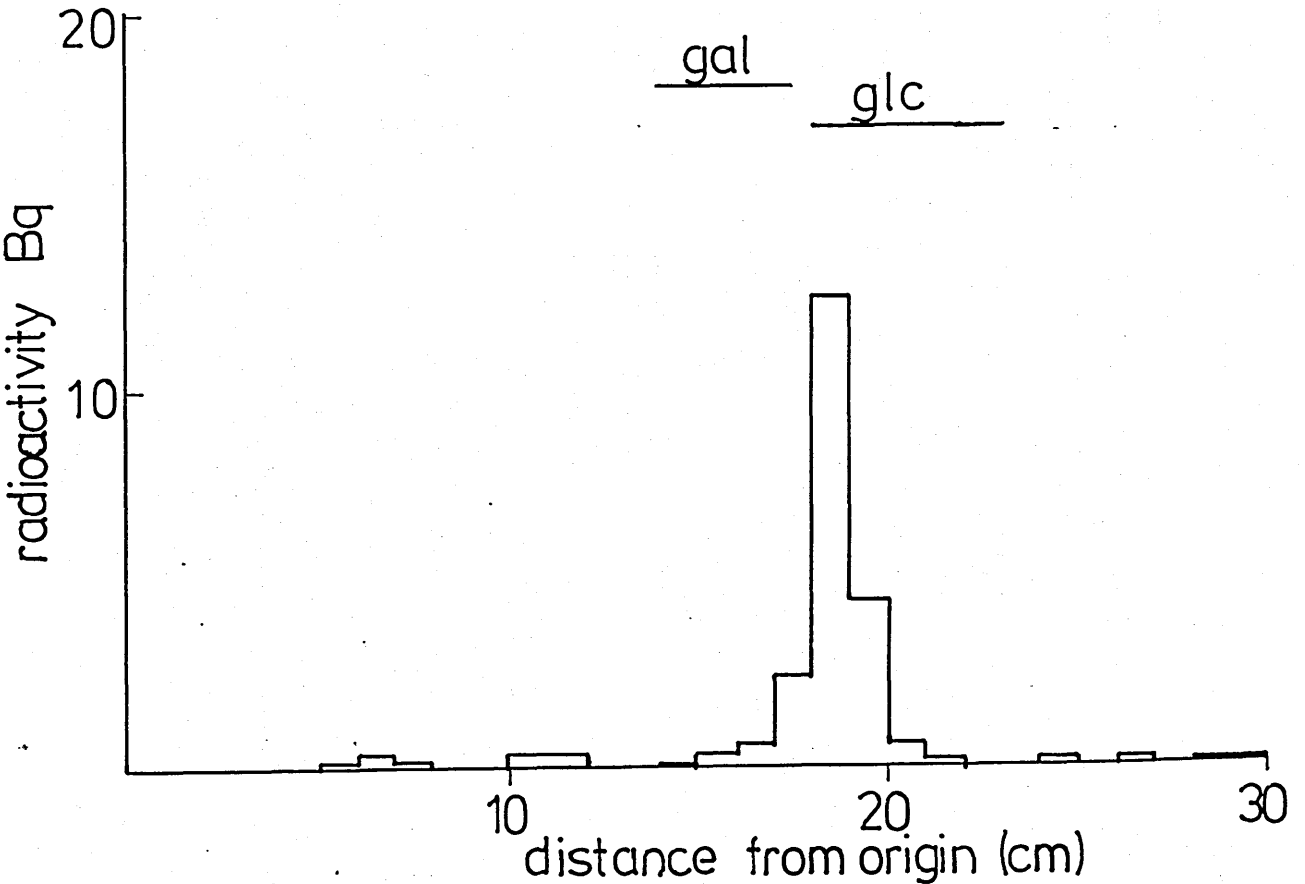
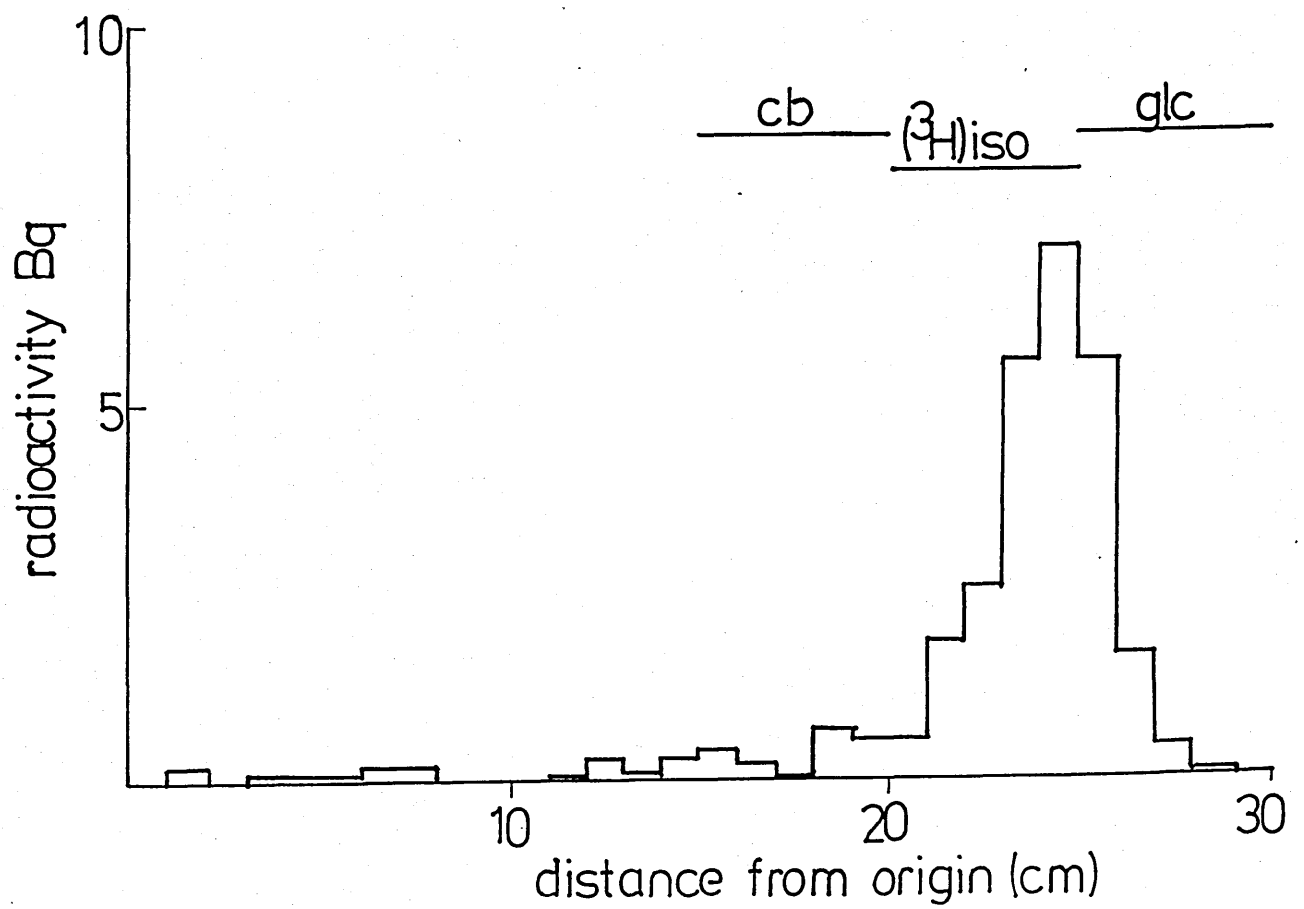

20
10
radioactivity Bq
gal
glc
10
20
30
distance from origin (cm)

Fig.2


10
5
radioactivity Bq
cb
(3H)iso
glc
10
20
30
distance from origin (cm)

One radioactive peak was observed (Fig. 2) co-chromatographing with isoprimeverose and possibly also glucose, suggesting it could be mono or disaccharide, or a mixture of both. To determine which, an extra separation step was introduced before final identification by paper-chromatography.

Analysis of [^{14}C]glucose-labelled product by gel-filtration and descending paper chromatography

A [^{14}C] glucose-labelled pellet, containing 138 Bq, was hydrolysed overnight with driselase (Ch.2 p.58), and the supernatant was run in water on a 1 x 15 cm Bio-gel P2 gel-filtration column. Fractions of 0.5 cm^3 were collected, and half of each was counted in xylene scintillant. The main peak was identified between fractions 49 and 55 (Fig. 3), and the remaining 0.25cm^3 of each of these fractions were pooled. The samples were rotary evaporated to dryness, diluted in 0.25 cm^3 H_2) and spotted onto Whatman no. 1 paper before running in solvent B for 30 hours. Glucose, cellobiose and [^{3}H]isoprimeverose were used as markers.

Most [^{14}C]glucose product co-chromatographed with glucose, and a small peak ran with cellobiose (Fig.4). This suggests that there is little or no incorporation from UDP-D-[U^{14}C]glucose into xyloglucan.

Analysis of [^{14}C]glucose-labelled product by driselase and laminarinase hydrolysis

To determine whether the [^{14}C]glucose-labelled product was likely

Figure 3

Bio-gel P2 profile of driselase hydrolysate of [^{14}C]glucose-labelled product of incubation of UDP-D-[U^{14}C]glucose with particulate enzyme preparation from suspension-cultured bean.

Figure 4

Paper-chromatographic analysis of [^{14}C]glucose-labelled hydrolysate isolated by gel-filtration on Bio-gel P2 (See Fig. 3)

Fig. 3

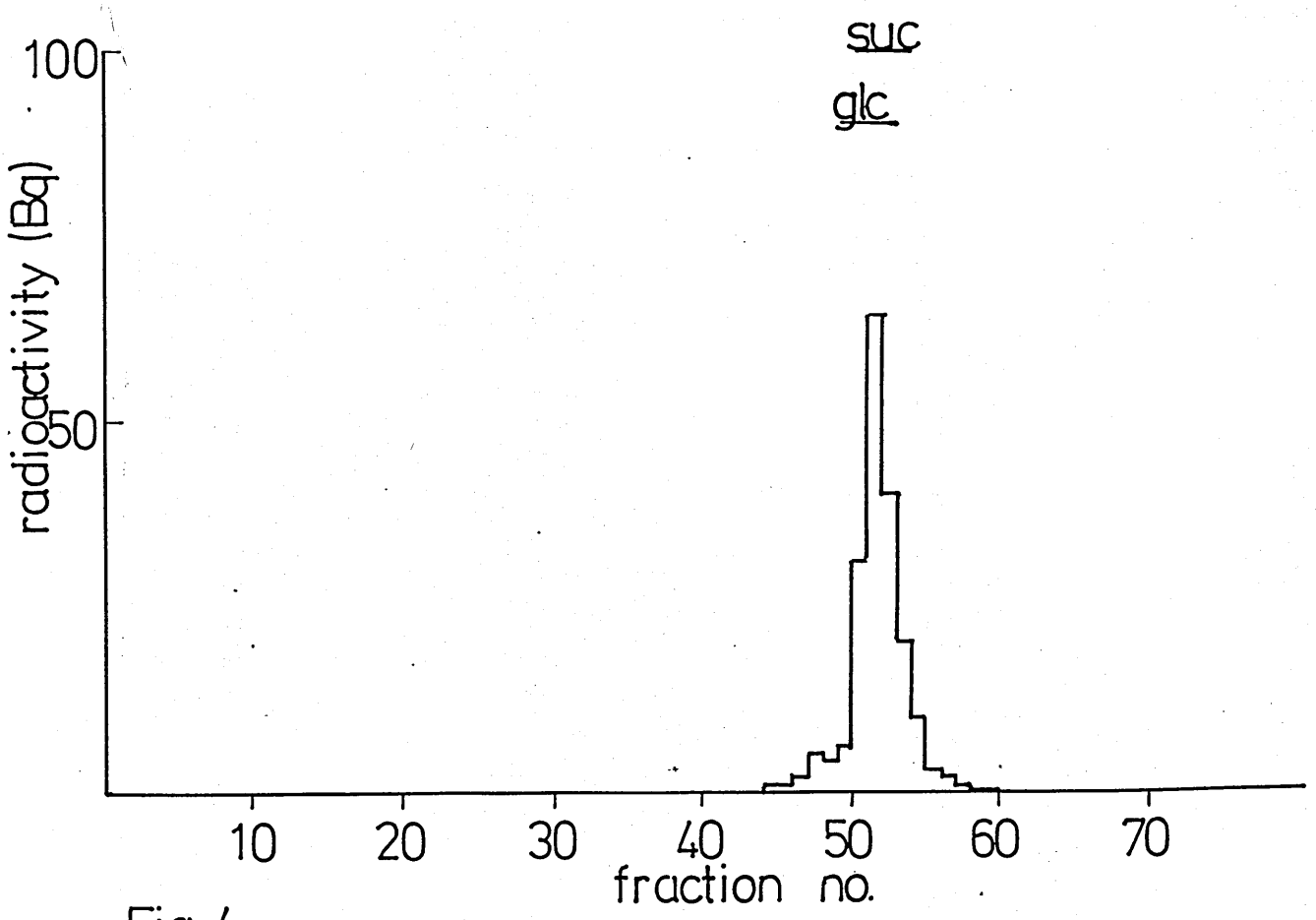
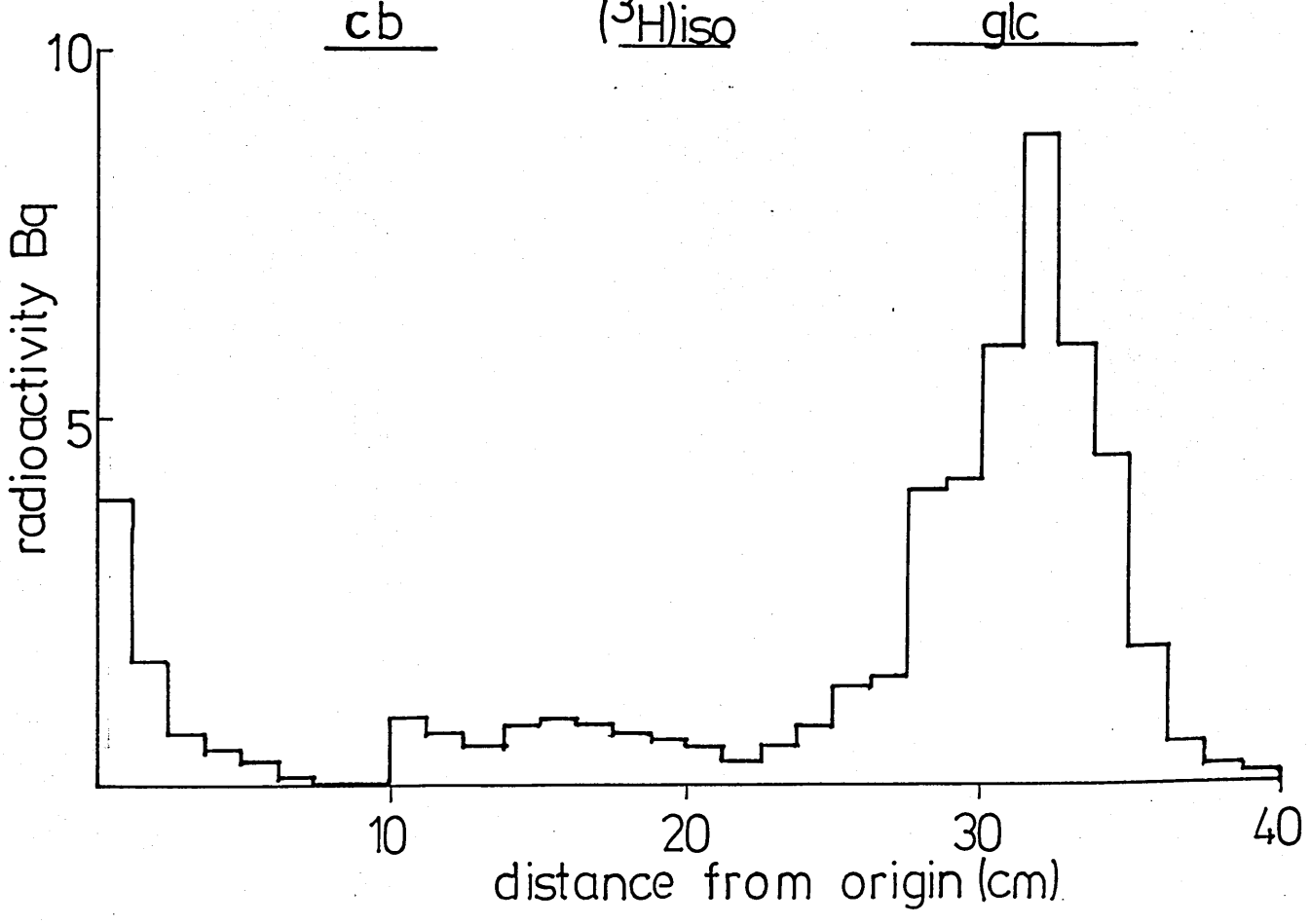

suc
glc
100
50
radioactivity (Bq)
10
20
30
40
50
60
70
fraction no.

Fig. 4


cb
(^{3}H)iso
glc
10
5
radioactivity Bq
10
20
30
40
distance from origin (cm)

to be β(1-3) or β(1-4) linked, a comparison of laminarinase and driselase hydrolysis products was carried out. Laminarinase is a β(1-3) glucanase and has little effect on xyloglucan (See Ch.5 p.149), but hydrolyses β(1-3) glucans to give a series of products including laminaribiose, laminaritriose, laminaritetrose, etc.

Two [^{14}C]glucose-labelled pellets containing 55 Bq were obtained from 15 min. standard incubations. Hydrolysis with driselase or laminarinase was carried out for 6 h (Ch.2 p. 58) and commerical laminarin was also hydrolysed with driselase and laminarinase to provide a series of markers. The hydrolysates were run in solvent B overnight.

Hydrolysis with either driselase or laminarinase resulted in breakdown of the product (Fig. 5 a & b), but separation on this system did not allow identification of the mono-, di- or trisaccharide peaks. The laminarin was hydrolysed by driselase (Fig. 5b) which is evidently capable of hydrolysing β(1-3) or β(1-4) glucans. In an earlier experiment (Ch.5 p.149) it was established that laminarinase has very limited activity against xyloglucan, so from the results of this experiment it would appear that at least one of the [^{14}C]glucose-labelled products is a β(1-3) glucan.

