GLUCURONYLTRANSFERASE ACTIVITY ASSOCIATED WITH HEMICELLULOSE AND PECTIN BIOSYNTHESIS IN *PISUM SATIVUM*

by

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I wish to dedicate this work to my parents.

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ABBREVIATIONS

Ara	Arabinose
Araf	Arabinose (furanose form)
BSA	Bovine serum albumin
Bq	Bequerel
^{o}C	Degrees centigrade
Ch.	Chapter
EDTA	Ethylene diamine tetraacetic acid
EM	Electron microscopy
x g	Multiples of the gravitational field
Gal	Galactose
GalA	Galacturonic acid
Glc	Glucose
GlcA	Glucuronic acid
h	Hour
IDP	Inosine diphosphate
Km	Michaelis constant
Man	Mannose
min	Minute
mw	Molecular weight
OD	Optical density
SAM	S-adenosyl methionine
TCA	Trichloroacetic acid
UDP	Uridine diphosphate
v/v	Volume for volume
w/v	Weight for volume
w/w	Weight for weight
Xyl	Xylose
%	Percent
[S]	Substrate concentration
v	Velocity of reaction
DNP-L	Di-nitro-phenyl-lysine
BD	Blue dextran
Me	Methyl group

Unless otherwise stated, all units follow the SI convention.

ABSTRACT

A particulate enzyme preparation made from 1-week-old dark-grown pea (*Pisum sativum*) seedlings has been shown to incorporate glucuronic acid from UDP-D-[U-^{14}C]-glucuronic acid into a hemicellulosic product. Optimum conditions for the incorporation include the presence of Mn^{2+} ions between 4 and 10 mmol dm^{-3} and a pH of approximately 7.5. UDP-D-xylose at 1 mmol dm^{-3} allows incorporation to continue for at least 8 h. In its absence, the reaction stops within 30 min. Analysis of the product by partial and total acid hydrolysis, followed by paper chromatography or electrophoresis, indicates that the polysaccharide produced is a glucuronoxylan. Freezing the particulate enzyme at -20°C for 24 h lowers the activity by 50%.

The distribution of the glucuronyltransferase along the epicotyl has also been investigated and the results indicate that the enzyme is involved mainly in secondary wall biosynthesis. However, a role in primary wall synthesis cannot be ruled out.

Upon solubilisation in Triton X-100 (a non-ionic detergent), the glucuronyltransferase activity becomes stable at -20°C. Although the UDP-D-xylose stimulatory effect is not altered, the optimum Mn^{2+} concentration drops to below 2 mmol dm^{-3} and the polysaccharide product appears to be slightly different.

Sucrose-density gradient centrifugation indicates that the glucuronyltransferase activity is located in the Golgi apparatus. However, the possibility that it is present in the plasmamembrane cannot be discounted.

CONTENTS

Chapter 1.

INTRODUCTION

I The Structure of Plant Cell Walls in the Higher Plants.

In higher plants, the cell walls consist of a skeleton of cellulosic microfibrils embedded in an amorphous matrix of polysaccharides, protein, glycoprotein and lignin (Ericson and Elbein, 1980). In living cells these components may undergo change and turnover as a result of developmental control and external forces (Gertel and Green, 1977). The composition and physical properties of cell walls are therefore dependent on the stage of growth and the state of differentiation.

Cellulose microfibrils

The cellulose microfibril component is found in all cell walls of higher plants. Its function is to support the wall in the same way as glass fibre in glass-reinforced plastic and steel strands in reinforced concrete (Northcote, 1972). The microfibrils are not necessarily static, but may move over each other during growth and differentiation as a result of developmental or external influences. This is common during the construction of the primary cell walls which are laid down by undifferentiated cells undergoing extension. The ability of microfibrils to move gives the primary walls a plasticity which is necessary if extension is to occur. When the extension ends however, secondary walls are laid down producing a rigid wall with little

microfibril mobility. As a result, the plasticity is lost, but the wall retains a certain elasticity.

The chemical and physical structure of microfibrils is under constant review. It is known that areas of crystallinity and non-crystallinity exist, and that β(1-4) linked glucan chains are the major components.

Matrix polysaccharides

Matrix polysaccharides are present in cell walls during all stages of cell-wall biosynthesis. They consist of both linear and highly-branched polysaccharides and occupy much of the space between the microfibrils. The matrix polysaccharides differ in size, structure and composition throughout the plant kingdom. Such differences also occur within a single species, between different tissues and between primary and secondary cell walls.

In the past there have been attempts to classify matrix polysaccharides by virtue of their extraction properties or by their structure. As a result two main groups have been established - the pectins and the hemicelluloses.

The pectic polysaccharides are soluble in water (Talmadge *et al*, 1973; Ericson and Elbein, 1980), in solutions of Ca^{2+} chelating agents (Northcote, 1969; Ericson and Elbein, 1980) including EDTA, ammonium oxalate and sodium hexametaphosphate (Lamport, 1970) or in dilute acid (Talmadge *et al*, 1973).

The hemicelluloses are dissolved from the insoluble wall residue left after pectin extraction, by the use of strong aqueous alkali (Lamport, 1970). Differential precipitation of hemicellulosic polysaccharide from the alkali allows the identification of hemicelluloses A and B (Blake *et al*, 1971).

Classification by extraction has led to confusion as a result of the overlap in the extraction properties of molecules belonging to the two groups (Ericson and Elbein, 1980). The terms 'pectic' and 'hemicellulosic' describe the methods of extraction better than the polysaccharide fractions obtained (Lamport, 1970) as shown by Roberts and Loewus (Robert and Loewus, 1966) who extracted hemicellulose from sycamore maple cell walls. On analysis, the 'hemicellulose' was found to contain sugars usually only found in pectic polysaccharides, and was decidedly 'pectic' in composition.

As well as the problems they cause in classification, chemical extraction procedures can chemically modify cell-wall components. Dilute acid may hydrolyse the methyl ester linkages to uronic acids, and acetylester linkages to other sugars (Talmadge *et al*, 1973). It may also hydrolyse some glycosidic linkages, particularly those of arabinose, fucose and rhamnose (Davidson *et al*, 1967). Aqueous alkali used in hemicellulose extraction causes transelimination of uronyl residues and the β-elimination of any glycosidic bonds to serine or threonine residues that may be present (Newkom and Deuel, 1958).

To try and overcome some of these problems, the extraction of certain matrix polysaccharides (particularly the pectins) has been achieved by the use of hydrolytic enzymes, including pectinases (Talmadge *et al*, 1973). The use of this method brings other problems, for it relies on a high degree of purity and specificity of the enzymes used. Work of this kind, however, has been useful in establishing inter-polymeric linkages and has helped in developing an understanding of cell-wall structure (Albersheim, 1978).

A classification using polysaccharide structure has been compiled by Aspinall (Aspinall, 1980) using results from extensive studies on wood and cereal polysaccharides. The matrix polysaccharides fall into groups similar to those formed in the classification by extraction, but Aspinall maintains that structural features of polysaccharides are not sufficiently specific to groups of plants to provide a basis for chemical taxonomy. In spite of the problems associated with the above methods of classification, certain matrix polysaccharides are commonly described as pectins, and others as hemicelluloses.

The structures of the matrix polysaccharides (some of which are described below) have been elucidated by analysis of the extracted polysaccharides, and several analytical methods have become well established.

Commonly used is the highly efficient Hakomori methylation with gas-liquid-chromatography and mass spectrum

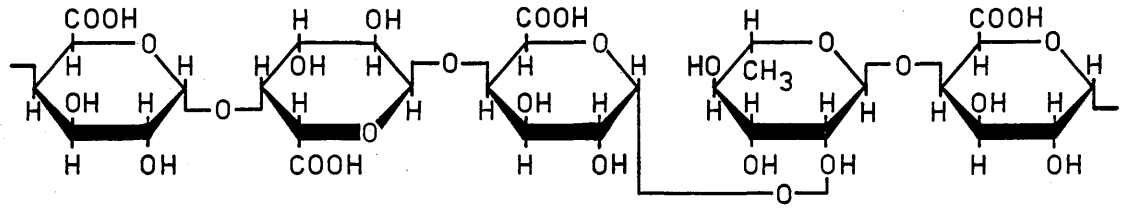
analysis for the separation and characterisation of methylated sugar derivatives (Hakomori, 1964). Another popular method involves the isolation of oligosaccharides after acid or enzymic hydrolysis, with degradation involving periodate oxidation. By using several methods in the analysis of a given polysaccharide, drawbacks in one method can often be overcome by the capabilities of another.

(a) The Pectins

The pectic polysaccharides are laid down during cell plate synthesis and form an integral part of the primary wall and middle lamella. Although pectins contain many closely-related polysaccharides, the major component is the galacturonorhamnan. This comprises a linear chain of α(1-4) linked D-galacturonic acid residues into which α-L-rhamnose is inserted at intervals (Fig. 1). Interpolymeric links between galacturonans usually take the form of ionic bridges between the carboxyl groups of D-galacturonic acid and Ca^{2+} ions. The removal of these Ca^{2+} ions by the use of chelating agents facilitates pectin extraction (Page 61), and the regulation of cell wall Ca^{2+} levels and pH may be important in the control of cell-wall plasticity. Other residues are often found in side chains. These include D-galactose, L-arabinose, D-xylose, L-fucose and D-glucuronic acid. The D-galactose and L-arabinose residues are found in multiple units attached to galacturonorhamnans, and in totally neutral fractions in the form of arabinans, galactans and arabinogalactans types I and II (Aspinall, 1980).

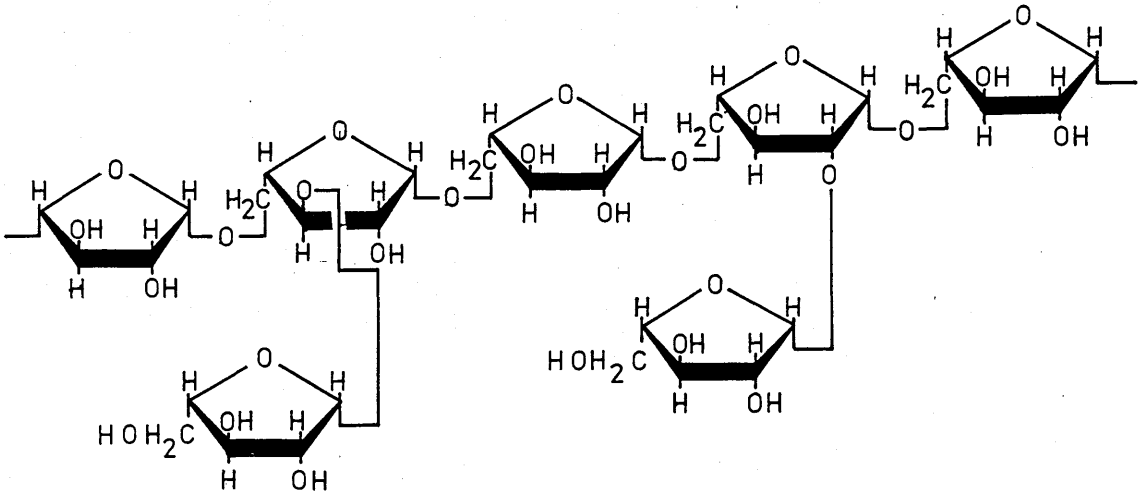
Fig. 1

(a) GALACTURONORHAMNAN



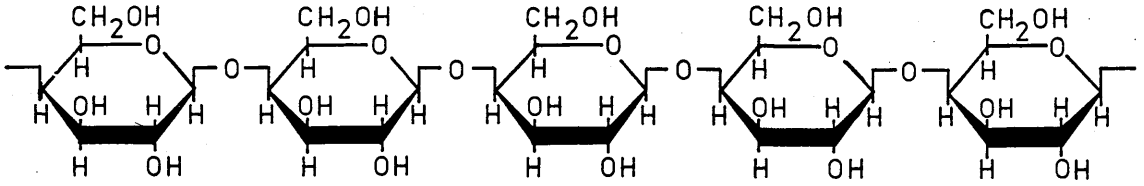
The backbone contains rhamnose and galacturonic acid residues, but the ratio of galacturonic acid to rhamnose is not clear. Other residues are found in small side chains.

(b) ARABINAN



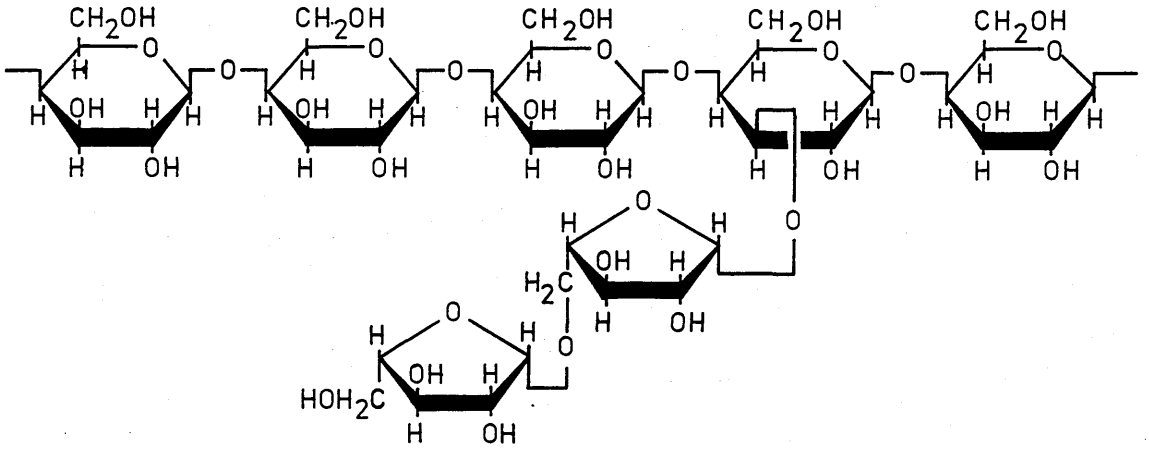
Pure arabinans contain α(1-5) linked arabinose residues in a linear backbone to which other arabinose residues are attached by α(1-3) and α(1-2) linkages.

(c) GALACTAN



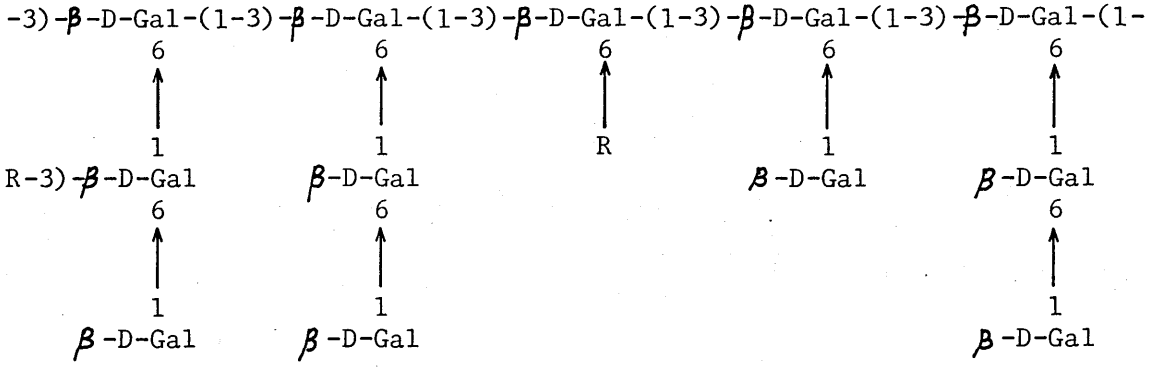
A β(1-4)-linked polygalactose chain

(d) ARABINOGALACTAN TYPE I



These polysaccharides consist of β(1-4)-linked galactose residues to which arabinose residues are attached in small side chains.

(e) ARABINOGALACTAN TYPE II



R= L-Ara-(1- or β-L-Ara-(1-3)-L-Ara-(1-

Highly-branched polysaccharides with ramified chains of galactose residues mutually joined by 1-3 and 1-6 linkages.

Arabinans have the general structure shown in Fig. 1. The α-arabinofuranose side chains are usually attached to the α(1-5) linked arabinofuranosyl backbone by 1-3 links. Some arabinans however contain side chains with α(1-2) linkages (Aspinall, 1980).

Pure galactans are of infrequent occurrence (Aspinall, 1980) and those that have been isolated in the past are probably arabinogalactans type I from which arabinofuranose side chains have been removed by acid hydrolysis. The structures of pure galactan and arabinogalactan type I are given in Fig. 1.

Unlike arabinogalactans of type I in which the galactan backbone is linear with β(1-4) linkages, those of type II have a branched structure with the galactan chain containing both β(1-3) and β(1-6) linkages (Fig. 1). The neutral pectic polysaccharides mentioned above may be constructed at sites away from the rhamnogalacturonan chain to which they are eventually attached (Stoddart and Northcote, 1967).

(b) The Hemicelluloses

The hemicelluloses consist mainly of D-glucose, D-glucuronic acid, D-galactose, D-galacturonic acid, D-mannose, D-xylose and D-arabinose found in different combinations and configurations by glycosidic linkages. There are three major groups:

(i) mannans, glucomannans and galactoglucomannans

(ii) xylans

(iii) xyloglucans.

For the purposes of this introduction, non-cellulosic β-D-glucans are classified separately.

(i) Mannans, glucomannans and galactoglucomannans

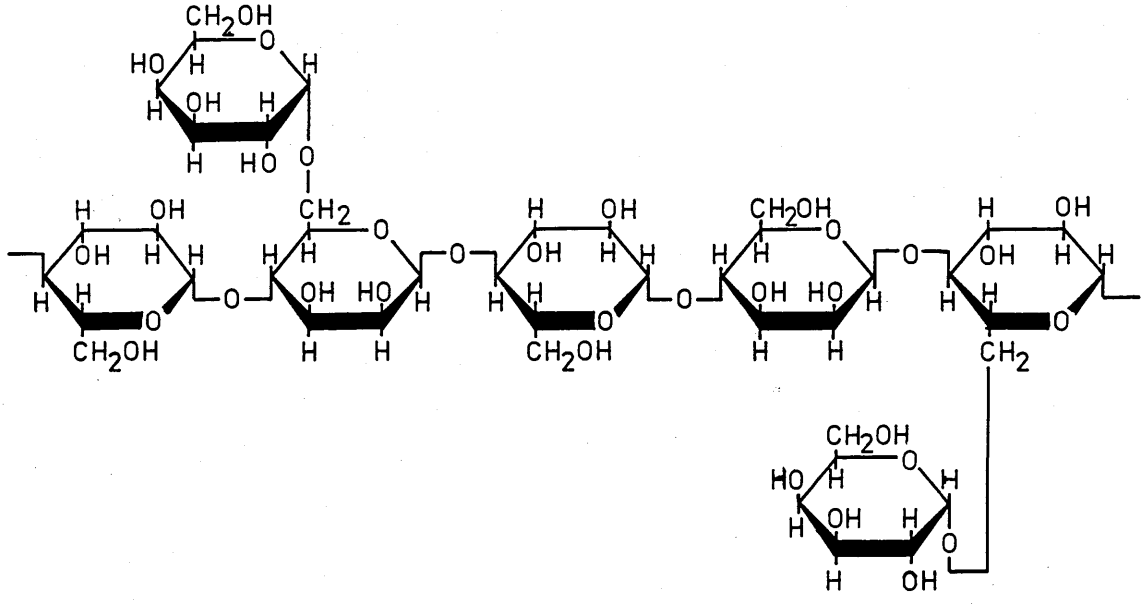
The majority of mannose-containing polysaccharides from the cell walls of higher plants are glucomannans and galactomannans. In gymnosperms, between 12 and 15% of the cell wall (dry weight) is composed of these polymers. In deciduous woods, however, only glucomannans are found and there, they account for only 3% or less of the wall (Timell, 1964 and 1965). The general structure is shown in Fig. 2; the α-D-galactose residues, if present, are attached to mannose and maybe to glucose in the main chain. The glucose and mannose residues appear to be distributed irregularly, although the ratio of glucose to mannose is 1:3 in gymnosperms and 1:2 in deciduous woods (Timell, 1964 and 1965). In coniferous woods, acetylation is also common (Aspinall, 1980).

(ii) Xylans

Between 20 and 30% of the dry weight of polysaccharide in angiosperms is composed of xylans. These polysaccharides are the most common matrix polysaccharides in this plant

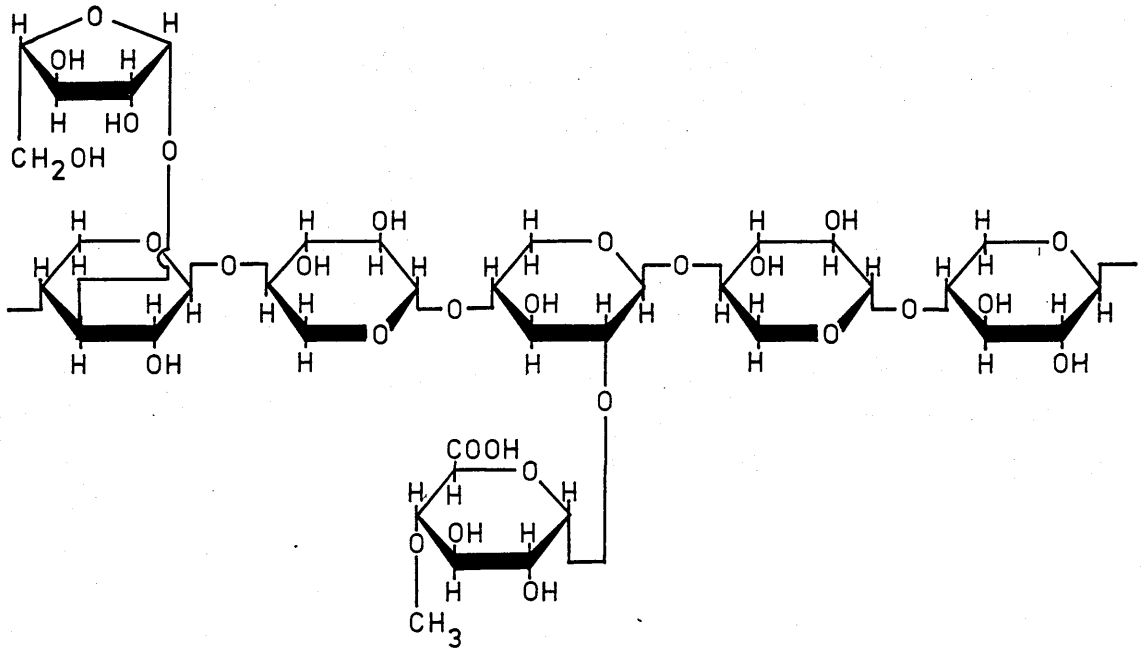
Fig. 2 GENERAL STRUCTURE OF HEMICELLULOSIC POLYSACCHARIDES

(a) GALACTOGLUCOMANNAN



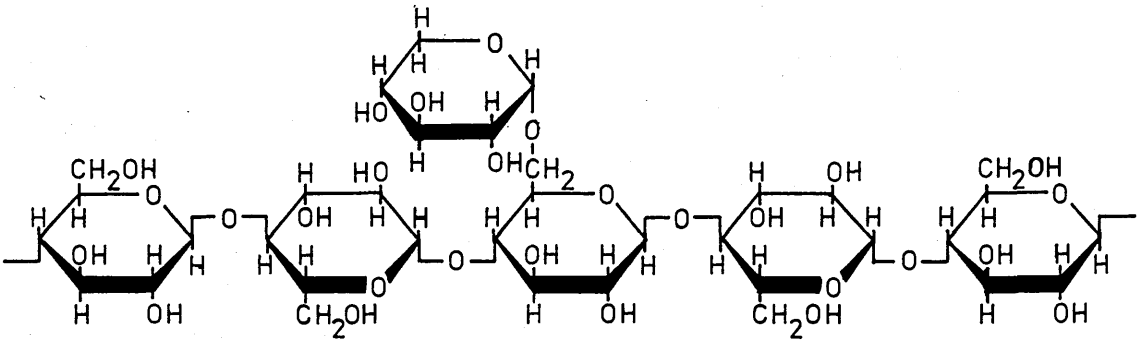
A β(1-4) linked backbone containing both mannose and glucose residues to which galactose residues are attached via α(1-6) residues.

(b) ARABINO-(4-O-methyl GLUCURONO)-XYLANS



A β(1-4)-linked xylan to which 4-O-methyl glucuronic acid and arabinose residues are attached. Xylose residues may be acetylated at C-2 and C-3.

(c) XYLOGLUCAN



A β(1-4)-linked glucan to which xylose residues are attached via α(1-6) links.

group, and are found predominantly in secondary cell walls, although they have also been identificd in the primary cell walls of suspension-cultured cells (Burke *et al*, 1974; Wada and Ray, 1978). Xylans normally consist of a linear β(1-4) linked xylan backbone with little branching, to which side chains are attached (Fig. 2). Table 1 gives a summary of the side chains found in xylans and illustrates their abundance in different plants.

Apart from the attachment of side chains, the xylan backbone may be modified by acetylation at C_2 and/or C_3, especially in glucuronoxylans (Bouveng, 1961a & b).

(iii) Xyloglucans

Xyloglucans are the predominant hemicelluloses in primary cell walls of dicotyledonous plants. They consist of a β(1-4) linked glucan backbone to which α-D-xylose residues are attached (Fig. 2). Although usually simple, the side chains can be complex (Albersheim, 1978) and can contain fucose and galactose as well as xylose.

(c) Noncellulosic β-D-glucans

The noncellulosic β-D-glucans are extracted with both pectic and hemicellulosic polysaccharides and comprise two main types:

(i) Callose

Table I

Sidechains in xylans (from Aspinall, 1980)

Sidechains	Source	Approximate proporations
α-L-Araf-(1-3)-	Gymnosperms	5-20%
	Dicots	Usually absent
	Monocots:	
	1^{ry} walls	30-40%
	2^{ry} walls	8-25%
4-O-Me-α-D-Glc A-(1-2)-	Gymnosperms	14-18%
	Hardwoods	8-12%
	Other dicots	Usually 10%
	Monocots	Less than 5%
α-D-Glc A-(1-2)-	Monocots	Approx. 5%
α-D-Xyl -(1-3)-L-Araf(1-	Dicots	Artifacts
β-D-Xyl -(1-2)-L-Araf(1-3)-	2^{ry} walls in monocots	Individually less than 5%.
β-D-Gal -(1-5)-L-Araf(1-		
β-D-Gal -(1-4)-D-Xyl (1-		

This is a linear β(1-3) linked D-glucan (Fig. 3) which occurs around sieve plates and on the sides of sieve plate tubes. It is also found in cotton hairs, pollen tubes and wound tissue.

(ii) β-D-glucans with mixed linkages.

These are linear β-D-glucans and in grasses are found with β(1-3) and β(1-4) linkages in the proportions 1:2 and 1:3. Unlike cellulose and callose they are usually soluble in aqueous solvents (Aspinall, 1980) although some can only be extracted using alkali. This, together with the results of experiments involving β-glucanase (Nevins *et al*, 1977), indicate that the glucans are linked covalently to other macromolecules in the wall (Aspinall, 1980).

Lignin

Lignin is an aromatic insoluble polymer of coumaryl, coniferyl and sinapyl alcohols produced by a process of dehydration (Freudenberg, 1968). It is found in primary and secondary cell walls and is abundant in schlerenchyma, xylem vessels and tracheids, making them strong and rigid. No one structural formula can describe lignin due to the complexity caused by the variable types of linkage. However, a general formula has been suggested by Freudenberg for the structure of a fragment of spruce lignin (Fig. 4).

Fig. 3

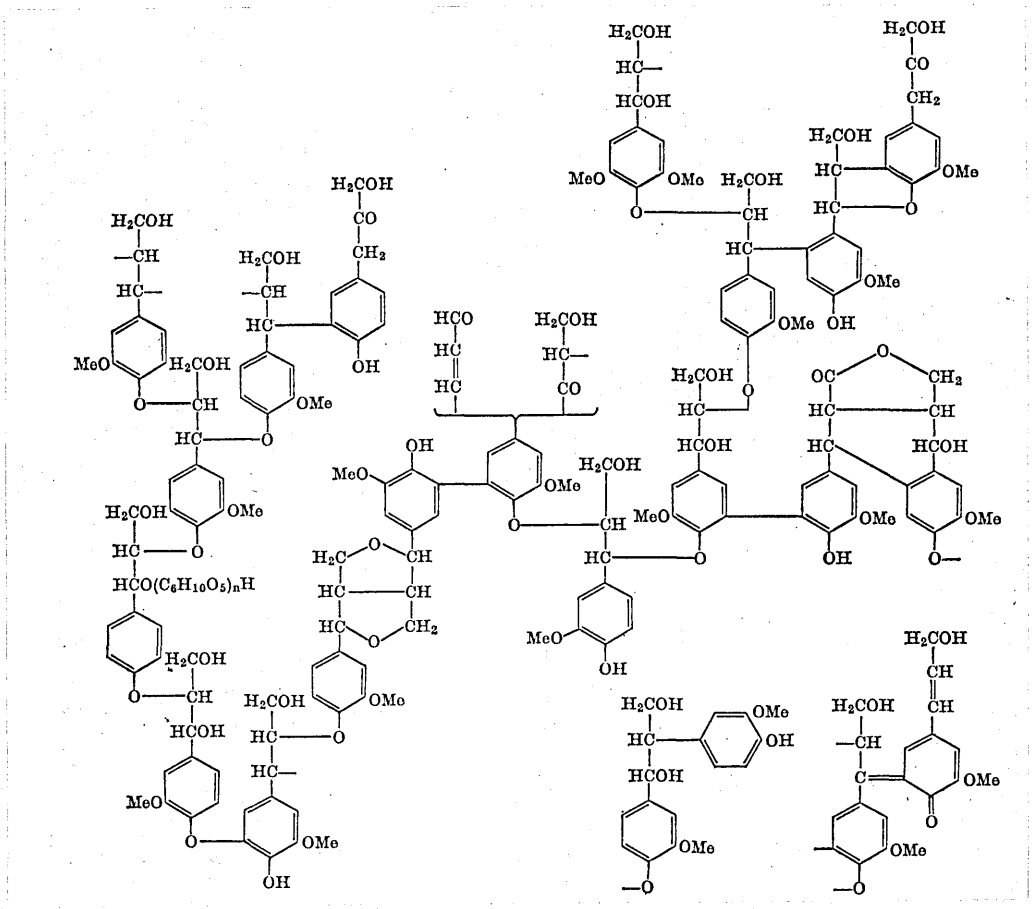
Callose (A β(1-3)-linked glucan)

-3)-β-D-Glc-(1-3)-β-D-Glc-(1-3)-β-D-Glc-(1-3)-β-D-Glc-(1-3)-β-D-Glc-(1

Fig. 4

Lignin (A general structure showing the different types of linkage present).

Taken from Northcote, 1969.



Protein

Although approximately 90% of the dry weight of plant-cell walls consists of polysaccharide and lignin, a substantial amount of protein is also present. The exact function of this protein is not clear. Most cell wall enzymes so far discovered are hydrolases (such as cellulase) and may be involved in cell-wall extension. Other protein may function in the recognition between plants and rhizobia (lectins). Certain proteins appear to be involved in cell wall structure (extensin), and it is possible that enzymes within the wall catalyse the synthesis of glycosidic bonds between polymers that have been synthesised either within the cell or at the plasma membrane. Cell wall protein is rich in hydroxyproline.

A Model of Cell Wall Structure

The following model of cell wall structure is based on the work of Albersheim and co-workers. The model applies to primary-cell walls of dicotyledonous plants and involves the main polysaccharides known to be present. Even though 10% of the cell wall consists of protein, that protein is not included in the model because, at the time, no covalent bonds had been found between protein and polysaccharide. However, recent work (Fry - unpublished work) suggests that extensin, a cell wall protein, may be involved in cell wall structure without such linkages.

The wall model summarised in Fig. 5 is simple, and is based on a cross-linked network of polysaccharides. Cellulose microfibrils are coated by a monolayer of xyloglucan which is, in turn, covalently attached to pectic rhamnogalacturonan via neutral arabinogalactan bridges. The authors, however, have recently emphasised that the model is over simplified and that the cell wall is a much more complex structure.