The main radioactive peaks from the driselase and laminarinase digestions were eluted and rerun on a system which separates mono- and disaccharides (Solvent D, Ch.2 p.60). In this case driselase

Figure 5

Paper chromatographic analysis of enzyme-hydrolysed [^{14}C] glucose-labelled product from 15 min standard incubations of UDP-D-[U^{14}C] glucose with a particulate enzyme preparation from suspension-cultured bean:-

a) Following laminarinase hydrolysis with laminarinase-hydrolysed laminarin as marker

b) Following driselase hydrolysis with driselase hydrolysis with driselase-hydrolysed laminarin as marker

Fig. 5a

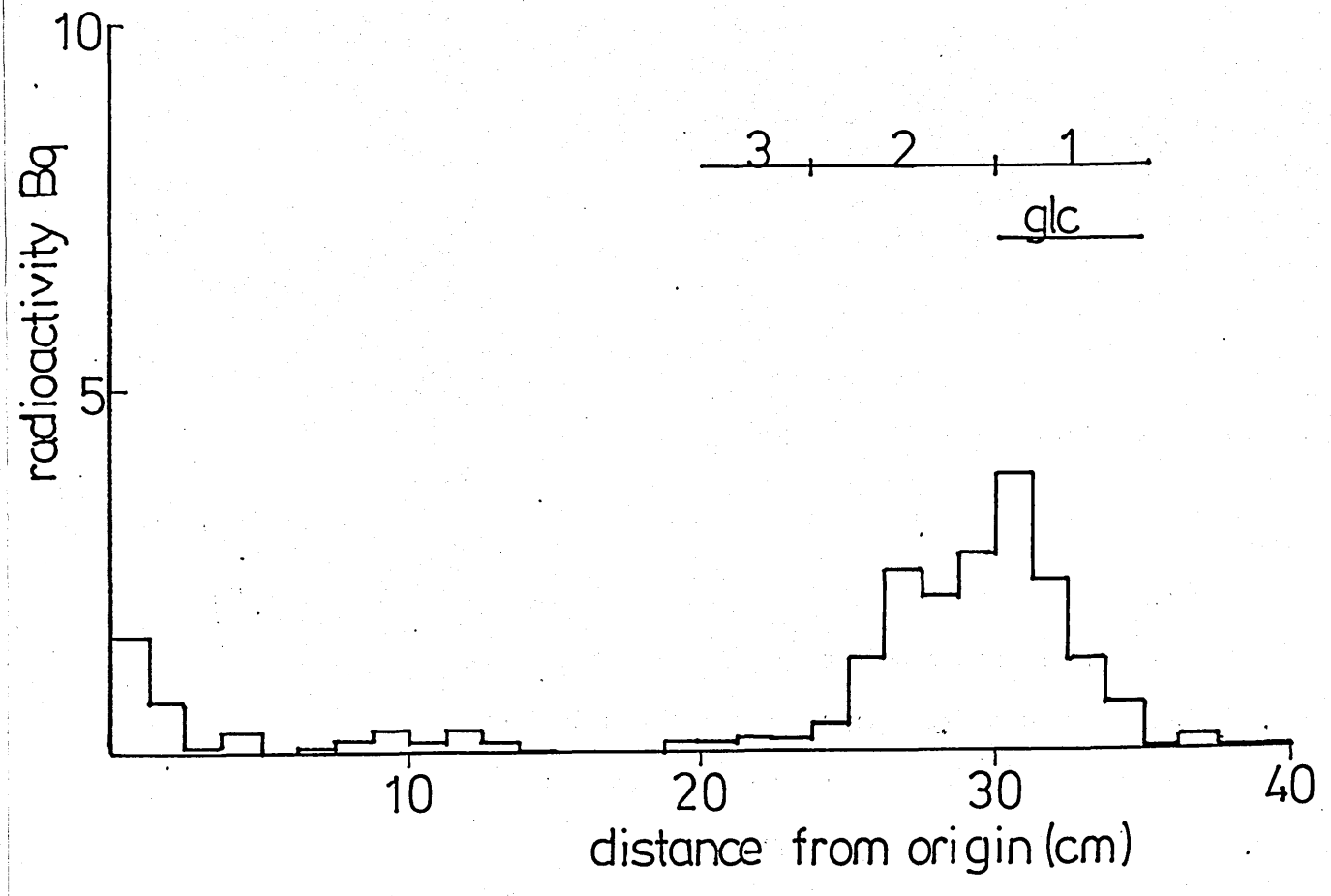

10
5
radioactivity Bq
3
2
1
glc
10
20
30
40
distance from origin (cm)

Fig. 5b

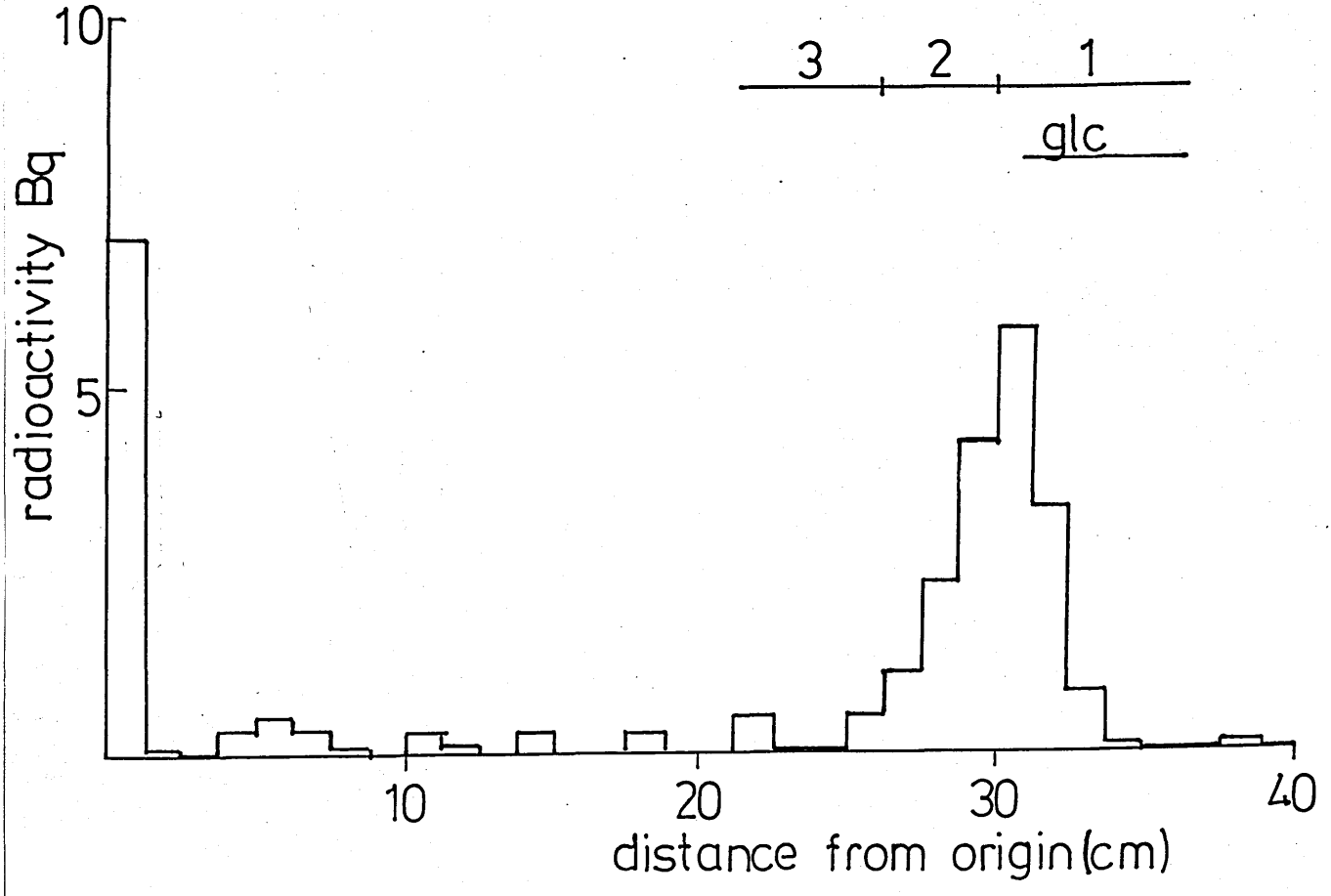

10
5
radioactivity Bq
3
2
1
glc
10
20
30
40
distance from origin (cm)

Figure 6

Paper chromatographic analysis of eluted mono- and disaccharide peaks from driselase and laminarinase-hydrolysed [^{14}C]glucose-labelled product:-

a) driselase-hydrolysed product

b) laminarinase-hydrolysed product

Fig. 6a

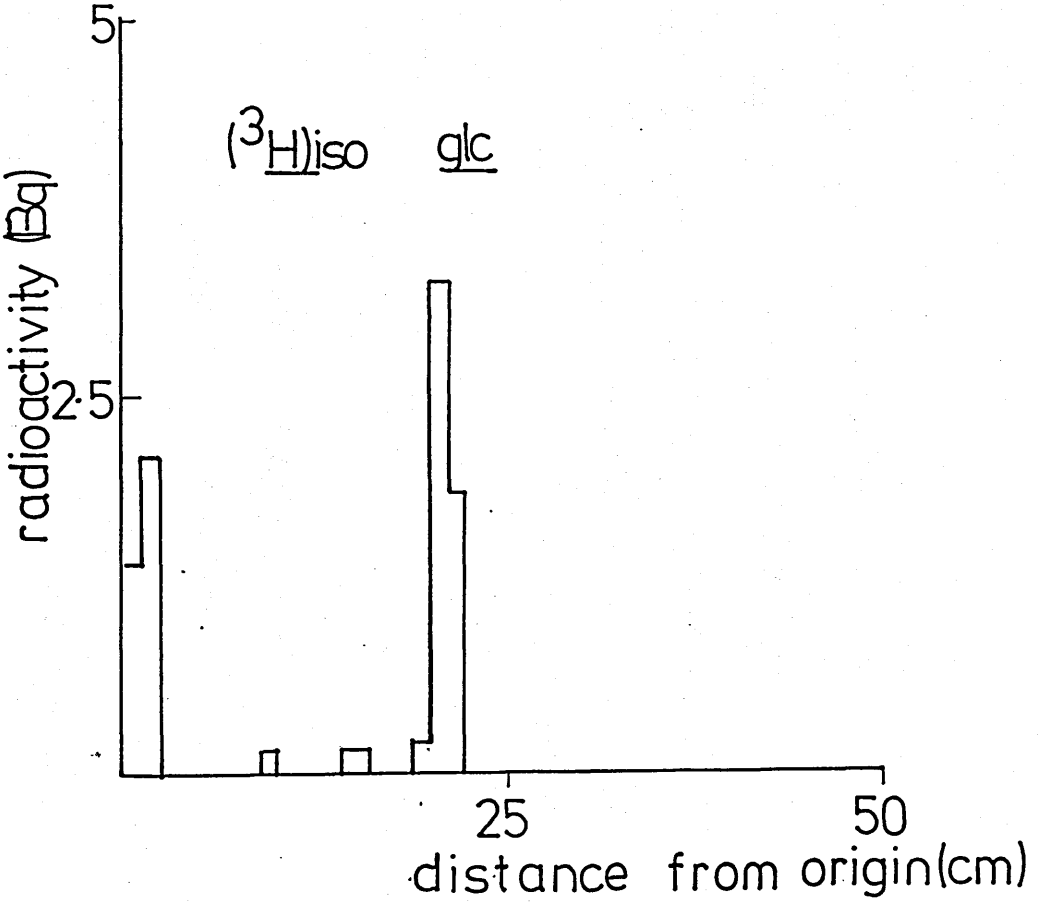
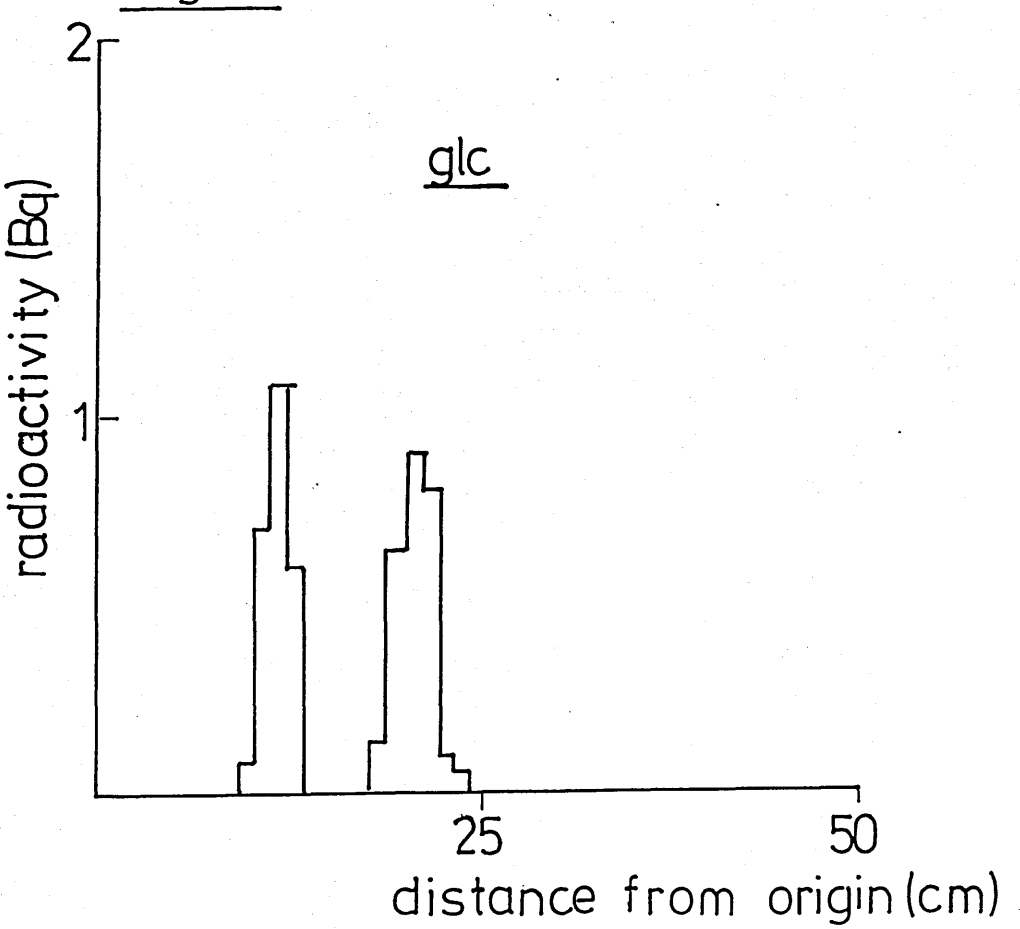

5
2·5
(^{3}H)iso glc
radioactivity (Bq)
25
50
distance from origin(cm)

Fig. 6b


2
1
glc
radioactivity (Bq)
25
50
distance from origin (cm)

hydrolysis resulted in the main peak co-chromatographing with glucose, with little evidence of isoprimeverose (Fig. 6a). The laminarinase hydrolysis gave two similarly-sized peaks, one running with glucose, the other probably being laminaribiose (Fig. 6b).

These results again suggest that the main product of the incorporation from UDP-D[U^{14}C]glucose is β(1-3) glucan, though it is possible that smaller quantities of other products are formed.

Discussion

It would appear that the majority of radioactivity from UDP-D-[U^{14}C]glucose is being incorporated into β(1-3) glucan rather than β(1-4) glucan. β(1-3) glucan is the major constituent of callose, which is formed in response to injury, and gives rise to great difficulties in studying glucan synthesis in plant tissue. The level of callose synthesis rises steeply so that it often becomes the major pathway for the utilisation of UDP-glucose. This is presumably occurring when suspension-cultured bean tissue is homogenised with a mortar and pestle.

It may be possible to find incubation conditions favouring β(1-4) glucan synthesis, but this was not attempted in this study.

Chapter 8

INVESTIGATION OF [^{14}C]GALACTOSE INCORPORATION FROM UDP-[U^{14}C]GALACTOSE BY BEAN SUSPENSION CULTURE

Galactose occurs in all reported xyloglucan molecules as a side chain attached α(1-2) to the xylose side chains, yet its incorporation into xyloglucan from the donor sugar nucleotide has not been reported. Studies of incorporation are, as with glucose, likely to be complicated by epimerase activity resulting in the interconversion of UDP-glucose and UDP-galactose, and also by the range of galactose-containing cell wall-polysaccharides.

Incorporation of galactose from UDP-galactose was investigated using a particulate enzyme preparation from bean suspension-cultures. Initial experiments involved varying sugar nucleotide concentrations.

Time course of incorporation of radioactivity from UDP-D-[U^{14}C]galactose

A time course of galactose incorporation up to 60 min was carried out. Incubations contained UDP-D-[U^{14}C]galactose (21 μmol.dm^{-3}, 925 Bq) UDP-glucose (2 mmol.dm^{-3}), UDP-xylose (20 μmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3}).

Incorporation increased with time, and zero time values were low. Overall incorporation was low (Table 1) suggesting low enzyme activity or missing elements in the incubation mixture.

In a second experiment a wider range of nucleotide combinations was used.

Incorporation of radioactivity from UDP-D-[U^{14}C]galactose using a range of nucleotide mixtures

Incubations were carried out with three sugar nucleotide

Table 1

Time course of incorporation from UDP-D-[U ^{14}C]galactose into polysaccharide by particulate enzyme preparation from suspension-cultured bean, from incubations containing UDP-D-[U ^{14}C] galactose (21 μmol.dm^{-3}, 925 Bq), UDP-xylose (20 μmol.dm^{-3}), UDP-glucose (2 mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3})

Time (min)	Radioactivity incorporated (Bq)
0	1.2 $\pm$ 0.1
15	2.8 $\pm$ 0.8
60	3.6 $\pm$ 0.1
120	3.7 $\pm$ 0.2

combinations in an attempt to improve incorporation of radioactivity Incubations included: 1. UDP-D-[$U^{14}C$]galactose (925 Bq, 0.84 $\mu mol.dm^{-3}$), UDP-glucose (2$mmol.dm^{-3}$), UDP-xylose (2 $mmol.dm^{-3}$) and $MnCl_2$ (10 $mmol.dm^{-3}$); 2. UDP-D-[$U^{14}C$]galactose (925 Bq, 20.8 $\mu mol.dm^{-3}$) UDP-glucose (2 $mmol.dm^{-3}$), UDP-xylose (20 $\mu mol.dm^{-3}$) and $Mncl_2$ (10 $mmol.dm^{-3}$), or 3. UDP-D- $U^{14}C$ galactose (925 Bq, 0.84 $\mu mol.dm^{-3}$) UDP-glucose (2 $mmol.dm^{-3}$) and UDP-xylose (2 $mmol.dm^{-3}$). Incubations were terminated at 60 min.

Incorporation was greatest with high UDP-glucose concentration combined with low UDP-galactose and UDP-xylose concentrations, and it was lowest where $MnCl_2$ was absent from incubations (Table 2). However, it was generally low, and still insufficient to permit product analysis. As Tris/HCl can be a rather harsh buffer, resulting in reduced enzyme activity, incorporation was examined using a potassium phosphate buffer which included various enzyme protectants (See ch.2 p.51).