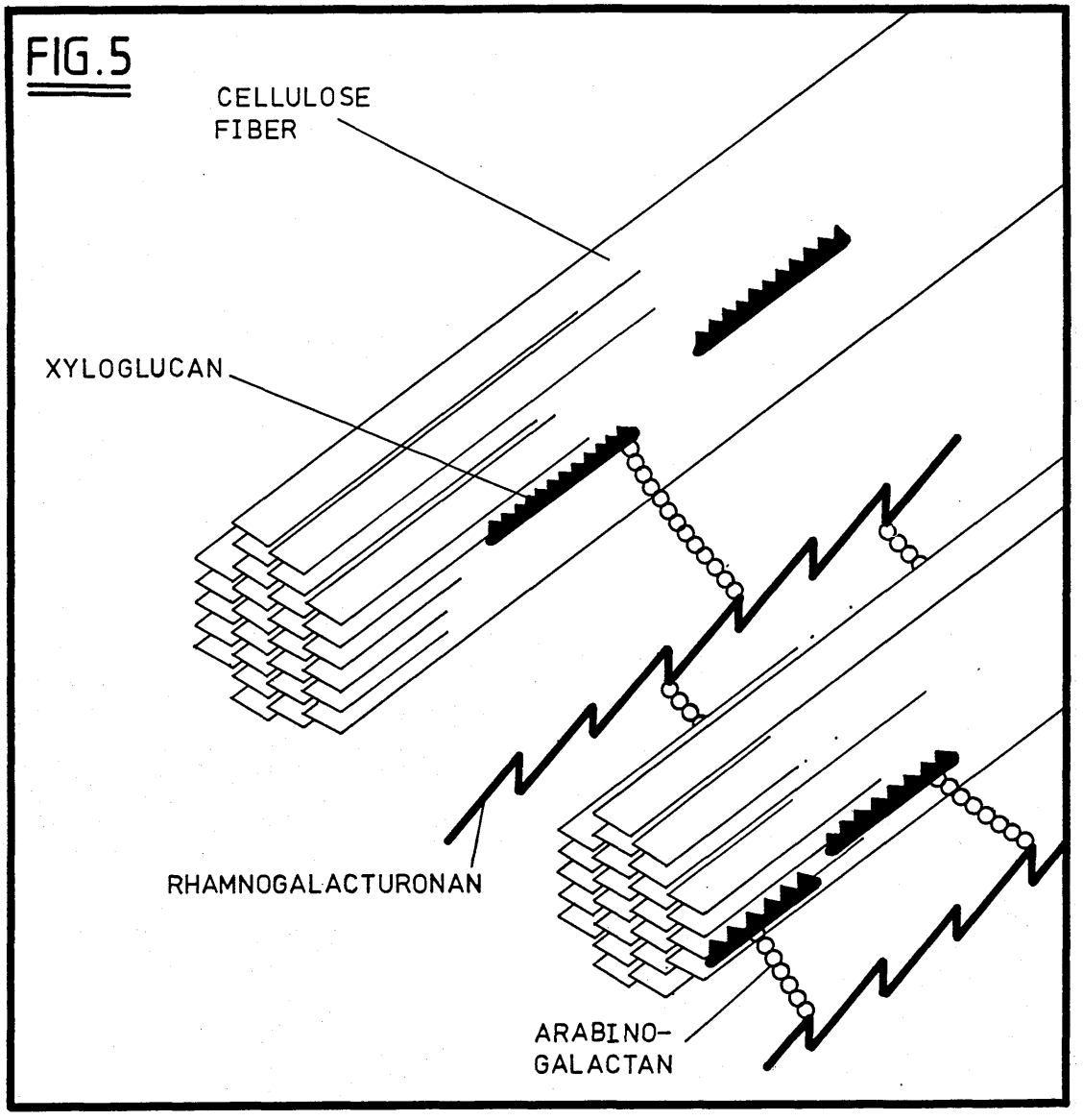


Fig. 5
Structural model of the primary-cell walls of dicotyledonous plants based on the work of Albersheim (1978).

The model gives an indication of the known linkages between polysaccharides and does not portray the relative proporations of the molecules.

II The Biosynthesis of Matrix (Non-cellulosic) Polysaccharides of the Cell Wall in Higher Plants.

The experimental procedure and problems associated with it.

Most biosynthetic experiments have utilised cell-free (*in vitro*) enzyme systems and involve three separate procedures:

(a) incubation of the enzyme system with radioactively-labelled possible substrates such as sugar nucleotides;

(b) fractionation of radioactively-labelled polymeric products using the classical pectin and hemicellulose extraction procedures;

(c) measurement of the incorporation of radioactivity into the polymeric products, followed by chemical analysis.

There are, however, a number of problems associated with this approach.

(a) *In vitro* enzyme systems are physically and chemically different from *in vivo* ones and cannot be expected to behave in the same way. Disruption of cell constituents during particulate enzyme preparation may alter the environment of the enzymes being studied. For example, concentrations of co-factors and co-enzymes normally required may be reduced or increased, and substrate availability may change.

(b) Pectin and hemicellulose solubilisation methods were

originally developed to extract polysaccharides from fully-formed cell walls in order to facilitate the chemical analysis of cell wall components. In higher plants, however, non-cellulosic cell-wall polysaccharides are synthesised within membrane-bound organelles of the cell and not within the cell wall. Consequently, a molecule that normally has hemicellulosic properties after insertion into the cell wall may be decidedly pectic in the *in vitro* system.

(c) The acceptor molecules to which radioactive sugars are transferred *in vitro* are often polymers. As a result, the size and chemical properties (such as extraction) of the radioactive product are likely to reflect those of the original acceptor, rather than those of the newly added sugars (Kauss, 1974).

(d) Most particulate-enzyme-preparations contain enzymes capable of interconverting sugar nucleotides (Feingold and Avigad, 1980). For instance, the incubation of UDP-D-[U-^{14}C]-glucuronic acid or UDP-D-[U-^{14}C]-xylose with some particulate enzyme systems can result in the incorporation of ^{14}C-arabinose into polysaccharides.

(e) One radioactively-labelled sugar nucleotide may be utilised by different enzyme systems within the same enzyme preparation, resulting in the synthesis of separate polysaccharides. UDP-D-[U-^{14}C]-glucuronic acid, for example, is used in the biosynthesis of both glucuronoxylans and glucuronogalactans (Kauss, 1974).

Problems (d) and (e) can be recognised by careful chemical analysis of the product. They may then be eliminated by choosing conditions under which the activity of the enzyme of interest is optimised, and the activity of unwanted enzymes is minimised. Problems (a), (b) and (c) are difficult to deal with and are often accepted as factors associated with *in vitro* investigations.

An early cell wall polysaccharide to be synthesised in a cell-free system was callose, a β(1-3) linked glucan (Feingold *et al*, 1958), and the biosynthesis of other polysaccharides was subsequently investigated in a similar manner. The remainder of section II describes briefly some of the research undertaken to elucidate the mechanism of cell wall-matrix polysaccharide biosynthesis.

Studies concerning the biosynthesis of pectins

As described in section I, pectins usually consist of a partially methylated β(1-4) linked polygalacturonic acid backbone to which neutral side chains are covalently attached.

The early biosynthetic studies concentrated on the polymerisation of the galacturonic acid component. Results from some groups (Feingold *et al*, 1964) prompted workers to experiment with the use of sugar nucleotides as possible substrates. Subsequently, UDP-D-galacturonic acid was shown to donate galacturonic acid to acidic pectin in mung beans

(Villemez *et al*, 1965; Villemez *et al*, 1966). Later investigations showed that TDP-D-galacturonic acid could also serve as a donor in tomatoes (Lin *et al*, 1966) indicating that galacturonic acid transferase is not necessarily specific for UDP-D-galacturonic acid . This discovery was followed by the isolation of TDP-galacturonic acid from sugar beet and the identification of enzymes capable of interconverting TDP-D-glucose and TDP-D-galactose within the same tissue.

Further investigations into galacturonan biosynthesis concentrated on the methylation of the galacturonic acid carboxyl groups. The methylation was originally thought to occur prior to polymerisation of the acidic backbone (Albersheim and Bonner, 1959), but Kauss and Hassid disproved this hypothesis (Kauss and Hassid, 1967(b); Kauss & Swanson 1969). Working with a particulate enzyme preparation from mung beans, they demonstrated that methylation occurred after polymerisation of the main chain, and used S-adenosyl methionine as the methyl donor. Other investigations demonstrated that only unmethylated NDP-D-galacturonic acid could be used in pectin biosynthesis, thereby supporting their conclusions. Like other enzymes involved in the biosynthesis of cell wall-matrix polysaccharides, those studied by Kauss and colleagues were membrane bound. Subsequent experiments suggested that pectin polymerisation and methylation occurred within a structured compartment, and this was eventually shown to be membranous.

The biosynthesis of the neutral pectin components is not as well understood as that of the galacturonic acid polymers. *In vitro* studies on neutral pectin biosynthesis have involved the incubation of radioactive sugar nucleotides with particulate enzyme preparations followed by product analysis. Some of the substrates used and the polymers produced are given in Table II. *In vivo* investigations have included pulse-chase experiments which have been used to examine schemes of pectin biosynthesis such as that outlined in Fig. 6 (Ericson and Elbein, 1980).

Studies concerning the biosynthesis of hemicellulose

The biosynthesis of hemicellulose *in vitro* has been studied by incubation of enzyme preparations with radioactive sugar nucleotides, followed by the analysis of the polysaccharide products.

(a) Xylans

Xylans are a predominant hemicellulose in angiosperms, and are found with or without side chains containing 4-O-methyl D-glucuronic acid, D-arabinose and D-xylose. Early research indicated that UDP-D-xylose could donate D-xylose to xylans in particulate enzyme preparations from corn cobs (Bailey and Hassid, 1966) and since then much work has concentrated on the synthesis of the various side chains. Arabinose side chains have been synthesised by a particulate enzyme preparation from *Zea mays* (Pridham and Hassid, 1966)

Table II

Nucleotide sugars used as substrates for the "in Vitro" synthesis of pectic substances (from Ericson and Elbein, 1980).

Substrate	Product	Enzyme source	References
UDP-apiose	Apiogalacturonan	Duckweed	Kindel (1973)
UDP-arabinose	Arabinan	Mung beans	Odzuck and Kauss (1972)
UDP-galactose	Galactan	Mung beans	Panayotatos and Villemez (1973)
UDP-xylose	Xylan	Mung beans	Odzuck and Kauss (1972)
UDP-galacturonic acic	Polygalacturonic acid	Mung beans	Villemez et al.(1965)
		Tomatoes	Lin et al. (1966)
TDP-galacturonic acid	Polygalacturonic acid	Tomatoes	Lin et al. (1966)
CDP-galacturonic acid	Polygalacturonic acid	Tomatoes	Lin et al. (1966)
S-adenosyl-methionine	Methyl esters of pectin	Mung beans	Kauss (1974)

Fig. 6

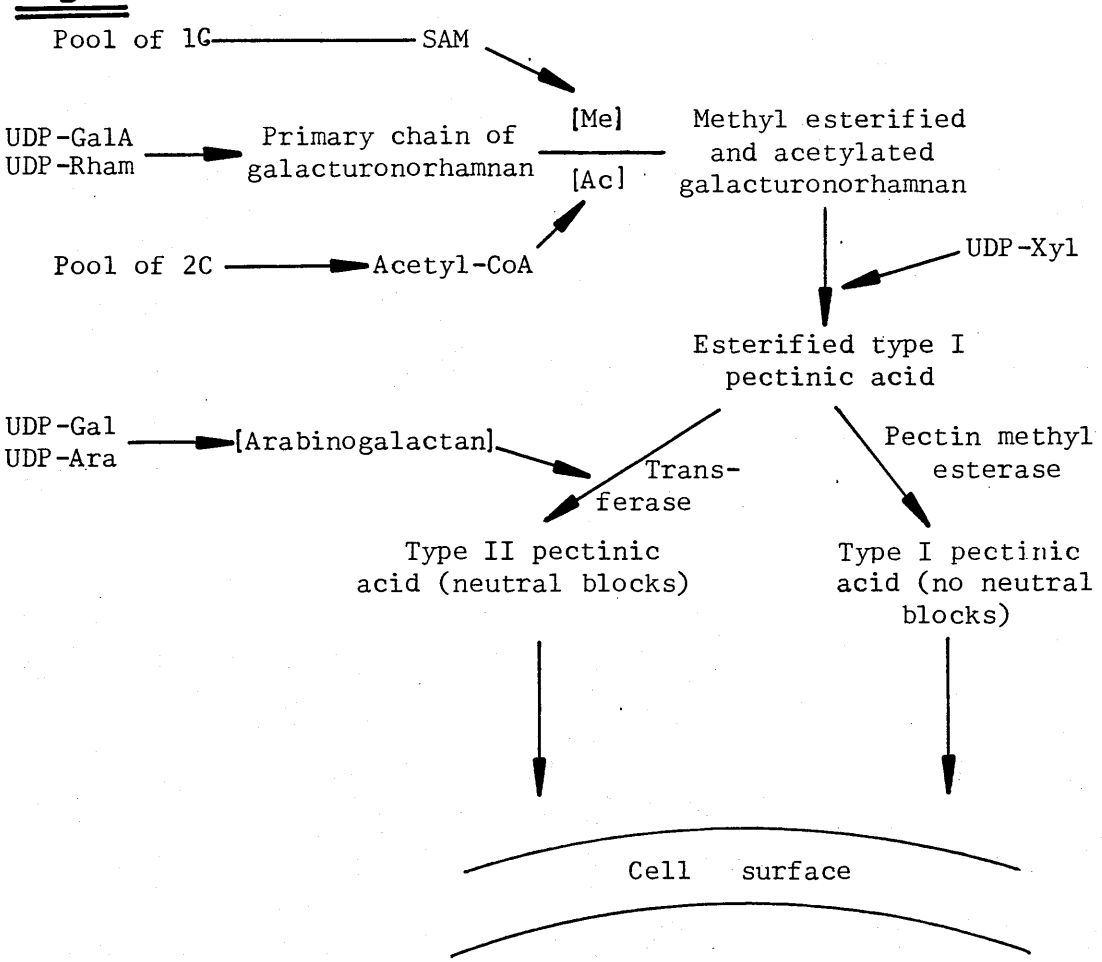


Fig. 6

Postulated series of reactions involved in the biosynthesis of certain pectins. (Taken from Ericson and Elbein, 1980).

using UDP-L-arabinose as a substrate, and glucuronic acid side chains can be synthesised from UDP-D-glucuronic acid in preparations from corn cobs (Kauss, 1967).

The methylation of glucuronic acid side-chains (Fig. 2) has been investigated in detail. In early experiments (Kauss and Hassid, 1967(a)) a particulate-enzyme preparation from immature corn cobs was shown to transfer methyl groups from S-adenosyl methionine to glucuronic acid units in hemicellulose B. The results showed that the methylation occurred at the macromolecular level at a pH optimum of 8 under the specific stimulation of $CoCl_2$ or $MnCl_2$.

(b) Xyloglucans

These constitute a large proportion of the hemicellulose in the primary walls of dicotyledonous plants (Valent and Albersheim, 1974; Wilder and Albersheim, 1973). Experiments by Ray (1980) recently showed that UDP-D-xylose and UDP-D-glucose will act as substrates for glucosyl and xylosyl transferases involved in xyloglucan biosynthesis. Both of the enzyme systems were also located on membranes of the golgi apparatus.

Interaction between the transferases was investigated in pea by Ray (1980) and in soy bean by Hayashi and Matsuda (1981). In pea, the UDP-D-xylose xylosyl transferase was stimulated by UDP-D-glucose and the results indicated that the UDP-D-glucose was used in synthesising the β(1-4) glucan backbone.

This, in turn, acted as an acceptor for xylose residues transferred by the xylosyl transferase.

Similar interaction of enzymes has also been shown to be involved in the synthesis of other polysaccharides, including glucomannans.

(c) Glucomannans

Observations that GDP-D-mannose stimulated the incorporation of glucose from GDP-D-glucose into an alkali-insoluble material (Elbein *et al*, 1964) in mung beans, prompted further research into glucomannan biosynthesis. Mannose from the stimulatory GDP-D-mannose was found to be incorporated into the same polymer as the glucose. Furthermore, the incorporation of mannose was inhibited by GDP-D-glucose (Elbein and Hassid, 1966; Elbein, 1969). Linkage analysis identified the product as a β(1-4) linked glucomannan.

Subsequent work included the solubilisation of both the glucosyl and mannosyl transferases in Triton X-100 (Heller and Villemez, 1972; Hinman and Villemez, 1975; Villemez, 1971) though purification of the solubilised enzymes was unsuccessful.

The involvement of lipid intermediates

It is generally accepted that lipid-bound sugar

intermediates are involved in the synthesis of bacterial cell wall polysaccharides. Their role is summarised in Fig. 7. In plants, however, it is not clear if such intermediates exist. Some particulate enzyme preparations can synthesise lipid-soluble materials (Elbein, 1969) but it seems likely that these are actually involved in glycoprotein biosynthesis (Ericson and Elbein, 1980).

Fig. 7

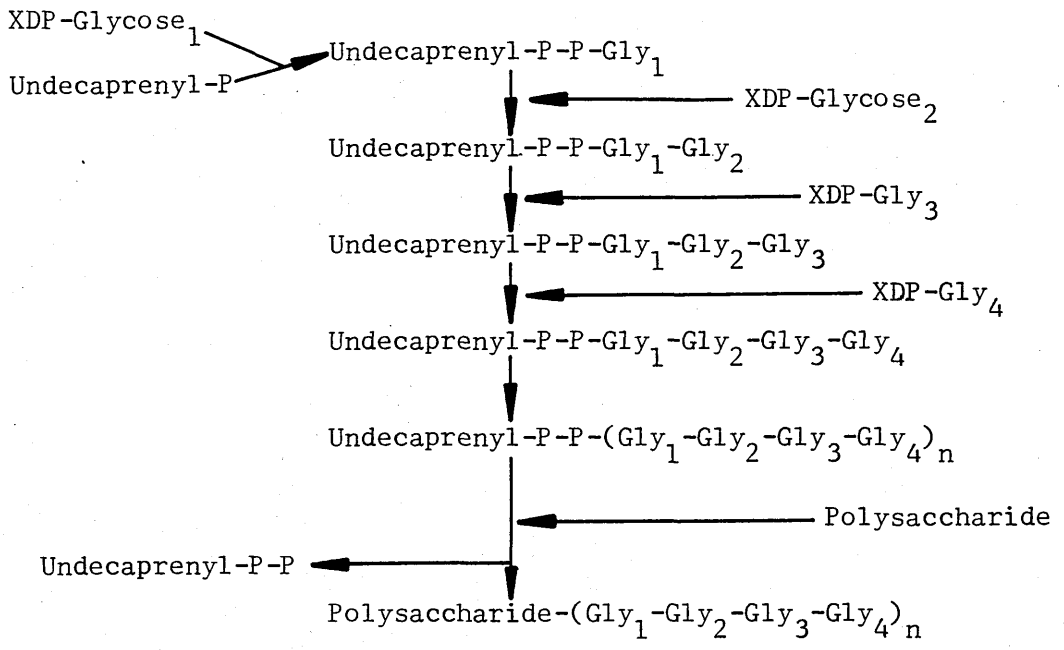


Fig. 7

General scheme of reactions showing the role of lipid intermediates in bacterial cell wall synthesis. (Taken from Ericson and Elbein, 1980).

III The Subcellular Localisation of Matrix-Polysaccharide Biosynthesis

Early observations made using electron microscopy indicated that golgi vesicles of root-cap cells contributed material to the cell wall (Mollenhauer *et al*, 1961), and prompted speculation that cell wall polysaccharide biosynthesis involved the golgi apparatus. Initial attempts to confirm this involved separation of organelle by centrifugation, but were unsuccessful (Lamport, 1970) due to massive disruption of subcellular particles during the homogenisation procedures.

The destruction of organelles had resulted in the synthetase activity of interest being associated with organelle fragments with a wide range of sedimentation coefficients. As a result, subsequent experiments involved more gentle homogenisation methods, such as chopping with razor blades (Villemez *et al*, 1968). This resulted in more efficient fractionation of organelles and, initially, it was suggested that the plasma membrane was the likely site of polysaccharide synthesis (Villemez *et al*, 1968). Certain pulse-chase experiments indicated otherwise (Northcote and Pickett-Heaps, 1966). In these investigations, a pulse of radioactively-labelled sugar was chased through a tissue with non-labelled sugar. Sections of tissue were taken at different times during the procedure, and analysed by autoradiography. The results showed that radioactivity from ^{3}H-D-glucose rapidly

associated with the golgi body, and then moved to the cell wall within 30 min (Northcote and Pickett-Heaps, 1966). Isolation and chemical analysis of the now radioactively-labelled wall revealed the presence of ^{3}H-D-galactose, ^{3}H-L-arabinose and ^{3}H-D-galacturonic acid residues, all of which are present in pectins. Similar studies with xylem and phloem tissue were also carried out and indicated that xylans are deposited into the cell wall from the golgi apparatus (Wooding, 1968; Northcote, 1969; Pickett-Heaps, 1966).

As a result of such early work, the golgi apparatus was generally recognised as a likely site of cell wall polysaccharide biosynthesis. The experiments above, however, could not conclusively prove the theory. To do this, the organelle involved in synthesis would have to be purified and identified. Consequently, methods of separating organelles have been sought, and a most useful technique involves the use of sucrose density-gradient ultracentrifugation. Examples of such work include that of Ray and co-workers (Ray *et al*, 1976; Ray, 1980) who have shown that the two enzyme systems responsible for xyloglucan biosynthesis are present in the golgi apparatus.

The principle of plant-cell-fractionation by sucrose density-gradient ultracentrifugation

The theory behind this technique is straightforward. A

particulate enzyme preparation is laid over a concentration gradient of sucrose dissolved in the appropriate buffer. Ultracentrifugation induces the organelles to sediment and, initially, they become separated as a result of differences in their sedimentation coefficients (rate-zonal centrifugation). If centrifugation is continued, the organelles move to regions of the gradient where the density of the medium equals their own. At this point they stop moving, now separated by virtue of their differing densities (isopycnic centrifugation).

Although simple in theory, the separation of organelles by density gradient centrifugation presents several problems.

(a) Organelle disruption during homogenisation

Animal cells are easily ruptured by gentle homogenisation, but plant cells require more severe treatment due to the protection afforded by their cell walls. Unfortunately, the shearing forces required to break these walls are often capable of severely damaging organelles within. Trials using different homogenisation techniques have indicated that the most gentle method involves chopping the tissue with razor blades (Honda, 1955). Less gentle methods may be employed only if the organelle under investigation is particularly strong (e.g. mitochondria).

(b) Organelle disruption after homogenisation

As soon as a plant cell has been ruptured, organelles from the cytoplasm enter a new environment composed of cell contents and homogenisation medium. Having survived the shearing forces of tissue homogenisation, many organelles may burst as a result of osmotically-induced swelling. This can be overcome by including an osmoticum such as sucrose, mannitol or sorbitol in the homogenisation medium. A more complicated series of problems arises from the liberation of denaturing enzymes and toxic molecules, which were originally compartmentalised within the cell.

Two classes of organelle denaturing enzymes exist, those which degrade lipids and those which denature proteins. The two groups of enzymes responsible for the majority of lipid degradation are the lipolytic enzymes and the lipoxygenases (Galliard,1955).

The lipolytic enzymes consist of two types. The first is phospholipase D which hydrolyses phospholipids to phosphatidic acid. The second encompasses the lipolytic acyl hydrolases which liberate free fatty acids.

The lipoxygenases catalyse the formation of hydroperoxides from unsaturated fatty acids. Linoleic and linolenic acids (the major fatty acids in plants) are the best natural substrates for such lipoxygenase activity. Although some damage to membranes is caused directly by the lipid degrading

enzymes, the majority is due to the effects of fatty acids released by their activity. By associating with the phospholipids within membranes, fatty acids can cause the general disruption of membranes, and can increase the availability of lipid acyl ester bonds to lipolytic acyl hydrolases. They can also act as substrates for lipoxygenase enzymes, resulting in increased lipoxygenase activity.

Any proteases released during homogenisation may degrade enzymes which are to be studied. They may also cause significant damage to organelles in which protein is structurally important. Examples of such organelles include mitochondria and chloroplasts.

Certain molecules are toxic to enzymes because of their ability to react with proteins. The most troublesome toxins are the phenols and quinones. Phenols have the ability to form hydrogen bonds with proteins. The binding is so strong that dialysis and gel-filtration will not induce separation, and is essentially irreversible. Quinones are formed by oxidation of phenols by polyphenol oxidase and can condense readily with reactive groups of proteins, including sulphydryl and amino groups. Both phenols and quinones can interact ionically and hydrophobically with proteins, again causing inhibition. Other toxic molecules include polyionic pectins, terpenes, resin acids and carotenoid pigments (Loomis, 1955).

Methods employed to minimise damage to organelles

(a) Homogenisation technique, and rapid application to the gradient.

Gentle homogenisation in the presence of an osmoticum is most effective in preventing the mechanical rupture of membranes. This in turn reduces the quantity of harmful toxins and enzymes released from liposomes and peroxisomes. The immediate application of the homogenate to a density gradient followed by centrifugation is also beneficial, for the sooner organelles enter the gradient, the sooner they will be separated from those toxins and enzymes which are free in solution.

(b) The regulation of the characteristics and constituents of the homogenisation and gradient media

(i) Bovine serum albumin (BSA). At a concentration of between 0.1 and 1% (w/v) BSA will bind to free fatty acids and will prevent them from damaging membranes. It will also dilute the native proteins in the organelles and will reduce the risk of damage by proteases and toxins.

(ii) Chelating agents. Certain heavy metal ions can be toxic to enzymes and can be removed by chelating agents, including ethylene diamine tetraacetic acid (EDTA). Such molecules are also included in gradient media to remove calcium. As a result, calcium-dependent phospholipases are

inhibited.

(iii) Antioxidants. The presence of reducing agents such as dithiothreitol (DTT) and mercaptoethanol can reduce the activity of some enzymes including polyphenol oxidase.

(iv) Binding agents. Certain compcunds may stabilise membranes, for example polyvinylpolypyrolidone (PVP).

(v) pH. High values of pH can reduce the binding of phenolics to proteins, and can lower the activity of some phospholipases.

Identificatien of organelles within a density gradient

Organelles within a gradient fraction are identified by assaying that fraction for the presence of features or markers that are characteristic to those organelles (Quail, 1979). Some markers are morphological while others are biochemical, some are endogenous to an organelle while others may be imposed (Table III). The greater the specificity and the larger the number of markers used in identifying an organelle, the higher the degree of accuracy in that identification.

Morphological analysis usually involves electron microscopy. Although it is useful in complementing biochemical analysis, this method is not quantitatively accurate and

TABLE III

Properties of Organelles

Organelle	Density ($g\ cm^{-3}$)	Equivalent Sucrose Concentration % (w/w)	Markers
Nuclei	1.32	66	DNA
Mitochondria (intact)	1.18-1.2	41-45	cytochrome-c-oxidase
Chloroplasts (intact)	1.21-1.24	46-52	Chlorophyll
Eticplasts (intact)	1.26	56	Carotenoids
Glyoxisomes (intact)	1.25	53	Catalase
Peroxisomes (intact)	1.25	53	Catalase
Smooth Endoplasmic Reticulum	1.11-1.12	26-29	NAD(P)H-cytochrome-c-reductase
Rough Endoplasmic Reticulum	1.15-1.18	35-41	16S and 26S RNA
Golgi	1.12-1.15	29-35	Latent IDPase. Glucan synthetase I
Plasma Membrane	1.13-1.18	30-40	Glucan synthetase II
Vacuole (intact)	1.0-1.2		Phosphodiesterase

cannot be used as a substitute (Quail, 1979). Examples of morphological markers are given in Table III.

Commonly used biochemical markers include enzyme activities. If a certain enzyme activity is specific to an organelle, then the organelle can be identified by assaying for the presence of that enzyme. This method is often complicated by the existance of isoenzymes in different organelles, and the release of enzymes from organelles during ultracentrifugation. Even so, a 'dictionary' of marker enzymes has been compiled and is being added to constantly. Examples of such markers are given in Table III.

Another method of identification involves the use of organelle density. This method is not as accurate as that which employs markers, but can be useful. Inaccuracies may arise if the membranes of an organelle have a different density to that of the intact organelle.

IV The Use of Detergents in the Solubilisation of Membrane-Bound Proteins

Biological membranes consist of phospholipid bilayers in which proteins, glycoproteins, glycolipids and steroids may be embedded. The relative proportions of these constituents depend to a great extent on the metabolic activity of the membrane. For instance in mitochondrial inner membranes or chloroplast lamellae, both of which have much metabolic activity, there is a very high level of protein (approximately 60% of the dry weight of the membrane). However, in myelin sheath membranes which act as insulators around nerve axons, phospholipids are the main component.

The most suitable description of bicmembrane structure that accounts for both the specificity and diversity of membrane components, is given in the Fluid-Mosaic Model (Fig. 8). The dynamic or fluid properties of the membrane are explained in terms of a phospholipid bilayer, the mosaic characteristics being due to the protein element.

The membrane proteins, as described by this model, can be divided into two classes - the extrinsic and the intrinsic proteins. In both cases the proteins are globular, like water-soluble plasma proteins. This is in contrast to the description of membrane proteins in the original Danielli-Davson model of bicmembranes.

Fig.8
Fluid Mosaic Model

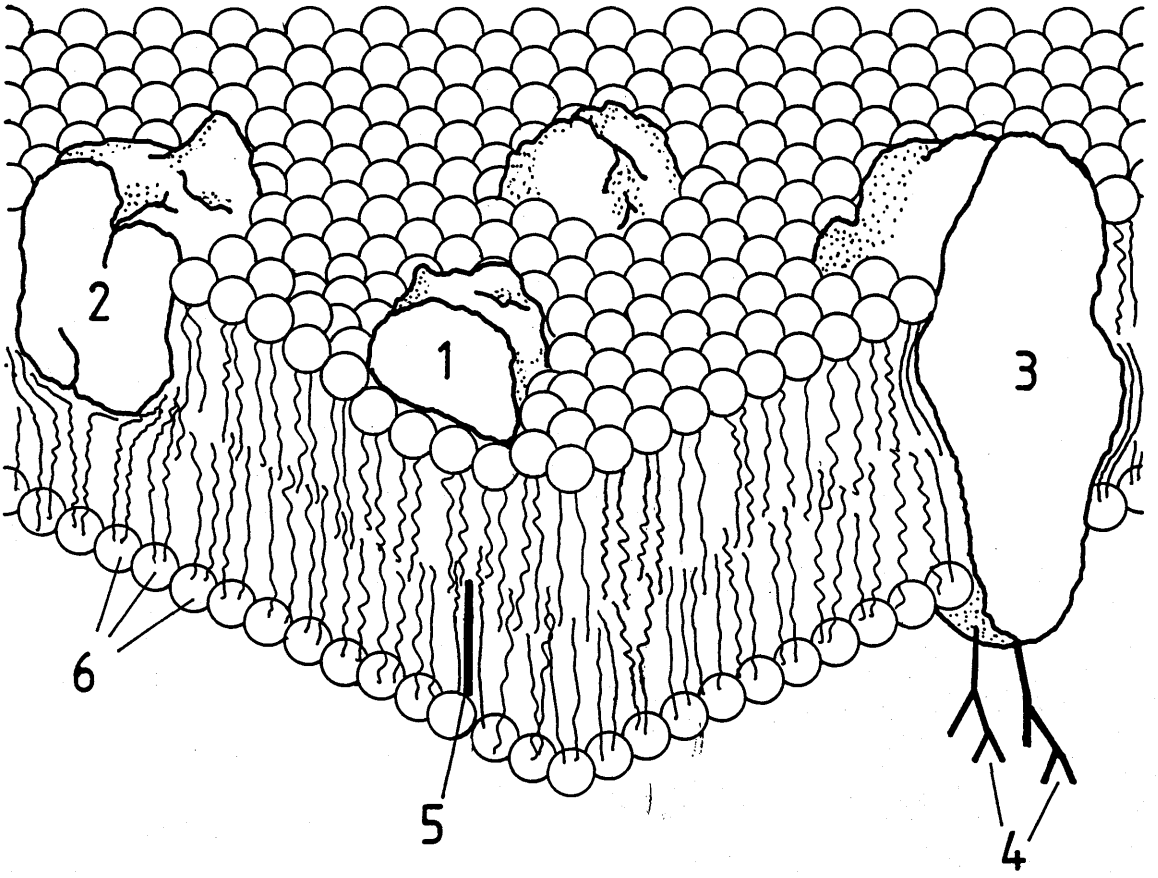


Fig. 8

Fluid-mosaic model of biological membranes.