Incorporation from UDP-D-[$U^{14}C$]galactose using potassium phosphate as homogenisation and resuspension buffer

A particulate enzyme preparation was prepared using potassium phosphate buffer for homogenisation and resuspension (ch.2 p.51). Incubations comprised UDP-D-[$U^{14}C$]galactose (925 Bq, 20.84 $\mu mol.d\ m^{-3}$), UDP-glucose (20 $\mu mol.dm^{-3}$), UDP-xylose (20 $\mu mol.dm^{-3}$) and $MnCl_2$ (10 $mmol.dm^{-3}$).

Zero time values were low, and incorporation was slightly higher than in previous experiments (Table 3). Incorporation was still not at a sufficiently high level to allow investigation of the incubation product however.

Table 2

Incorporation from UDP-D-[U ^{14}C] galactose into polysaccharide by particulate enzyme preparation from suspension-cultured bean from 60 min incubations containing:-

a) UDP-D-[U ^{14}C]galactose (0.84 μmol.dm^{-3}, 925 Bq), UDP-glucose (2 mmol.dm^{-3}), UDP-xylose (2 mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3})

b) UDP-D-[U ^{14}C]galactose (21 μmol.dm^{-3}), 925 Bq), UDP-glucose (2 mmol.dm^{-3}), UDP-xylose (20 μmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3})

c) UDP-D-[U ^{14}C]galactose (0.84 μmol.dm^{-3} , 925 Bq), UDP-glucose (2 mmol.dm^{-3}) and UDP-xylose (20 μmol.dm^{-3})

Incubation mixture	Incorporation at 60 min (Bq)
a	4.8 $\pm$ 0.1
b	6.2 $\pm$ 0.9
c	3.5 $\pm$ 0.5

Table 3

Incorporation from UDP-D-[U ^{14}C] galactose into polysaccharide by particulate enzyme preparation from suspension-cultured bean following homogenisation and resuspension in potassium phosphate buffer: incubations contained UDP-D-[U ^{14}C]galactose (21 μmol.dm^{-3}, 925 Bq), UDP-glucose (2 mmol.dm^{-3}), UDP-xylose (20 μmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3})

Incubation time (min)	Radioactivity incorporated (Bq)
0	1.3 $\pm$ 0.1
60	8.4 $\pm$ 2.5

No further attempts at improving incorporation from UDP-D-[U^{14}C]galactose were made due to the low rates of incorporation and potential problems in identification of the product, even if reasonable rates of incorporation could eventually be achieved.

Chapter 9

INVESTIGATION OF [^{14}C]FUCOSE INCORPORATION FROM GDP-D-[U^{14}C]FUCOSE BY BEAN SUSPENSION CULTURE

Fucose occurs as a terminal side chain in many xyloglucans (Aspinall et al, 1977; Bauer et al, 1973; Hayashi et al, 1980; Kato & Matsuda, 1980), and its transfer to the xyloglucan molecule has been investigated recently by Camirand and Maclachlan (1986). Using a preparation of pea microsomal membranes, they studied the transfer of [^{14}C]fucose from GDP-D-[U^{14}C]fucose to an insoluble product with properties characteristic of xyloglucan. Transfer of [^{14}C]fucose was catalysed in the presence or absence of UDP-xylose or UDP-glucose.

The transfer of fucose from GDP-fucose was investigated for the bean particulate enzyme preparation.

Incorporation of radioactivity from GDP-D-[U^{14}C]fucose in three incubation mixtures

Incorporation of [^{14}C]fucose was measured from three incubation mixtures which contained either GDP-D-[U^{14}C]fucose only, GDP-D-[U^{14}C]fucose with UDP-glucose, UDP-galactose and UDP-xylose, or GDP-D-[U^{14}C]fucose with UDP-glucose and UDP-galactose. All non-radioactive sugar nucleotides were present at a concentration of 20 μmoldm^{-3} and incubations also contained 10 mmoldm^{-3} $MnCl_2$ GDP-D-[U^{14}C]fucose (925 Bq) was present at a concentration of 1.25 μmoldm^{-3}. Incubations were terminated at 0 and 15 min.

Incorporation was greater where sugar nucleotides other than GDP-fucose were present in incubations (Table 1), but although their

Table 1

Radioactivity incorporated from GDP-D-[$U^{14}C$] fucose into polysaccharide by particulate enzyme preparation from bean suspension culture. Incubation mixtures were terminated after 15 min and comprised:

a) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq) and $MnCl_2$ (10 $mmol.dm^{-3}$)

b) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-glucose (20 $\mu mol.dm^{-3}$) and UDP-galactose (20 $\mu mol.dm^{-3}$)

c) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq), $MnCl_2$ (10 $mmol.dm^{-3}$), UDP-glucose (20 $\mu mol.dm^{-3}$), UDP-galactose (20 $\mu mol.dm^{-3}$) and UDP-xylose (20 $\mu mol.dm^{-3}$).

Incubation mixture	Incorporation at 15 min (Bq)
a	19.1 ± 0.22
b	27.2 ± 0.7
c	27.5 ± 3.3

presence in incubations stimulated incorporation by up to 44%, it was not essential for incorporation.

Extended time course of incorporation from GDP-D-[U^{14}C]fucose

A time course of incorporation up to 120 min was performed with increased UDP-glucose in incubations, which now contained GDP-D-[U^{14}C] fucose (925 Bq, 1.25 μmol.dm^{-3}), UDP-xylose (20 μmol.dm^{-3}), UDP-glucose (2 mmol.dm^{-3}), UDP-galactose (20 μmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3}). Incubations were terminated at 0, 15, 60 and 120 min.

Incorporation was maximal at 15 min. and then decreased gradually (Fig. 1). To determine whether the concentration of any specific sugar nucleotide was limiting, incubations were carried out with the concentration of one or two nucleotides at a time varied.

Effect of varying sugar nucleotide concentrations on incorporation from GDP-D-[U^{14}C]fucose - 1.

Incubations were carried out with high concentrations of all non-radioactive sugar nucleotides, or with high UDP-glucose combined with low UDP-xylose and low UDP-galactose. The effect of absence of $MnCl_2$ from incubations was also assessed. Incubations comprised GDP-D-[U^{14}C]fucose (925 Bq, 1.25 μmol.dm^{-3}) with either 1. UDP-glucose (2 mmol.dm^{-3}), UDP-galactose (2 mmol.dm^{-3}), UDP-xylose (2mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3}); 2. UDP-glucose (2 mmol.dm^{-3}), UDP-galactose (20 μmol.dm^{-3}), UDP-xylose (20 μmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3}); or

Figure 1

Extended time course of incorporation of radioactivity from GDP-D-[U^{14}C]fucose into polysaccharide by particulate enzyme preparation from bean, from an incubation mixture containing GDP-D-[U^{14}C]fucose (1.2 μmol.dm^{-3}, 925 Bq), UDP-xylose (20 μmol.dm^{-3}), UDP-glucose (2 mmol.dm^{-3}), UDP-galactose (20 μmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3}).

Figure 2

Time course of incorporation of radioactivity from GDP-D-[U^{14}C] fucose into polysaccharide by particulate enzyme preparation from bean, from three incubation mixtures containing GDP-D-[U^{14}C] fucose (1.25 μmol.dm^{-3} 925 Bq), (●——●) UDP-glucose (2 mmol.dm^{-3}), UDP-galactose (2 mmol.dm^{-3}), UDP-xylose (2 mmol.dm^{-3}) and $MnCl_2$ (10 mmol.dm^{-3}); (■——■) UDP-glucose (2 mmol.dm^{-3}), UDP-galactose (20 μmol.dm^{-3}), UDP-xylose (20 mmol.dm^{-3}), and $MnCl_2$ (10 mmol.dm^{-3}); (▲——▲) UDP-glucose (2 mmol.dm^{-3}), UDP-galactose (20 μmol.dm^{-3}) and UDP-xylose (20 μmol.dm^{-3}).

Fig. 1

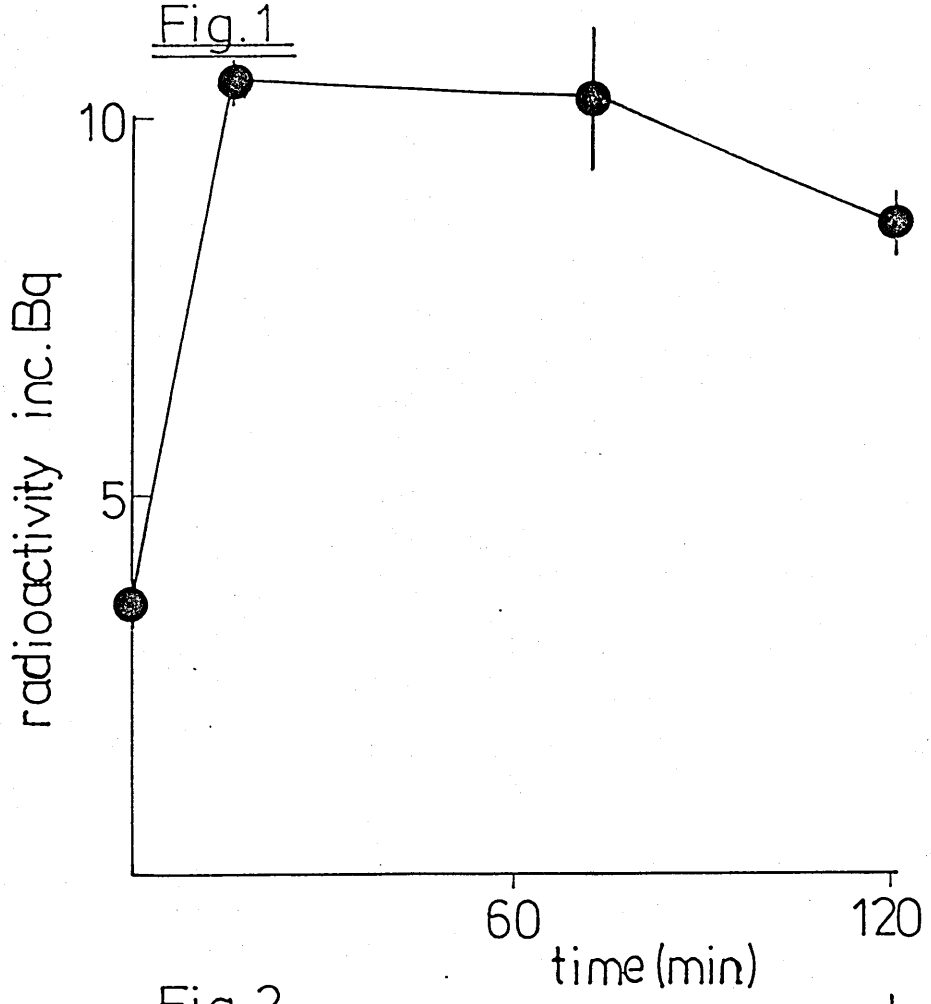
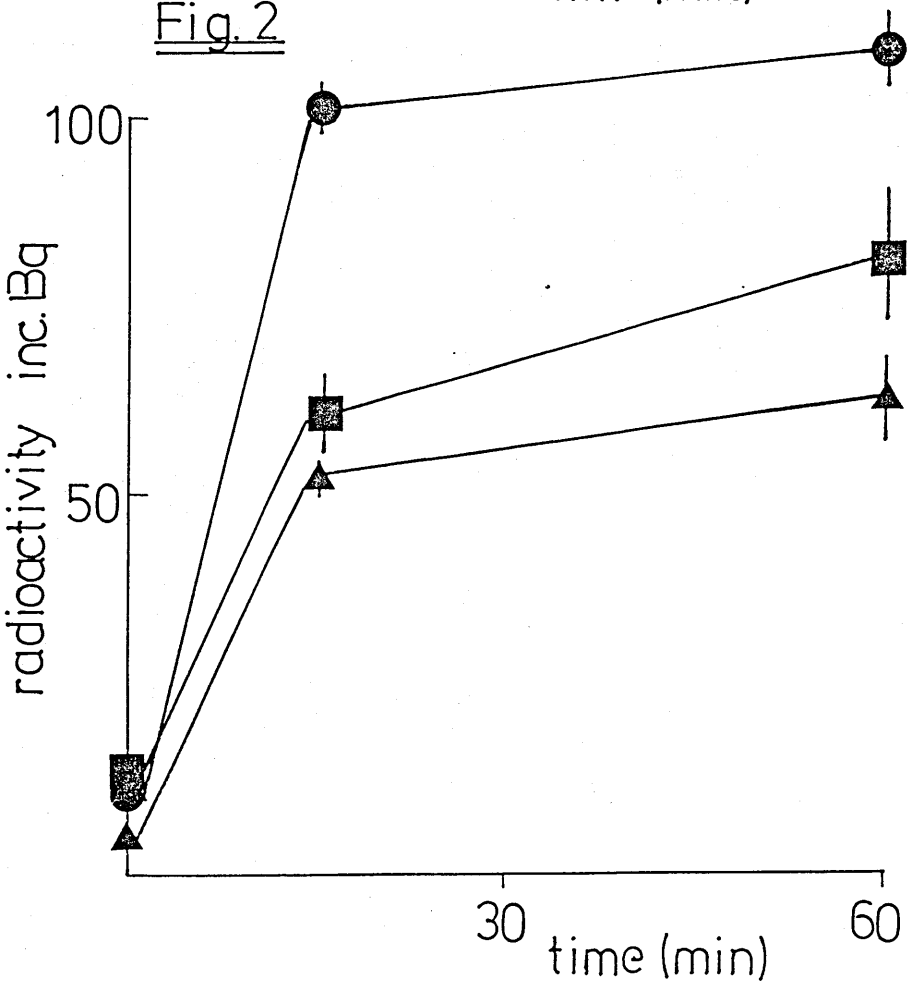

10
5
radioactivity inc. Bq
60
120
time (min)

Fig. 2


100
50
radioactivity inc. Bq
30
60
time (min)

3. UDP-glucose (2 mmoldm^{-3}), UDP-galactose (20 μmoldm^{-3}) and UDP-xylose (20 μmoldm^{-3}). Incubations were terminated at 0, 15 and 60 min.

Incorporation levels were highest where non-radioactive sugar nucleotides were all present at 2 mmoldm^{-3} (Fig.2). Absence of $MnCl_2$ from incubations resulted in slightly lower incorporation, but did not make an appreciable difference. It would appear that a low concentration of either UDP-xylose, UDP-galactose or both limits incorporation. To determine whether this was indeed the case, incubations were performed with low concentrations of one or other of these sugar nucleotides.

Effect of varying nucleotide concentrations on incorporation from GDP-D-[$U^{14}C$]fucose - 2.

Incorporation from five incubation mixtures was measured to study the effect of specific sugar nucleotides and absence of $MnCl_2$ from incubations. All incubations contained GDP-D-[$U^{14}C$]fucose (950 Bq, 1.25 μmoldm^{-3}) and UDP-glucose (2 mmoldm^{-3}), with varying combinations of UDP-xylose and UDP-galactose at 2 mmoldm^{-3} or 20 μmoldm^{-3} to give incubations with either low UDP-xylose, low UDP-galactose or low concentration of both UDP-xylose and UDP-galactose. $MnCl_2$ was also omitted from some incubation mixtures, details of which are given in figure legends (Table 2). Incubations were terminated at 0 and 60 min.

Incorporation was reduced in incubations where $MnCl_2$ was absent,

Table 2

Radioactivity incorporated from GDP-D-[$U^{14}C$]fucose into polysaccharide by particulate enzyme preparation from bean suspension culture. Incubations were terminated after 60 min and comprised:-

a) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$), 925 Bq), UDP-xylose (2 $mmol.dm^{-3}$), UDP-glucose (2 $mmol.dm^{-3}$), UDP-galactose (2 $mmol.dm^{-3}$) and $MnCl_2$ (10 $mmol.dm^{-3}$)

b) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$), UDP-xylose (2 $mmol.dm^{-3}$), UDP-glucose (2 $mmol.dm^{-3}$) and UDP-galactose (2 $mmol.dm^{-3}$)

c) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq), UDP-xylose (2 $mmol.dm^{-3}$), UDP-glucose (2 $mmol.dm^{-3}$), UDP-galactose (20 $\mu mol.dm^{-3}$) and $MnCl_2$ (10 $mmol.dm^{-3}$)

d) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq), UDP-xylose (20 $\mu mol.dm^{-3}$), UDP-glucose (2 $mmol.dm^{-3}$), UDP-galactose (2 $mmol.dm^{-3}$) and $MnCl_2$ (10 $mmol.dm^{-3}$)

e) GDP-D-[$U^{14}C$]fucose (1.25 $\mu mol.dm^{-3}$, 925 Bq), UDP-xylose (20 $\mu mol.dm^{-3}$), UDP-glucose (2 $mmol.dm^{-3}$), UDP-galactose (20 $\mu mol.dm^{-3}$) and $MnCl_2$ (10 $mmol.dm^{-3}$)

Incubation mixture	Incorporation at 60 min (Bq)
a	64.3 ± 0.5
b (minus $MnCl_2$)	42.3 ± 3.2
c (low UDP-galactose)	31.7 ± 1.5
d (low UDP-xylose)	53.0 ± 0.4
e (low UDP-xylose and low UDP-galactose)	33.0 ± 0.5

where UDP-galactose was at low concentration, or where both UDP-galactose and UDP-xylose were at low concentration, but it was reduced far less by low concentration of UDP-xylose (Table 2). Incorporation was reduced to a greater extent by low UDP-galactose concentration than by absence of $MnCl_2$ from incubations.

This experiment was repeated with the non-radioactive sugar-nucleotides absent from incubations, rather than at low concentration in incubations. Absence of non-radioactive sugar nucleotides had no greater effect on incorporation than their presence at low concentration.

If the fucose from GDP-D-[U^{14}C]fucose is being incorporated into xyloglucan, this result may have implications for the mechanism of synthesis for the xyloglucan molecule. Attempts were therefore made to characterise the product of the incubation with GDP-D-[U^{14}C]fucose.

Camirand and Maclachlan (1986), who investigated biosynthesis of fucose-containing xyloglucan by pea microsomal membranes, identified a specific nonasaccharide fragment following cellulase digestion of the product. This nonasaccharide fragment, which Camirand and Maclachlan claim is characteristic of the [^{14}C]fucose-labelled product, is formed with regularity in the xyloglucan structure, but only in the presence of GDP-fucose.

Identification of this nonasaccharide was attempted for the bean suspension-culture particulate enzyme product. First however, it

was necessary to confirm that the GDP-D-[U^{14}C]fucose did not undergo epimerisation or other modification prior to incorporation into polysaccharide.