A model showing the way in which membrane-bound proteins are attached to the lipid components of the membrane. Note that the lower surface would represent the inside of the golgi apparatus or endoplasmic reticulum, and the outer surface of the plasmalemma.

1): Extrinsically bound protein.

2): Intrinsically bound protein .

3): Intrinsically bound glycoprotein passing right through the membrane.

4): Carbohydrate component of the glycoprotein (3).

5): Position of steroid molecules.

6): Lipidic component of the bilayer.

Extrinsic proteins interact electrostatically with the charged phosphate groups of the membrane surface. As a result, they can be easily removed by high salt concentrations (as in the case of cytochrome-C) and subsequently purified using normal methods applicable to water-soluble proteins.

Intrinsic proteins interact hydrophobically with the lipid molecules of the membrane, with only their hydrophobically-coated regions emerging into the aqueous environment. The interaction between protein and lipid may be specific, and often deforms the orderly arrangement of adjacent alkyl chains. Some intrinsic proteins can span the membrane whereas others emerge only from one surface, depending on their function.

The insolubility of intrinsic-membrane proteins in water makes their purification difficult, because all methods of protein purification require solubility in an aqueous environment. Extraction of membrane lipids by conventional means, such as the use of organic solvents, usually results in the irreversible aggregation and denaturation of proteins, and is not a useful procedure. However, additives which prevent direct interaction between water and the hydrophobic surface of intrinsic proteins, while allowing solubility, can be employed. Such additives are often called detergents.

Detergents

Detergents are molecules which have the ability to

solubilise normally insoluble molecules in water. They are commonly used in solubilising intrinsic membrane-bound proteins to permit purification and analysis. Two main classes exist:

(a) Ionic detergents

Ionic detergents contain one or more charged groups as well as hydrophobic regions and can be cationic (positively charged), anionic (negatively charged) or zwitterionic (containing both positively and negatively charged groups). Most are denaturants at the concentrations and temperatures used in membrane solubilisation and examples include S.D.S. (sodium dodecyl sulphate) (Fig. 9) and alkyl sulphonates.

(b) Non-ionic detergents

Non-ionic detergents do not contain charged groups (although polar groups may be present) and do not have such a denaturing effect on proteins. This allows solubilisation and characterisation of enzymes in the native state. Because such detergents can break lipid-lipid and lipid-protein interactions, but not protein-protein interactions, they are useful in studying any subunit structure, although artifacts may arise as a result of artificial aggregation. A commonly used example of a non-ionic detergent is Triton X-100 (TX-100) which has been used extensively in this project (Fig. 9).

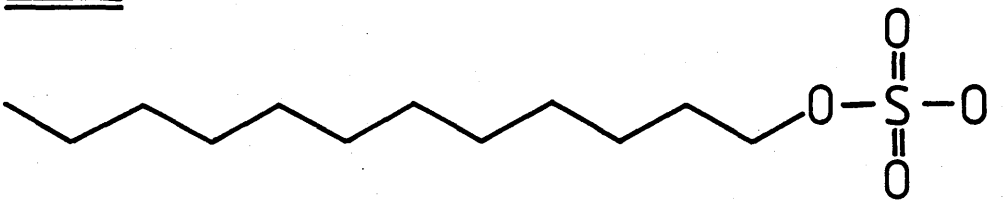
Fig. 9

The chemical structures of S.D.S. (a), and Triton X-100 (b).

Fig. 9(c).

A temperature-concentration phase diagram of S.D.S. in NaCl (0.1 mol. dm^{-3}), $NaPO_4$ (0.05 mol.dm^{-3}) pH 7.4. (Taken from Becker et al. 1975)

Fig 9 (a) Sodium dodecyl sulphate



(b) Triton X-100

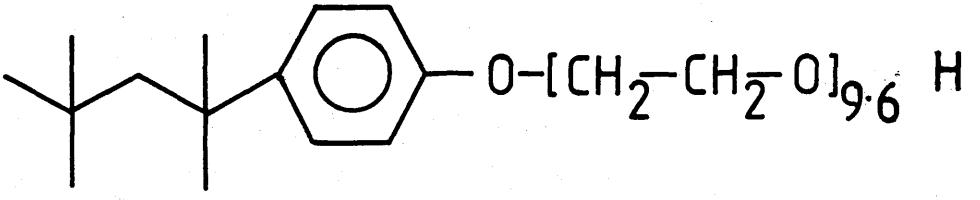
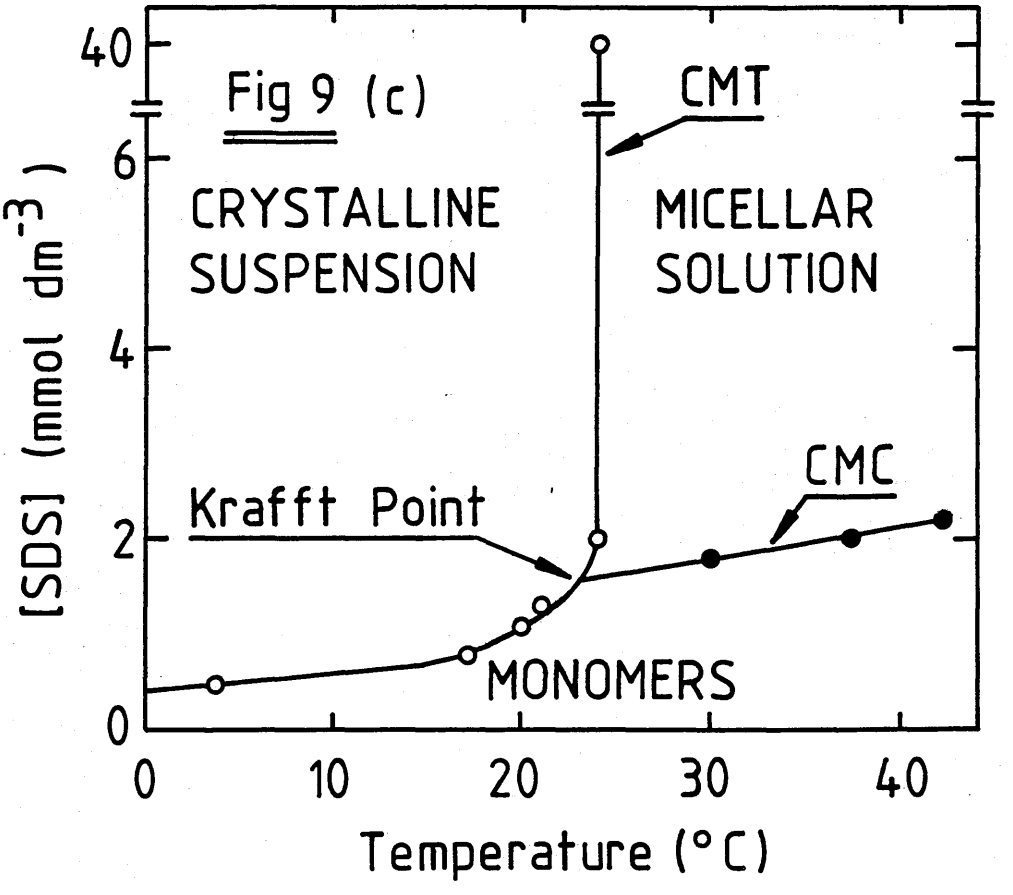


Fig 9 (c)



Selection of a detergent

There are many different detergents available to biochemists for use in membrane-bound-protein solubilisation, but there are no real criteria available upon which to base a selection. However, certain properties are often desired if purification is to be carried out after the initial solubilisation.

(a) The critical micelle concentration (CMC)

At low concentrations in aqueous solution, detergents (which consist of a single alkyl chain joined to a polar head group) are present as monomers in solution and are in equilibrium with monomers forming a monolayer at the air/water interface. If the detergent concentration increases above a certain level known as the Critical Micelle Concentration (CMC), and the temperature is above a value known as the Critical Micelle Temperature (CMT) then added molecules associate to form elipsoidal micelles (Fig. 9). These micelles are defined as thermodynamically stable colloidal aggregates, spontaneously formed by amphiphiles above a narrow concentration range (CMC) at temperatures above the critical micelle temperature (CMT). The effects of temperature and concentration are illustrated in Fig. 9.

If rapid removal or displacement of detergent is desired, then a high CMC is required. However, if the binding of

detergent to protein is to be carried out, then a low CMC is needed so as to minimise the ratio of free detergent to bound.

(b) Micelle size

The size of micelles is important if protein purification is to be achieved. A small micelle size is more likely to result in one molecule of protein per micelle of detergent, thus giving a greater potential for the separation of solubilised proteins.

(c) Effect on enzyme activity

If a detergent totally inactivates an enzyme, then it is unsuitable for solubilising that enzyme if the biochemical characteristics of that enzyme are to be studied. The exact effect of a detergent on enzyme activity differs from enzyme to enzyme and can only be established by trial and error. Some detergents will, however, be more gentle than others as discussed earlier.

In the present research programme, the use of a detergent was required to solubilise a membrane-bound protein. Of the several types available Triton X-100 was found to be the most suitable as described in Chapter 5.

V Introduction to Experimental Work

Glucuronic acid is an important constituent of hemicellulose in the cell walls of most higher plants. It is most commonly found in the form of 4-O-methyl glucuronic acid side chains linked to the xylan backbone of glucuronoxylans.

In spite of the importance of glucuronoxylans, little work has been carried out to elucidate their mechanism of biosynthesis. Synthesis of the xylan chain has been studied in immature corn (*Zea mays*) cobs by Bailey and Hassid (1966) and in woody dicots (sycamore (*Acer pseudoplatanus*) and poplar (*Populus robusta*)) by Dalessandro and Northcote (1981). However, the only analysis of the incorporation of glucuronic acid into hemicelluloses is the work of Kauss (1967) and Kauss and Hassid (1967a) on immature corn cobs. They showed that UDP-D-glucuronic acid could be used as the donor of glucuronic acid, and that methylation from S-adenosylmethionine occurred at the macromolecular level. In this work, only a relatively small amount of the glucuronic acid was shown to be linked to xylose, and the evidence indicated that the majority was linked to galactose. In dicotyledonous plants, glucuronic acid has been shown to be incorporated from UDP-D-glucuronic acid into polysaccharide in a few cases (Villemez *et al*, 1968; Villemez and Clark, 1969) but no detailed analysis of the incorporation into glucuronoxylan has been carried out.

Since glucuronic acid is present in polysaccharides other than glucuronoxylan in some tissues (Northcote, 1969), and since it might also be present in glycoproteins, incorporation of glucuronic acid into a general 'hemicellulose' fraction may not involve glucuronoxylan synthesis. It is necessary to provide more definite evidence that glucuronic acid has indeed been incorporated into glucuronoxylan. This evidence may be provided by a structural analysis of the product. Additional evidence may be supplied by showing an interaction between the two sugar-nucleotide substrates needed for glucuronoxylan synthesis, UDP-D-glucuronic acid and UDP-D-xylose. This second approach is also a step in the direction of the complete synthesis of the glucuronoxylan molecule *in vitro*. A similar approach has been adopted in studies on the synthesis of xyloglucan (Ray, 1980) and glucomannan (Heller and Villemez, 1972a&b)

The present thesis reports the results of experiments *in vitro* concerning the incorporation of glucuronic acid from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose by particulate and detergent-solubilised enzyme preparations from dark-grown pea seedlings. Structural analysis indicates that the product is glucuronoxylan, and this conclusion is supported by the observation that incorporation from UDP-D-[U-^{14}C]-glucuronic acid is stimulated by UDP-D-xylose.

Chapter 2.

MATERIALS AND METHODS

Chemicals and Sundries

UDP-D-[U-^{14}C]-glucuronic acid (925 KBq cm^{-3}, 9.77GBq mmol^{-1}) and UDP-D-[U-^{14}C]-glucose (925 KBq cm^{-3}, 10.8 GBq mmol^{-1}) were purchased from Amersham International, White Lion Road, Amersham, Buckinghamshire, England HP7 9LL. Non-radioactively labelled sugar nucleotides and larch xylan were purchased from Sigma. These chemicals, and all others, were of analar or laboratory grades, and glass distilled water was used for all solutions.

Except for Triton X-100, (which was obtained from Koch-Light Laboratories Ltd, Colnbrook, England) all detergents used were kindly given by Dr. R.J. Cogdell of this department.

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

Plant Material

Peas (*Pisum sativum* var. Alaska) were supplied by Sinclair McGill (Ayr, Scotland) and were soaked for between 6 and 8h (hours) in tap water. If fungal contamination was a current problem, then the peas were soaked initially in 10% (v/v) NaOCl for 15min (minutes) and then rinsed 10 times in tap water before being left to swell. The peas were subsequently planted in trays (58 x 30 cm) which contained moist tissue

paper or vermiculite, and were grown in darkness at 22°C for approximately 7 days.

Homogenisation of Plant Tissue

Scissors were used to harvest pea epicotyls and to remove the hook and leaves above. A weighed quantity of epicotyl tissue was then homogenised in a specific volume of a suitable buffer in one of three ways:

(i) by using a polytron kinematica homogeniser at setting 5 for 10 seconds;

(ii) by chopping with a razor blade for 5 or 10 minutes at 4 strokes per second in a petri dish on ice;

(iii) by grinding for 5 minutes in an ice-cold mortar with a pestle.

The homogenate was expressed through 4 crossed layers of muslin and the filtrate was used in making the enzyme preparation. All solid material retained by the muslin was discarded.

Enzyme Preparation

(i) Particulate enzyme preparation for general use.

This method was employed to produce the particulate enzyme that was used to identify and characterise glucuronyltransferase activity (Ch. 3). Pea epicotyls (25 g)

were homogenised using a polytron homogeniser in 100 cm^3 buffer containing Tris - HCl (10 mmol dm^{-3}), pH 7.5 (Tris base at 10 mmol dm^{-3} brought to pH 7.5 with HCl). The muslin filtrate was centrifuged at 97,000 x g (average) for 30 min and the three resulting enzyme pellets were each resuspended in 0.5 cm^3 Tris-HCl (50 mmol dm^{-3}), pH 7.5.

(ii) Particulate enzyme preparation and enzyme solubilisation as referred to in Ch. 5.

Pea epicotyls (between 60 and 100 g) were homogenised in approximately 200 cm^3 buffer containing Tris-HCl (10 mmol dm^{-3}) and Mes (10 mmol dm^{-3}), pH 6.0 or Tris-HCl (10 mmol dm^{-3}), pH 7.5. The muslin filtrate was centrifuged at 97,000 x g (average) for 30 min and the membrane pellets were then resuspended in fresh homogenisation buffer to bring the final volume to 25 mm^3 per g of epicotyl tissue.

To produce particulate enzyme, the resuspended pellets were diluted with an equal volume of homogenisation buffer.

To achieve solubilisation in Triton X-100 (a non-ionic detergent), the resuspended pellets were diluted with an equal volume of homogenisation medium containing Triton X-100 at 20% (w/w). After mixing thoroughly with a glass homogeniser, the enzyme-Triton X-100 mixture was centrifuged at 230,000 x g (average) for 2 h and the supernatant was used as the solubilised enzyme preparation. To measure enzyme activity in the non-solubilised material, the 230,000 x g

pellet was resuspended in homogenisation buffer containing Triton X-100 at 10% (w/w).

(iii) Particulate enzyme preparation for application to sucrose density gradients.

Pea epicotyls were homogenised in approximately 10 cm^3 homogenisation medium by chopping with razor blades or by grinding with a pestle and mortar. Homogenisation media contained the same constituents as the gradient media being used, except that the sucrose was at 10% (w/w).

When homogenisation and gradient media made at pH 6.0 were used, then 25 g of pea epicotyls were chopped for 10 min. and, unless otherwise stated, the muslin filtrate was applied directly to the gradient.

When media made at pH 7.5 were used, then 11 g of pea epicotyls were chopped for 5 min and the muslin filtrate was pre-centrifuged at 2,000 x g for 5 min before application to the gradient.

(iv) Particulate enzyme preparation from sections along pea epicotyls.

Epicotyls of the same physiological age (the first node was within the top 1 cm) were cut into 1 cm lengths from the hook downwards (Fig. 30, Ch. 4). The tissue from each section was homogenised with a pestle and mortar in Tris-HCl

(10 mmol dm^{-3}), Mes (10 mmol dm^{-3}) pH 6.0 and each muslin filtrate was brought to 40 cm^3 with the same buffer. Centrifugation of the filtrates at 97,000 x g for 30 min resulted in a membrane pellet representing each epicotyl section. The pellets were resuspended with homogenisation buffer to final volumes of 0.85 cm^3 and those suspensions were used as particulate enzyme preparations.

The Composition, Centrifugation and Fractionation of Sucrose Density Gradients

Continuous sucrose density gradients were made from discontinuous 'step' gradients which had been left for between 24 and 48 h at approximately 4°C. The completed gradients contained sucrose at concentrations ranging from 10% (w/w) at the top to 50% (w/w) at the bottom.

The gradients made at pH 6.0 were all continuous and contained sucrose, bovine serum albumin (BSA, 0.1% w/v), dithiothreitol (DTT, 1 mmol dm^{-3}), KCl (10 mmol dm^{-3}) and ethylene diamine tetracetic acid (EDTA, 1 or 2 mmol dm^{-3}) in Tris-HCl (10 mmol dm^{-3}) and Mes (10 mmol dm^{-3}). The KCl was occasionally omitted.

All the continuous gradients that were made at pH 7.5 contained sucrose (10 - 50% w/w), BSA (0.1% w/v), DTT (1 mmol dm^{-3}), KCl (10 mmol dm^{-3}), EDTA (1 mmol dm^{-3}) and $MgCl_3$ (0.1 mmol dm^{-3}) in Tris-HCl (40 mmol dm^{-3}). The discontin-

uous gradients at this pH contained the same constituents except that $MgCl_2$ was included at either 0.1 mmol dm^{-3} (low Mg^{2+}) or 3 mmol dm^{-3} (high Mg^{2+}). The diagram of a discontinuous gradient is shown in Fig. 10. The step gradients contained three layers of gradient media containing 10%, 30% and 50% w/w sucrose respectively. After centrifugation, material at the 10-30% (top) and 30-50% (bottom) interfaces was collected. Each interface was diluted to 4 cm^3 in homogenisation media before assaying protein and various enzymes.

The sucrose density gradients at both pH 6.0 and 7.5 were formed in centrifuge tubes of 38 cm^3 capacity, and were centrifuged at 97,000 x g for 2 h by which time they had become isopycnic. Subsequent fractionation of continuous gradients was accomplished by the use of a Fisons Gradient Fractionator (Model 185) and a Gilson fraction collector. Fractions were kept at between 0 and 4°C before analysis and the density of sucrose was measured using a refractometer.

Centrifugation

Sedimentation of membraneous material at 97,000 x g (average) and non detergent solubilised material at 230,000 x g (average) involved the use of a Sorval OTD 65-B ultracentrifuge and AH627 or T865.1 rotors respectively.

Sedimentation of muslin filtrate at 2,000 x g prior to

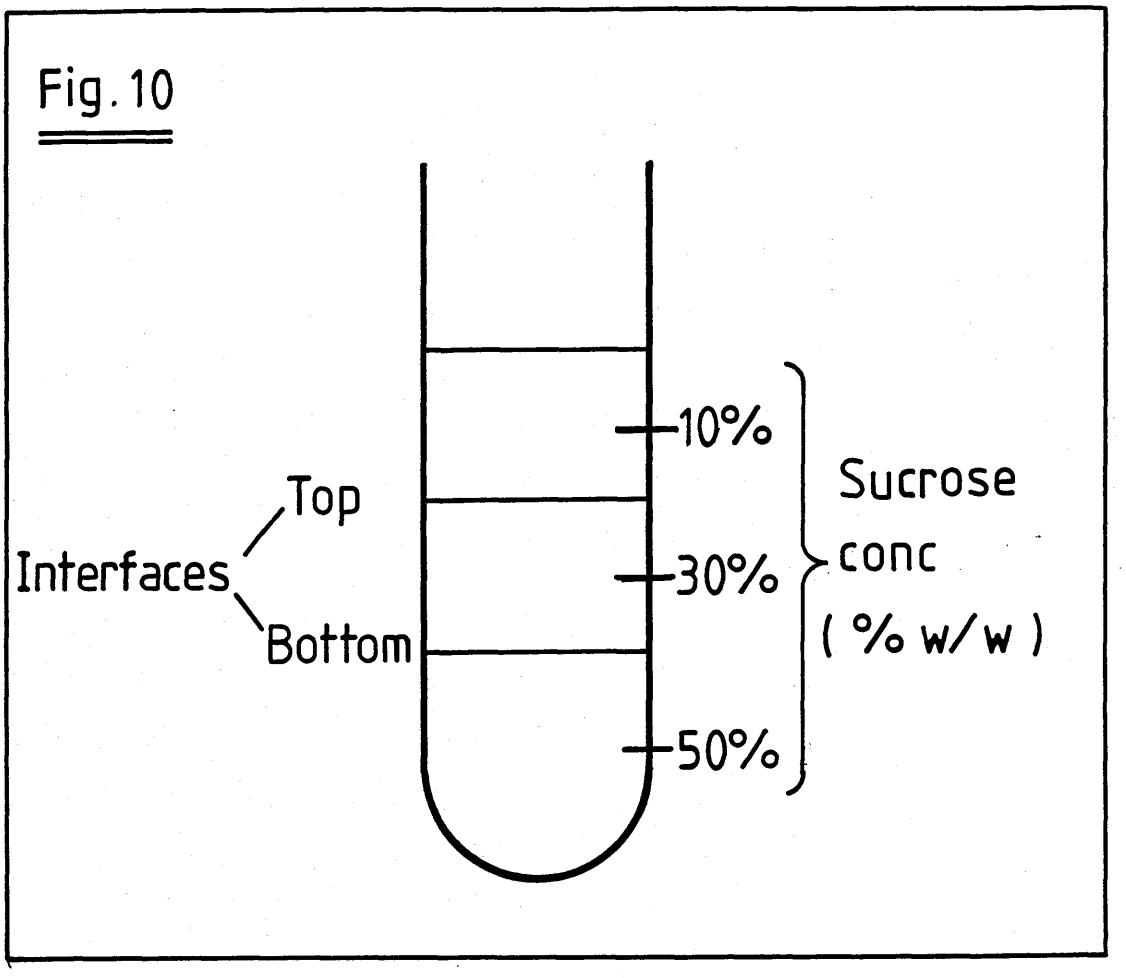


Fig. 10

A longitudinal section through a discontinuous-sucrose gradient to show the layers of gradient medium and the interfaces from which organelles were collected.

application to a sucrose density gradient involved the use of an MSE-18 centrifuge and an 8 x 50 cm^3 angle rotor.

Centrifugation at 10,000 x g during the 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 4,000 x g (approximately) using an MSE-minor S centrifuge and 10 cm^3 conical tubes.

Enzyme Assays

Glucuronyltransferase

The standard incubation mixture consisted of 50mm^3 of particulate enzyme preparation, $MnCl_2$ (10 mmol dm^{-3}) and UDP-D-[U-^{14}C]-glucuronic acid (50 pmol ; 462 Bq) in a total volume of 100mm^3. Incubations were carried out at 25°C and were terminated by the addition of 70% (v/v) ethanol (1 cm^3). During the preparation of labelled hemicellulose for chemical analysis, the incubation medium was scaled up by a factor of 10. In those incubations in which the enzyme was obtained from a sucrose density gradient fraction or a solubilised enzyme preparation, a carrier such as boiled enzyme or cellulose powder was added after termination to aid extraction. Incorporation of radioactivity into polysaccharide was then measured as described.

Incubation times are given below:

Ch. 3: 2 h.

Ch. 4: 4 h.

Ch. 5: 4 h at pH 6.0 and 2 h at pH 7.5.

Ch. 6: 4 h at pH 6.0 and 2 h at pH 7.5.

In all experiments designed to analyse the effect of pH on the incorporation of radioactivity from UDP-[U-^{14}C]-glucuronic acid into either 70% (v/v) ethanol insoluble polysaccharide or hemicellulose, the pea epicotyl tissue was homogenised in the way described for general use. The membrane pellets, however, were resuspended in Tris-HCl at a concentration of 1 mmol dm^{-3} at pH 7.5. Incubation components were standard (p. 57) except for the following alterations:

(i) In those incubations from which incorporation into 70% (v/v) ethanol insoluble polysaccharide was measured, $MgCl_2$ (10 mmol dm^{-3}) was included instead of $MnCl_2$. Regulation of pH was achieved by the addition of 30mm^3 Tris-HCl (0.1 mol dm^{-3}) at the required pH instead of H_2O. Incubations were terminated after 6 min.

(ii) In those incubations from which incorporation into hemicellulose was measured, pH was regulated by the inclusion of a buffer mixture of the required pH value instead of H_2O. If UDP Xylose (1 mmol dm^{-3}) was present, then the mixture contained Tris-HCl (20 mmol dm^{-3}), Mes (20 mmol dm^{-3}) and glycine (20 mmol dm^{-3}). If UDP-D-xylose was absent, then the buffer mixture was the same as in its presence except that each constituent was at 15 mmol dm^{-3} instead of 20 mmol dm^{-3}.

β Glucan Synthetase I (GS I)

This assay was based on that of Ray *et al* (1976). Incubations contained 50 mm^3 enzyme preparations (from a sucrose density gradient), Tris-HCl (30 mmol dm^{-3}) at pH 8.0, $MgCl_2$ (40 mmol dm^{-3}), UDP-D-glucose (0.5 μmol dm^{-3}) and UDP-D-[U-^{14}C]-glucose (0.435 μmol dm^{-3}) in a final volume of 100 mm^3. Incubations were of 5 min duration at 25°C and were terminated by the addition of 70% (v/v) ethanol (1 cm^3) and holding in water at 100°C for 1 min. The incorporation of radioactivity into 70% (v/v)-ethanol-insoluble material was measured after washing 3 times in 70% (v/v) ethanol.

β Glucan Synthetase II (GS II)

Incubations were as for GS I except that UDP-D-glucose was present at a final concentration of 0.5 mmol dm^{-3} and no Mg^{2+} was present.

Total IDPase

The composition of the incubation mixture was based on the methods employed by Bowles and Kauss (1976) and Ray *et al* (1969). Incubations contained IDP (2.25 mmol dm^{-3}), $MgCl_2$ (1.875 mmol dm^{-3}), Tris-HCl pH 7.5 (45.75 mmol dm^{-3}) and sucrose density gradient fraction (100 mm^3) that had been stored for between 2 and 4 days at between 0 and 4°C. The total volume of each incubation was 200 mm^3. Incubations were terminated and assayed for phosphate released as described

for 'Latent IDPase' (see below).

Latent IDPase

Incubations contained 50mm^3 enzyme preparation (from a sucrose density gradient), Tris-HCl (28 mmol dm^{-3}), $MgCl_2$ (2.5 mmol dm^{-3}) and IDP (3.0 mmol dm^{-3}) in a final volume of 200mm^3. After 5 min at 25^{0}C, the incubations were terminated by the addition of 1.3 cm^3 trichloroacetic acid (11.5% (w/v)) and the phosphate released was measured immediately.

In order to determine the latent IDPase activity, enzyme was either stored at 0^{0}C for 4 days before performing the assay, or Triton X-100 was included in the incubation medium to a final concentration of 0.1% (w/w).

NADH-cytochrome C reductase

Incubations contained 50 mm^3 of the enzyme preparation, 20 mm^3 of NADH solution and 900 mm^3 of cytochrome-c solution. The rate of increase in the absorbance at 550 nm over a period of 2 min was measured at room temperature.

NADH solution consisted of NADH (24 mmol dm^{-3}) dissolved in Tris-HCl (50 mmol dm^{-3}) pH 7.5.

Cytochrome-c solution consisted of cytochrome-c (0.53 mg cm^{-3}), Tris-HCl (50 mmol dm^{-3}) pH 7.5, some ethanol (0.13%

w/v), Antimycin A (0.12 mmol dm^{-3}) and KCN (1.1 mol dm^{-3}).

Incubations were started by addition of the enzyme preparation.

The Extraction of ^{14}C Labelled Polysaccharides after Incubation

Terminated incubation mixtures were centrifuged at 10,000 x g for 0.5min. The pellets were washed 3 times in 70% (v/v) ethanol (0.5 cm^3) to remove the majority of UDP-D-[U-^{14}C]-glucuronic acid, (or UDP-D-[U-^{14}C]-glucose in the case of glucan synthetase I and II assays,) and any low molecular weight products. Measurement of incorporation of radioactivity into 70% (v/v)-ethanol-insoluble material was achieved by counting on a scintillation spectrophotometer.

Extraction of pectin from the ethanol-washed material employed a method adapted from that described by Stoddart *et al* (1967). Each pellet was resuspended in 0.5 cm^3 buffer containing EDTA (0.05 mol dm^{-3}) and sodium phosphate (0.05 mol dm^{-3}) pH 6.8. The mixture was heated to 100°C in a boiling water bath for 15 min, centrifuged at 10,000 x g, and the supernatant removed and stored. The same procedure was repeated on the residue, and the supernatant from the second extraction was added to that from the first. The radioactivity incorporated into this total pectin fraction was measured by liquid scintillation counting. However, the radioactivity incorporated into high molecular weight-pectin was measured by separating high-and low-molecular-weight pectin within the

extract on a Sephadex G-75 or G-100 column and quantifying the radioactivity within the high molecular weight peak (see Fig. 11).

After pectin extraction, the remaining insoluble material was washed twice in the EDTA/sodium phosphate buffer. Hemicellulose was subsequently extracted by a method adopted from that described by Boffey and Northcote (1975); the insoluble residue was vortex mixed with 24% (w/v) KOH (1 cm^3), and left for 48 h at room temperature. Alkali-insoluble material was removed by centrifugation at 10,000 x g for 5 min. The supernatant was adjusted to pH 4.5 by addition of glacial acetic acid (1 cm^3) and adjusted to 73% (v/v) ethanol by addition of 96% (v/v) ethanol (6 cm^3). A small quantity of cellulose powder (Machery Nagel and Co., 516 Düren, Germany) was added to help in sedimenting the hemicellulose precipitated during the following 48 hours. At the end of this time the precipitates were centrifuged at 4,000 x g for 15 min. The supernatants were decanted off and the precipitates washed twice in 70% (v/v) ethanol before counting for radioactivity.

Measurement of Phosphate

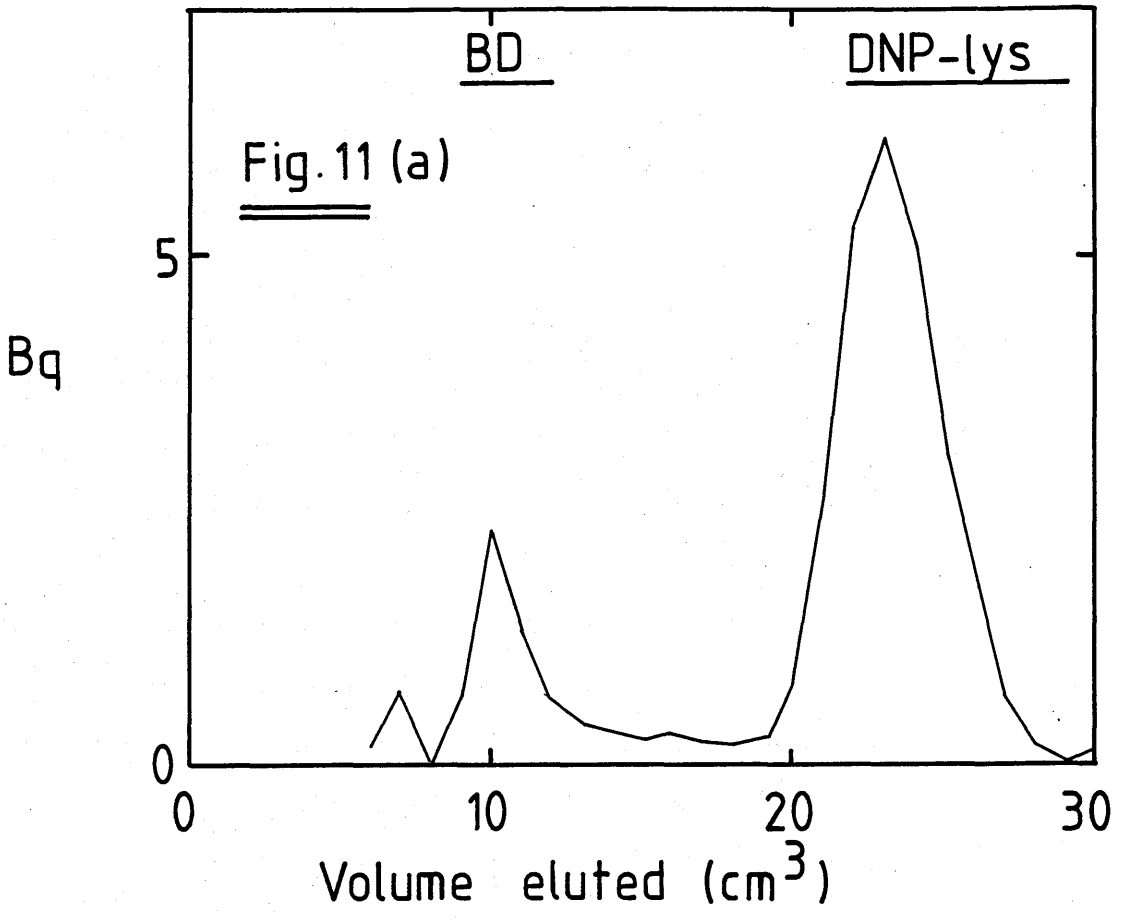
Phosphate was measured by the method of Taussky and Shorr (1953). Terminated IDPase incubations were centrifuged at 10,000 x g for 10 min to sediment precipitated protein. The supernatant was then transferred to a test tube and 0.5

Fig. 11(a)
An elution profile of total pectin (labelled with radioactivity from UDP-D-[U-^{14}C]glucuronic acid by the action of particulate enzyme at pH 7.5 for 2h in the presence of UDP-D-xylose at 1mmol.dm^{-3}) which has been passed through a Sephadex G-75 gel-filtration column.

The profile shows a high-molecular-weight peak of radioactivity which co-elutes with Blue Dextran, and a low-molecular-weight peak of material which is fully included by the gel.

Fig. 11(b)
An elution profile of total pectin (labelled with radioactivity from UDP-D-[U-^{14}C]glucuronic acid by the action of Triton X-100-solubilised enzyme at pH 6.0 for 4h in the presence of UDP-D-xylose at 1mmol.dm^{-3}) which has been passed through a Sephadex G-100 gel-filtration column.

The profile is similar to the one in Fig. 11(a) except for the relative sizes of the peaks.


BD
DNP-lys
Fig. 11 (a)
Bq
5
0
0
10
20
30
Volume eluted (cm3)

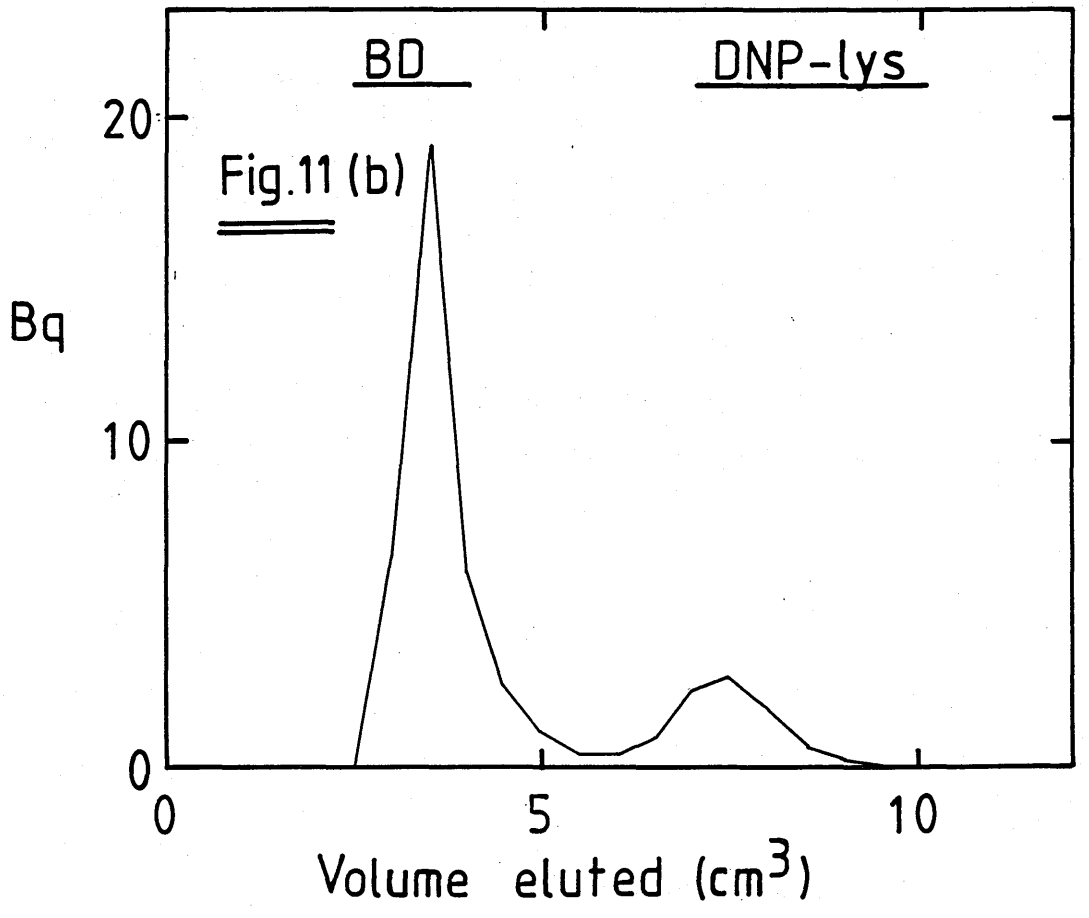
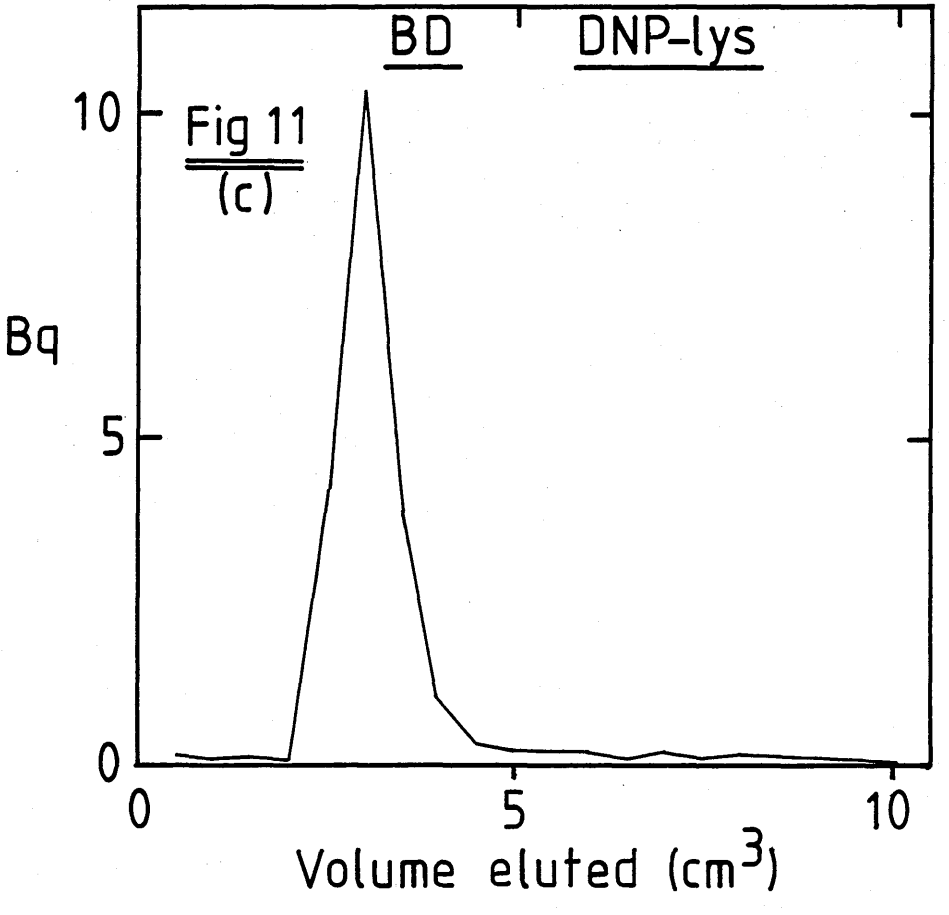

BD
DNP-lys
Fig.11 (b)
Bq
20
10
0
0
5
10
Volume eluted (cm3)

Fig. 11 (c)
An elution profile of total pectin (labelled by the same method as the pectin used in Fig. 11 (a) and then dialysed against water for 4 days) which has been passed through a Sephadex G-100 gel-filtration column.

The effect of dialysis has been to remove all the low molecular weight material.



cm^3 of H_2O and 1 cm^3 ferrous sulphate-ammonium molybdate reagent were added. After waiting for the colour to develop (2 min), the absorbance at 720 nm was measured.

The ferrous sulphate-ammonium molybdate reagent was made by the following procedure. 10 cm^3 ammonium molybdate stock solution (50 g $(NH_4)_6Mo_7O_{24}.4H_2O$ dissolved in 10 N sulphuric acid to a final volume of 500 cm^3) was diluted to approximately 70 cm^3. To this was added $FeSO_4.7H_2O$ (5g). After dissolving, the volume was brought to 100 cm^3.

A standard curve was made using KH_2PO_4 in H_2O and an example of such a curve is given in Fig. 12.

Measurement of Protein

To measure protein in enzyme preparations, the Tannin assay method (of Mejbaum-Katzenellenbogen and Drobryszycka (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.

Fig. 12 (a)
Standard curve for the phosphate assay. See text for details.

Fig. 12 (b)
Standard curve for the protein assay utilising the 'Tannin' method. See text for details.

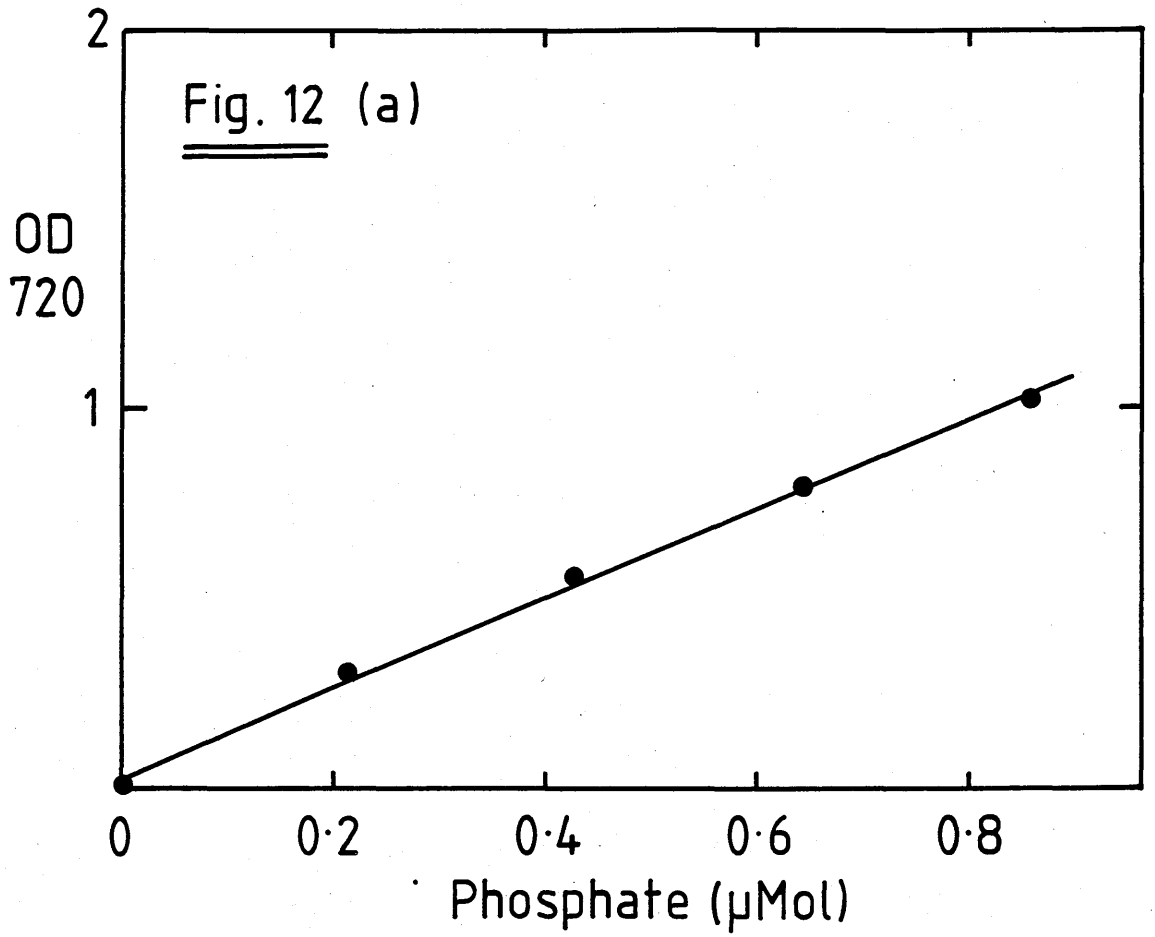


Fig. 12 (a)
OD
720
2
1
0
0·2
0·4
0·6
0·8
Phosphate (μMol)

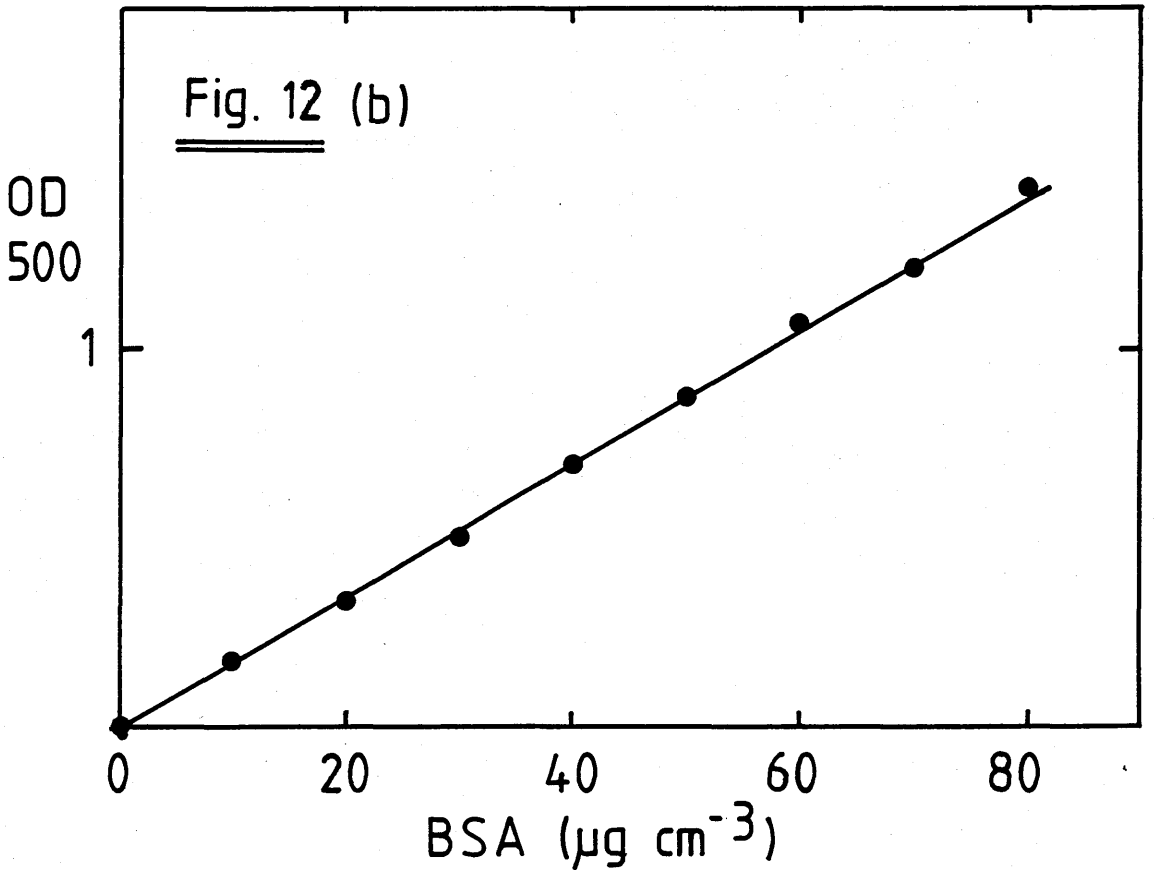


Fig. 12 (b)
OD
500
1
0
20
40
60
80
BSA (μg cm^{-3})

A standard curve was made using bovine serum albumin. An example is given in Fig. 12.

Hydrolysis of Polysaccharide

Partial acid hydrolysis was carried out in trifluoroacetic acid (2 mol dm^{-3}) at 100°C in sealed Reacti-vials (Pierce and Warriner (U.K.) Ltd., Chester, U.K.) for 1 h or more. Total acid hydrolysis involved the use of a similar procedure, except that the sealed vessels were autoclaved at 120°C. After hydrolysis, the non-hydrolysed material was centrifuged down at 10,000 x g for 5 min and the hydrolysate was evaporated to dryness under reduced pressure, under a stream of nitrogen, or while being spotted onto thin layer or paper chromatographic sheets. In some cases, non-hydrolysed material was monitored for radioactivity. If this was to be done, the residue was first resuspended in 0.5 cm^3 Tris-HCl (100 mmol dm^{-3} pH 7.5) to neutralise any T.F.A. and prevent quenching.

Separation of Oligosaccharides

Oligosaccharides produced by partial acid hydrolysis were separated by descending chromatography on Whatman No. 1 paper for 30 h in Solvent I (ethyl acetate/formic acid/acetic acid/water, 18:1:3:4 by vol.) (Ray and Rottenberg, 1964). Marker sugars were detected by the method of Trevalyan *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 of ethanol (150 cm^3) containing 0.5 cm^3 of 0.2% NaOH. On drying the sugars showed up as grey/brown spots.

Separation of Monosaccharides

This was performed by descending chromatography on Whatman No. 1 paper. The solvent (Solvent II) was ethyl acetate/pyridine/water (8:2:1 by vol.). Marker sugars were detected as described above.

Thin-layer Electrophoresis

Electrophoresis was carried out on plastic sheets pre-coated with silica-gel G (Camlab, Cambridge, U.K.) at 300 V for between 3 and 5 h. The buffer consisted of pyridine/acetic acid/water (1:10:89, by vol). Marker sugars 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.

Elution of Samples from Paper-Chromatographic Strips

If the strips had been saturated with toluene/PPO scintillation fluid for scintillation counting, the scintillant

was first washed out of the strips by three washes with sulphur-free toluene. The remaining toluene was then allowed to evaporate. The strips were agitated in water ($3 cm^3$/strip) for 5 min. The water was then decanted and centrifuged at 4,000 x g for 10 min to remove paper particles. The strips were washed again in the same way, and the washings treated as above. The washings were then combined and dried by rotary evaporation at 40°C.

Reduction of Glucuronic Acid in Oligosaccharides

The dried oligosaccharide was resuspended in redistilled methanol (1 cm^3) which was then adjusted to 1 mol dm^{-3} with respect to HCl by the addition of concentrated HCl. After heating to 100°C for 2 h in a sealed Reacti-vial, the products were evaporated to dryness under reduced pressure. $NaBH_4$ reduction was then carried out for 30 min at room temperature by addition of $NaBH_4$ ($50 mm^3$ of a 1 mol dm^{-3} solution). The reduction was terminated by addition of $200 mm^3$ of acetic acid, and the sample was evaporated under reduced pressure to remove methyl borate and acetic acid. The products were hydrolysed for 2 h in trifluoroacetic acid (2 mol dm^{-3}) at 100°C in a sealed Reacti-vial. After being evaporated to dryness under reduced pressure, the hydrolysate was redissolved in water and analysed by paper chromatography in solvent II.

Estimation of Radioactivity

Pellets of 70% (v/v)-ethanol-insoluble material produced in incubations, or of precipitated hemicellulose, were vortex-mixed with 5.5 or 11 cm^3 of scintillation fluid/water (10:1, v/v). The scintillation fluid used was Packard liquid-scintillation cocktail type 299, Triton/toluene scintillant (Brett, 1981) or Koch-Light xylene based scintillant.

Total pectin extracts (1 cm^3) were mixed with 10 cm^3 of one of the above scintillation fluids.

Strips of chromatography paper or of silica gel on its plastic backing were placed in a scintillation vial with between 0.5 and 1 cm^3 of the scintillation fluid consisting of PPO in toluene (sulphur free) (4g dm^3) (Harris and Northcote, 1970).

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

Typical background counts were 0.5 Bq.

Dialysis of Pectin Extracts

In order to separate high-molecular-weight pectin from low-molecular-weight material on a large scale, the total pectin extract was dialysed against distilled water for five days with continuous stirring. The water was changed every day and contained a few drops of 4% sodium azide to prevent

microbial contamination. After the dialysis was complete, the dialysed pectin was condensed by rotary evaporation and analysed by gel-filtration to ensure that all low-molecular-weight material had been removed.

Cadoxen Solubilisation of Hemicellulose

Cadoxen was prepared by the method of Wood and McCrae (1978). To make approximately 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°C for 18 h. The mixture was then allowed to settle and the supernatant (100% Cadoxen) decanted and stored at 4°C. Solubilisation of hemicellulose was achieved by mixing the dried hemicellulose in 0.1 cm^3 Cadoxen (100%) for 12 h followed by dilution with 0.1 cm^3 water. Non-solubilised hemicellulose was removed by centrifugation at 12,000 x g.

Digestion of Hemicellulose by the Action of Proteases

Digestion by pronase was carried out at 25°C for 2 h in sodium acetate buffer pH 7.5. The pronase was present at 1 mg cm^{-3}. Incubations were terminated by the addition of TCA.

Digestion by Proteinase-K was carried out at 25°C for 2 h in Tris-HCl pH 7.5. Proteinase-K was present at a concentration of 0.5 mg cm^{-3}. Digestion was terminated by drying

under N_2 followed by the addition of 100mm^3 Cadoxen (100%).

Gel Filtration

All gel filtration columns had a void volume of approximately 10 cm^3.

Analysis of pectin involved the use of Sephadex G-75 or G-100 (medium grade) gel media. Such gel columns were run at an elution rate of approximately 0.5 cm^3 min^{-1} at room temperature. The running buffer was the same as that used for pectin extraction.

'Cadoxen'-solubilised hemicellulose was analysed by using Sepharose CL-2B or CL-6B gel filtration media. The use of the cross-linked from of Sepharose was necessary in order to prevent solubilisation of the gel-filtration media by the Cadoxen. 50% Cadoxen was used for elution.

Treatment of Pea Tissue for E.M. Analysis

Pea material was fixed in 3% glutaraldehyde in 0.1 mol dm^{-3} cacodylate buffer pH 7.2 for between 12 and 16 h at room temperature. This was followed by 3 x 10 min rinses in 0.1 mol dm^{-3} cacodylate buffer pH 7.5. Post fixation was carried out in 1% osmium tetroxide dissolved in 0.1 mol dm^{-3} cacodylate buffer pH 7.5 for 4 h at room temperature followed by block

staining of the tissue in 1% (aqueous) uronyl acetate for 1 h at room temperature. Dehydration was performed using a graded series of acetone concentrations and the prepared material was finally embedded in Spurr's resin which was polymerised for between 16 and 24 h at 70°C. Sections were cut on an L.K.B. Ultratome III, picked up on 300 mesh copper grids and stained for 15 min in saturated ethanolic uranyl acetate (Gibbons and Grimstone, 1960) and for 5 min in lead citrate (Reynolds, 1963). The sections were subsequently examined in a Phillips 301 microscope at 80 KV.

Micrographs were taken on Kodak Eastman fine-grain release positive film and prints were made on Ilford 'Ilfoprint' paper.

Ion Exchange

Ion exchange was carried out using Amberlite IR-120 (H) resin to remove Na^+ ions from monosaccharide solutions. The resin (10 cm^3) was packed into a gel-filtration column, regenerated in 0.1 M HCl and washed thoroughly in H_2O. 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 all the deionised monosaccharide material.

Presentation of Results and Statistical Analysis

Incubations were performed in either duplicate or triplicate and the mean and standard deviations were calculated using a Texas Instruments TI- Programmable 57 calculator. The formula used in the calculation of the standard deviation is given below:

$$\text{Standard deviation} = \sqrt{\text{Variance}} = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{N}}$$

N = the number of data points (3 for incubations in triplicate)

x_i = the value of each data point in the population.

Chapter 3.

IDENTIFICATION OF A GLUCURONYLTRANSFERASE INVOLVED IN THE BIOSYNTHESIS OF GLUCURONO-XYLANS IN PEA (*PISUM SATIVUM*)

I The Incorporation of Radioactivity from UDP-D-[U-^{14}C]-Glucuronic Acid into 70% (v/v)-Ethanol-Insoluble Polysaccharide.

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

In the early stages of this research programme, the effects of pH, buffer concentration, cofactors and other chemicals on incorporation were not known. As a result, Tris-HCl (100 mmol dm^{-3}) at a neutral pH was chosen in which to homogenise plant tissue and resuspend membrane pellets. Incubations were as described (Ch. 2, p. 57) but contained Mg^{2+} and not Mn^{2+}. Mg^{2+} was originally chosen because of its stimulatory effects in other polysaccharide synthetase systems (Kennedy and Brett, 1984).

Time courses of incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into polysaccharide are given in Fig. 13 and show that incorporation ceased within 15 minutes. If the enzyme preparation was boiled prior to incubation, then no increase in incorporation occurred with time. The freezing of the enzyme pellets at -20 ^{0}C for 24 and 48 h before use lowered the incorporation during incubations of 30 min duration by 70% and 90% respectively. These results, together with the time courses indicated that the incorporation was due to enzyme activity.

Fig. 13 (a) and (b).

Time courses of the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into 70% (v/v)-ethanol-insoluble polysaccharide.

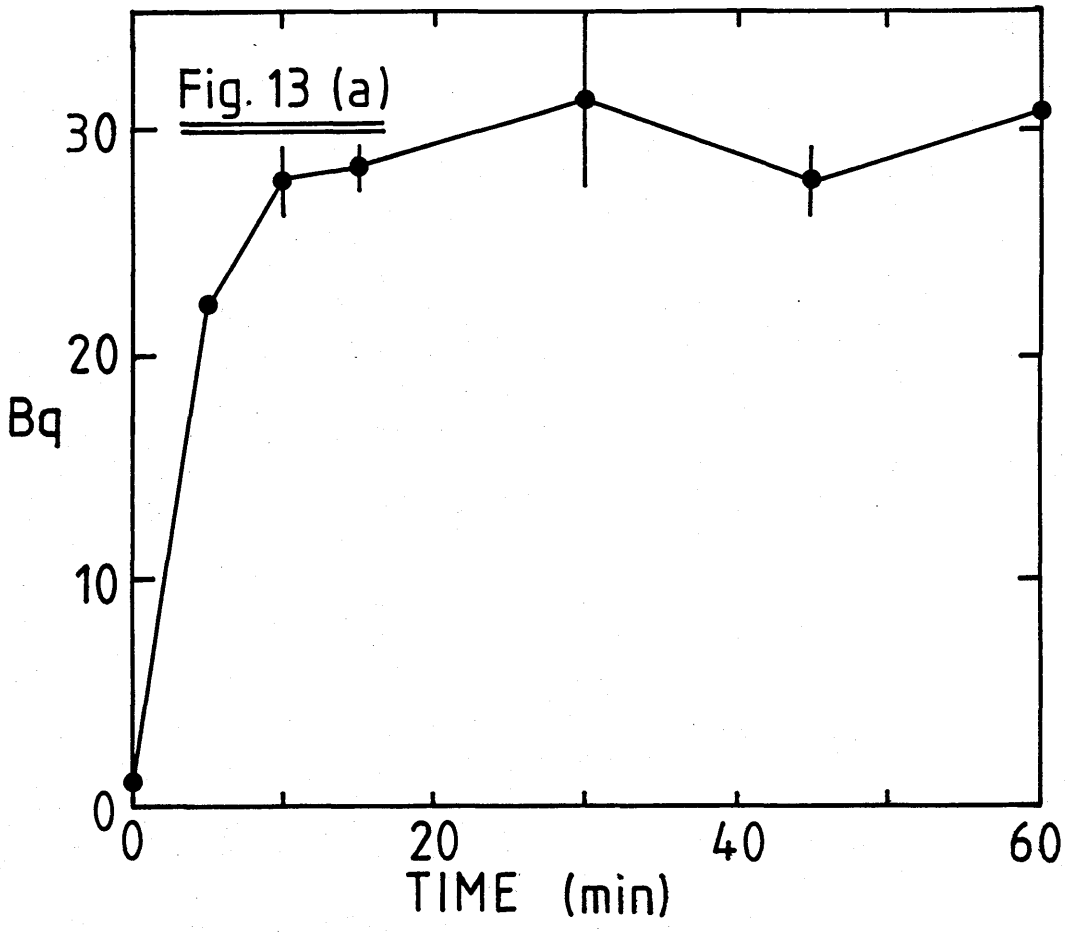


Fig. 13 (a)
30
20
10
0
Bq
0
20
40
60
TIME (min)

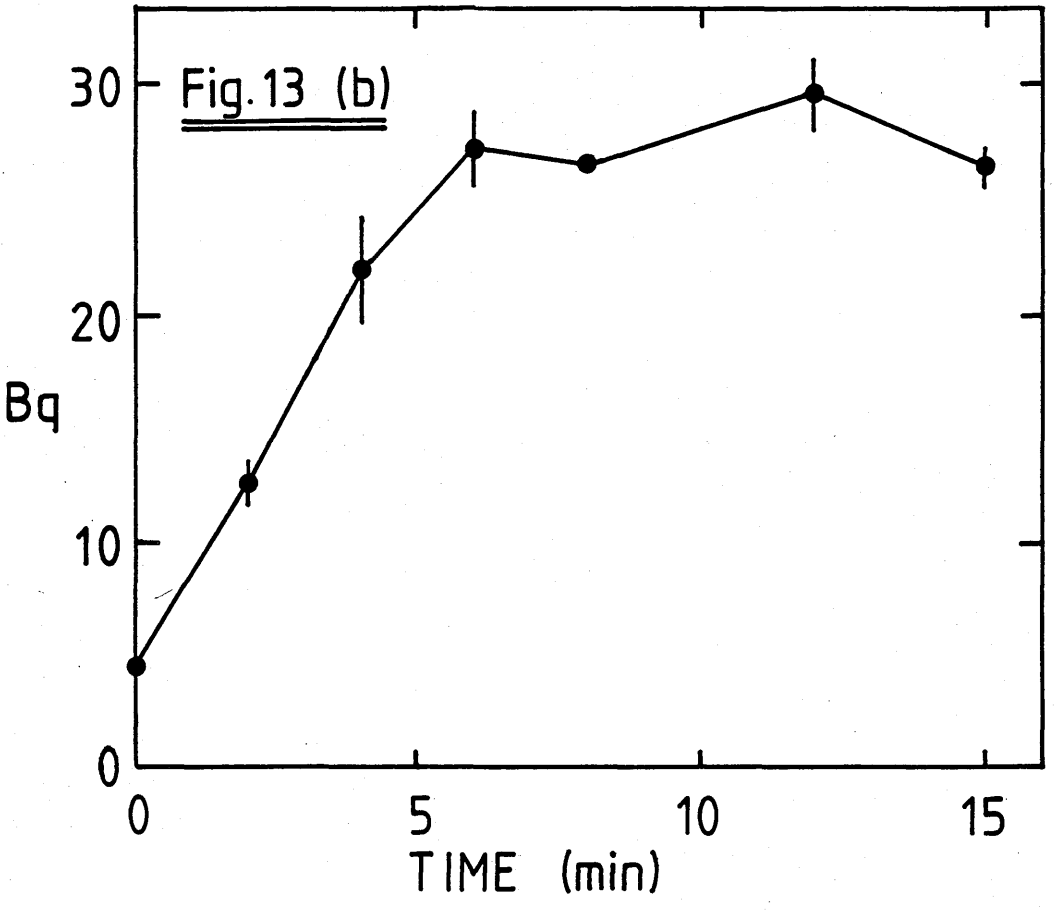


Fig. 13 (b)
30
20
10
0
Bq
0
5
10
15
TIME (min)

Increasing the concentrations of the buffers used in homogenisation and pellet resuspension appeared to inhibit incorporation slightly. Consequently, the concentration of Tris-HCl used in the remaining experiments in this chapter was 10 mmol dm^{-3} for homogenisation and 50 mmol dm^{-3} for pellet resuspension.