Analysis of product of incubation with GDP-[U^{14}C]fucose by total acid hydrolysis and descending paper chromatography

A 60 minute GDP-fucose incubation sample from a standard incubation, containing approx. 15 Bq, was subjected to complete acid hydrolysis as described (Ch.2 p. 55), rotary evaporated to dryness, then run for 16 h in solvent D with mannose and fucose as markers. The sugar markers were detected using silver nitrate, and radioactive strips counted in toluene scintillant.

The radioactively-labelled sample clearly co-chromatographed with fucose with no other peaks of radioactivity (Fig.3). This indicates that there is no epimerisation or other modification taking place during incubation.

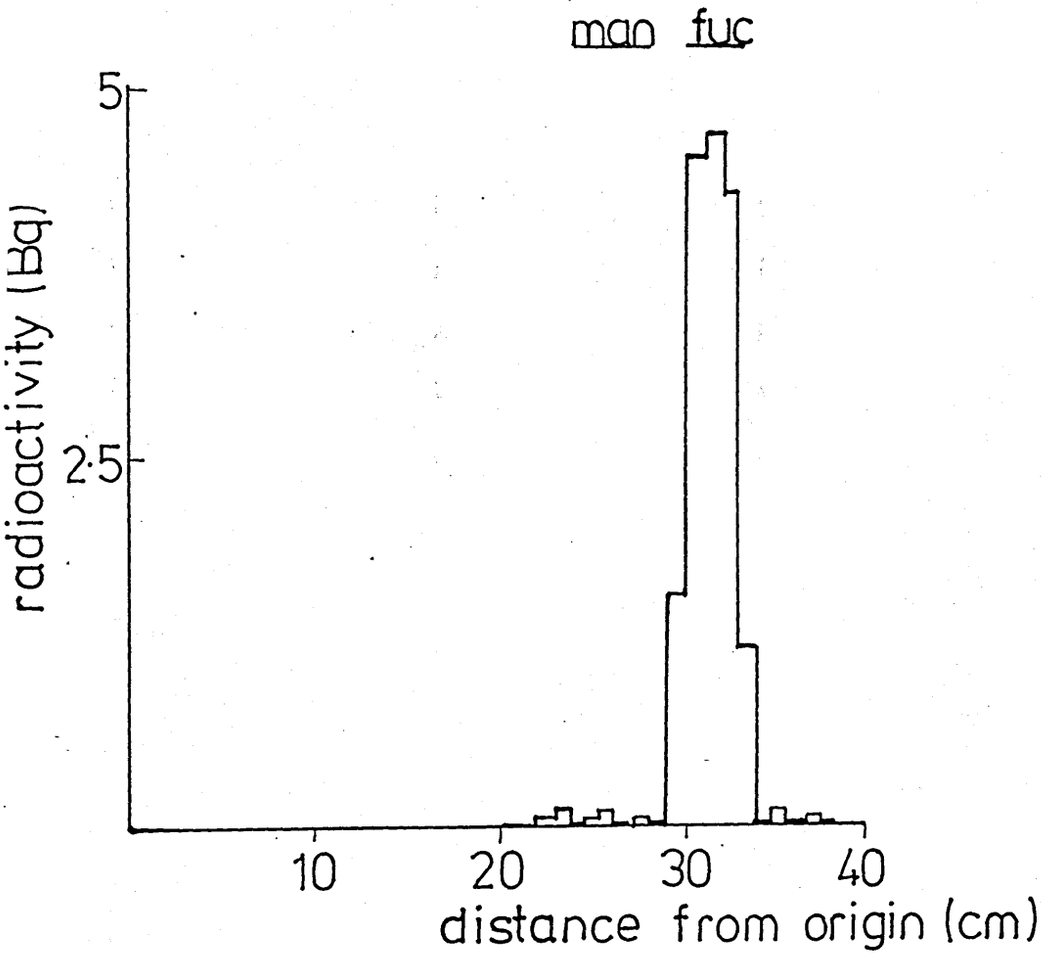
Comparison of Trichoderma viride and driselase hydrolysates of Tamarind xyloglucan

To determine which of two available enzyme systems would be most suitable for production of larger sized polysaccharide hydrolysates for use in the isolation of the fucose-containg nonasaccharide fragment, two (0.5 mg) samples of purified tamarind were hydrolysed. Hydrolysis with Trichoderma viride and driselase was carried out (Ch.2 p.58),

Figure 3

Paper chromatographic analysis of complete acid hydrolysates of polysaccharide labelled with radioactivity incorporated from GDP-D-[U^{14}C] fucose during 60 min incubation from standard incubation mixture.

Fig.3


man fuc
5
2·5
radioactivity (Bq)
10
20
30
40
distance from origin (cm)

and then the supernatants were separated on a 150 x 0.5 cm Bio-gel P2 gel-filtration column. Fractions of 0.5 cm^3 were collected and peaks detected using phenol/sulphuric acid (Ch.2 p. 64). Peaks were measured using a spectrophotometer measuring at 480 nm.

The two samples gave a similar range of hydrolysates (Fig. 4a & b) with two main peaks, one corresponding to di- or monosaccharides. The hydrolysis with Trichoderma viride (Fig. 4b) gave a higher percentage of larger-sized hydrolysis products, so this enzyme was used for analysis of the [^{14}C]fucose-labelled product.

Hydrolysis of [^{14}C]fucose-labelled product with Trichoderma viride

Three [^{14}C]fucose-labelled pellets from 60 minute incubations using standard assay conditions (Ch.2 p.53) and containing approx. 100 Bq were incubated with Trichoderma viride for either 90 min, 6 h or 24 h. Supernatants were applied to a 150 x 0.5 cm Bio-gel P2 column, and 0.5 cm^3 fractions counted in xylene scintillant. A sample of [^{14}C]glucose was run along with the 6 h hydrolysate as a marker.

The three samples gave similar radioactive traces (Fig. 5a-c) i.e. three main peaks, the middle one possibly representing a combination of two products. With increasing hydrolysis time up to 24 h, the proportion of the smallest peak relative to the two larger peaks increased, suggesting more complete hydrolysis with increasing time. The [^{14}C]glucose marker eluted from the column more slowly

Figure 4

Bio-gel P2 profile of enzyme hydrolysed tamarind xyloglucan:-

a) Following driselase hydrolysis

b) Following T.viride hydrolysis

Fig. 4a

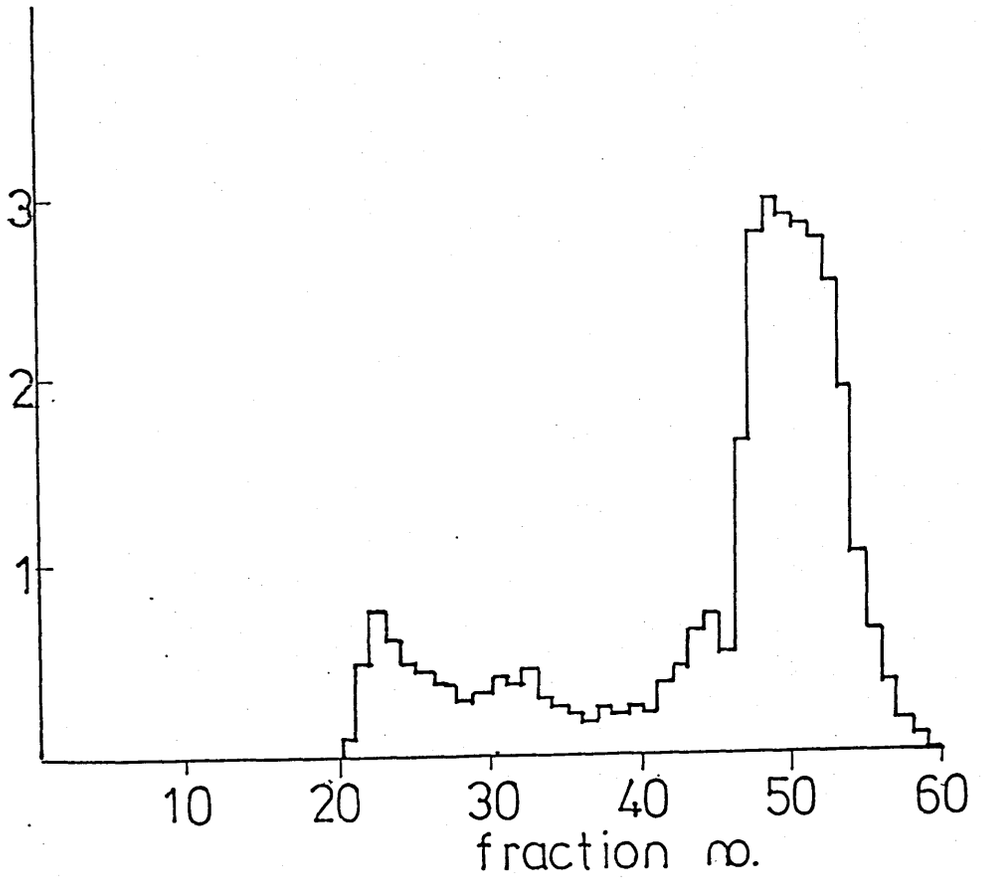
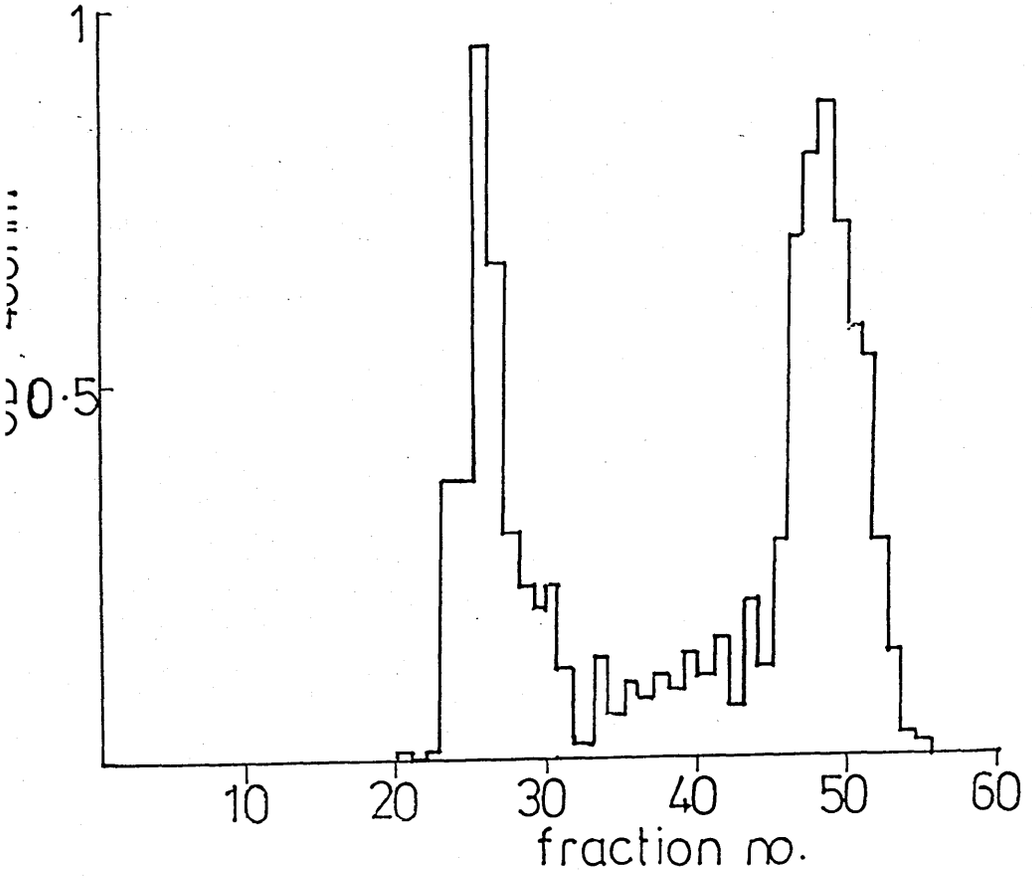

3
2
1
10
20
30
40
50
60
fraction no.

Fig. 4b


1
0·5
10
20
30
40
50
60
fraction no.

than all other peaks, suggesting the smallest peak must be at least a disaccharide (Fig. 5a & b).

Further characterisation of GDP-D-[U^{14}C]fucose incubation product by Trichoderma viride hydrolysis and gel-filtration

A [^{14}C]fucose-labelled sample from a 60 min. incubation using the standard assay was hydrolysed overnight with Trichoderma viride. The sample contained approx. 100 Bq, and after hydrolysis, it was applied to a Bio-gel P2 column (150 x 0.5 cm) as in the previous experiment. A combination of raffinose (1 mg in 0.5 cm^3 H_2O) and [^{14}C]glucose (7.4 kBq) was also run down the column and 0.5 cm^3 samples were collected. The samples were halved: radioactive products and [^{14}C]glucose were detected by scintillation counting, while raffinose was detected with phenol and sulphuric acid (Ch.2 p.64).

Four radioactively-labelled peaks were detected, the smallest eluting with the raffinose marker, suggesting it may be a tri-saccharide (Fig. 6). Identification of the other peaks was not possible as suitable markers were not available.

Discussion

Incorporation from GDP-D-[U^{14}C]fucose into polysaccharide was stimulated by, but not dependent upon, the presence in incubations of UDP-glucose, UDP-galactose and UDP-xylose. The [^{14}C]fucose-

Figure 5

Bio-gel P2 profile of T. viride-hydrolysed polysaccharide (labelled with radioactivity from GDP-D-[U ^{14}C]fucose during 60 min incubations with standard incubations) hydrolysed for:-