The effect of pH on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid

The effect of pH on incorporation of radioactivity into 70% (v/v)-ethanol-insoluble material is shown in Fig. 14. The incubation times used were 6 minutes (as described in Ch. 2) in order to analyse the effect of pH on the rate of incorporation, and not on the total incorporation. The results shown indicate a pH optimum of less than 6 units.

The effect of divalent cations on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid

Because Mg^{2+} cations had been shown to stimulate the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into 70% (v/v)-ethanol-insoluble material, the effect of other divalent cations was also investigated. The results (portrayed in Table IV) show that at a concentration of 10 mmol dm^{-3}, $MnCl_2$ gives the greatest stimulation. This was the initial reason for including Mn^{2+} at 10 mmol dm^{-3} in subsequent experiments.

Fig. 14

The effect of pH on the incorporation of radioactivity from UDP-D-[U^{14}C]glucuronic acid into 70% (v/v)-ethanol-insoluble polysaccharide.

Fig. 14

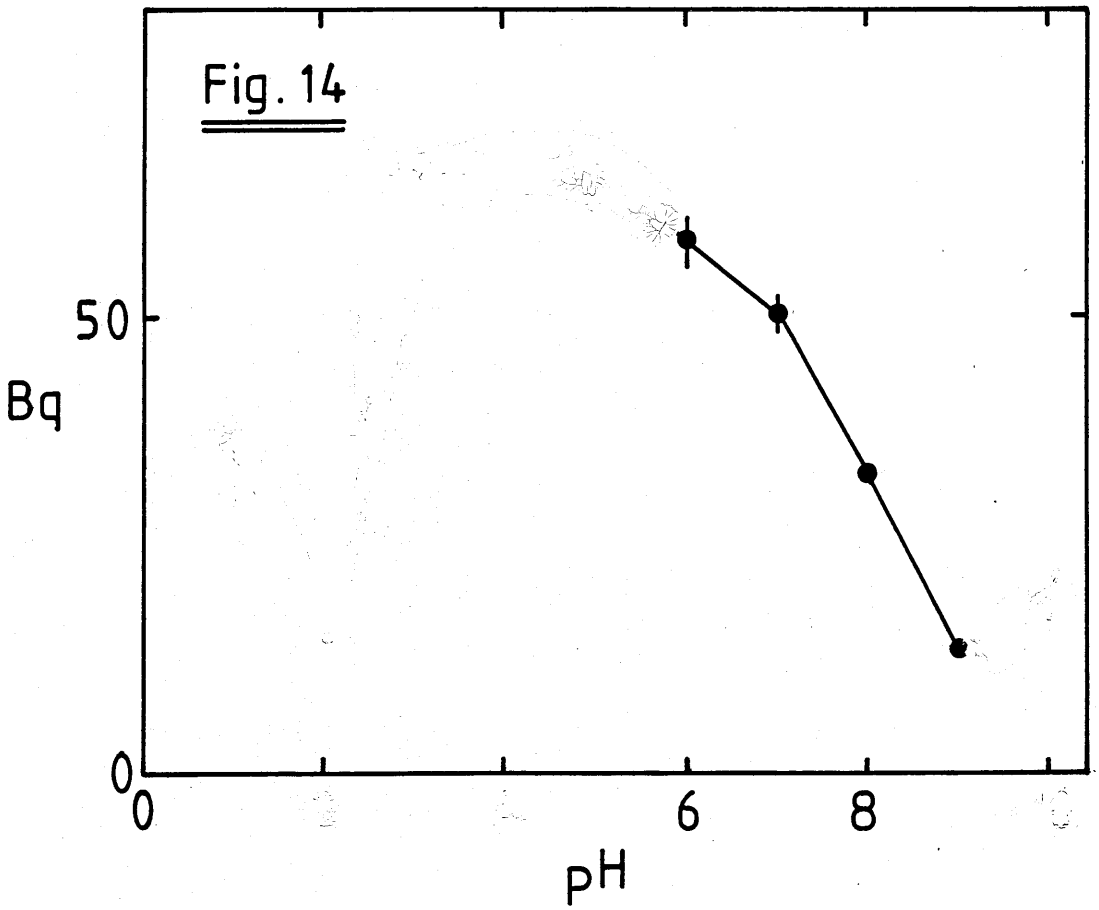


Table IV

Effect of divalent cations on the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into 70% (v/v)-ethanol-insoluble material in 5 min.

Divalent cation	Incorporation (Bq)
None	12.4 +/- 0.3
Mg^{2+}	46.0 +/- 0.5
Ca^{2+}	61.6 +/- 2.0
Ni^{2+}	65.0 +/- 5.8
Co^{2+}	70.0 +/- 2.0
Mn^{2+}	105.0 +/- 4.5

II Incorporation of Radioactivity from UDP-D-[U-^{14}C]-Glucuronic Acid into Hemicellulose and Pectin

The effect of UDP-D-xylose on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose and pectin

Theoretically, glucuronoxylan biosynthesis requires the synthesis of a xylan backbone prior to the addition of the glucuronic acid side chains. With this in mind, the effect of non-labelled UDP-D-xylose, a known substrate for xylan synthesis (Bailey and Hassid, 1966), was studied in the particulate enzyme system.

Time courses of standard incubations (see Ch. 2, p. 57) were performed in the presence and absence of UDP-D-xylose (1 mmol dm^{-3}). and the products were extracted for hemicellulose and total pectin (Figs. 15 & 16). The incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose stopped after 30 min unless UDP-D-xylose (1 mmol dm^{-3}) was present, in which case the incorporation continued for at least 8 h (Fig. 17). No other sugar nucleotide tested had this same stimulatory effect (Table V) and further experiments (Fig. 18) showed that UDP-D-xylose, when added to a final concentration of 1 mmol dm^{-3} after 75 min of incubation, still had the ability to increase incorporation. The effect of changing the UDP-D-xylose concentrations is shown in Fig. 19.

The stimulation of the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose by UDP-D-xylose was in keeping with the possibility that the radioactive

Fig. 15

Time course of the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into total pectin (a) and hemicellulose (b) in the presence (●——●) and absence (o——o) of UDP-D-xylose (1mmol.dm^{-3})

The apparent incorporation of radioactivity into pectin at t=0 probably reflects non-specific binding of sugar nucleotides during extraction in 70% (v/v) ethanol. These were then extracted with pectic substances. This problem was overcome in future experiments by separating high-molecular-weight pectin from low-molecular-weight materials by gel filtration.

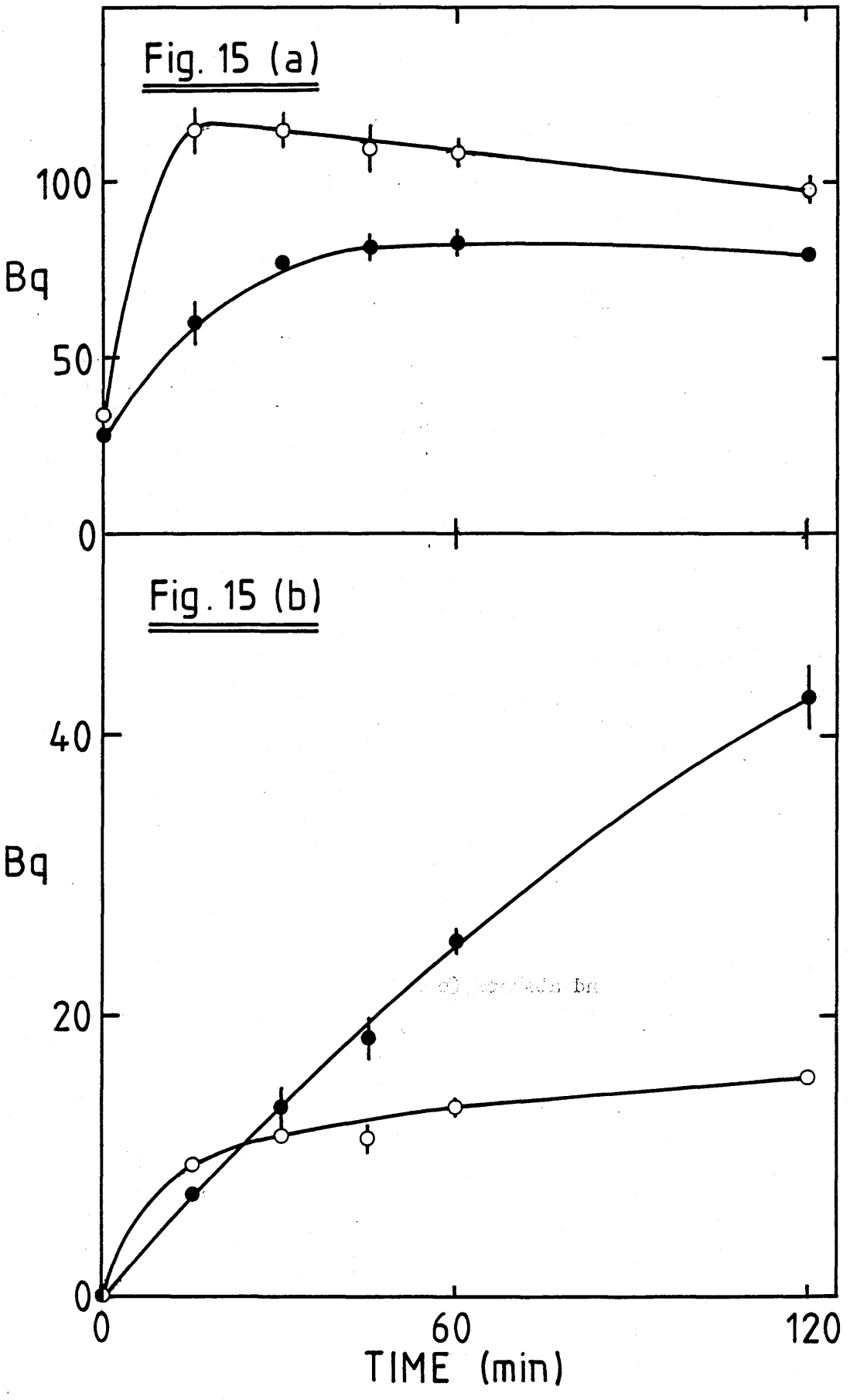


Fig. 15 (a)
100
Bq
50
0
Fig. 15 (b)
40
Bq
20
0
0
60
120
TIME (min)

Fig. 16

Time course of the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose in the presence (●—●) and absence (o—o) of UDP-D-xylose ($1 mmol.dm^{-3}$)

Fig. 17

Time course of the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose in the presence (●—●) of UDP-D-xylose ($1 mmol.dm^{-3}$).

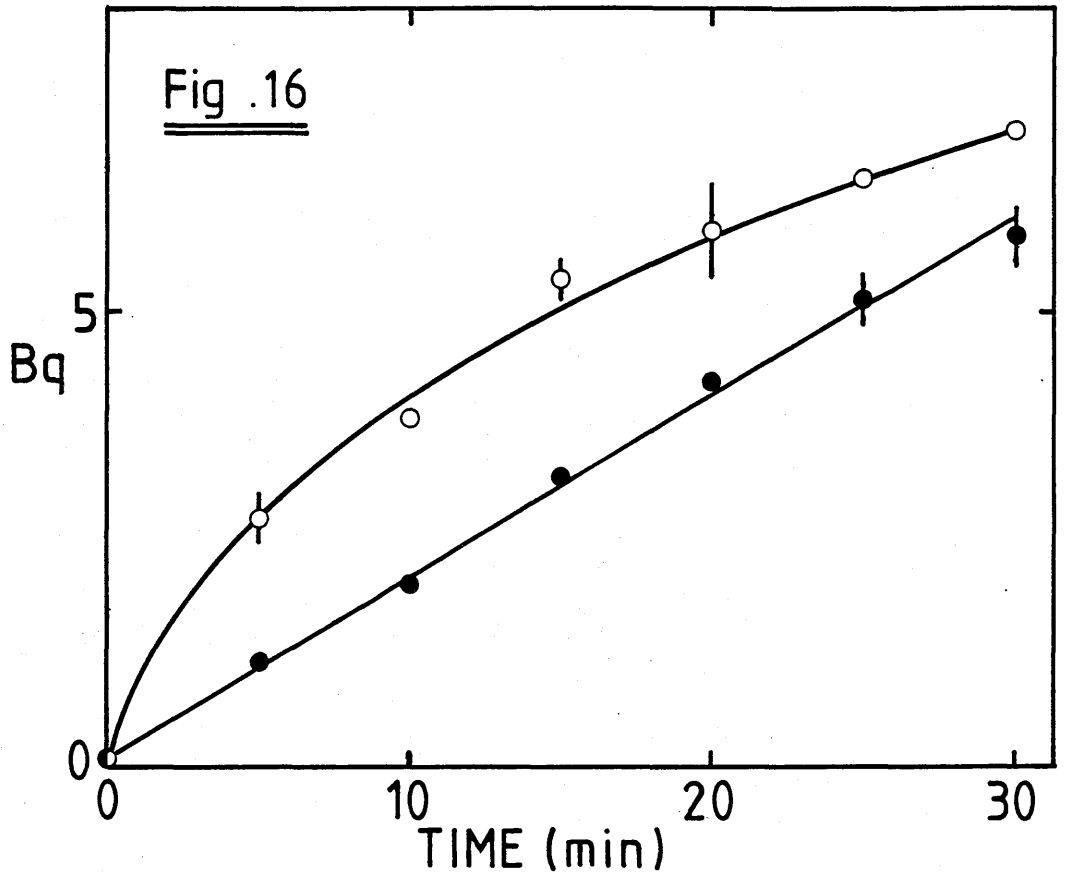


Fig .16
Bq
5
0
0
10
20
30
TIME (min)

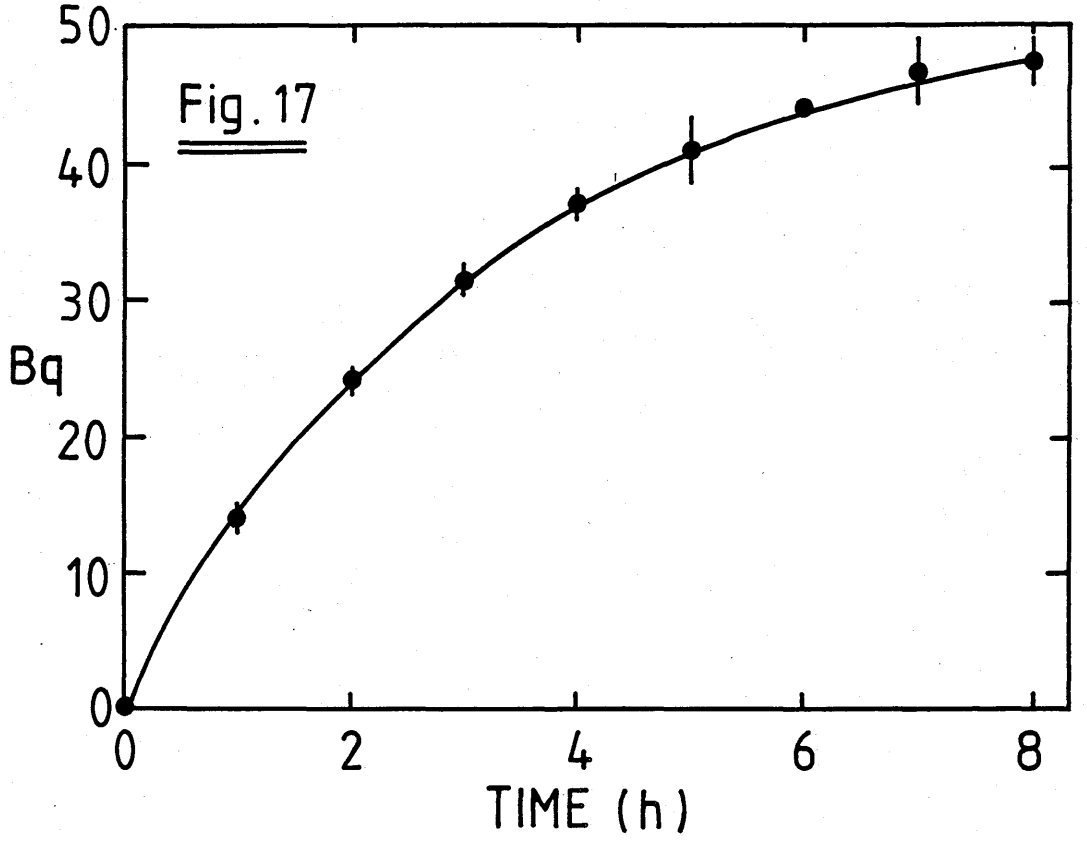


Fig. 17
50
40
30
Bq
20
10
0
0
2
4
6
8
TIME (h)

Table V

Effect of sugar nucleotides at a concentration of 1mmol.dm^{-3} on the incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose.

Incubations were of 2h duration.

Sugar nucleotide	Incorporation (Bq)	% increase in incorporation by the sugar nucleotide.
None	14.1 +/- 0.3	---
UDP-D-glucose	11.0 +/- 0.3	-22
GDP-D-glucose	19.2 +/- 0.6	+36
GDP-D-mannose	17.0 +/- 0.5	+20
UDP-D-galactose	9.0 +/- 0.4	-37
UDP-D-xylose	33.0 +/- 1.3	+123

Fig. 18

Time course showing the effect of adding UDP-D-xylose ($1 mmol.dm^{-3}$) after 75 min of incubation, on the incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose.

Fig. 19

Effect of UDP-D-xylose concentration on incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose during 2h incubations.

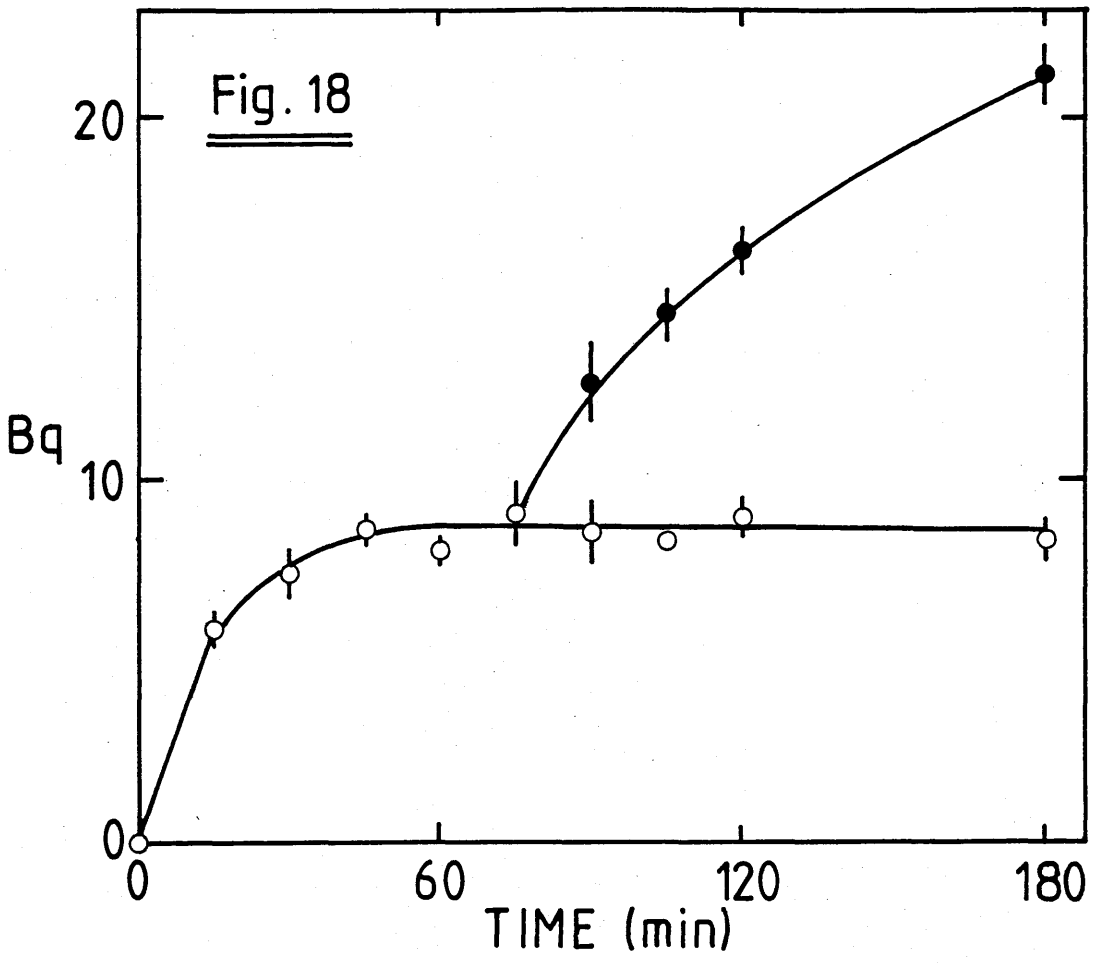


Fig. 18
20
10
0
Bq
0
60
120
180
TIME (min)

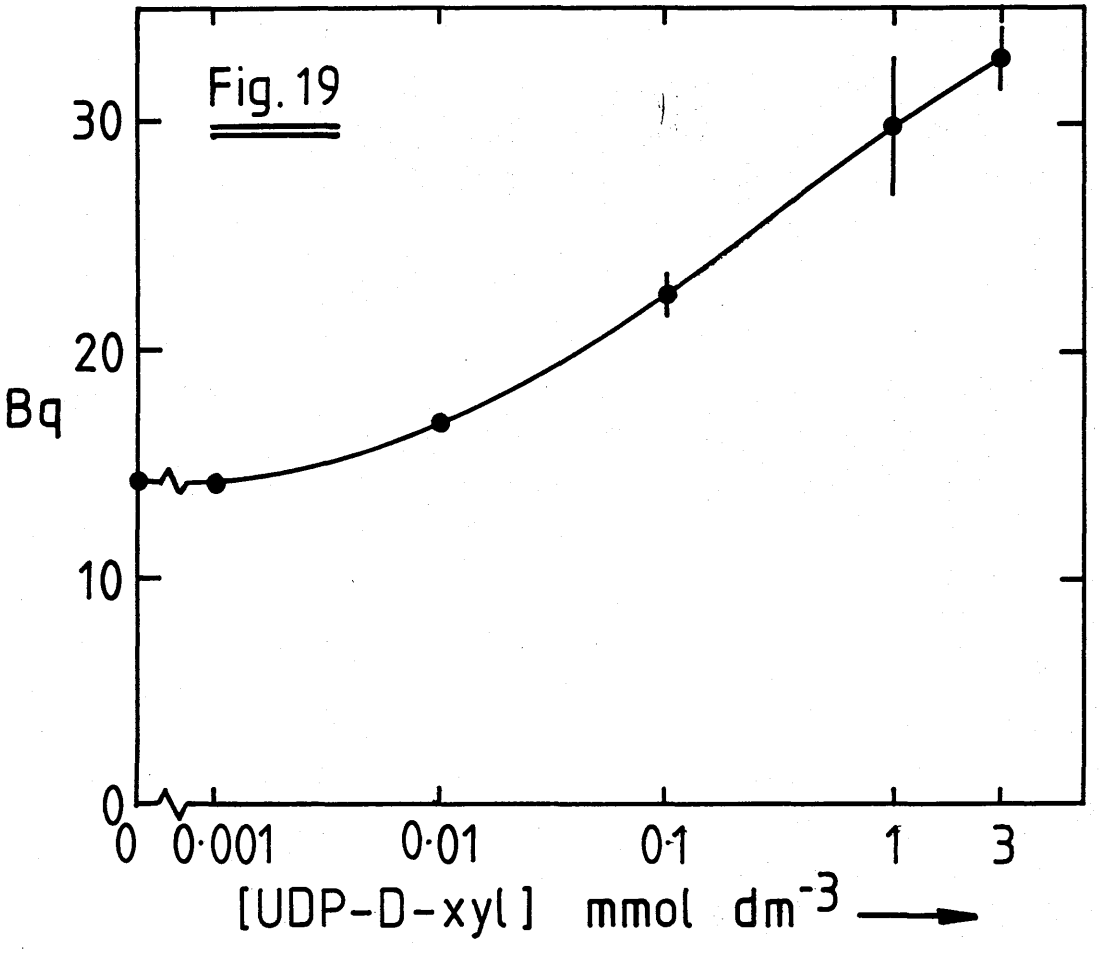


Fig. 19
30
20
10
0
Bq
0
0·001
0·01
0·1
1
3
[UDP-D-xyl] mmol dm-3

product was a glucuronoxylan. However, the only conclusive way of showing that a glucuronoxylan was being synthesised was to carry out structural analyses of the product. Such an analysis is described and discussed in a later part of this chapter.

The effect of divalent cations on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose in the presence of UDP-D-xylose.

Mn^{2+} divalent cations were again shown to be the most stimulatory of the divalent cations tested (Table VI) and because of this, Mn^{2+} (which is more common in plant cells than Co^{2+} or Ni^{2+} ions) was included in the standard incubation format. The greatest stimulation occurred at Mn^{2+} concentrations between 4 and 10 mmol dm^{-3} (Fig. 20), and at the concentration used in standard incubations (10 mmol dm^{-3}), the incorporation was 87% of the maximum observed.

The effect of pH on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose

In the presence and absence of UDP-D-xylose (1 mmol dm^{-3}) the optimum pH for the incorporation of radioactivity into hemicellulose fraction was between 5 and 6 (Fig. 21). The pH profile was also investigated at two different concentrations of Mn^{2+} (in the absence of UDP-D-xylose) and the results confirmed that the effect of pH was not due to a change in Mn^{2+} concentration.

The effect of UDP-D-glucuronic acid at different concentrations on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose

Incubations of 2 h duration were carried out in the

Table VI

Effect of divalent cations at a concentration of 10 mmol.dm^{-3} on the incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose.

Incubations were of 2h duration.

Divalent cation	Incorporation (Bq) -UDP-D-xylose	Incorporation (Bq) +UDP-D-xylose
None	3.2 +/- 0.4	2.4 +/- 0.2
Ni^{2+}	6.3 +/- 0.4	3.9 +/- 0.4
Ca^{2+}	4.8 +/- 0.4	5.5 +/- 0.2
Mg^{2+}	4.7 +/- 0.4	6.8 +/- 0.1
Co^{2+}	10.4 +/- 1.0	10.5 +/- 0.3
Mn^{2+}	11.2 +/- 0.2	22.6 +/- 0.4

Fig.20

Effect of Mn^{2+} concentration on the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose during 2h incubations.

UDP-D-xylose was present at 1mmol.dm^{-3}.

Fig. 20

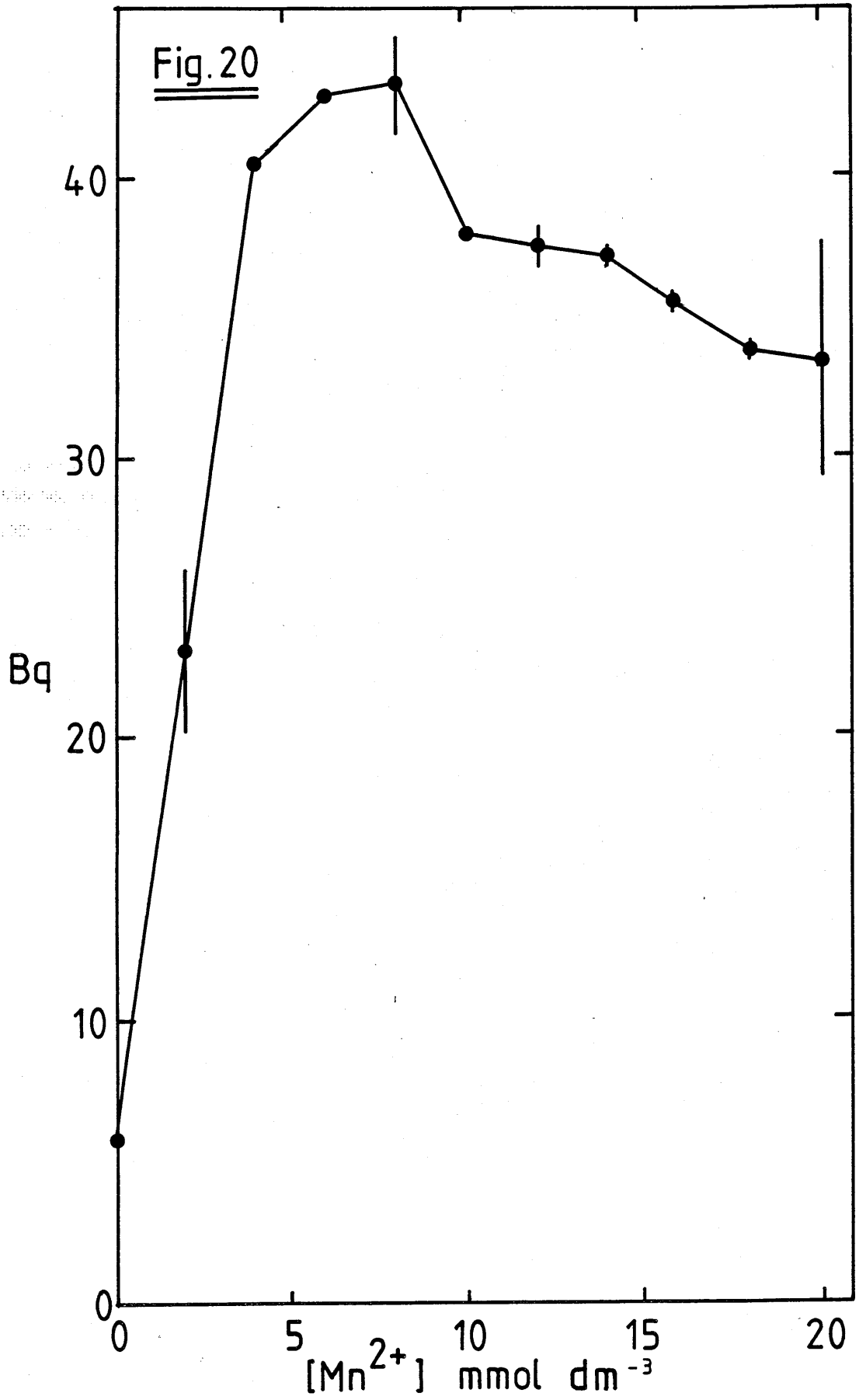


Fig.21

Effect of pH on the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose in the presence (●——●) and absence (o——o) of UDP-D-xylose (1mmol.dm^{-3}).

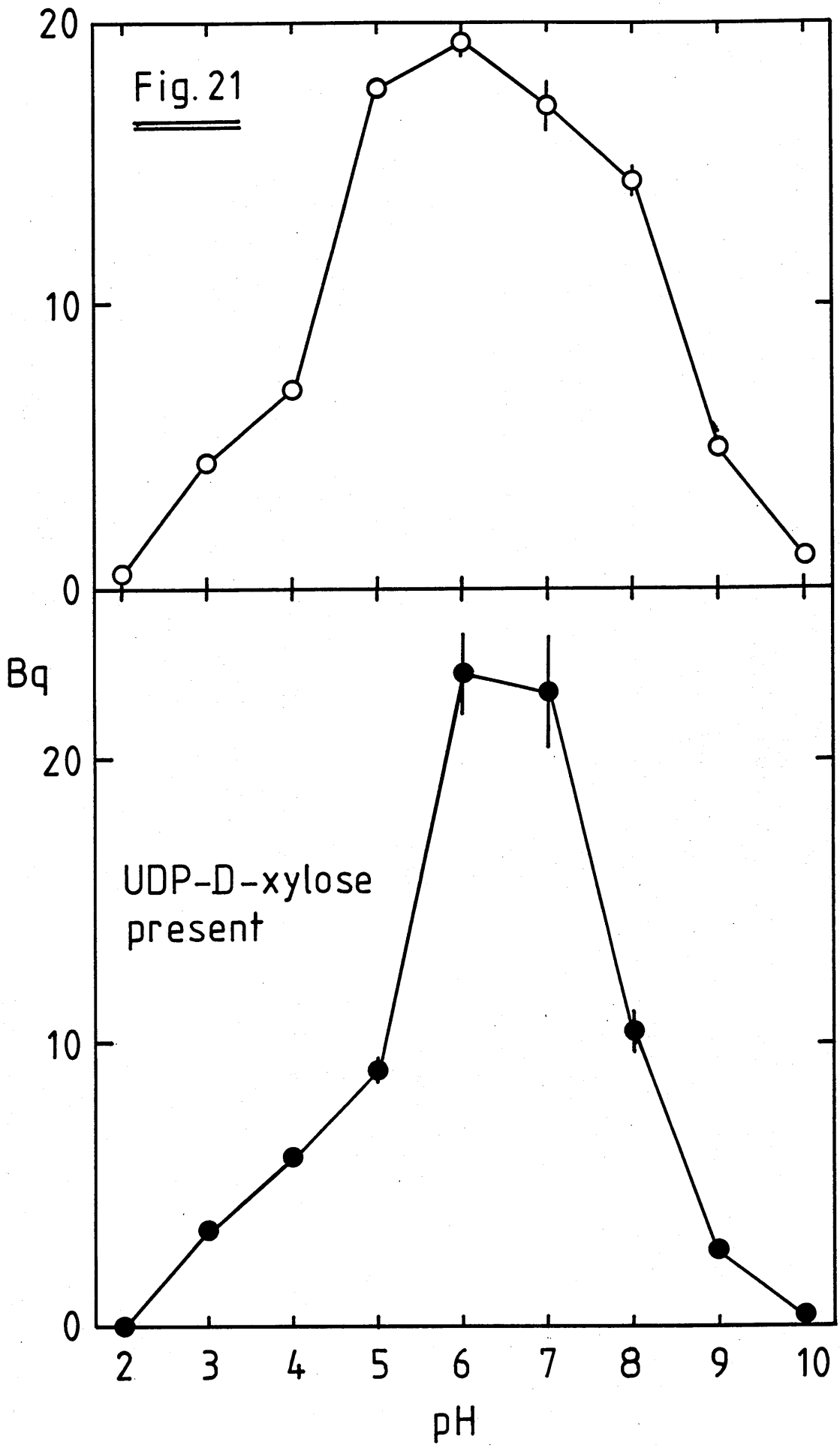


Fig. 21
20
10
0
Bq
20
10
0
UDP-D-xylose
present
2
3
4
5
6
7
8
9
10
pH

presence and absence of UDP-D-xylose at different concentrations of UDP-D-glucuronic acid. The results are given in Fig. 22 and it can be seen that the stimulation of incorporation by UDP-D-xylose diminishes as the concentration of UDP-D-glucuronic acid increases.

By making the assumption that in the presence of UDP-D-xylose, the incorporation of radioactivity is linear during the 2 h incubation period at all concentrations of UDP-glucuronic acid tested, one can tentatively suggest an apparent K_m value for the enzyme system by constructing a 'Hanes Plot' (Cornish-Bowden, 1979). Such a graph is given in Fig. 23 and suggests a K_m value of 8.5×10^{-5} mol dm^{-3} for the incorporation of glucuronic acid.

Other properties of the enzyme system which incorporates radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose

Pre-incubation of the enzyme preparation at 25 C before addition to the standard incubation mixture decreased significantly the incorporation of radioactivity during 2 h incubations (Fig. 24). These results indicated that the enzyme system was not particularly stable, and this conclusion is supported by the effects of freezing the enzyme pellets before use (Table VII). As a result, fresh enzyme was prepared for all experiments.

Fig. 22

Effect of UDP-D-glucuronic acid concentration on the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose in the presence (●——●) and absence (o——o) of UDP-D-xylose (1mmol.dm^{-3}) in 2h.

The results in Fig. 22 (a) are presented in the form of glucuronic acid incorporated as a function of the log of the concentration of UDP-D-glucuronic acid. This makes the assumption that all the radioactivity incorporated is in the form of glucuronic acid.

The results in Fig. 22 (b) show the % stimulation by UDP-D-xylose on the incorporation of glucuronic acid.

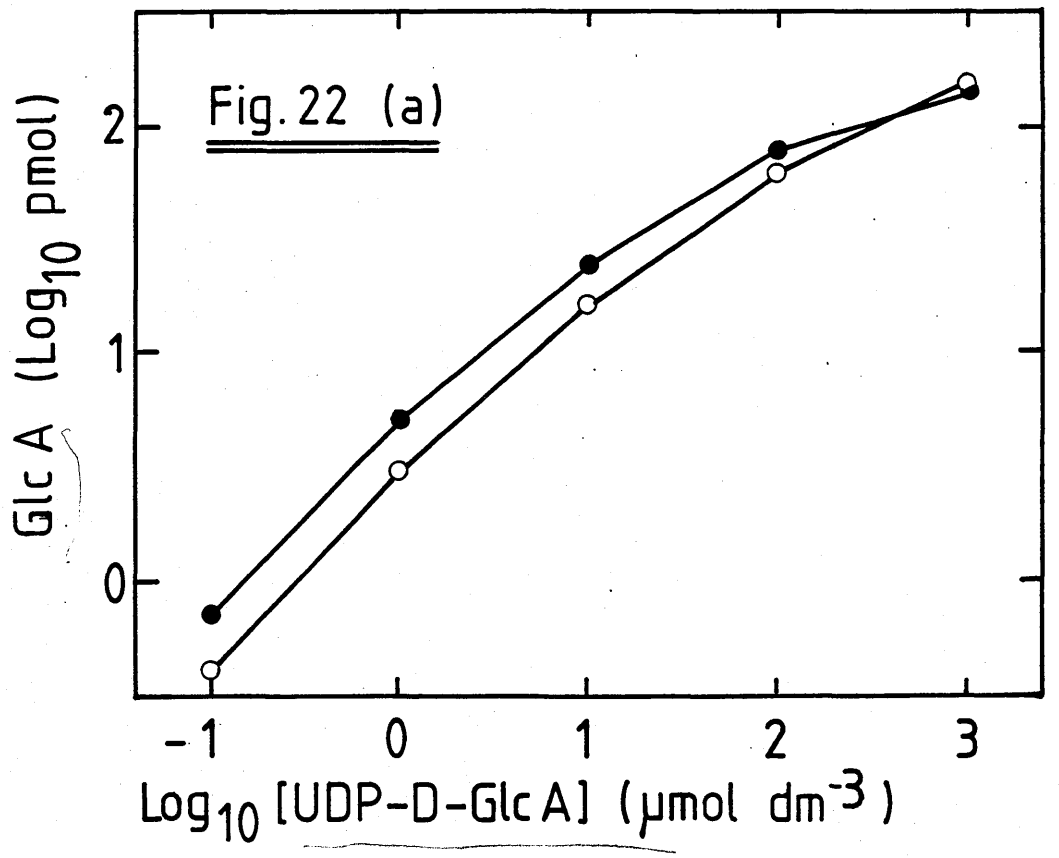


Fig. 22 (a)
Glc A (Log10 pmol)
2
1
0
-1
0
1
2
3
Log10 [UDP-D-Glc A] (µmol dm-3)

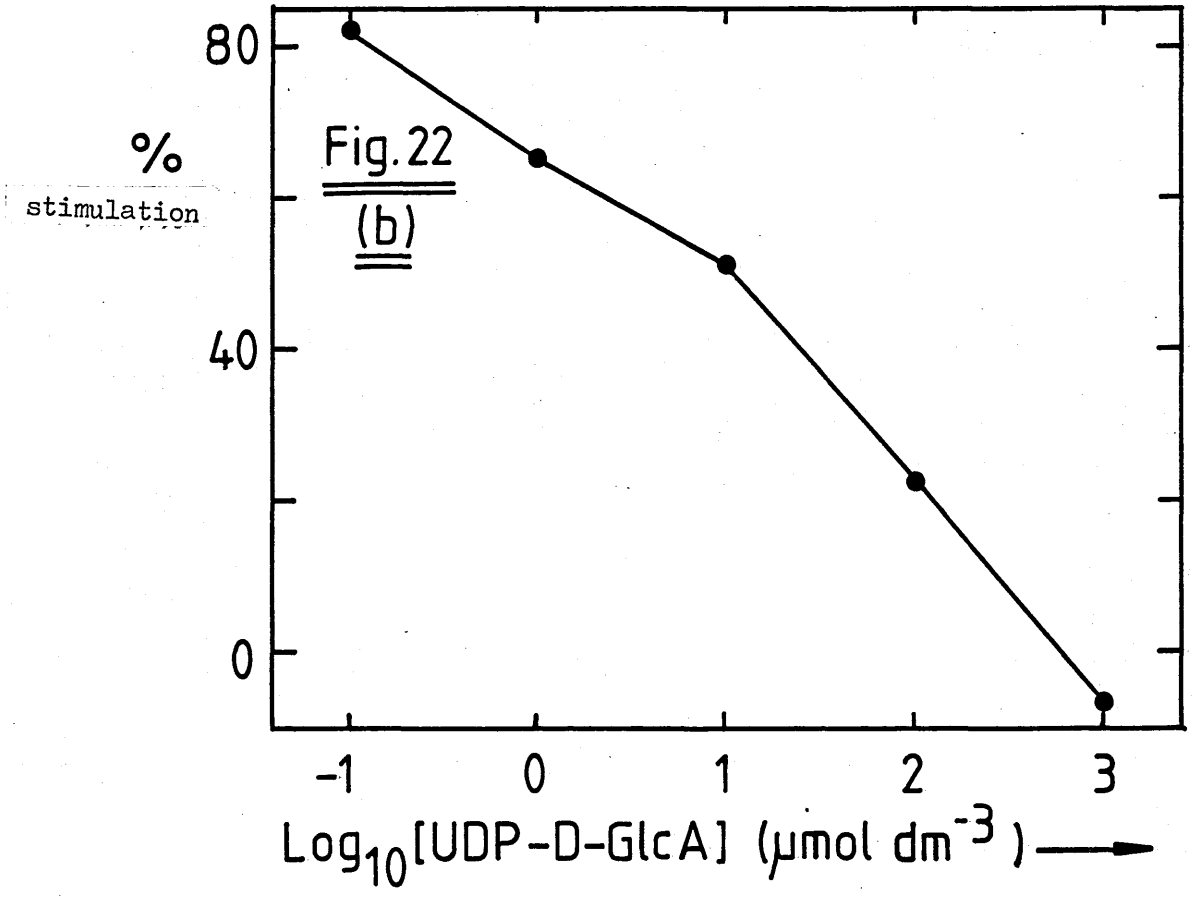


Fig. 22
(b)
%
stimulation
80
40
0
-1
0
1
2
3
Log10[UDP-D-GlcA] (µmol dm-3)

Fig.23

A 'Hanes Plot' that has been constructed with the data present in Fig. 22 to allow a valuation to be made of the Km and Vmax of the enzyme system.

Fig. 24

Effect of pre-incubation of the particulate enzyme at 25°C on the incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in the presence (●—●) and absence (o—o) of UDP-D-xylose in 2h

Fig. 23

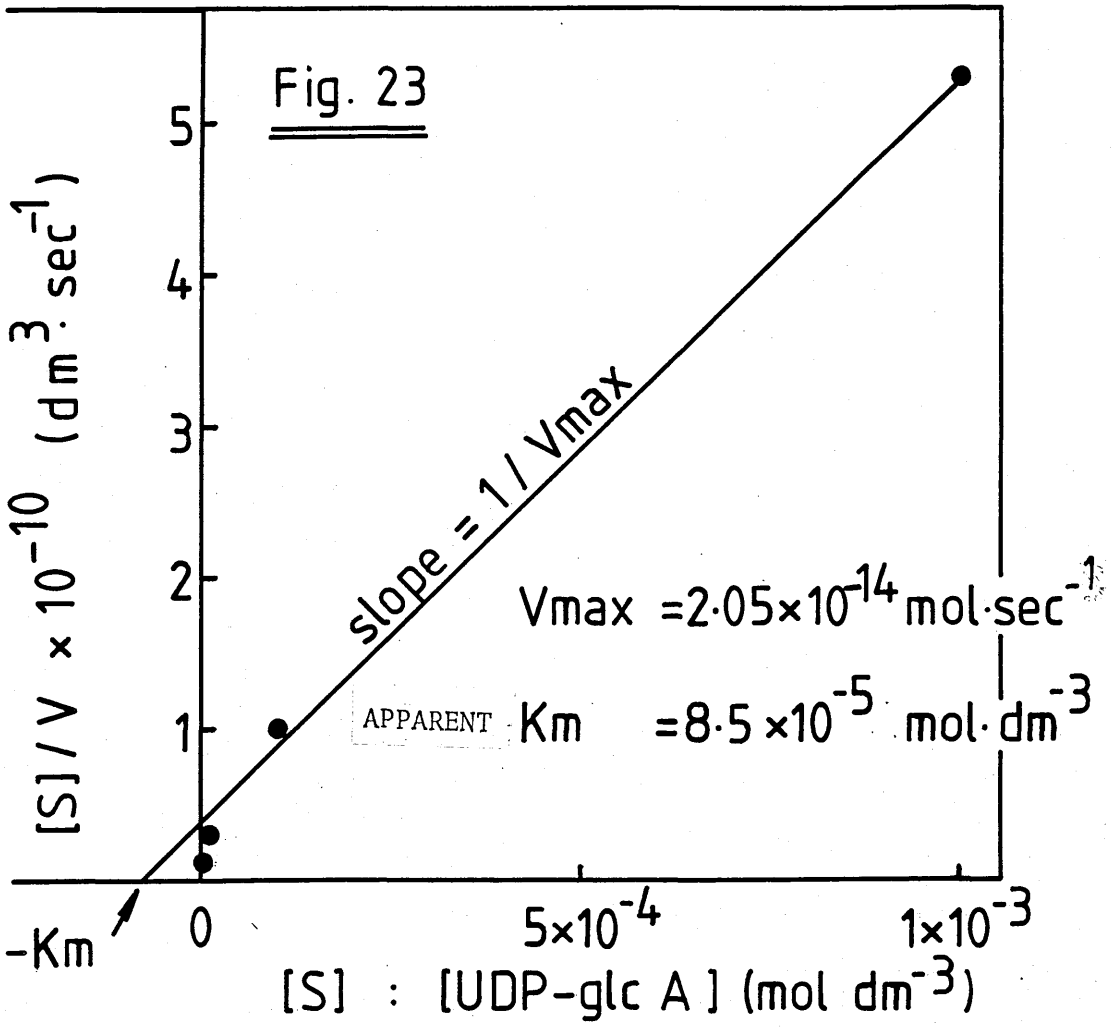

[S] / V × 10^-10 (dm^3 · sec^-1)
5
4
3
2
1
slope = 1 / Vmax
Vmax = 2·05×10^-14 mol·sec^-1
APPARENT Km = 8·5×10^-5 mol·dm^-3
-Km
0
5×10^-4
1×10^-3
[S] : [UDP-glc A] (mol dm^-3)

Fig. 24

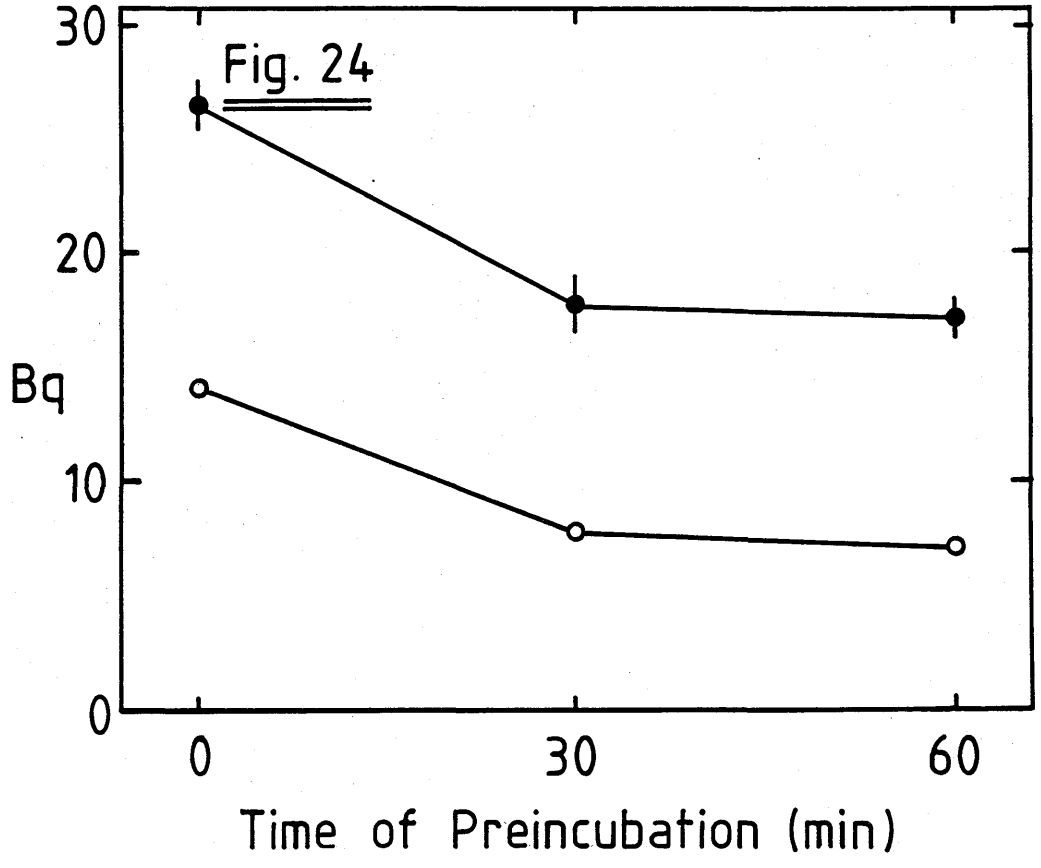

30
20
10
0
Bq
0
30
60
Time of Preincubation (min)

Table VII

Effect of freezing 97000xg membrane pellets at -20^{o}C on the ability of the subsequent enzyme preparations to incorporate radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose.

Incubations were performed in the presence of UDP-D-xylose (1mmol.dm^{-3}).

(a) Enzyme preparation made from non-frozen membrane pellets.
(b) Enzyme preparation made from membrane pellets that had been frozen for 24h.

Incubation time (min)	(a) (Non-frozen) (Bq)	(b) (Frozen) (Bq)
0	0.02 +/- 0.16	0.2 +/- 0.22
120	25.5 +/- 0.52	4.2 +/- 0.37

III Analysis of Hemicellulose Labelled with Radioactivity from UDP-D-[U-^{14}C]-glucuronic acid in the presence of UDP-D-xylose (1 mmol dm^{-3}).

The effect of protease activity on the water solubility and gel-filtration properties of the radioactive hemicellulose product

Proteinase-K digestion had no effect on the water solubility of the radioactive hemicellulose indicating that the hemicellulosic material was not a glycoprotein. Similar results were found with pronase digestion.

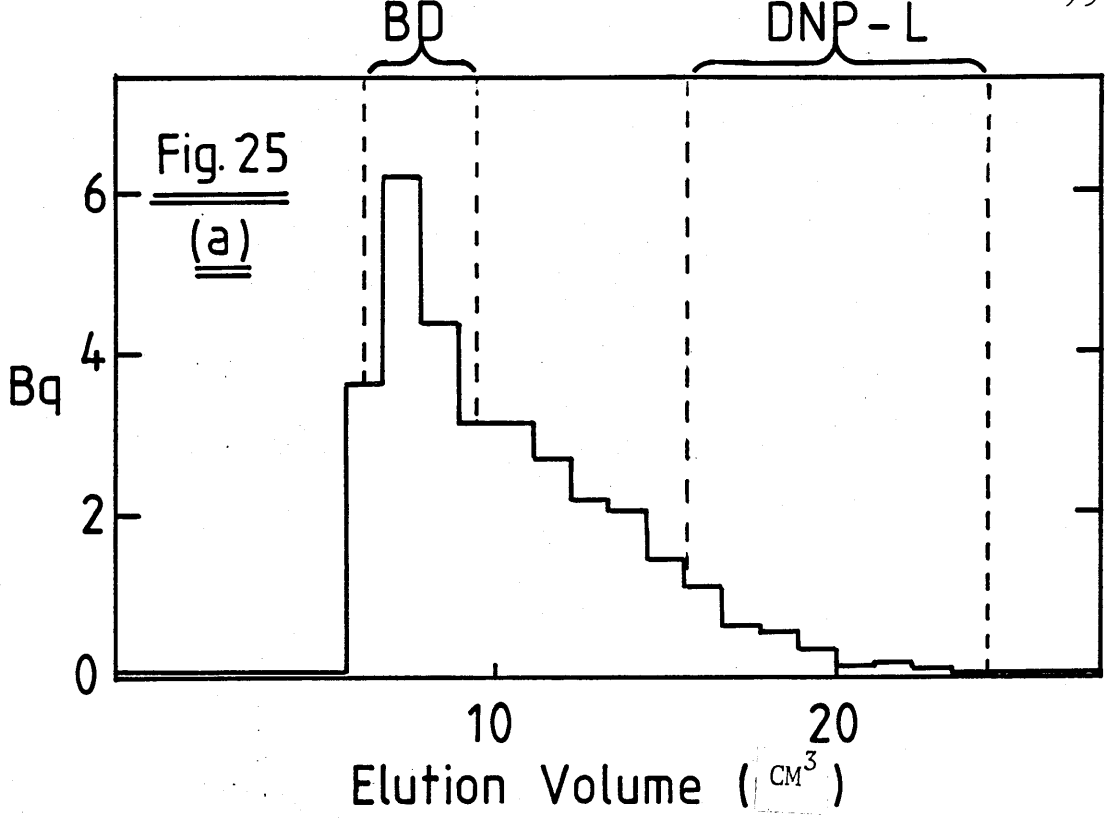
Analysis of the hemicellulose by gel filtration before and after Proteinase-K digestion supports the above conclusion (Fig. 25). A slight change in the shape of the gel filtration profiles can be seen, but because all the radioactivity is of a very high molecular weight, it is unlikely to be glycoprotein. Nevertheless, the possibility exists that some protein may be attached to the hemicellulose. The discrepancy in the total recoveries in each of the two gel filtration profiles is probably due to inaccurate pipetting of the suspended hemicellulose prior to digestion.

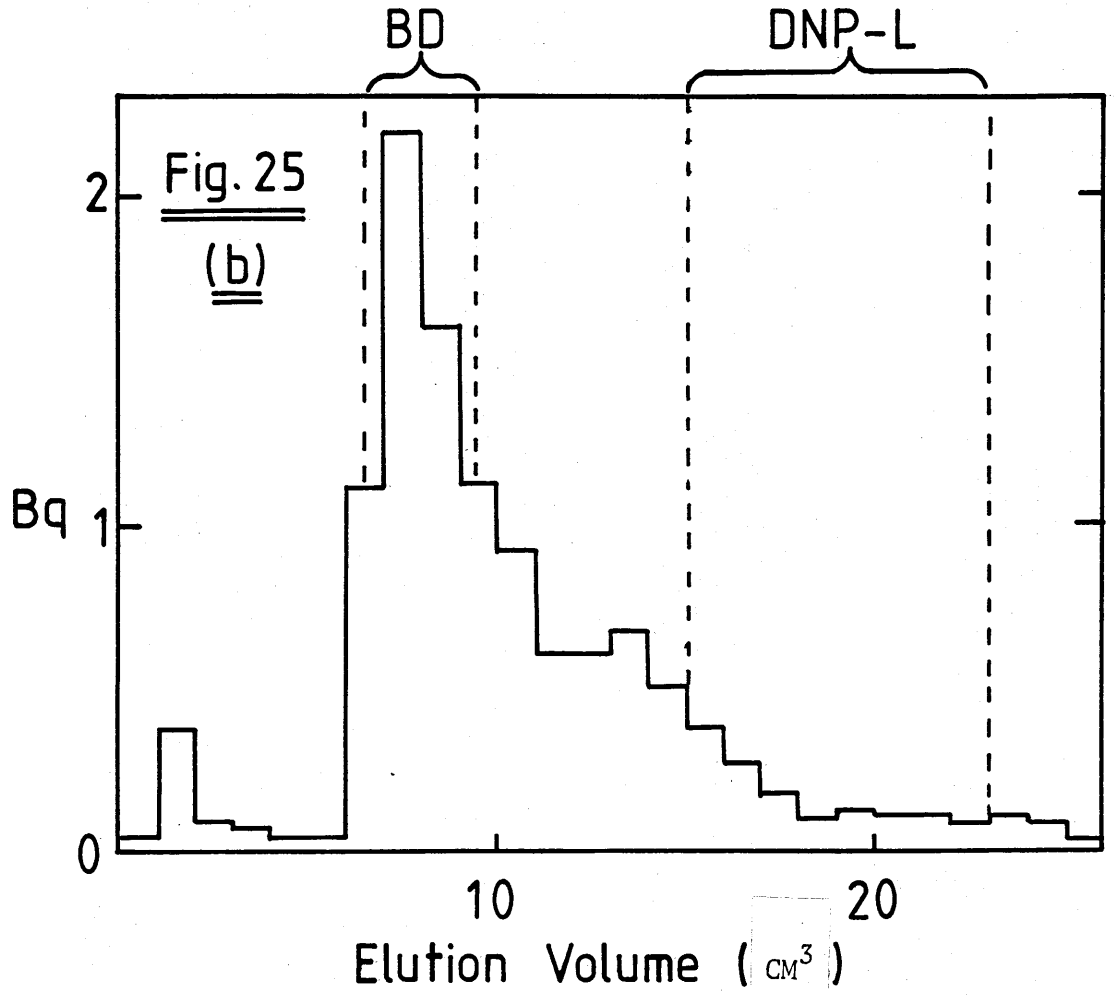
Identification of the radioactively-labelled sugar residues in the hemicellulosic product

Radioactively labelled hemicellulosic product was subjected to total acid hydrolysis and then analysed by thin

Fig. 25

Sepharose CL-2B profile of cadoxen-solubilised hemicellulose (labelled with radioactivity from UDP-D-[U-^{14}C]glucuronic acid during 2h incubations in the presence of UDP-D-xylose) before (a) and after (b) digestion by Proteinase-K . See text for details.


BD
DNP-L
Fig. 25
(a)
6
4
2
0
Bq
10
20
Elution Volume (CM3)


BD
DNP-L
Fig. 25
(b)
2
1
0
Bq
10
20
Elution Volume (CM3)

layer electrophoresis as described in Ch. 2 (p. 68). The results shown in Fig. 26 indicate that the major product is glucuronic acid. NaOH treatment is obviously necessary to hydrolyse the lactone form of the glucuronic acid back into the uronate form and this is emphasised by the differences in the electrophoretic profiles. Although the results shown in Fig. 26 seem clear cut, the total yield after hydrolysis is low (64% in (a) and 52% in (b)). This is probably due to degradation of ^{14}C -glucuronic acid during the original hydrolysis, since when UDP-D-[U-^{14}C]-glucuronic acid (463 Bq) was subjected to the same experimental procedure, the final yield of [U-^{14}C]-glucuronic acid after thin layer electrophoresis was only ablut 50% of the quantity used at the start.

Identification of the radioactively-labelled oligosaccharides produced during the partial acid hydrolysis of radioactively-labelled hemicellulose

Radioactively-labelled hemicellulose synthesised in the presence of UDP-D-xylose (1 mmol dm^{-3}) was partially hydrolysed in 2N trifluoroacetic acid for 2 hours as described (Ch. 2, p. 67). The hydrolysate was analysed by descending paper chromatography in solvent I and the results are given in Fig. 27. Several peaks are visible, and those of most interest are peaks I, II and III. Shorter periods of hydrolysis result in a relatively greater amount of radioactivity in peaks I and II, and less in peak III. This indicates that peaks I and II break down with continued hydrolysis and form the material in peak III which is acid-stable.

Fig. 26

Thin-layer-electrophoretic analysis of total acid hydrolysates of hemicellulose labelled with radioactivity incorporated from UDP-D-[U-^{14}C]glucuronic acid during 2h incubations in the presence of UDP-D-xylose (1mmol.dm^{-3}).

(a) Profile of total-acid hydrolysate.
(b) Profile of total-acid hydrolysate that has been subjected to NaOH treatment.

NaOH treatment involved the hydrolysis of glucuronolactone to glucuronic acid. This was performed by subjecting the glucuronolactone (in the total acid hydrolysate) to NaOH (0.33 mol.dm^{-3}) for 1h before electrophoresis.