a) 90 min

b) 6 h

c) 24 h

Fig. 5a

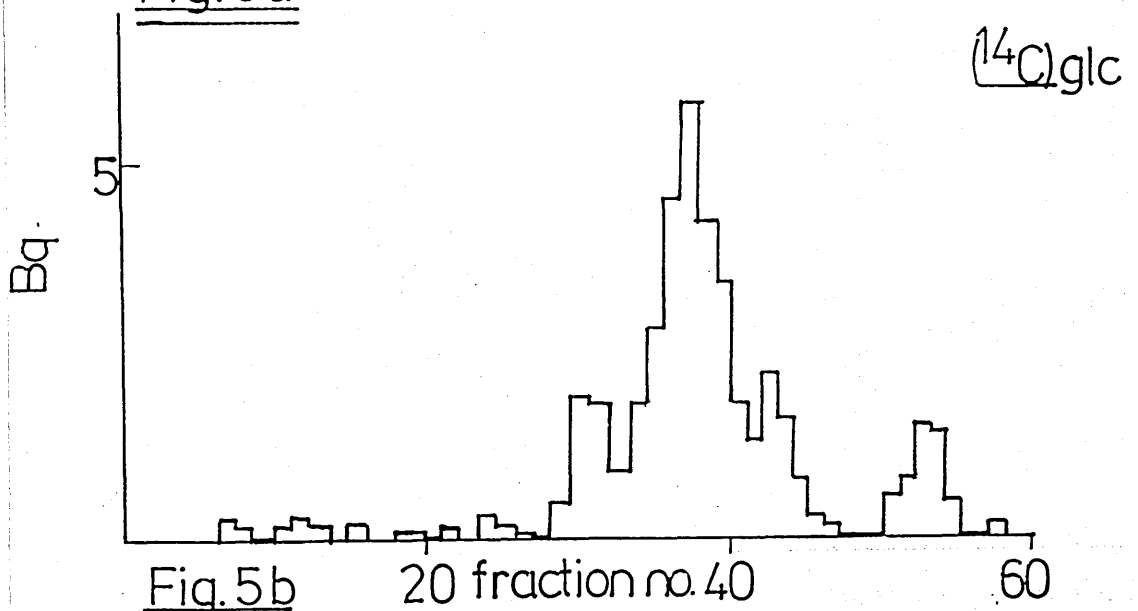


Fig. 5b

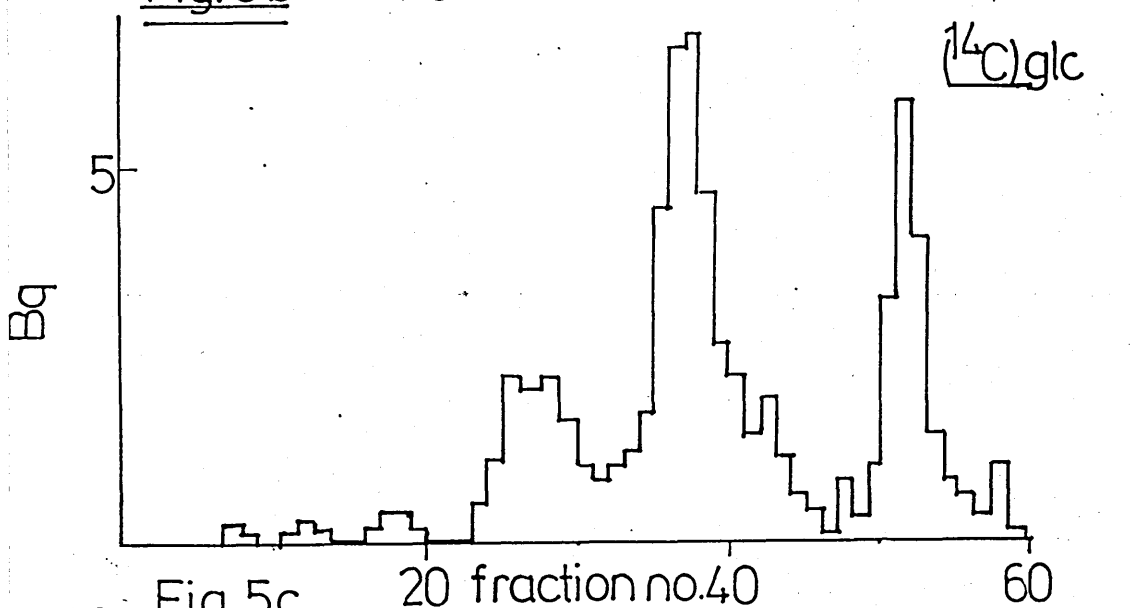


Fig. 5c

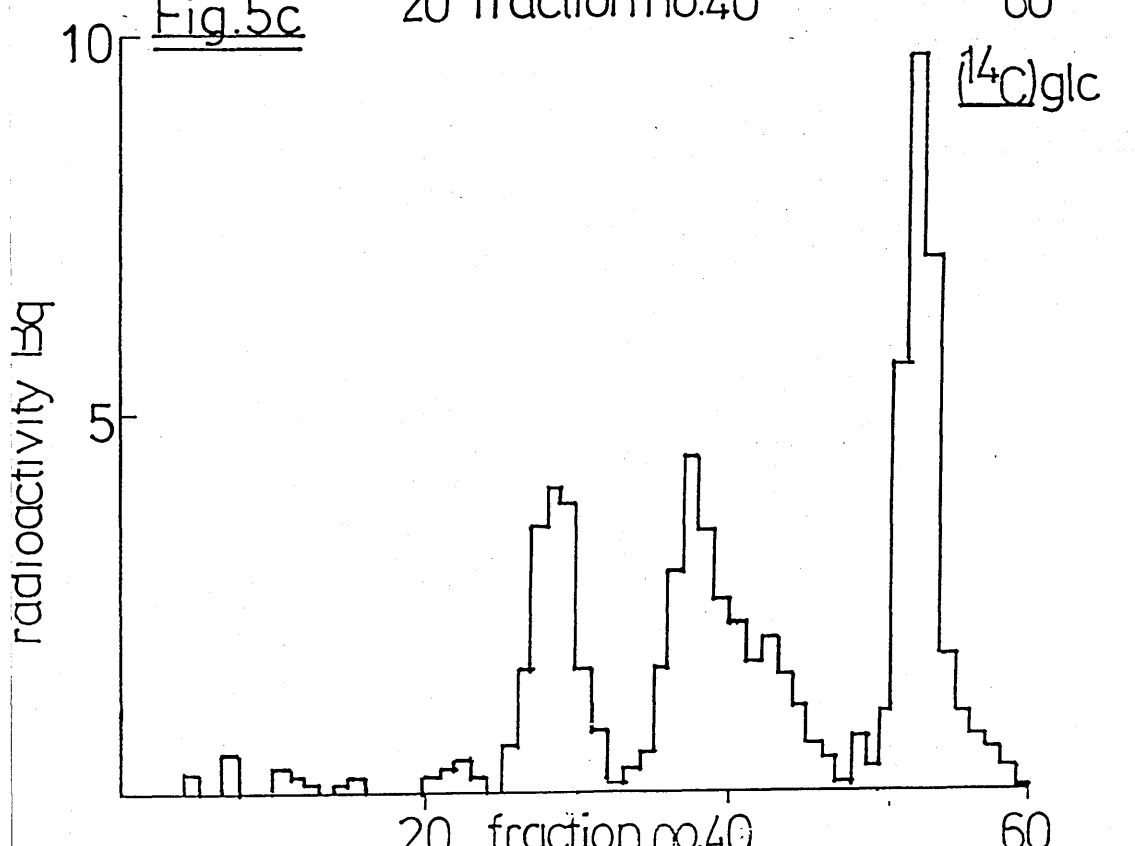
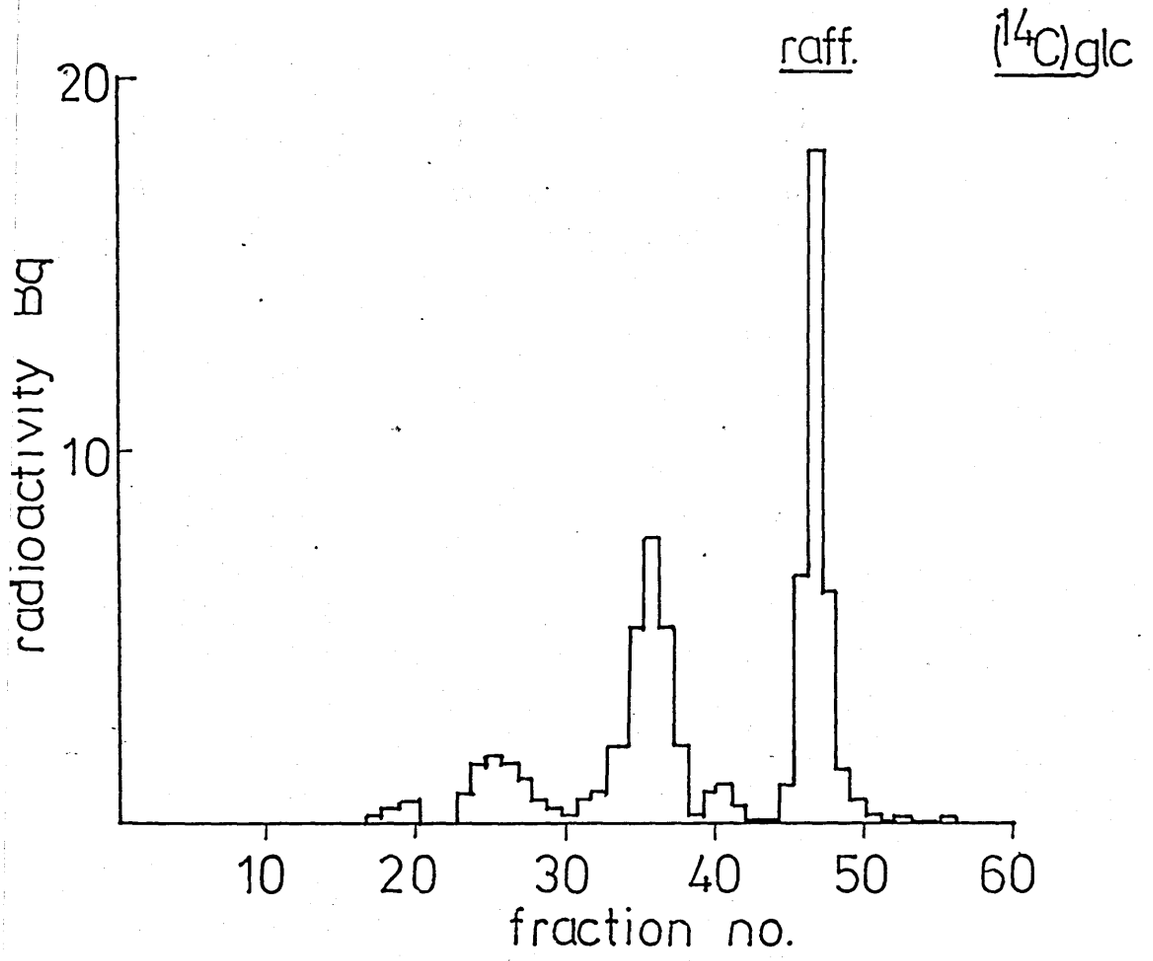


Figure 6

Bio-gel P2 profile of T.viride hydrolysed polysaccharide (labelled with radioactivity from GDP-D-[U^{14}C]fucose during 60 min standard incubation) hydrolysed for 60 min.

Fig.6


raff.
(14C)glc
20
10
radioactivity Bq
10
20
30
40
50
60
fraction no.

labelled polysaccharide was hydrolysed by Trichoderma viride cellulase to give three or four characteristic peaks, the proportions of which vary with hydrolysis time. Fig. 6 shows four radioactively-labelled peaks, the smallest of which (Fractions 39-41) may occur as a shoulder in 90 min and 6 h hydrolysates in Fig.5a & b (around fractions 4-45). Raffinose and [^{14}C]glucose markers indicated that the peak of smallest molecular weight hydrolysate may be a di- or tri-saccharide. Unfortunately larger molecular weight markers were not available to permit clear identification of peaks, so it was not possible to say whether any of the peaks corresponded to the characteristic [^{14}C]fucose-labelled nonasaccharide peak described by Camirand and Maclachlan (1986).

If radioactivity from GDP-D-[U^{14}C]fucose is being incorporated into xyloglucan, which seems quite likely, the results of experiments performed with varying nucleotide combinations may have implications for the mode of incorporation from GDP-D-[U^{14}C]fucose. A low concentration of UDP-galactose in incubation mixtures reduced incorporation far more than a low concentration of UDP-xylose, suggesting that the presence of UDP-galactose is much more important for incorporation from GDP-D-[U^{14}C]fucose. It is likely therefore that much of the [^{14}C]fucose incorporated is added onto a preformed glucan backbone with xylose side chains rather than total synthesis of the polymer occurring. This was also the conclusion of Camirand and Maclachlan (1986) who found that pea microsomes supplied with GDP-D-[U^{14}C] fucose, UDP-xylose and UDP-glucose produced an alkali-soluble product which showed no detectable size increase with time, suggesting they are

not initiated de novo but represent endogenous "primers" which are acceptors for transglycosylation reactions. Double labelling experiments with [^{14}C]fucose and [^{3}H]xylose led Camirand and Maclachlan to the conclusion that approximately 15-30 times as much fucose was incorporated into pre-existing xyloglucan as into newly elongated regions.

Chapter 10

EFFECT OF CELL DIFFERENTIATION ON POLYSACCHARIDE BIOSYNTHESIS BY PARTICULATE ENZYME PREPARATION FROM SUSPENSION-CULTURED BEAN

1. Preliminary Experiments

The differentiation of xylem vessels from non-differentiated tissue has been demonstrated in several plant tissue culture systems (Torrey, 1975), Kohlenbach & Schmidt (1975), Dudley & Northcote (1978), Fukuda & Komamine (1980) and it provides a useful system where changes in enzyme activities are accompanied by visible changes in cell form.

Dudley and Northcote (1978) have established a system for inducing differentiation of xylem and phloem cells in suspension-cultured bean cells. Differentiation occurred on transfer of cells from a 'maintenance' medium which favoured cell division, to an 'induction' medium in which differentiation, but little cell division took place. Phaseolus vulgaris hypocotyls were grown on solid agar containing salts and vitamins (Gamborg et al, 1968) supplemented with 2% sucrose, 20% coconut milk, 2 $mgdm^{-3}$ 2, 4-D and 1% agar. Callus tissue was excised and sub-cultured three times on the same medium before being transferred to a liquid medium, identical to the original maintenance medium but without agar. After no more than four sub-cultures, cells were transferred to an induction medium, similar to the maintenance medium but containing 3% sucrose, 0.2 $mgdm^{-3}$ kinetin plus 1 $mgdm^{-3}$ NAA, instead of coconut milk and 2, 4-D. This transfer resulted in cell differentiation, which could be detected by histological and enzyme techniques.

The technique for achieving cell differentiation in suspension-cultures as outlined above has one major drawback, which is that sub-culture onto induction medium results in much slower cell growth, providing far less tissue for use in enzyme preparations. To overcome this problem and allow this system to be used for investigation of the effect of differentiation on the glycosyltransferases involved in xyloglucan biosynthesis, a modification of the standard particulate enzyme preparation was made. This involved omission of the centrifugation step which requires a minimum of 30 g tissue, and instead tissue was ground in an equal weight of buffer, filtered through muslin, and then 0.5 cm^3 of the homogenate was added to incubations without any centrifugation. As this resulted in a preparation with about 10% of the total tissue weight per incubation and dilution of sugar nucleotides to about one-fifth of their concentration in the standard preparation, the feasibility of using the modified preparation was assessed by comparing incorporation rates for the two preparations. Enzyme preparations were compared for xylan-xylosyltransferase, β 1-3 glucan-glucosyltransferase, xyloglucan-xylosyltransferase and xyloglucan-fucosyltransferase systems, and at the same time inducation of differentiation of suspension-cultured bean cells was attempted to determine optimum conditions.

Comparison of incorporation of radioactivity by four synthetase systems using a particulate and a crude enzyme preparation from suspension-culture tissue

Enzyme preparations were made by the standard method i.e. homogenisation followed by ultracentrifugation, or by

homogenising only ("homogenate preparation") (Ch.2 p.51). Homogenate (0.5 cm^3) or resuspended pellet (50 mm^3) was added to incubations, which were of 0, 15 or 60 min duration, and extractions were carried out as previously. Incubation contents are detailed in Chapter 2 (p.51).

For the xylan synthetase there was little difference in amounts of radioactivity incorporated by the two preparations, despite the reduced tissue content and dilution of sugar nucleotides (Fig. 1). The fucosyltransferase (Fig.2) and glucosyltransferase (Fig. 3) systems showed only slight reduction in activity in the homogenate preparations, and only the xyloglucan-xylosyltransferase activity was substantially reduced in the homogenate preparation (Fig. 4). Taking sugar nucleotide and tissue concentration into account, enzyme activity is greater in the crude homogenate preparation, perhaps reflecting damage to enzyme complexes brought about by the processes of centrifugation and resuspension.

Induction of differentiation in bean suspension cultures

Attempts were made to induce bean suspension-cultures to differentiate, following the methods of Dudley and Northcote (1978). Initially more than ten different callus lines were used to set up suspension-cultures on maintenance medium. These were transferred onto induction media with varying hormone and sucrose concentrations. The length of time between sub-culturing and the amount of tissue transferred were also varied. Cells were examined by light microscopy

Figure 1

Comparison of incorporation of radioactivity from UDP-D-[$U^{14}C$] xylose into xylan by two enzyme preparations from suspension-cultured bean, using assays as described in chapter 2:-

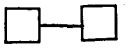
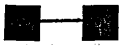
() = standard preparation; () = homogenate preparation.

Figure 2

Comparison of incorporation of radioactivity from GDP-D-[$U^{14}C$] fucose into xyloglucan by two enzyme preparations from suspension-cultured bean, using assays as described in chapter 2:-

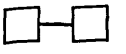
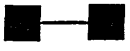
() = standard preparation; () = homogenate preparation.

Fig.1

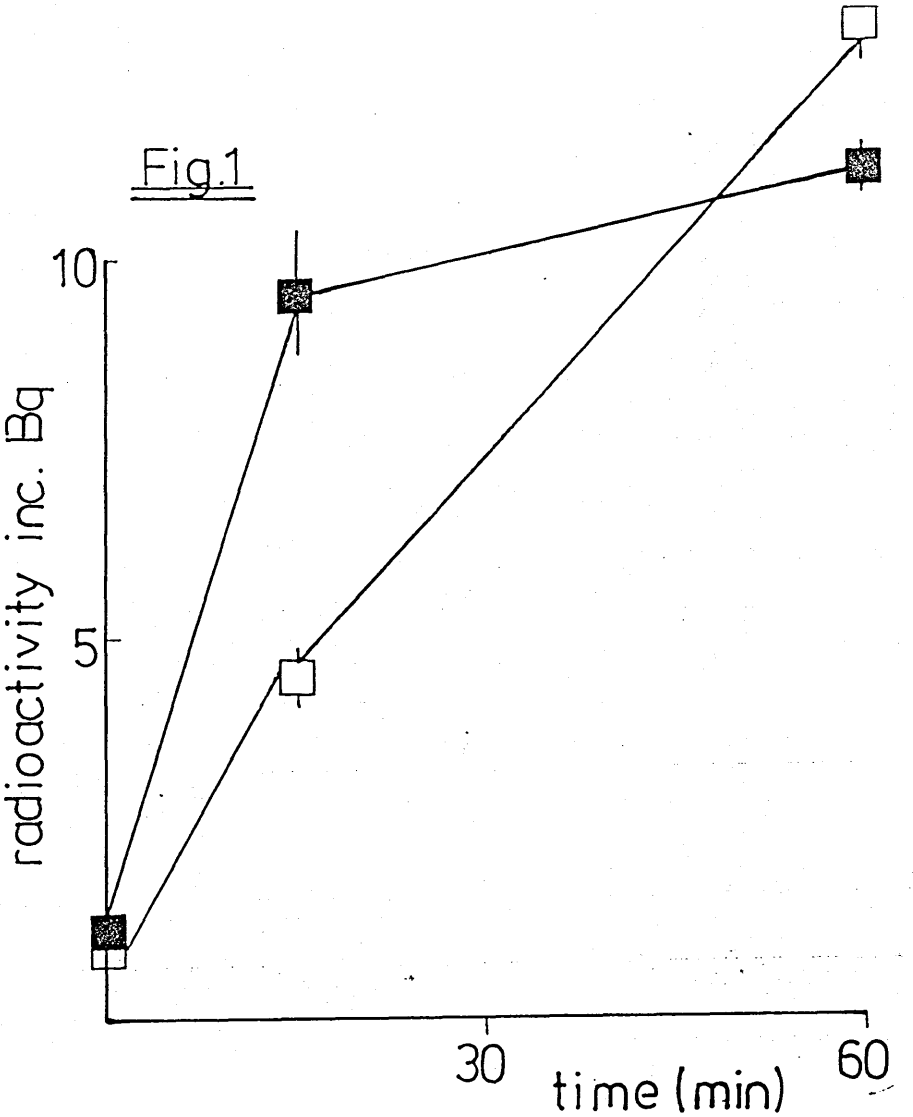

10
5
radioactivity inc. Bq
30
time (min)
60

Fig.2

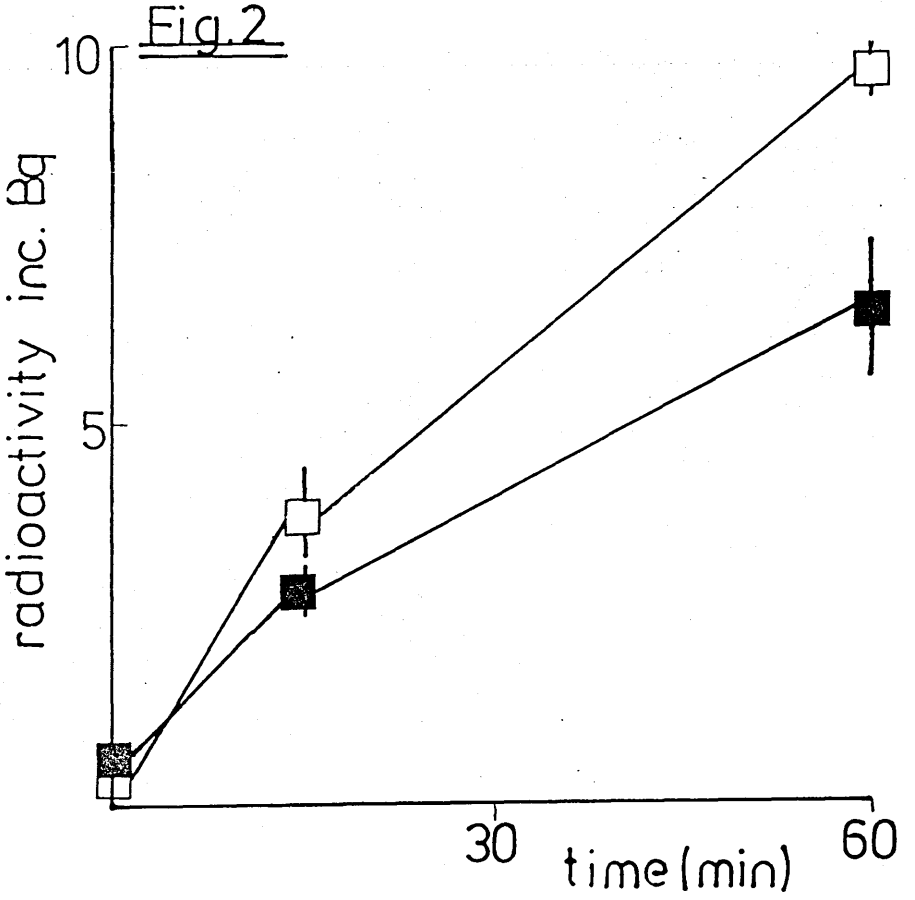

10
5
radioactivity inc. Bq
30
time (min)
60

Figure 3

Comparison of incorporation of radioactivity from UDP-D-[$U^{14}C$] glucose in to β 1-3 glucan by two enzyme preparations from suspension-cultured bean, using assays as described in chapter 2:- (□—□) = standard preparation; (■—■) 0 = homogenate preparation.

Figure 4

Comparison of incorporation of radioactivity from UDP-D-[$U^{14}C$] xylose into xyloglucan by two enzyme preparations from suspension-cultured bean, using assays as described in chapter 2:- (□—□) = standard preparation; (■—■) = homogenate preparation.

Fig.3

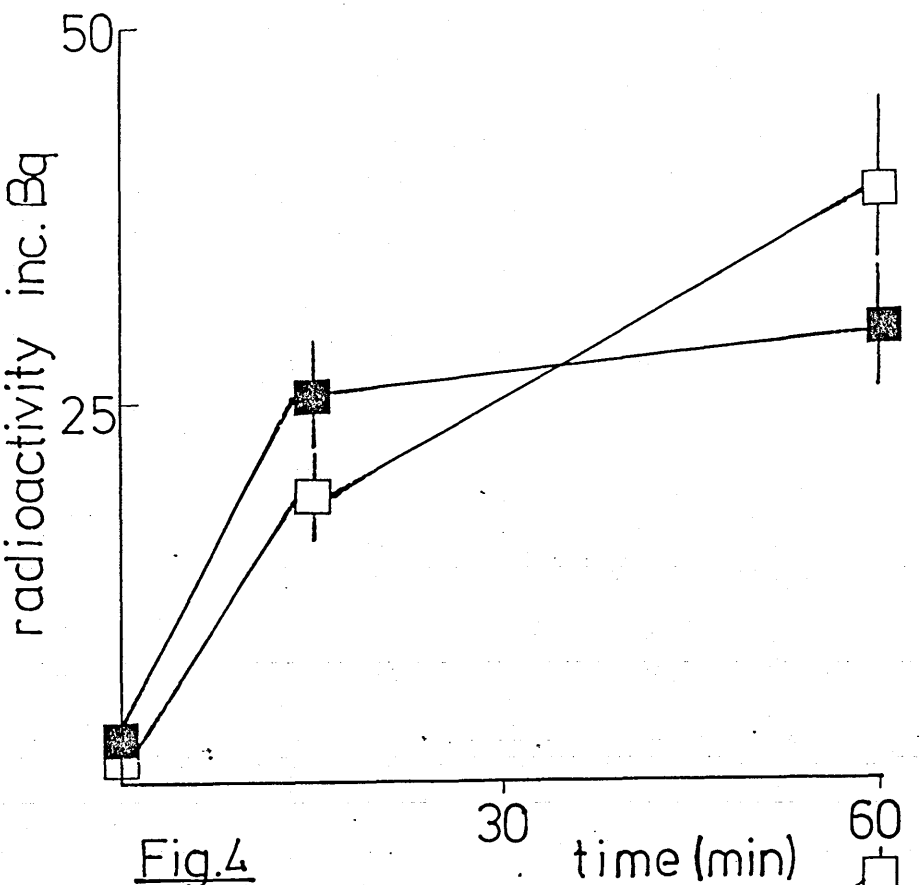
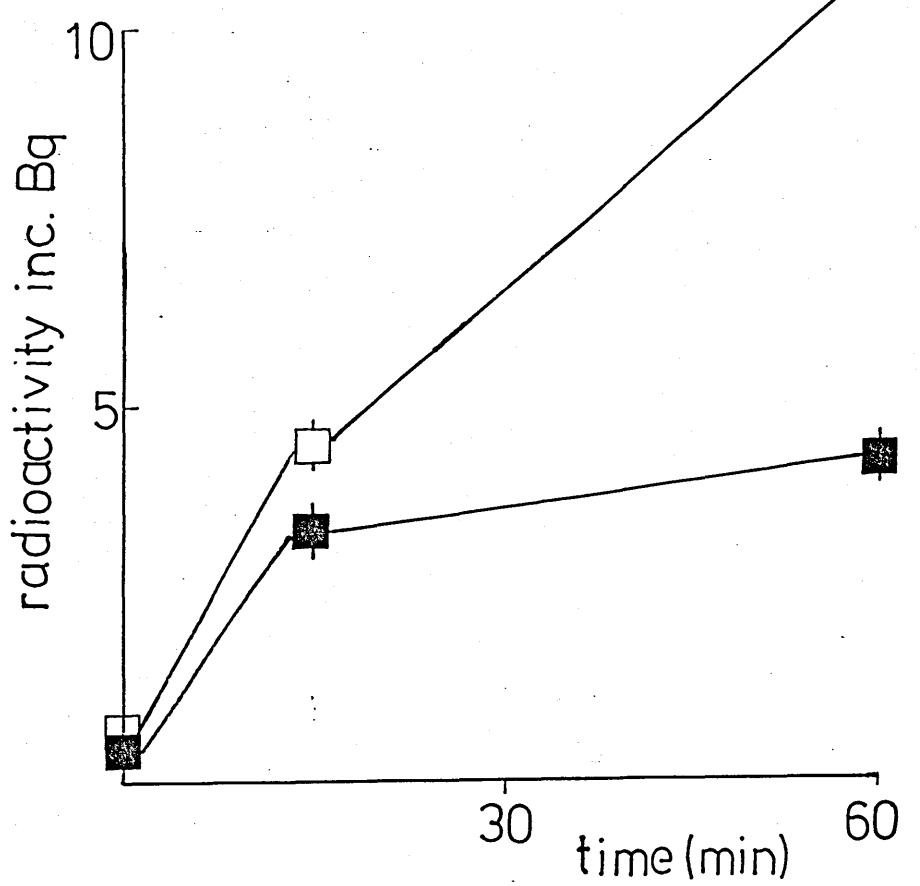

50
25
radioactivity inc. Bq
30
60
time (min)

Fig.4


10
5
radioactivity inc. Bq
30
60
time (min)

and by binocular microscopy using phloroglucinol/HCl as a lignin stain (Jensen, 1962) (Ch.2 p. 67) - this produces a red colour due to condensation of the coniferyl aldehyde group in lignin. While occasional single xylem vessels were observed in some cultures, these probably originated from the original callus, and no appreciable difference was found. A further series of experiments was performed with differentiation investigated also by means of a phenol assay, using a modification by Stafford (1960) of the method of Geirer (1954) for native lignin preparation (Ch.2 p.69). The presence of phenolic compounds is assumed to be a crude indication of lignin biosynthesis and hence of differentiation. The quantities of phenols present in suspension-culture samples detected by this method are expressed in terms of μg of guiaucol, a lignin derivative, estimated by use of a standard curve.

Investigation of differentiation in suspension-cultured bean - 1.

Flasks of induction medium (200 cm^3) were prepared with 1 $mg.dm^{-3}$ NAA, and either 0.2, 0.5 or 1 $mg.dm^{-3}$ kinetin. Approximately 20 cm^3 suspension-cultured tissue was transferred from maintenance medium into two flasks of each hormonal combination and two flasks of fresh maintenance medium as a control. Following 7 or 14 days incubation at 25°C, flasks of each treatment were examined by light microscopy following staining with phloroglucinol/HCl, and by performing a phenol assay on duplicate 50 mg (dried weight) tissue samples.

No xylem vessels were evident following staining. Phenol assay

results were recorded in Table 1. Phenol levels were maximal after 7 days where the ratio of NAA:kinetin was 5:1. However after 14 days the highest phenol level recorded was for the control flask. Overall levels of phenol were low, so the experiment was repeated using an extra hormone concentration and one time only.

Investigation of differentiation in suspension-cultured bean - 2.

The previous experiment was repeated with several changes:- hormone concentrations of 1 $mgdm^{-3}$ NAA with either 0.125, 0.2 or 0.5 $mgdm^{-3}$ kinetin were used, 60 cm^3 of tissue was transferred into each flask, and the flasks were left for six days only.

There was no staining with phloroglucinol/HCl and no evidence of vessels under light microscope. Phenol assay results, recorded in Table 2, showed low levels of phenol in all samples, and values from hormone treatments were lower than control values. As tissue in this and in the previous experiment was transferred directly into the experimental flasks, some dilution of the induction medium may have occurred, altering hormone concentrations. In the next experiment therefore, samples were washed in the appropriate induction medium before transfer. Samples were incubated for 8 days, and two sucrose concentrations were used.

Investigation of differentiation in suspension-cultured bean - 3.

Tissue samples (approx. 60g) were incubated for 8 days with

Table 1

Phenol content, expressed in $\mu g.cm^{-3}$ guaiacol equivalent, in 50 mg (dried weight) bean suspension-culture samples, following incubation of tissue in various induction media.

Medium used: ratio NAA : kinetin	Duration of incubation 7 day	14 day
Maintenance medium	3.2	3
1:1	1.2	0.3
2:1	2.5	2.8
5:1	3.7	1.3

Table 2

Phenol content, expressed in $\mu g.cm^{-3}$ guaiacol equivalent, in 50 mg (dried weight) bean suspension-culture samples, following incubation of tissue in various inducation media for six days.

Medium used: ratio NAA: kinetin	Phenol content: $\mu g.cm^{-3}$ guaiacol
Maintenance medium	3 ± 0.3
2:1	2.3 ± 0.4
5:1	2.3 ± 0.3
8:1	2 ± 0

induction medium containing 1 mg.cm^{-3} NAA with 0.125, 0.2 or 0.5 mg.dm^{-3} kinetin, or with maintenance medium only. Two sets of flasks were used, with 3% sucrose in the induction medium as previously, or with 6% sucrose, giving a total of 8 flasks.

No vessels or staining with phloroglucinol/HCl were observed, and phenol levels were generally low. Increased sucrose concentration appeared to result in increased phenol levels, but differences between hormone concentrations were narrowed down, making selection of an optimum concentration difficult (Table 3). As absorbance was often higher with a hormone ratio of 5:1 (i.e. 1 mg.cm^{-3} NAA, 0.2 mg.cm^{-3} kinetin), a narrower range of concentrations around this value was used, with 3% sucrose in media.

Investigation of differentiation in suspension-cultured bean - 4.

Tissue (60 cm^3) was washed and added to flasks with 4:1, 5:1 or 6:1 NAA:kinetin. Flasks were incubated for 8 days. No differentiation was detected by phloroglucinol/HCl staining or by light microscopy, and phenol assays (Table 4) showed maximum levels in the maintenance medium control. Of the hormone ratios used, 5:1 NAA: kinetin gave the highest phenol reading.

It was felt that the inconsistency of the results obtained from the phenol assay could be due in part to the size of the tissue sample taken i.e. two 50 mg samples from each flask represents a very small proportion of the total contents. An experiment was therefore

Table 3

Phenol content, expressed in $\mu g cm^{-3}$ guaiacol equivalent, in 50 mg (dried weight) bean suspension-culture samples, following incubation of tissue in various induction media for eight days at two sucrose concentrations.

Medium used: ratio NAA : kinetin	% sucrose in medium 3%	6%
Maintenance medium	2.2 ± 0.8	2.6 ± 0.4
2:1	1.2 ± 0.01	3.3 ± 0.2
5.1	2.7 ± 0.1	3.2 ± 0.4
8:1	1.2 ± 0.1	3 ± 0.5

Table 4

Phenol content in $\mu g cm^{-3}$ guaiacol equivalent in 50 mg (dried weight) bean suspension-culture samples, following incubation of tissue for eight days in various media.

Medium used: NAA ; Kinetin	$\mu g cm^{-3}$ guaiacol equivalent
Maintenance medium	4.5 ± 0.5
4:1	3.3 ± 0.5
5:1	3.8 ± 0.1
6:1	2.8 ± 0.1

performed to attempt at least partially to eliminate this variation. Two 50 mg tissue samples were taken from four identical treatments and assayed for phenol.

Investigation of differentiation in suspension-cultured bean - 5.

Flasks were set up containing 60 cm^3 washed material, four with CMCB and four with 5:1 NAA/kinetin, and were incubated for 8 days. No vessels were detected with phloroglucinol/HCl or observed under light microscope. Phenol assay results (Table 5) showed clear variation between identical treatments, but overall higher phenol levels occurred in control flasks.

There is therefore little evidence of differentiation under the conditions used, and this system is unsuitable for investigation of biosynthetic enzymes during or after differentiation. Several further attempts at achieving differentiation were made, using varying hormone concentrations and different cell lines. None were successful, so experiments with solid callus cultures were commenced as an alternative source of material.

Differentiation of 'nodules' of vascular tissue in callus cultures has been well documented (Wetmore & Rier (1963); Haddon & Northcote (1975, 1976)), but this sytem has several obvious disadvantages over the suspension-culture system: in general calluses grow relatively slowly between sub-cultures, variation exists between cultures, and

Table 5

Phenol content, expressed in $\mu g cm^{-3}$ guaiacol equivalent in 50 mg (dry weight) bean suspension-culture samples, following incubation for eight days on induction (5:1 NAA:Kinetin) or maintenance medium.

Replicate	Maintenance medium	Induction medium
1	3.0 $\pm$ 0.8	2.5 $\pm$ 0.1
2	2.5 $\pm$ 0.1	2.4 $\pm$ 0.1
3	2.6 $\pm$ 0.1	2.0 $\pm$ 0.2
4	2.7 $\pm$ 0.1	2.5 $\pm$ 0.1

only one surface of the callus is in direct contact with the hormone-containing medium. The slow callus growth rate is likely to result in low enzyme activity, making comparisons of enzyme activity in differently treated calluses difficult.

A preliminary study was made of enzyme activities in calluses kept on maintenance medium. The enzyme preparation was similar to that devised for use with differentiated suspension cultures, i.e. homogenisation of tissue in an equal weight of buffer followed by filtration and direct addition to incubations, rather than centrifugation.

Investigation of incorporation of radioactivity by four synthetase systems using a crude enzyme preparation from solid callus cultures

Enzyme preparations were prepared from calluses which had been sub-cultured three to four times, and were about six weeks old. The tissue was homogenised in an equal weight of buffer in a mortar with acid-washed sand, and then the homogenate was filtered through muslin and added directly to incubations (0.5 cm^3). Enzyme assays as described in chapter two (p. 51) were used to investigate the xyloglucan-xylosyltransferase, β1-3 glucan-glucosyltransferase, xyloglucan-fucosyltransferase and xylan-xylosyltransferase systems. Incubations were terminated at 0, 15 and 60 min.

Incorporation rates were generally low. There was evidence of activity for the xyloglucan-xylosyltransferase (Fig. 5) and β 1-3 glucan-glucosyltransferase systems (Fig. 6), with incorporation

Figure 5

Incorporation of radioactivity from UDP-D-[U^{14}C]xylose into xyloglucan by a homogenate preparation from bean callus, using standard conditions.

Figure 6

Incorporation of radioactivity from UDP-D-[U^{14}C]glucose into β 1-3 glucan by a homogenate preparation from bean callus, using standard conditions.

Fig.5

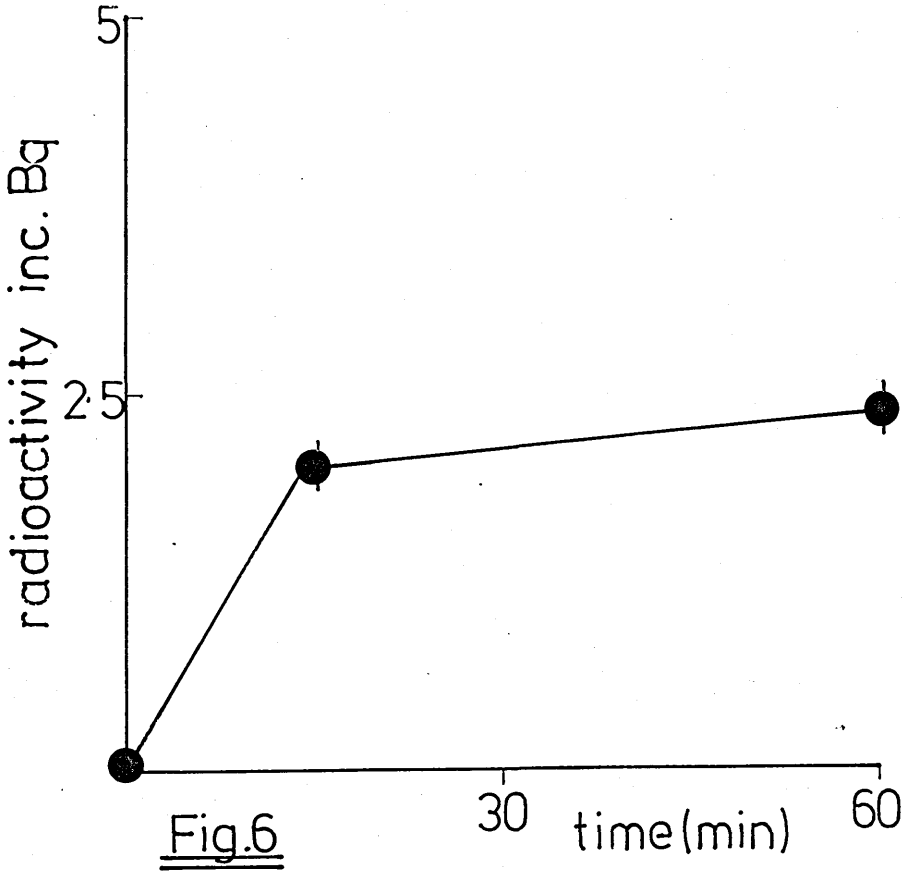
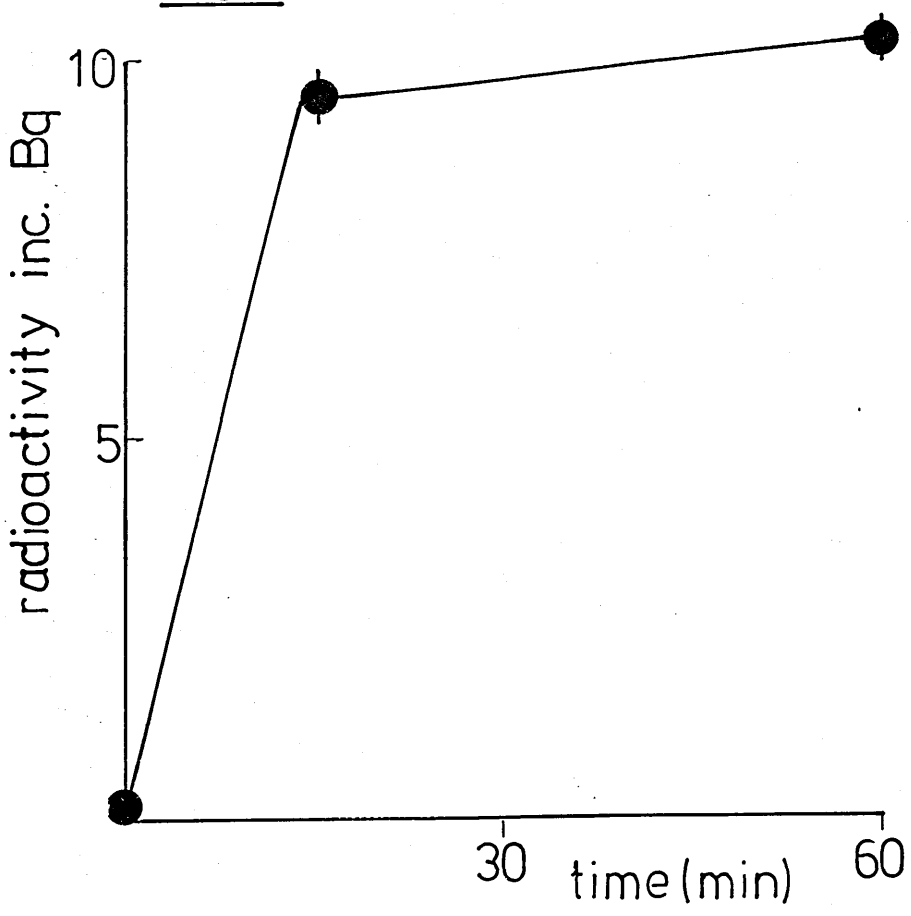

5
2·5
radioactivity inc. Bq
30
time(min)
60

Fig.6


10
5
radioactivity inc. Bq
30
time(min)
60

increasing with time. There was little evidence of incorporation for the fucosyltransferase (Table 6) or xylan-xylosyltransferase systems (Table 7). This variability in enzyme activity may simply reflect variation between calluses, which though of a common origin can differ in size, rate of growth, friability, appearance, etc.