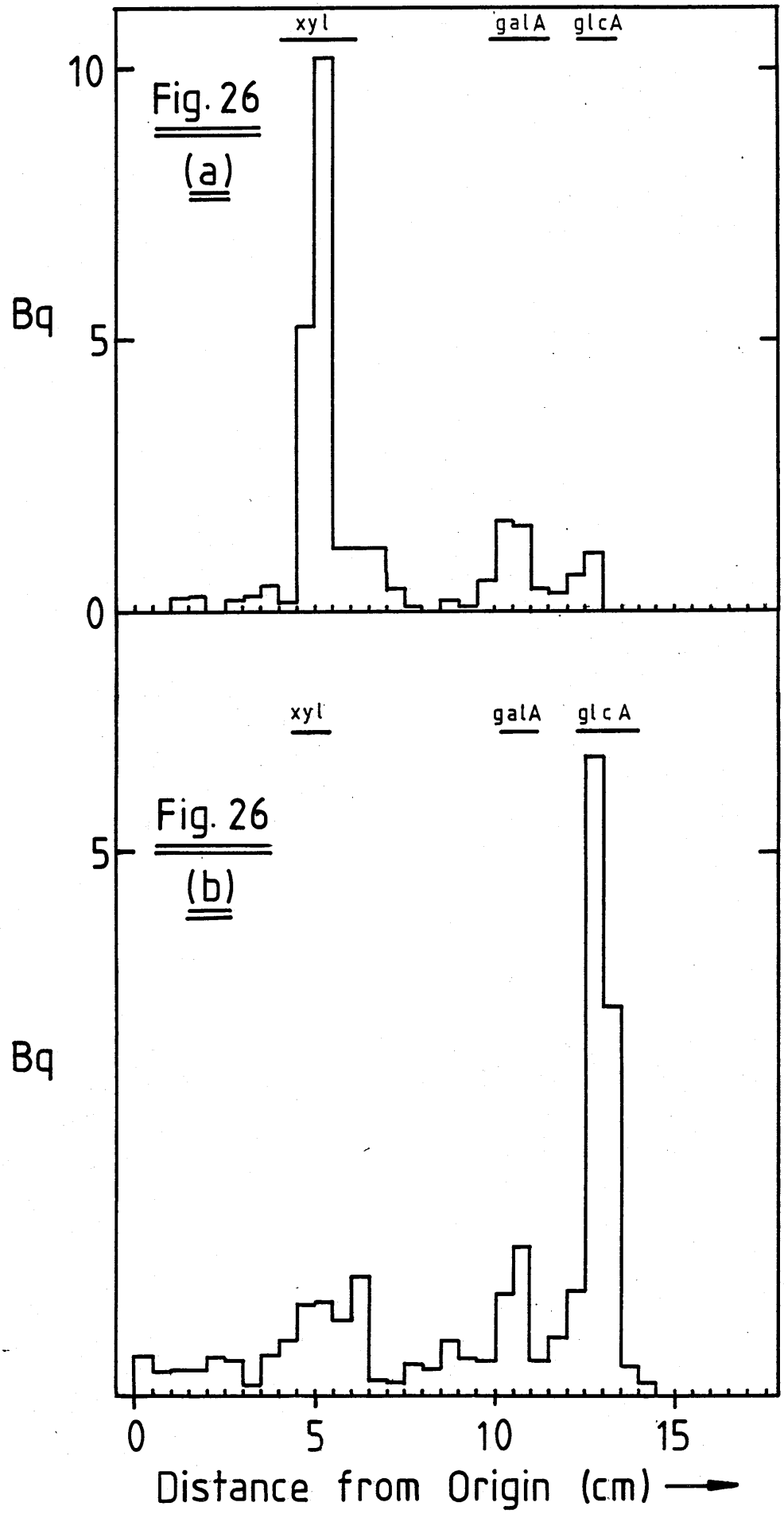

xyl
galA
glcA
10
Fig. 26
(a)
Bq
5
0
xyl
galA
glcA
Fig. 26
(b)
5
Bq
0
5
10
15
Distance from Origin (cm)

Fig. 27

Paper-chromatographic analysis of partial acid hydrolysates of hemicellulose labelled with radioactivity incorporated from UDP-D-[U-^{14}C]glucuronic acid during 2h incubations in the presence of UDP-D-xylose (1mmol.dm^{-3}).

(a) Profile of a 1h hydrolysate.

(b) Profile of a 2h hydrolysate.

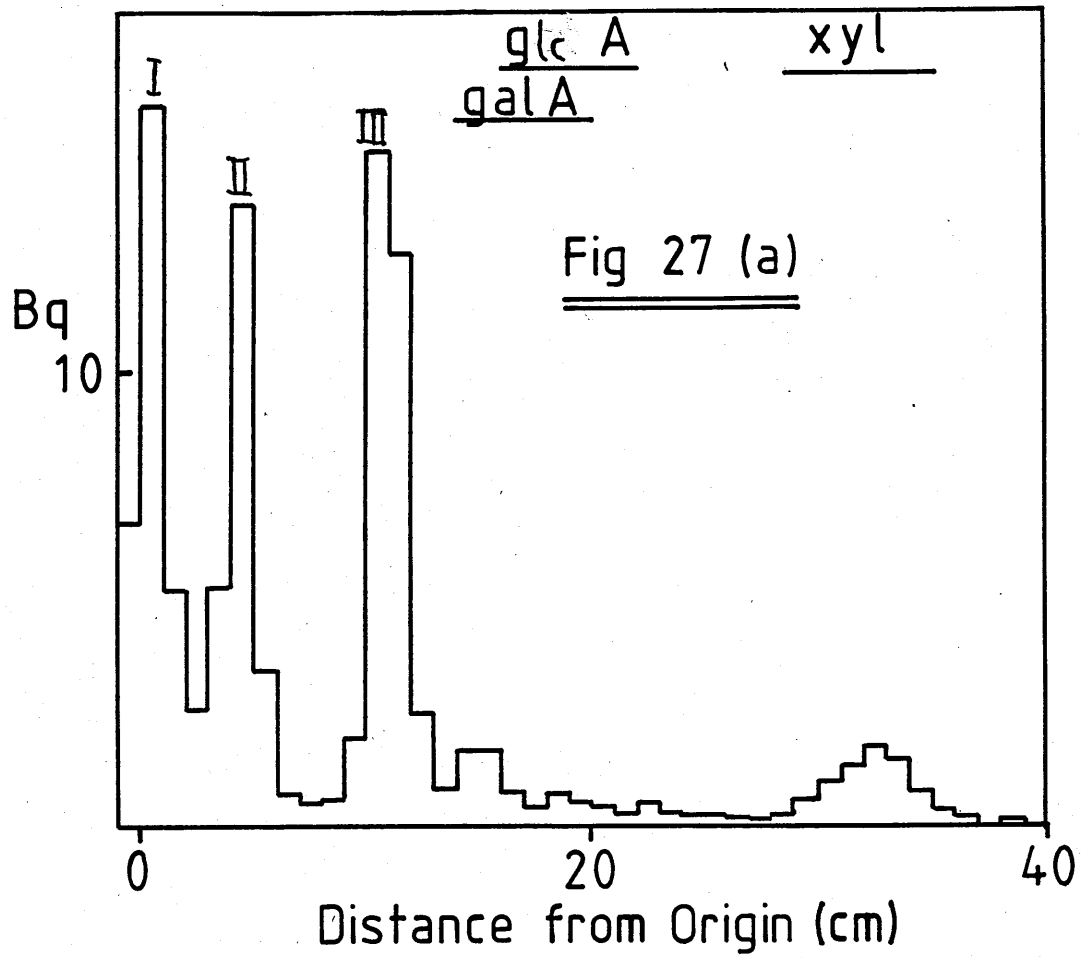


Fig 27 (a)

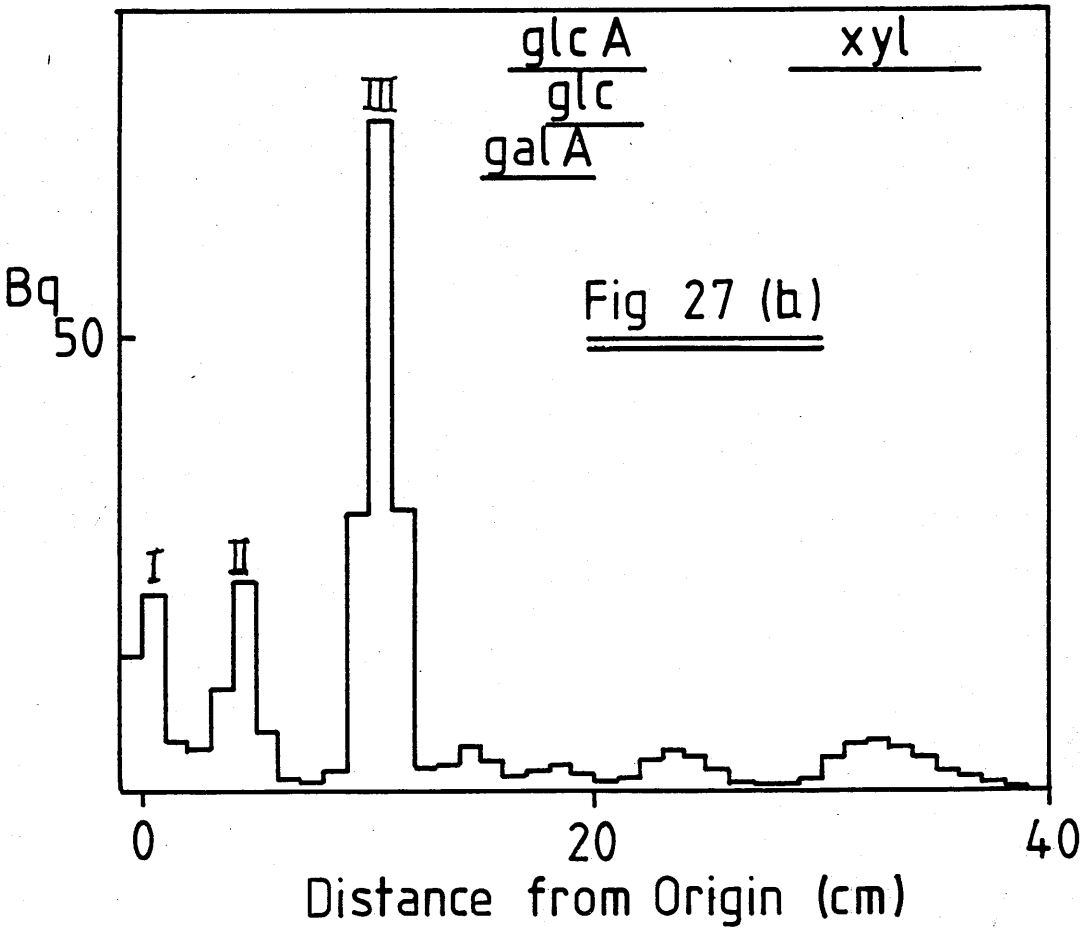


Fig 27 (b)

Studies on the cell walls of oat-coleoptile cell walls by Ray and Rottenberg (1964) involved partial acid hydrolysis and analysis of glucuronoxylan fragments using the same chromatographic system. The R_{xylose} values for certain oligosaccharides are illustrated in Table VIII. If the present enzyme system was transferring [U-^{14}C]-glucuronic acid onto a xylan polymer, then one would expect partial acid hydrolysis to create an acid-stable disaccharide of glucuronic acid $\alpha 1 \rightarrow 2$ linked to xylose, and an acid-unstable trisaccharide of glucuronic acid $\alpha 1 \rightarrow 2$ linked to xylose $\beta 1 \rightarrow 4$ linked to xylose. Peak III in Fig. 27 is definitely acid-stable indicating a covalent bond between a uronic acid and some other saccharide, and peak II in Fig. 27 is acid-unstable. However, the R_{xylose} values of Peaks II and III are 0.14 and 0.32 respectively, which is a little different to those given for the corresponding di- and tri-saccharides of interest in Table VIII. This difference was originally thought to be due to the difficulties experienced with this system as reported by Ray and Rottenberg (1964) but was shown at a later stage in the project to have been due to a slight error when making the chromatographic solvent. Instead of pure formic acid, only 90% (w/w) had been used and this was enough to produce the differences shown. If the solvent was made correctly, then peak III ran with an identical R_{xylose} value to that of glucuronic acid linked $\alpha 1 \rightarrow 2$ to xylose. Peak II also corresponded to glucuronic acid linked $\alpha 1 \rightarrow 2$ to xylose linked $\beta 1 \rightarrow 4$ to xylose. For the purposes of this thesis, the original solvent I (made with 90% (w/w) formic acid) has been used throughout to allow direct comparability

Table VIII

R_{xylose} values of mono- and oligosaccharides after separation by descending paper chromatography in solvent I.

Mono- and oligosaccharides	R_{xylose}
GlcA-(1-2)-Xyl-(1-4)-Xyl	0.19
GlcA-(1-2)-Xyl	0.38
MeGlcA-(1-2)-Xyl-(1-4)-Xyl	0.38
GalA	0.63
GlcA	0.68
MeGlcA-(1-2)-Xyl	0.84
MeGlcA	1.37

Taken from Ray and Rottenberg, 1964.

between the experimental results.

Further analysis of the radioactively-labelled residues in peaks II and III of Fig. 27

Glucuronic acid is known to be difficult to hydrolyse from polysaccharides (Kauss and Hassid, 1967a), but if it is reduced to glucose, the molecule can be freed easily by acid hydrolysis. The radioactive oligosaccharides in peaks II and III were eluted and then treated as described in Ch. 2 (p. 68). Methanolysis of the uronic acids was achieved by subjecting them to methanol containing HCl (1 mol dm^{-3}) at 100 C for 1 h. The methyl ester produced was subsequently dried under vacuum and reduced with $NaBH_4$ to convert it to glucose. Acid hydrolysis liberated any ^{14}C-glucose which was then identified by paper chromatography.

The results for the analysis of peak III by the above procedure are given in Fig. 28(a). The wide band of radioactivity in strips 19-36 of the chromatogram were eluted, de-ionised and re-run in solvent II to give a peak that co-chromatographed with D-glucose (Fig. 28(b)). The large peak of radioactivity (strips 0-10) was also re-eluted and run in solvent I where it was shown to consist of peak III oligosaccharide and some glucuronic acid. This indicates that the reduction and methylation process was not totally efficient, probably as a result of water in the HCl which would hydrolyse any methyl-ester linkages.

Fig. 28
Identification of the radioactive monosaccharides present in peak III of Fig. 27.

(a) Analysis of the radioactivity in peak III of Fig. 27 by paper chromatography in solvent II after treatment as described in the text.
(b) Analysis of radioactivity in strips 19 to36 of the chromatogram in Fig. 28 (a) by paper chromatography in solvent II.

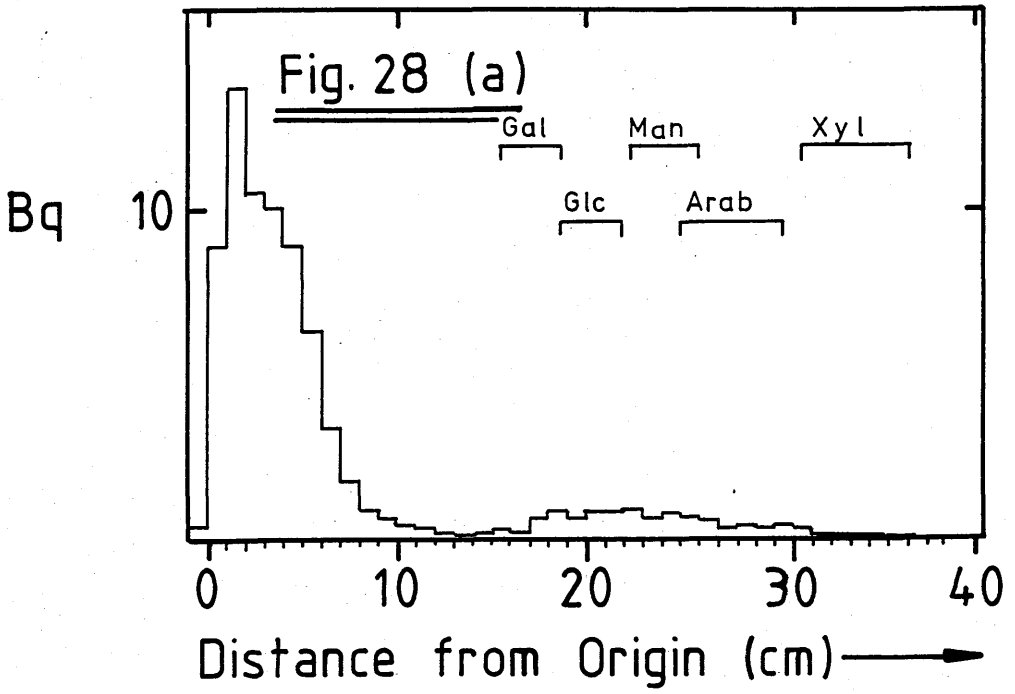
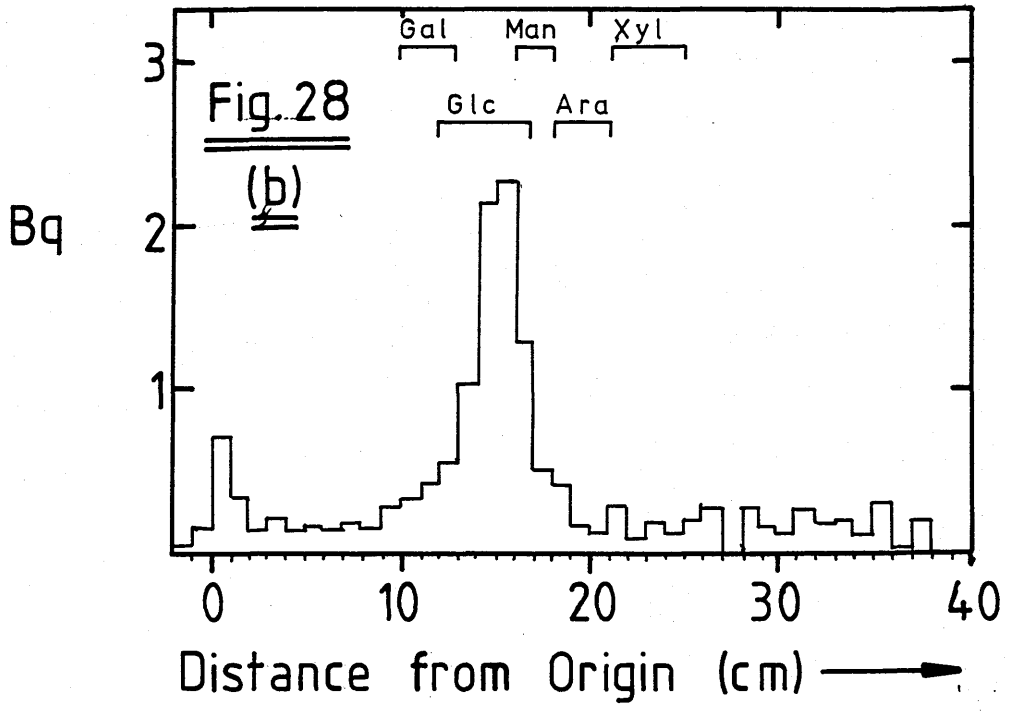


Fig. 28 (a)
Gal
Man
Xyl
Glc
Arab
Bq
10
0
10
20
30
40
Distance from Origin (cm)


Gal
Man
Xyl
Fig. 28
(b)
Glc
Ara
Bq
3
2
1
0
10
20
30
40
Distance from Origin (cm)

The results for the analysis of peak II were similar to those for peak III. Fig. 29(a) shows the first chromatogram and Fig. 29(b) gives the profile of material from strips 8-16 of Fig. 29(a) after being re-eluted and re-run in the same solvent.

IV Conclusions

The results show that pea epicotyls contain a membrane-bound glucuronyltransferase that transfers glucuronic acid from UDP-D-glucuronic acid to a hemicellulosic product. The following evidence indicates that this product is glucurono-xylan.

The product is not a glycoprotein. This is shown by its resistance to digestion by two non-specific proteinases, Pronase and Proteinase-K. However, there may be protein covalently or otherwise attached to some of the radioactive product, suggested by the slight change in the profile of cadoxen-solubilised hemicellulose on sepharose CL-2B after digestion with Proteinase-K.

The effect of UDP-D-xylose on the incorporation of glucuronic acid from UDP-D-glucuronic acid suggests that the product is a glucuronoxylan. In the absence of UDP-D-xylose, the incorporation of glucuronic acid ceases after approximately 15 minutes. In the presence of UDP-D-xylose, the incorporation of glucuronic acid is prolonged for at least

Fig. 29

Identification of the radioactive monosaccharides that are present in peak II of Fig. 27.

(a) Analysis of the radioactivity in peak II of Fig. 27 by paper chromatography in solvent II after the treatment described in the text.

(b) Analysis of the radioactivity in strips 8-16 of the chromatogram in Fig. 29 (a) by paper chromatography in solvent II.

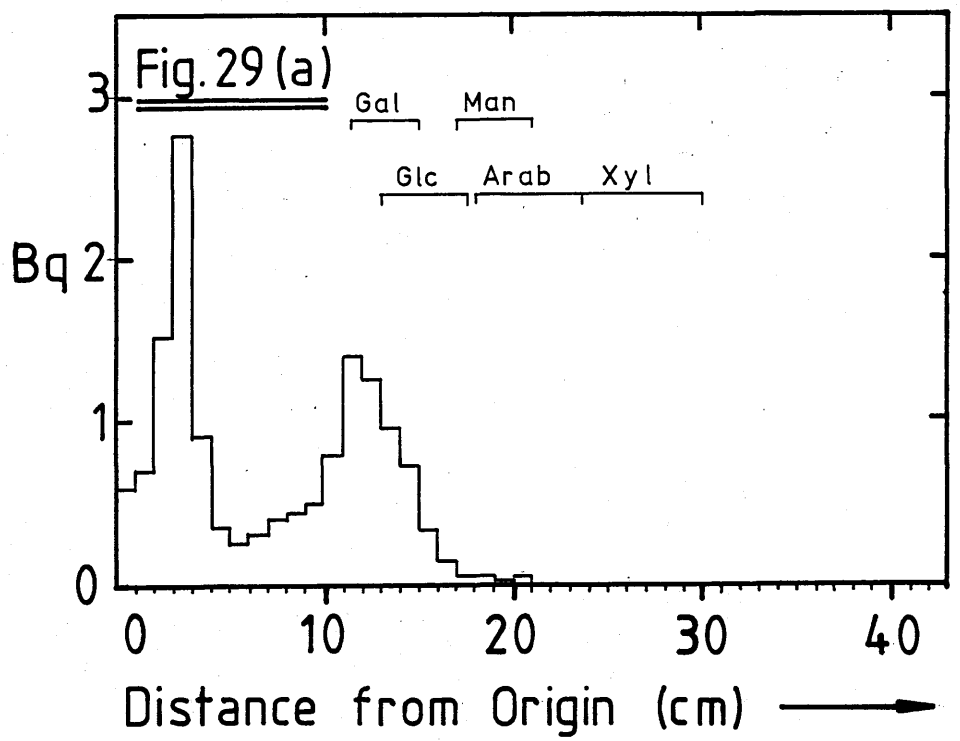


Fig. 29 (a)
Gal
Man
Glc
Arab
Xyl
Bq
3
2
1
0
0
10
20
30
40
Distance from Origin (cm)

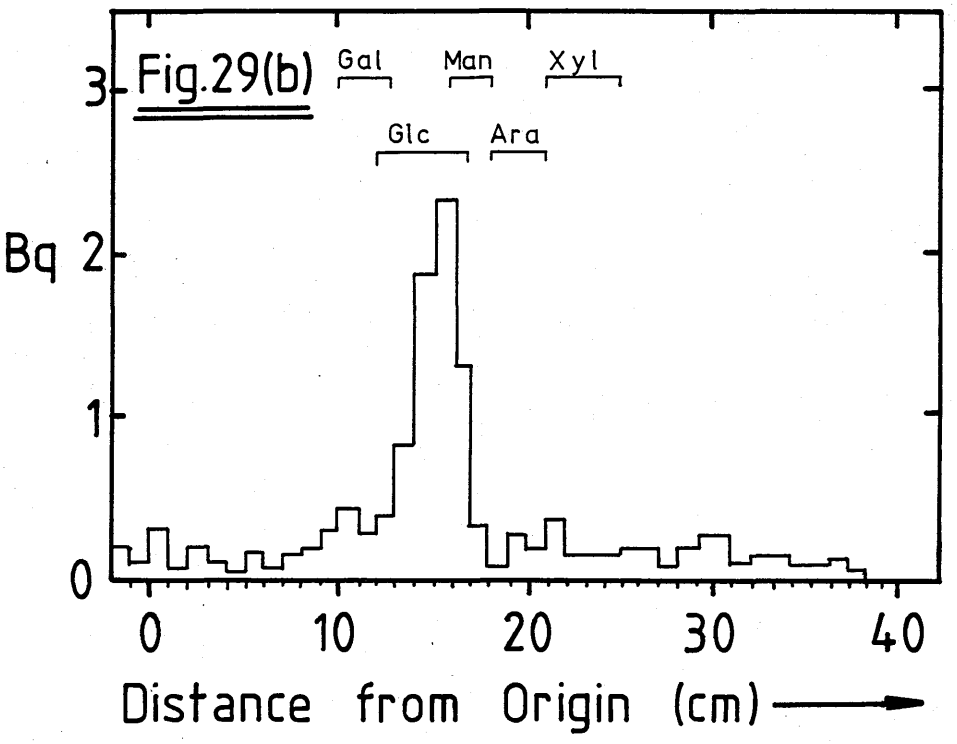


Fig.29(b)
Gal
Man
Xyl
Glc
Ara
Bq
3
2
1
0
0
10
20
30
40
Distance from Origin (cm)

8 hours. These observations might be explained in the following ways, (1) UDP-D-xylose might be an allosteric activator of the glucuronyl transferase: the termination of the glucuronic acid incorporation after 15 minutes would then have to be due to the degradation of endogenous UDP-D-xylose by another enzyme in the membrane preparation. (2) UDP-D-xylose might protect the glucuronyl transferase from denaturation, which would occur within 15 minutes of incubation in the absence of UDP-D-xylose. (3) UDP-D-xylose might be required as a second sugar donor for the formation of the product.

The first and second explanations can be ruled out by preincubation of the enzyme (Fig. 24) before addition of UDP-D-glucuronic acid. The preincubated enzyme is still able to incorporate glucuronic acid into the product, showing that neither degradation of an activator nor denaturation of the enzyme had occurred. Denaturation of the enzyme in the absence of UDP-D-xylose can be further ruled out by the fact that UDP-D-xylose causes incorporation of glucuronic acid to resume, even when added after 75 minutes of incubation with UDP-D-glucuronic acid. The third explanation, that UDP-D-xylose is a second sugar donor, seems the most likely one. This would imply that the product is a glucuronoxylan. If it is assumed that glucuronoxylan is also being synthesised in the absence of UDP-D-xylose, then the incorporation seen in the absence of added UDP-D-xylose, could be due to a small amount of endogenous UDP-D-xylose present in the membrane preparation, or it could be due to the presence in

the membrane preparation of a small amount of non-glucuronidated xylan which could act as an acceptor for a limited amount of glucuronic acid. Addition of UDP-D-xylose would bring about the formation of further xylan chains, each capable of accepting further glucuronic acid side chains.

The slight, but reproducible, inhibition of the glucuronyltransferase by UDP-D-xylose, which is observed at short incubation times (Fig. 16), may be due to competitive inhibition. This would not be surprising, since the UDP-D-glucuronic acid and UDP-D-xylose differ only in the presence of a carboxyl group in the former, and UDP-D-xylose is present at a much higher concentration than was UDP-glucuronic acid. The decrease in the effect of UDP-D-xylose on the glucuronyltransferase at high UDP-glucuronic acid concentration (Fig. 22) might have been due to the reverse effect, namely competitive inhibition of the xylosyl transferase by UDP-D-glucuronic acid.

The effect of bivalent cations on the glucuronyltransferase is similar to the effect of these cations on the methylation of glucuronic acid residues in glucuronoxylan of corn cobs (Kauss & Hassid, 1967(a)). It might be, therefore, that glucuronidation of xylans and the methylation of the resulting glucuronoxylans are controlled in parallel in the plant cell by the levels of bivalent cations.

Chapter 4.

DISTRIBUTION OF GLUCURONYLTRANSFERASE ACTIVITY ALONG DARK-GROWN PEA (*PISUM SATIVUM*) EPICOTYLS.

I Introduction

The experiments discussed in the present chapter were designed to assess the distribution of glucuronyltransferase activity along pea epicotyls. This permitted the distribution of the active transferase enzymes to be compared with the state of differentiation of epicotyl cells and tissues.

II Results and discussion

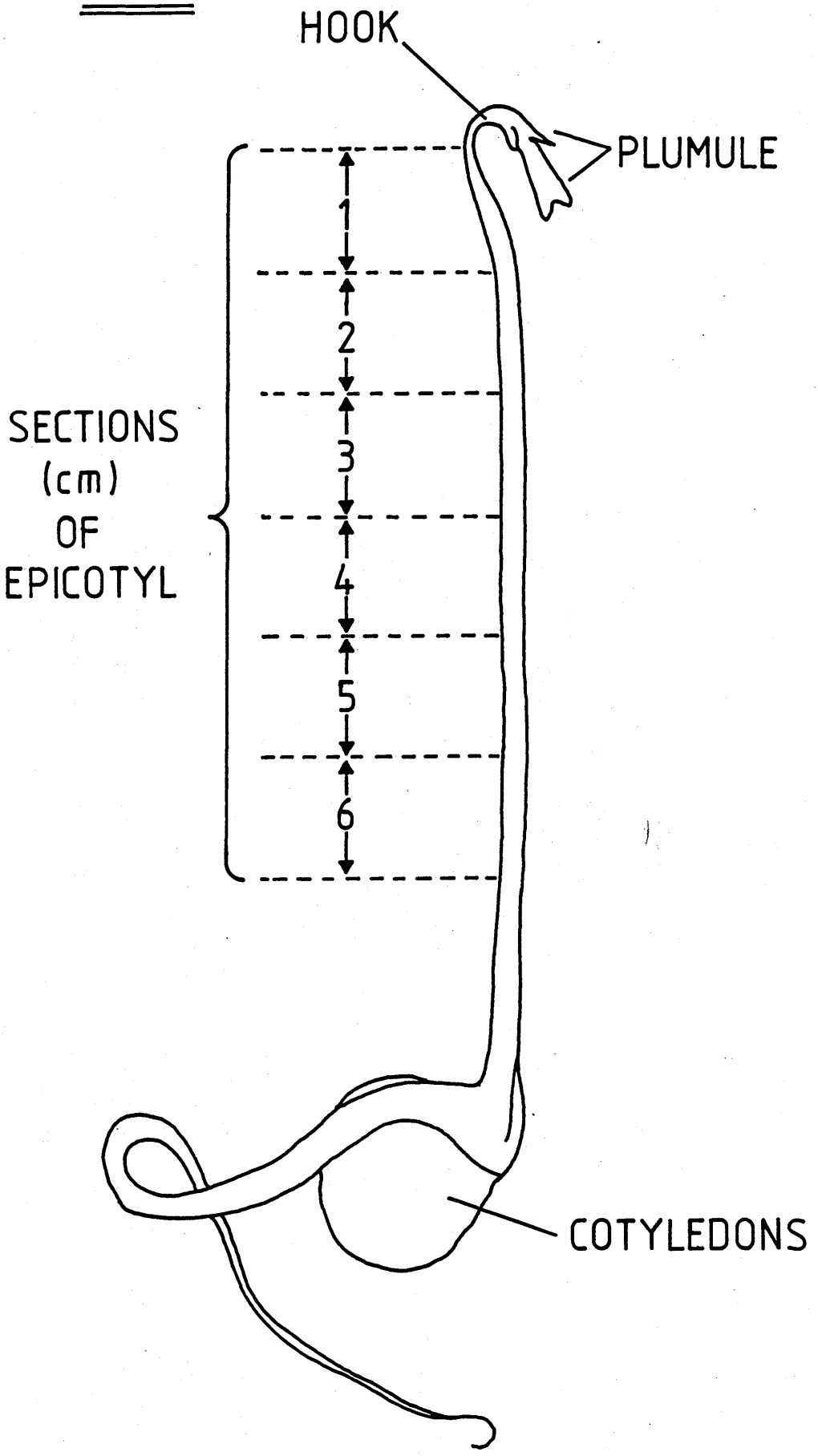
The results are summarised graphically in Figs. 30-35 and pictorially in Plates 1 to 8. In Ch. 3 it was shown that in the absence of UDP-D-xylose, the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose ceased within 30 minutes. In the presence of UDP-D-xylose, the incorporation was continuous for up to 8 h. Therefore, in 4 h incubations, any differences in incorporation brought about by enzyme preparations made from different regions of the epicotyl would reflect the distribution of active glucuronyltransferase.

The profile of total glucuronyltransferase activity was investigated (Fig. 31). The activity was greatest in the second 1 cm epicotyl section and continued at a high level in the third. It then decreased progressively along the remainder of the epicotyl. The profile of glucuronyltransferase activity per g tissue (Fig. 33) showed a smaller difference between the activities in sections 1 and 2. This was due to the low weight of tissue present in section 1.

Fig. 30

A diagram of a dark-grown pea epicotyl showing the sections from which particulate enzyme was prepared in order to assay the glucuronyltransferase along the epicotyl.