Before further investigation of enzyme activity in calluses was undertaken, induction of differentiation in callus cultures was attempted by transfer of tissue from maintenance to induction medium.

Investigation of differentiation in bean callus cultures - 1.

Solid callus was grown on maintenance medium for four sub-cultures before transfer to induction medium containing 3% sucrose, 1 $mg dm^{-3}$ NAA and a ratio of 2:1, 5:1 or 8:1 NAA/kinetin. (The same media were used as for suspension cultures but with 1% agar added before autoclaving.) Calluses were found to vary in texture, being basically hard and difficult to cut, or soft and friable resulting in a much greater surface area coming into contact with the medium. Samples of both types of callus were used for comparison in the first experiment, and calluses were left for 21 days before examining.

Calluses were examined by phloroglucinol/HCl staining for lignin, and by phenol assay. Distinct pink patches and spiral thickenings were observed in callus on induction medium with 2:1 NAA/kinetin on staining. A few pink patches were also observed at other hormone concentrations but no staining occurred in control samples on

Table 6

Incorporation of radioactivity from GDP-D-[U^{14}C]fucose by homogenate preparation from bean callus, using standard assay conditions.

Incubation Time (min)	Incorporation of radioactivity (Bq)
0	0.1 ± 0
15	0.8 ± 0.1
60	0.7 ± 0.1

Table 7

Incorporation of radioactivity from UDP-D-[U^{14}C]xylose into xylan by homogenate preparation from bean callus, using standard assay conditions.

Incubation Time (min)	Incorporation of radioactivity (Bq)
0	0.2 ± 0
15	0.7 ± 0.1
60	0.7 ± 0.1

maintenance medium. Friable callus stained more noticeably than non-friable callus. Phenol assay results (Table 8) for friable callus reflected those of phloroglucinol staining i.e. maximum phenol levels in 2:1 NAA/kinetin, but for non-friable callus maximum phenol levels were recorded for 8:1 NAA/kinetin, and in general levels were lower. The experiment was repeated with friable callus only.

Investigation of differentiation in bean callus cultures - 2.

The previous experiment was repeated, using friable callus and two hormone concentrations - 2:1 and 5:1 NAA/kinetin. After 21 days, vessels were observed in 2:1 NAA/kinetin on staining, and also in 5:1 NAA/kinetin to a smaller extent. No vessels or staining was observed in the control flask. Phenol assay results (Table 9) did not reflect staining results, and phenol levels were low.

In the next experiment a time course of differentiation was carried out, at one hormone concentration.

Time course of differentiation in bean callus

Bean callus was sub-cultured into six flasks, three containing maintenance medium and three with 2:1 NAA/kinetin. Flasks were incubated for 7, 15 or 21 days and then examined by phloroglucinol/HCl staining and by phenol assay. No vessels were observed at any time in control flasks (i.e. maintenance medium) and in induction medium vessels were first observed at 15 days, then in greater number at 21

Table 8

Phenol content, expressed in $\mu g cm^{-3}$ guaiacol equivalent, in 50 mg (dried weight) bean callus cultures, following incubation of friable and non-friable callus for 21 days on various induction media.

Incubation medium ratio Naa/kinetin	Type of callus	
	Friable	Non-friable
Maintenance	- (contaminated)	2.3
2:1	3.7	2
5:1	3	2.4
8:1	2.8	2.5

Table 9

Phenol content in $\mu g cm^{-3}$ guaiacol equivalent in 50 mg (dried weight) bean callus cultures, following incubation for 21 days on various induction media.

Incubation medium: NAA/kinetin	Phenol content - μg guaiacol. cm^{-3}
Control (maintenance medium)	2 ± 0.5
2:1	2.2 ± 0.4
5:1	2.7 ± 0.9

days. Phenol assay results (Table 10) did not however reflect staining results and showed a decrease in phenol levels with time.

As it appeared obvious that differentiation was occurring but not being reliably detected by the phenol assay, a second biochemical test was introduced - an assay for phenylalanine ammonia lyase activity (or PAL assay). Phenylalanine is a precursor of lignin in dicotyledenous plants and is believed to be concentrated in tissues that are synthesizing lignin e.g. sycamore xylan, celery petioles and pea roots (Rubery & Northcote, 1968). Evidence of PAL activity, which is indicated by conversion of phenylalanine to cinnamic acid, is a more specific indication of differentiation than presence of phenols, but requires more precise detection, as PAL activity rises at the commencement of differentiation and declines afterwards. Timing of assays is therefore critical. Once differentiation has occurred, phenol levels remain high, so timing is far less critical.

The feasibility of using a PAL assay to detect differentiation in solid calluses was investigated by comparing phenol levels and PAL activity at four day intervals up to 20 days in control and induced calluses.

Comparison of PAL and phenol assays for bean calluses grown on maintenance and induction media - 1.

The course of differentiation in bean calluses transferred onto induction medium was assessed using PAL and phenol assays in conjunction

Table 10

Time course of differentiation in bean callus on induction medium (2:1, NAA:Kinetin) detected by measuring phenol content in 50 mg (dried weight) samples, expressed as $\mu g cm^{-3}$ guaiacol equivalent.

Incubation time (days)	Incubation medium	
	Control (maintenance medium)	Induction medium (2:1 Naa/kinetin)
7	2.2 ± 0.3	2.4 ± 0.1
15	2.3 ± 0.1	2.3 ± 0.2
21	1.2 ± 0.1	2.0 ± 0.4

with phloroglucinol/HCl staining for lignin. Culture flasks were set up using tissue from two calluses grown on maintenance medium which were transferred onto fresh maintenance medium or onto induction medium containing 2:1 Naa/Kinetin. Samples were incubated for 4, 8, 12, 16 or 20 days, and at each time interval one flask of each treatment was assayed for phenol and PAL, and examined using phloroglucinol/HCl.

On staining with phloroglucinol pink patches appeared in samples on induction medium from day 12 onwards. Phenol and PAL assay results were recorded in Table 11. These results show agreement between phloroglucinol staining and PAL activity (expressed in terms of percentage conversion of phenylalanine to cinnamic acid) i.e. greatest evidence of differentiation on day 12. For the phenol assay, phenols peaked on day 8 and were only marginally higher than in the starting material (i.e. the original callus).

Thus the PAL assay appeared to be more accurate than the phenol assay. This was further investigated by repeating the experiment, using the same conditions but examining more calluses around day 12.

Comparison of PAL and phenol assays for bean calluses grown on maintenance and induction media - 2.

Three calluses from a different culture line to that used in the previous experiment were transferred onto fresh maintenance medium, or onto induction medium, and then incubated for 8, 10, 12, 14, 16 or 20

Table 11

Time course of differentiation in bean callus on induction medium (2:1 NAA/kinetin) detected by phenol assay and PAL assay: phenols expressed as $\mu g cm^{-3}$ guaiacol equivalent and PAL activity as percentage conversion of phenylalanine to cinnamic acid.

Incubation time (days)	Phenol Assay		PAL Assay	
	Control	Induced 2:1 NAA/kinetin	Control	Induced 2:1 NAA/kinetin
0	2.8 ± 0.4		13.4	
4	2.2 ± 0.4	2.1 ± 0.01	13.2	15.2
8	2.7 ± 0.1	3 ± 0.3	14.5	15.5
12	1.3 ± 0	2.2 ± 0.3	18.5	22.8
16	1.5 ± 0	1.8 ± 0.2	16.3	19.3
20	1.2 ± 0.1	1.3 ± 0.01	14.3	15

days. Tissue was examined by phoroglucinol/HCl staining, and phenol and PAL assays were carried out.

Unfortunately, in this experiment some pink-staining patches were present in the starting material. Thereafter pink staining was only observed at day 12 in induced samples, though there was also evidence of thickened walls at day 16. Results of phenol and PAL assays (Table 12) showed PAL activity in induced samples peaking again on day 12. Phenol levels peaked on day 16, but were also high in the starting tissue. The experiment was repeated using different calluses and the same pattern emerged - maximum PAL activity on day 12 and maximum phenol levels on day 16.

The difference in the results of these two assays presumably reflects the different nature of the tests i.e. the PAL assay directly measures enzyme activity and reflects the course of lignin biosynthesis, while the phenol assay reflects the total phenol concentration which will continue to rise even as enzyme activity slows down, as it is a cumulative measurement. The PAL assay was therefore used in subsequent experiments as it would allow correlation of lignin biosynthesis with specific assays of wall biosynthetic enzymes. Results of PAL assays were also expressed in a more meaningful form, taking the protein content of enzyme preparations into account i.e. nmol cinnamic acid formed per mg protein.

Table 12

Time course of differentiation in bean callus on induction medium (2:1 NAA/kinetin) detected by phenol assay and PAL assay : phenols expressed as $\mu g cm^{-3}$ guaiacol equivalent and PAL activity as percentage conversion of phenylalanine to cinnamic acid.

Incubation time	Phenol Assay		PAL assay	
(days)	Control	2:1 NAA/kinetin	Control	2:1 NAA/kinetin
0	3.75 ± 1.3		15.6	
8	1.3 ± 0	2.2 ± 0	16.3	16.9
10	1.3 ± 0.5	2.4 ± 0.2	14.7	18.4
12	1.3 ± 0	2.4 ± 0.1	15.6	22.7
14	1.4 ± 0.1	2.4 ± 1	14.9	17.3
16	2.4 ± 0.5	3.6 ± 0.5	16.4	17.0
20	2.5 ± 0.5	2.6 ± 0	15.0	14.6

2. Investigation of the Effect of Differentiation on the Activity of Four Wall Biosynthetic Enzymes

Changes in the activities of enzymes involved in cell wall biosynthesis have been reported by several authors. Dalessandro and Northcote (1977) studied changes involved in nucleoside diphosphate sugar interconversions during differentiation of cambium to xylem in pine and fir. The specific activities of these enzymes varied according to the type of polysaccharide being synthesized. Haddon and Northcote (1975) correlated an increase in nodule formation in bean callus transferred from a maintenance to an induction medium with an increase in PAL and β 1-3 glucan synthetase activity. Dalessandro and Northcote (1985) found that the activity of a polygalacturonic acid synthase fell at least 6-10 fold as cells differentiated from vascular cambium to xylem, and was correlated with the cessation of pectin deposition.

The changes in activity during differentiation of enzymes involved in xyloglucan biosynthesis do not appear to have been investigated. An investigation of the activity of three wall synthase enzymes was therefore carried out using bean callus cultures and enzyme assays, partially optimised as earlier described (p.220). Time courses of differentiation were studied by performing a PAL assay and a specific enzyme assay at time intervals - generally at four day intervals up to 20 days. A protein assay was performed at each time interval to allow calculation of specific enzyme activities.

i. Investigation of xyloglucan-xylosyltransferase and xyloglucan-fucosyltransferase activity during differentiation in bean callus

Friable bean callus was transferred, after three to four sub-cultures on maintenance medium, onto induction medium containing 2:1 NAA/kinetin, or onto fresh maintenance medium. Calluses were incubated at 25°C in the dark for 0, 4, 8, 12, 16 or 20 days, and at each time interval PAL activity, xylosyltransferase activity and fucosyltransferase activity were assessed for callus tissue grown on maintenance and on induction medium. The protein content of the enzyme preparation for assaying transferase activity was calculated using a tannin assay. Standard xylosyltransferase, fucosyltransferase and PAL assays were used as described (Chapter 2, p.69), and transferase incubations were terminated at 0 and 15 min.

PAL activity (Fig. 7) in induced calluses showed two peaks, at 4 and 16 days incubation. Levels of PAL activity in control calluses on maintenance medium showed little variation apart from a slight rise in activity at 4 and at 20 days. The activity of the xylosyltransferase enzyme showed a similar pattern to that of the PAL enzyme, with two peaks, though the peak at 16 days was considerably larger (Fig. 8). The levels of enzyme activity in the control samples were similar to those of induced samples, apart from the lack of a peak of activity at 16 days. Fucosyltransferase activity similarly reflected PAL activity, with a small increase at 4 days and a larger peak at 16 days (Fig. 9).

Figure 7

Time course of PAL activity in bean callus on maintenance

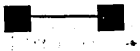
() or induction () medium.

Fig.7

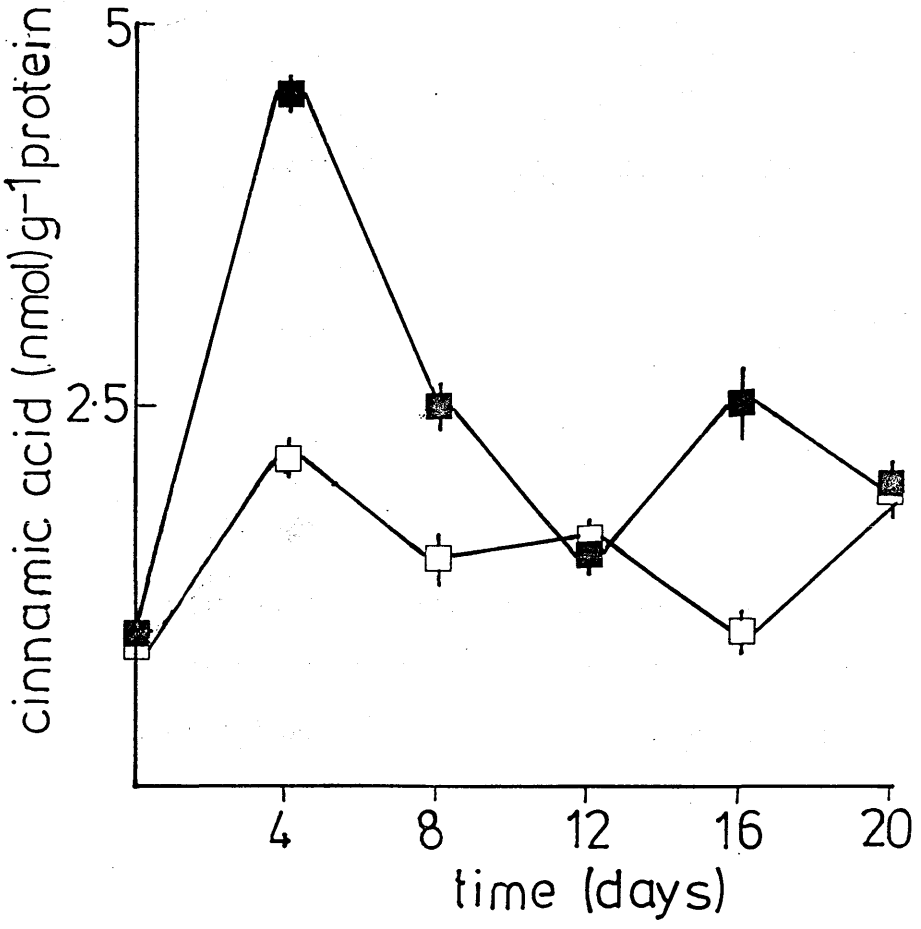


Figure 8

Time course of incorporation of radioactivity from UDP-D-[$U^{14}C$] xylose into xyloglucan by homogenate preparations from bean callus on (○—○) maintenance, or (●—●) induction medium.

Figure 9

Time course of incorporation of radioactivity from GDP-D-[$U^{14}C$] fucose into xyloglucan by homogenate preparations from bean callus on (○—○) maintenance, or (●—●) induction medium.

Fig. 8

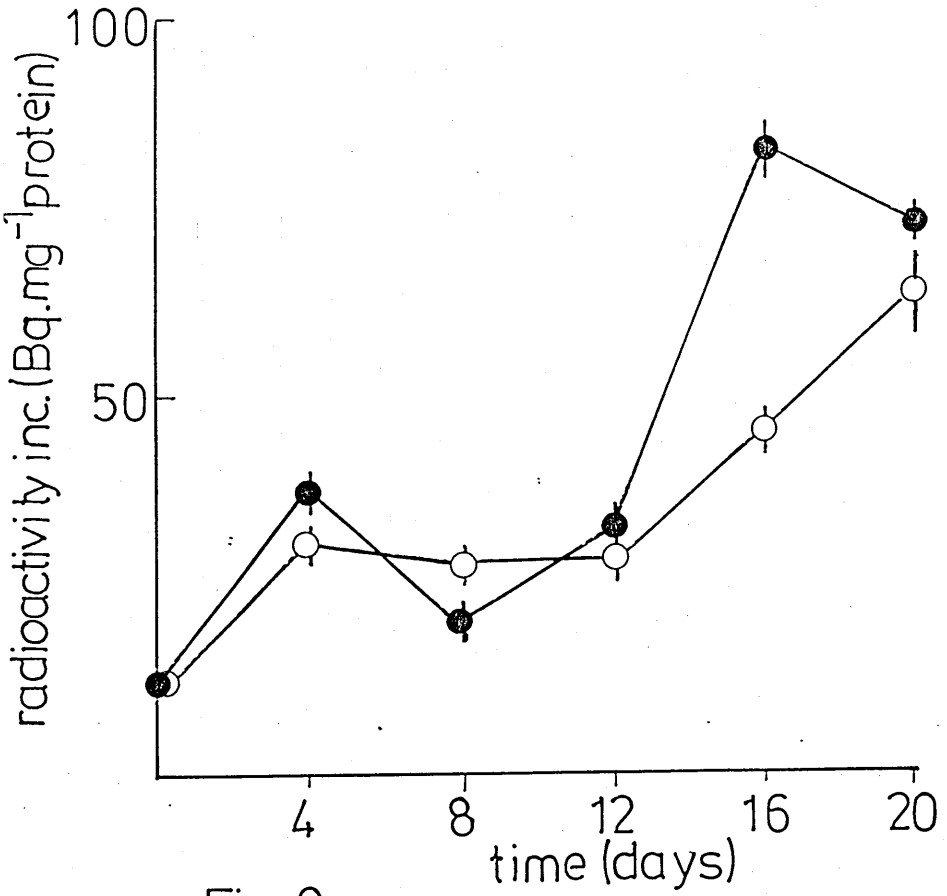
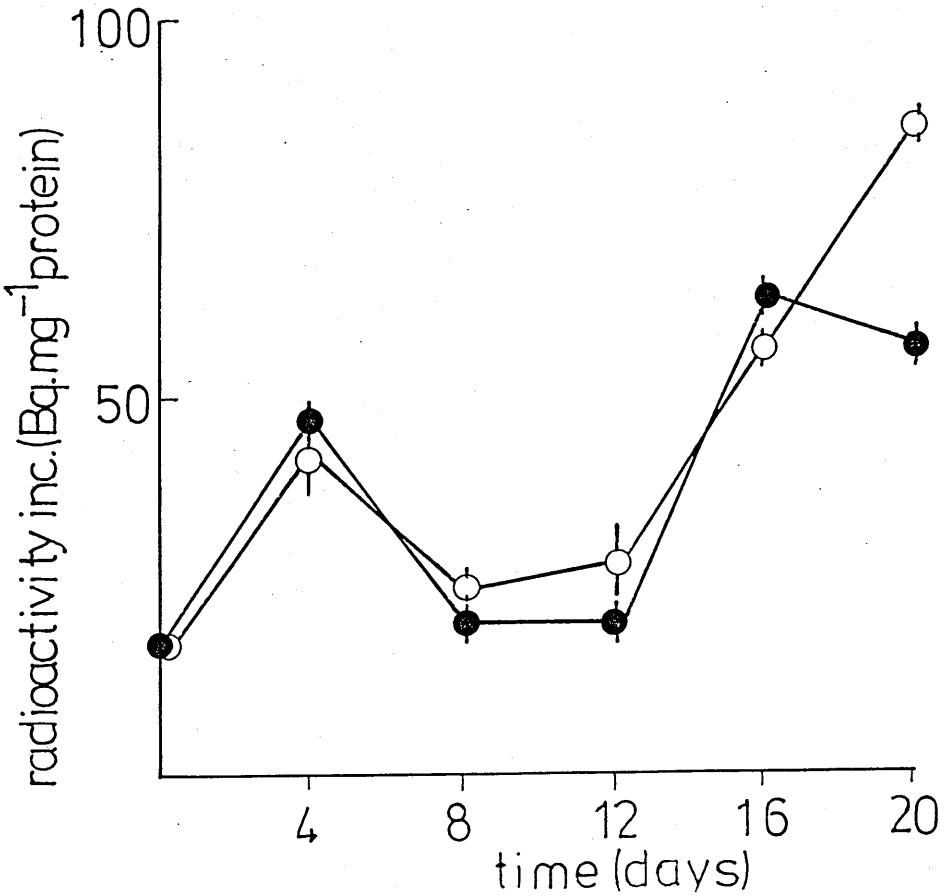

100
50
radioactivity inc.(Bq.mg^{-1}protein)
4
8
12
16
20
time (days)

Fig. 9


100
50
radioactivity inc.(Bq.mg^{-1}protein)
4
8
12
16
20
time (days)

In this case results from induced and control calluses were very similar, apart from a sharp rise in activity in control samples at 20 days.

ii. Investigation of β 1-3 glucosyltransferase activity during differentiation in bean callus

The method for investigation of enzyme activity during differentiation used in the previous experiment was followed to investigate β 1-3 glucosyltransferase activity. A different callus line was used, but otherwise conditions were identical. The assay for glucosyltransferase was as outlined in Chapter 2 (p.52).

The PAL assay for induced samples was similar to the previous experiment, with peaks in activity at 4 and 16 days (Fig. 10). The PAL activity for the control samples however also showed two peaks of activity, at 4 and at 20 days. The glucosyltransferase activity was high in the callus at day 0 (Fig.11). Sub-culture onto induction medium brought about a decline in activity followed by a peak of activity at 16 days, reflecting PAL activity. Sub-culture onto maintenance medium resulted in a reduction in enzyme activity to a steady background level.

Discussion

Surprisingly good rates of incorporation of radioactivity were

Figure 10

Time course of PAL activity in bean callus on maintenance (□——□) or induction (■——■) medium.

Figure 11

Time course of incorporation of radioactivity from UDP-D-[$U^{14}C$] glucose into β 1-3 glucan by homogenate preparation from bean callus on (□——□) maintenance or (■——■) induction medium.

Fig.10

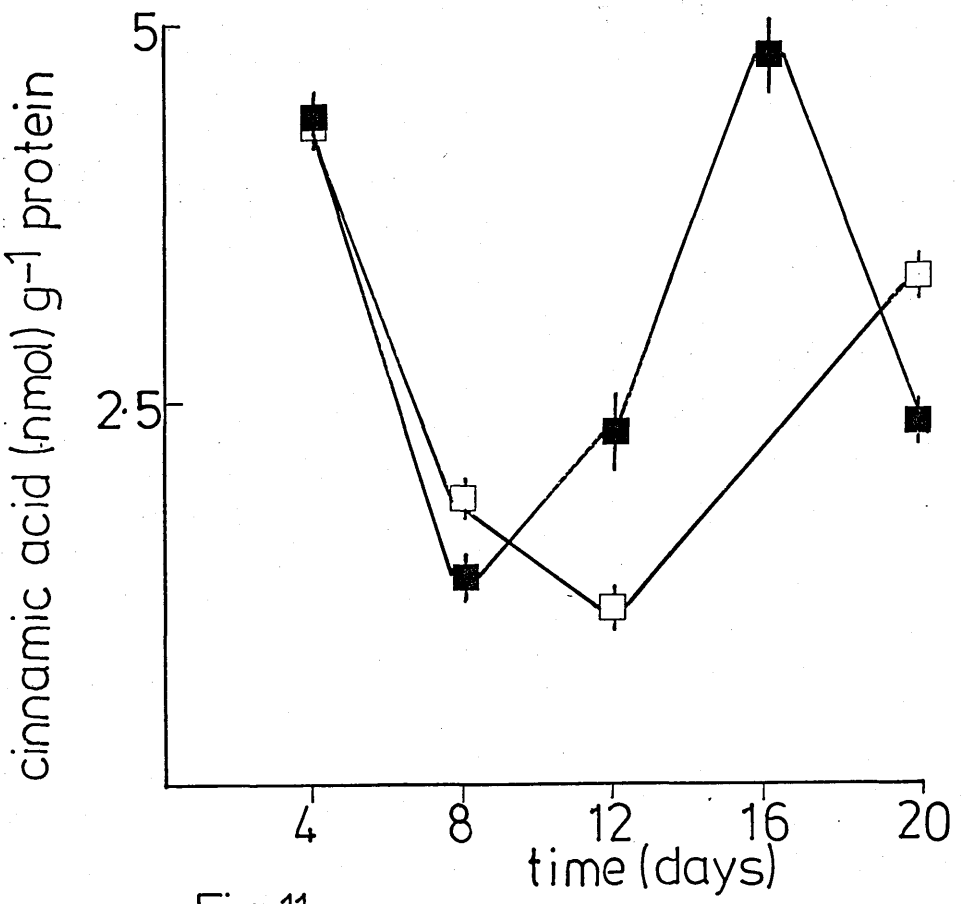
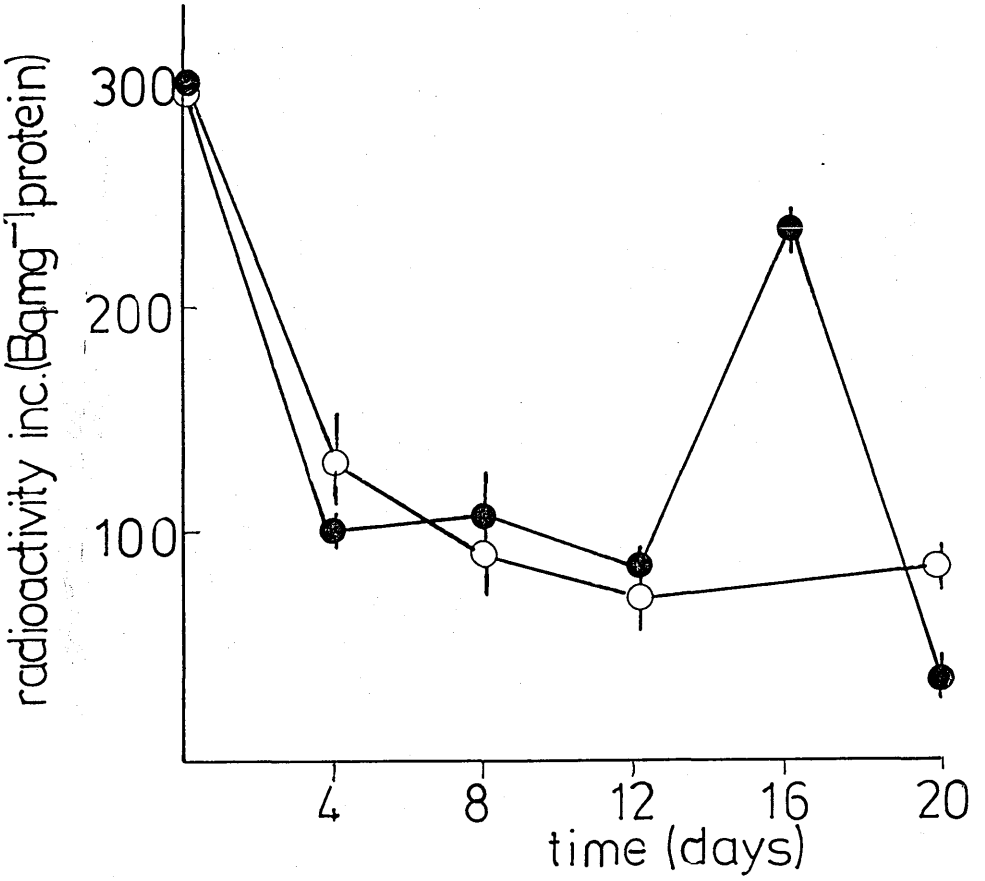

5
2·5
cinnamic acid (nmol) g⁻¹ protein
4
8
12
16
20
time (days)

Fig.11


300
200
100
radioactivity inc.(Bqmg⁻¹protein)
4
8
12
16
20
time (days)

obtained from homogenate preparations, considering that sugar nucleotides were diluted more than fivefold, and tissue volume was also reduced compared with centrifuged preparations. It is interesting that while centrifuged preparations showed time courses with incorporation of radioactivity still apparently increasing at 60 min of incubation, homogenate preparations showed considerable slowing of the rate of incorporation of radioactivity between 15 and 60 min, probably due to the presence in this type of preparation of hydrolases, proteases and other enzymes involved in the degradation of transferases, substrates and products. Incubations of short duration are clearly more suitable for use with this preparation. It is assumed in this study that the products of the incubations are the same as for centrifuged preparations. This was not investigated and requires confirmation before firm conclusions can be drawn from results obtained.

It is not clear why attempts to achieve induction of differentiation in suspension-cultures were unsuccessful despite the investigation of several variables. It is reported (Haddon & Northcote, 1975) that bean (solid) calluses varied considerably in their ability to form nodules on transfer to induction medium, one strain retaining the ability to form nodules for several years, another for nine months, yet a further strain losing the ability after five sub-cultures on maintenance medium. This innate variability may partly explain the lack of success.

As expected, homogenate preparations from calluses showed low

incorporation of radioactivity, presumably due to the slow growth rate of the calluses. This incorporation could probably be substantially improved by selection of fast-growing callus lines and optimisation of incubation conditions.

Differentiation in solid calluses was much more easily achieved than in suspension cultures, implying that that transfer to suspension resulted in the loss of ability of calluses to differentiate. Variation between calluses is a potential problem, as is spontaneous nodule formation in "control" calluses which cannot be controlled. Assay of differentiation by measurement of soluble phenols proved unreliable, not usually reflecting histochemical results or results from assays of PAL activity. Haddon and Northcote (1976) found PAL activity was more closely correlated with xylogenesis than formation of solid phenols, and Jeffs and Northcote (1966) reported that increases in soluble phenols only partially reflected increases in lignin content (around 10%). Haddon and Northcote (1976) also report that polyphenols accumulate in cultures kept for a long time on maintenance medium although no increase in differentiation or PAL activity occurs, indicating that some control values may be unreliable. Thus PAL activity was used as a "marker" for differentiation in investigations of wall biosynthetic enzymes in the present study.

PAL activity was measured in calluses from two experiments for comparison with transferase activity. In both experiments an unexpectedly high peak of activity occurred on day 4 in both induced

calluses and in one control (Figs. 7 & 10). This may be due to slightly elevated enzyme activity combined with low total protein content giving rise to abnormally high specific activity values. PAL activity values were initially recorded as percentage conversion of phenylalanine to cinnamic acid (results not shown) and these values do not reflect the values for specific activity - e.g. the conversion rate recorded for induced calluses from Figure 10 for day 4 was 15% while for day 16 it was 19%.

Assays for xylosyl- and fucosyltransferase activities (Figs. 8 & 9 respectively) show increases in activity of these enzymes on both induction and maintenance medium, with peaks at day 4 and day 16 reflecting PAL activity in the same callus. This result is perhaps surprising as xyloglucan is assumed to be a primary wall polysaccharide, the synthesis of which might be expected to decrease on commencement of differentiation. This is in contrast to the results of Dalessandro & Northcote (1985), but may reflect the difference in location of these polysaccharides in the cell wall recently reported by Moore *et al*, 1986.

The activity of β 1-3 glucosyl transferase (Fig. 11) on induction medium reflected the PAL activity, peaking on day 16. Activity in the callus at day 0 was high then declined on both media. Haddon and Northcote (1975) used β 1-3 glucosyltransferase activity as a marker for phloem formation in *Phaseolus aureus* and they also found activity on maintenance medium peaked around day 16, while PAL activity increased from day 12

onwards. The similarity in these results suggests that phloem formation is being measured in the bean callus in the present study also (though this does not explain the high enzyme activity on day 0).

Chapter 11

DISCUSSION

The results of the preliminary investigation in this study (chapter 3) indicated that suspension-cultured tissue may be more suitable than hypocotyls for the study of enzymes involved in primary wall biosynthesis. This was clearly confirmed in subsequent chapters where much of the variability of results found with hypocotyl preparations was eliminated by using suspension-cultured tissue. In this study, six wall biosynthetic enzymes were investigated:- xylose from UDP-xylose was incorporated by a particulate enzyme preparation from suspension-cultured bean into a product which was identified by analysis of hydrolysis products as xyloglucan; under slightly different incubation conditions xylose from UDP-xylose was incorporated into another product, identified as a xylan. Characterisation of the former product was carried out more thoroughly than for the latter, but it was clear that two quite different products were being formed by the transfer of xylose from UDP-xylose under conditions which were not very different (i.e. different buffer and duration of centrifugation). This result highlights the complexities of this sort of study, where altering a single parameter can result in predominant activity of a different enzyme.

The particulate enzyme preparation which contains xylosyltransferase activity also appears to contain fucosyltransferase activity, transferring fucose from GDP-fucose into a product thought to be xyloglucan - identification of the incubation product in this case was more complicated due to the absence of an easily identifiable product of hydrolysis. Galactosyltransferase activity was found to vary between

different batches of the same particulate enzyme preparation; zero time values were high and incorporation rates were low, and identification of the incubation products was not attempted. Similar lack of success in investigating galactosyltransferase is also reported by Camirand and Maclachlan (1986). It is interesting that xylosyltransferase and fucosyltransferase activity can apparently proceed in the absence of galactosyltransferase activity, suggesting addition of galactose is not required for continued growth of the molecule.

It has been reported that most membrane preparations from pea, supplied with UDP-glucose will synthesize β 1-3 glucan (Raymond et al, 1978). This is also true of pea protoplasts, and in wounded or stressed plants where β 1-3 glucan synthesis predominates. It is perhaps not surprising therefore that glucose from UDP-glucose appears to be transferred by the bean particulate enzyme preparation principally into a product identified as β 1-3 glucan. However, the fact that xylose transfer from UDP-xylose is enhanced in incubations pre-incubated with UDP-glucose suggests that at least some incorporation from UDP-glucose is into xyloglucan.

It is not known how heteropolysaccharide biosynthesis is initiated, but it has been proposed that the initiation steps may involve addition of sugars to a protein 'primer' (Tandecarz & Cardini, 1978). The transfer of xylose from UDP-xylose by particulate enzyme preparation from bean appears to involve a protein primer, as the product of the transfer is closely associated with protein, probably through a covalent

association. Thus, as demonstrated, cellulose-derived oligosaccharides are unable to act as acceptors for the xylosyl-transferase. Little is understood of the enzyme interactions occurring once biosynthesis has been initiated, but a model has recently been proposed by Waldron and Brett (1985) which may help to explain how the enzymes interact in the synthesis of heteropolysaccharides. This model, already described in Chapter 1, is based on the degrees of precision with which synthesis may proceed: "imprecise" synthesis would involve enzymes specific enough to define residues and linkages, but unable to give further regularity to the product. Side chains would be irregularly attached and of variable length, and the backbone would be synthesized independantly of the side chains. "Precise" synthesis would involve synthesis of identical, repeating sub-units linked in a precise manner but of undefined number. The backbone of such a "precisely" synthesized molecule would be unable to grow without concomitant addition of side chains and non-glycosyl substituents. Xyloglucan biosynthesis by particulate enzyme preparations from bean suspension cultures appears to proceed by an "imprecise" mechanism, as xylose can be transferred to a preformed glucan backbone following pre-incubation with UDP-glucose, even though UDP-glucose is removed from the incubation medium. This also applies to the transfer of fucose from GDP-fucose which proceeds in the absence from incubations of UDP-glucose and UDP-xylose, suggesting it is added, along with UDP-galactose, to a pre-formed chain.

The investigation of xyloglucan biosynthesis during differentiation was of a preliminary nature due to the low number of experiments performed.

This was due to the unforeseen difficulties in bringing about induction of differentiation and also to the low level of enzyme activity in calluses. It had been hoped to investigate factors involved in the control of xyloglucan biosynthesis, such as varying hormonal regimes. It was also not possible to carry out identification of the products of the callus enzyme assays for differentiated tissue, due to the low levels of radioactivity incorporated. Development of these aspects would clearly have added to this study, and it is hoped that this work may be continued to develop the obvious potential of this system.

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