Fig. 30



Sectioning Epicotyl

Fig. 31

Profile of total incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose by enzyme prepared from the different regions of the epicotyl. Incubations were performed in the presence (●——●) and absence (o—o) of UDP-D-xylose.

Fig. 32

A graph to illustrate the % stimulation of incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose by UDP-D-xylose, and the elongation capacity of individual epicotyl sections in water over a period of 24h.

(△——△): %stimulation by UDP-D-xylose.

(○——○): % elongation in water.

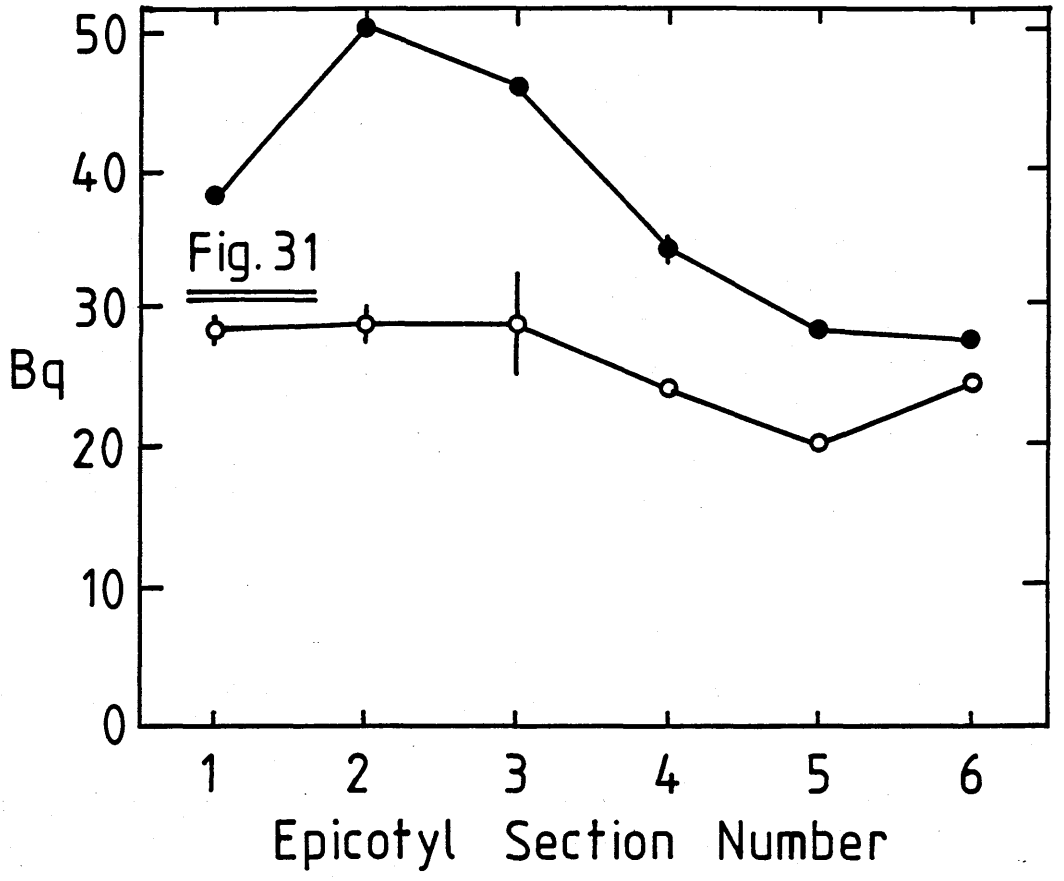


Fig. 31
Bq
50
40
30
20
10
0
1
2
3
4
5
6
Epicotyl Section Number

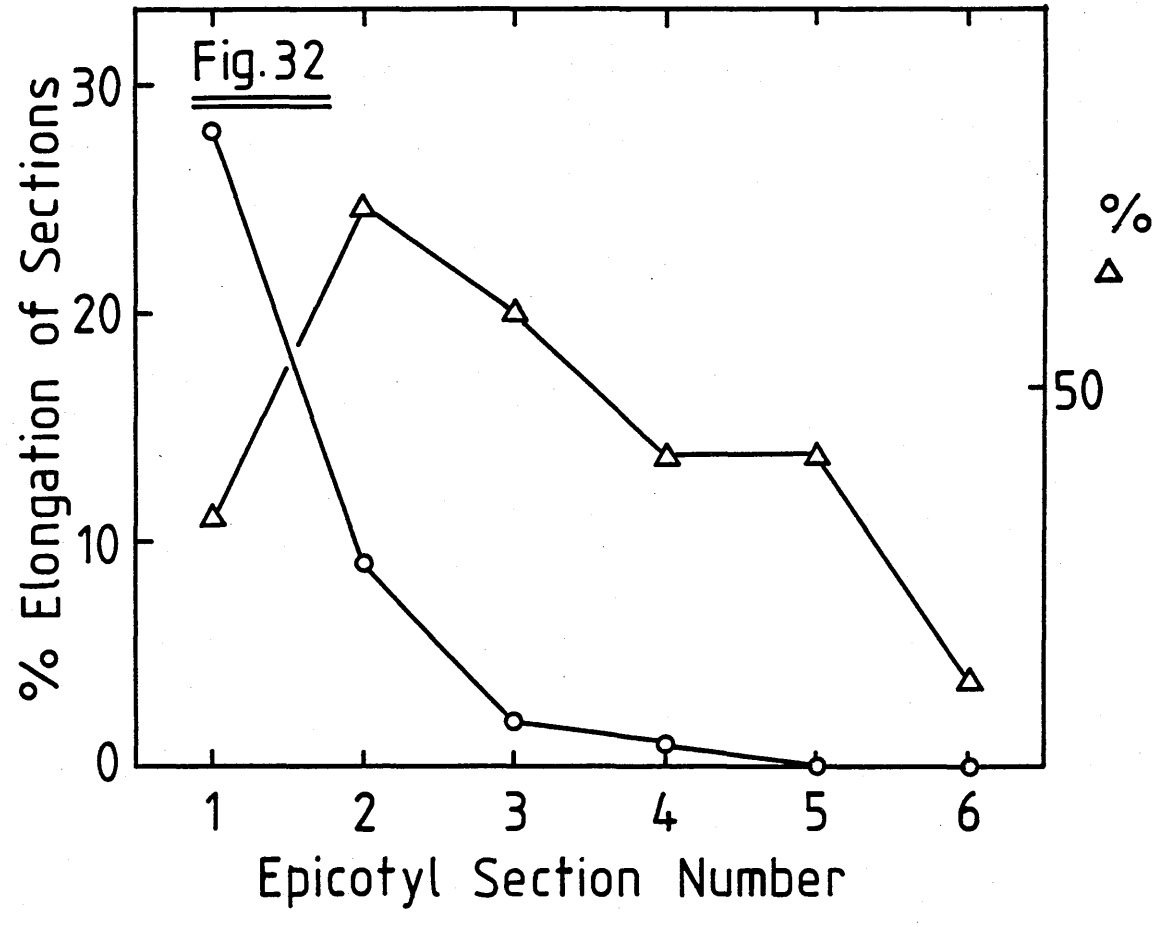


Fig. 32
% Elongation of Sections
30
20
10
0
%
50
1
2
3
4
5
6
Epicotyl Section Number

Fig. 33

Profile of the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid by enzyme prepared from different regions of the epicotyl. Incubations were performed in the presence (●—●) and absence (o—o) of UDP-D-xylose.(1mmol.dm^{-3}).

(a) Incorporation into hemicellulose,

(b) Incorporation into pectin.

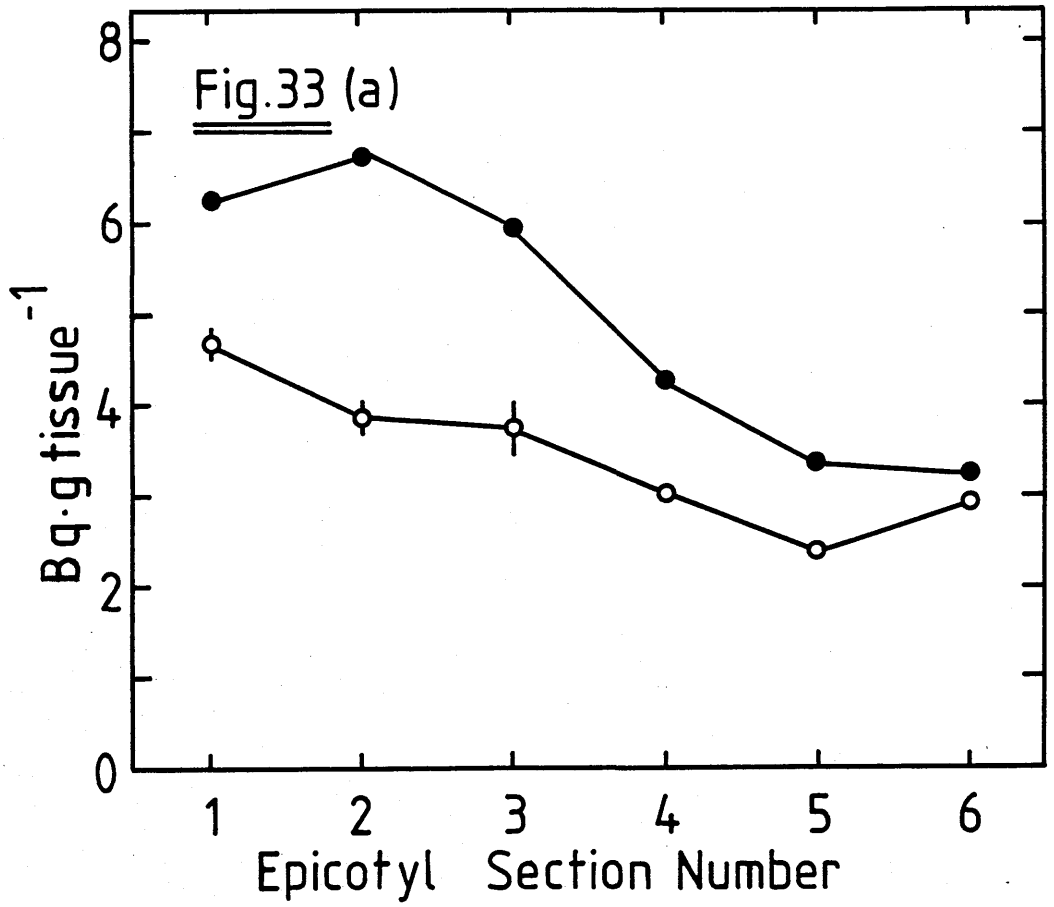


Fig.33 (a)
Bq·g tissue^{-1}
0
2
4
6
8
1
2
3
4
5
6
Epicotyl Section Number

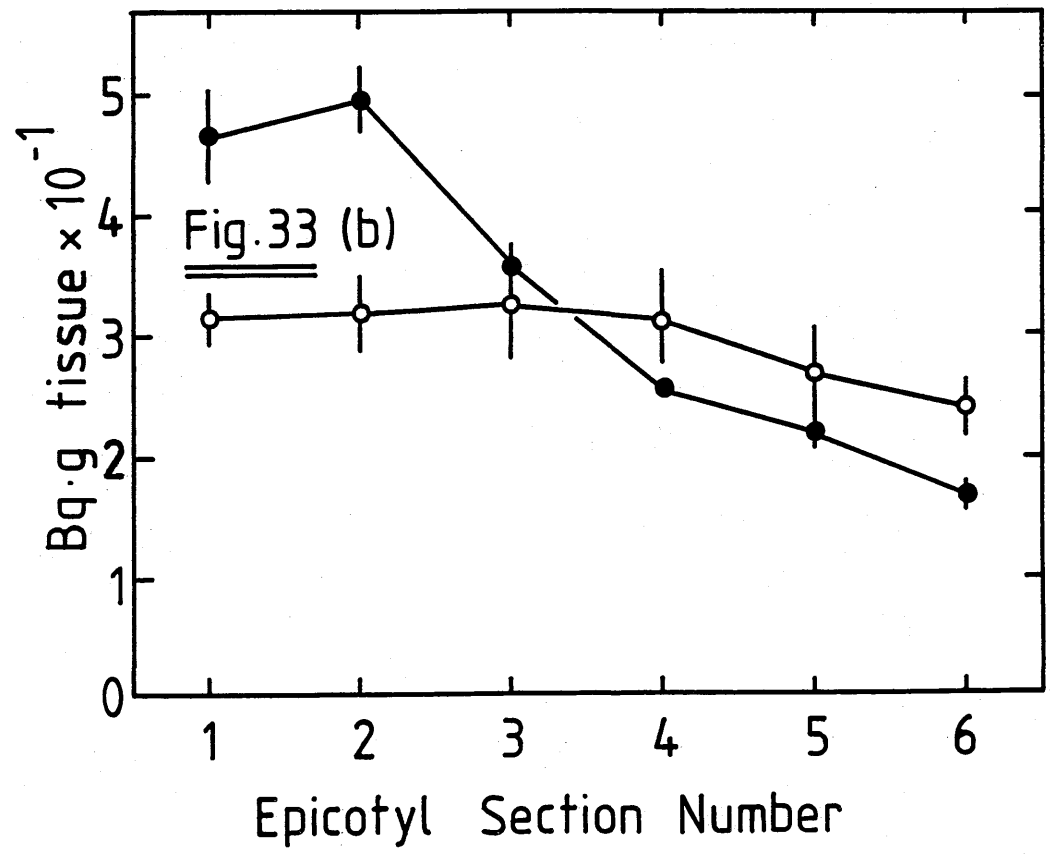


Fig.33 (b)
Bq·g tissue x 10^{-1}
0
1
2
3
4
5
1
2
3
4
5
6
Epicotyl Section Number

The profile of tissue elongation was rather different (Fig. 32). Elongation capacity was greatest in the first 1 cm epicotyl section. It then dropped to a low level in the second, and became negligible in the remaining sections. This corresponded to the states of cell wall structure shown in Plates 1 to 8.

Membrane-bound protein had the distribution shown in Fig. 34. In the top 1 cm section the protein concentration was high, but as cells elongated, the concentration fell (sections 2, 3 and 4). This decrease resulted in a dramatic increase in the specific activity of glucuronyl transferase in sections 2 to 4 relative to section 1 (Fig. 35).

The incorporation of radioactivity into high-molecular-weight pectin (Fig. 33) was similar to the incorporation into hemicellulose.

III Conclusions

In Ch. 5 it will be shown that at pH 6.0, radioactivity from UDP-D-[U-^{14}C]-glucuronic acid is not incorporated into only glucuronoxylan hemicellulose. However, the results presented in this chapter demonstrate that the UDP-D-xylose stimulatory effect is still present and this indicated that glucuronoxylan biosynthesis is occurring. The results confirm that glucuronoxylan is present in the secondary wall of this tissue. This conclusion may be made because primary cell-wall synthesis is thought to occur in young elongating

Fig.34

Protein present in the enzyme preparations made from the different regions of the epicotyl.

Protein concentration (△——△)

Protein per g tissue (○——○).

Fig. 35

Specific activity of the enzyme preparations incorporating radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose in the presence (●——●) and absence (o——o) of UDP-D-xylose.

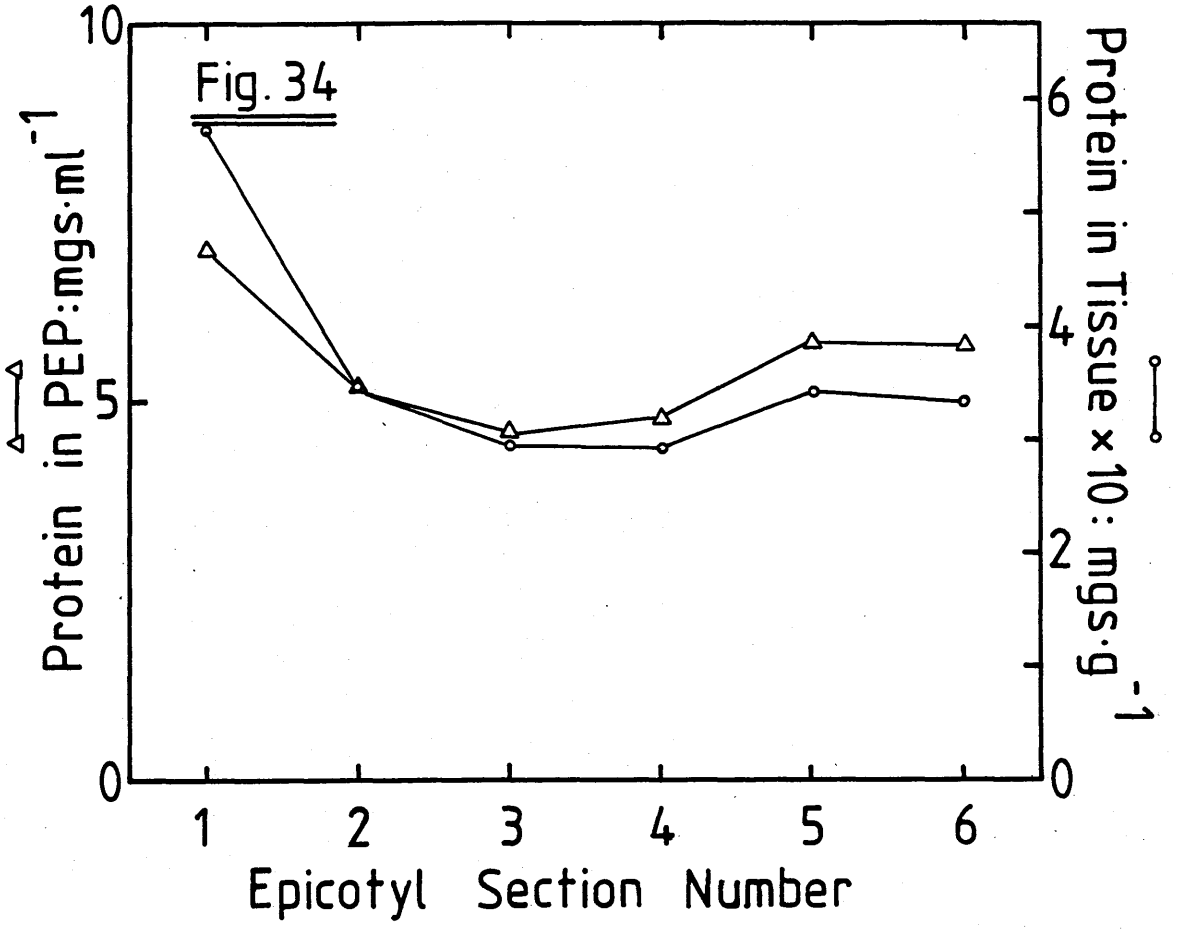


Fig. 34
Protein in PEP: mgs·ml⁻¹
Protein in Tissue × 10 : mgs·g⁻¹
10
5
0
6
4
2
0
1
2
3
4
5
6
Epicotyl Section Number

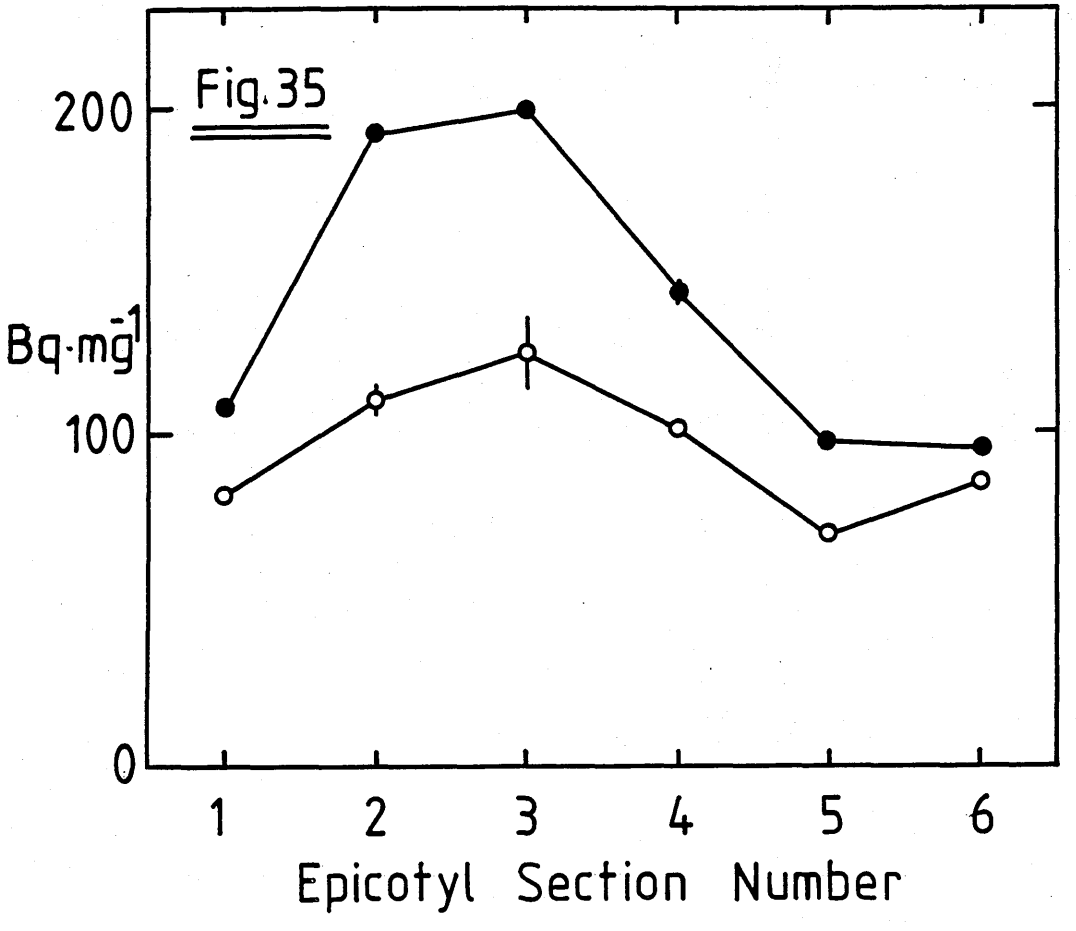


Fig. 35
200
100
0
Bq·mg⁻¹
1
2
3
4
5
6
Epicotyl Section Number

cells and to cease after elongation has ended, while secondary walls are generally laid down in non-elongating cells. The glucuronyltransferase activity is, however, present in large amounts in the elongating epicotyl sections. This suggests either (1) that glucuronoxylan is present in the primary cell wall (as identified by Darvill et al, 1980), or (2) that some cells that are present in the elongating tissue have already started secondary cell-wall biosynthesis, or (3) that glucuronyltransferase which is present in the elongating zone is not active *in vivo*. Suggestions (1) and (2) seem to be the most likely reasons for the activity present in section 1.

The photographs in plates 1 to 8 show the cell walls of cells from different regions of the epicotyl.

The vascular tissue (plates 1,3,4,6,8) always contains cells with much thicker cell walls than those of the parenchyma (plates 2,5,7). Also, the thickness of the walls of cells in the vascular tissue increases dramatically as the distance from the apex increases (plates 3 and 8). This reflects the increase in secondary thickening parenchyma cells have relatively thin cell walls and these do not increase greatly in thickness as the distance from the apex increases (plates 2 and 7). Layering of cell wall constituents is less evident in these cells when compared with cells of vascular tissue.

Plate 1

Transverse section of vascular tissue taken from the top of the first epicotyl section. (x 6000)

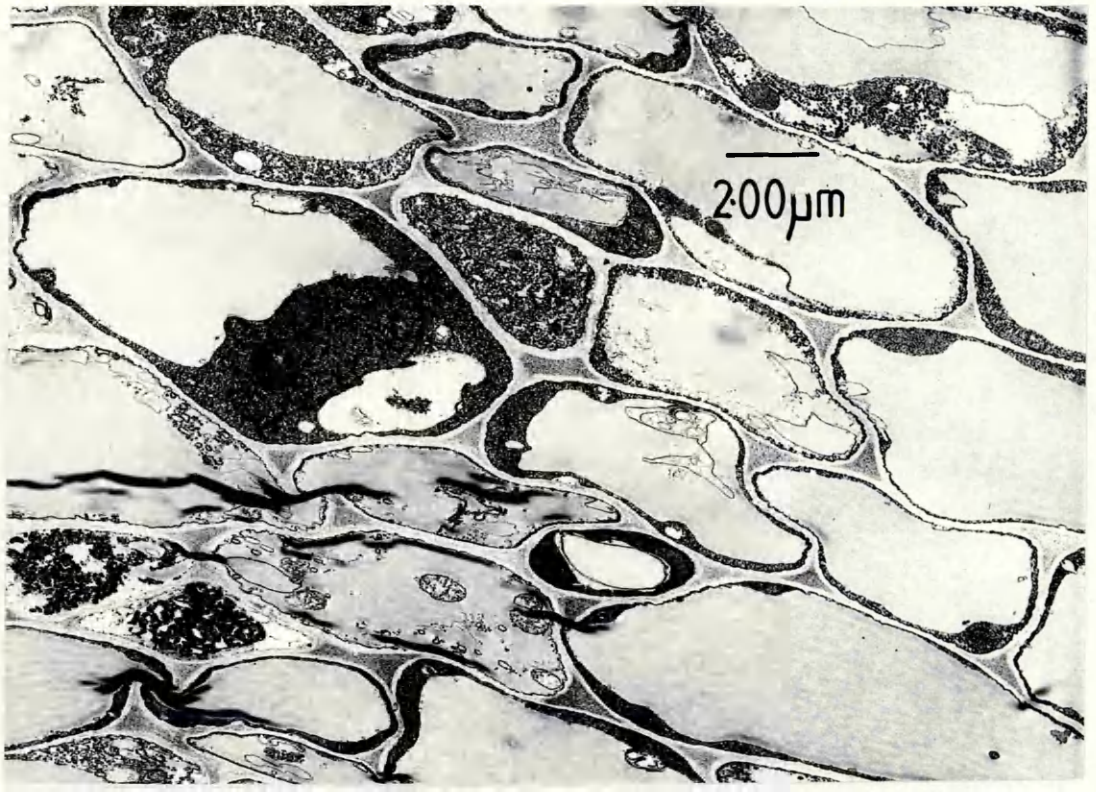


Plate 2

Transverse section of a parenchymal-cell wall taken from the top of the first epicotyl section. (x 43200)

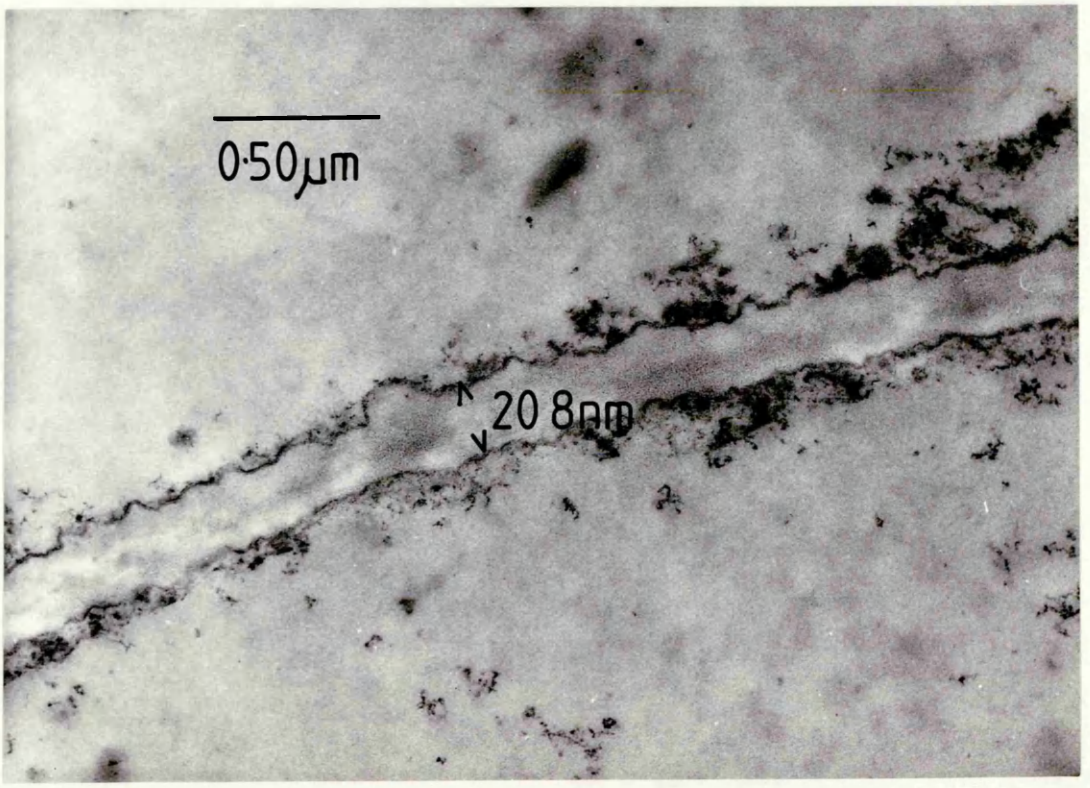


Plate 3

Transverse section of cell walls of vascular tissue taken from the top of the first epicotyl section. (x 25200)

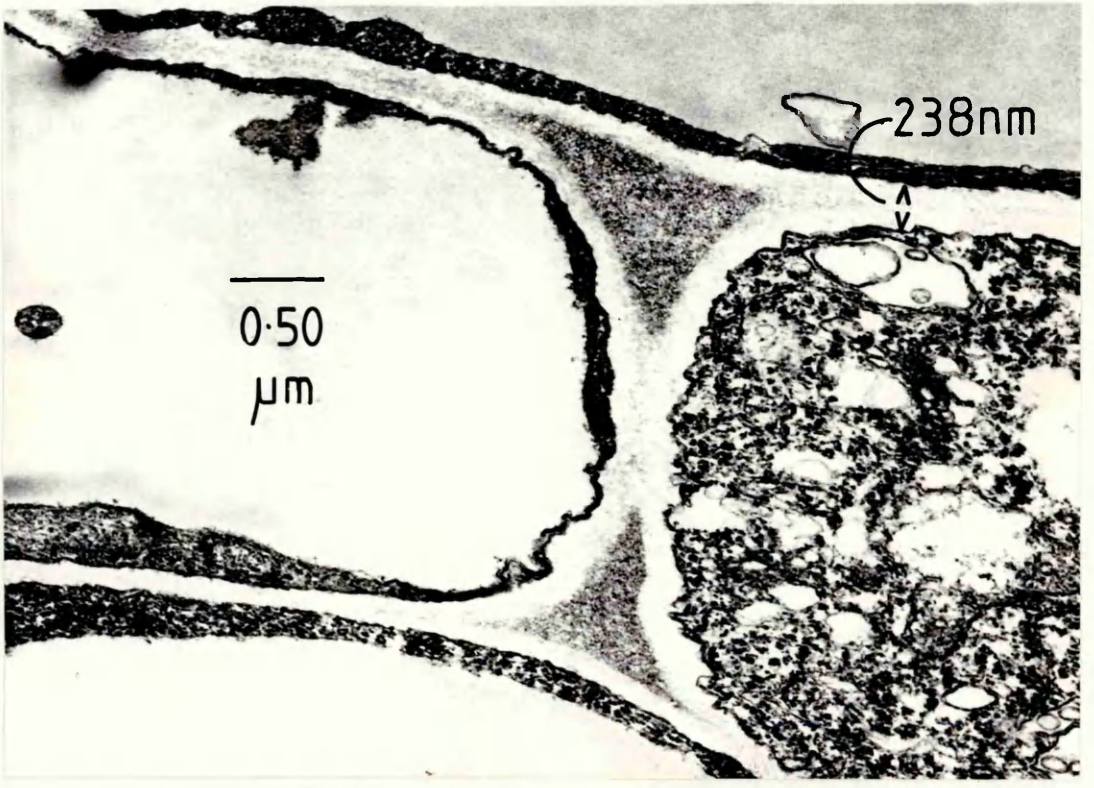


Plate 4

Transverse section of vascular tissue taken from the bottom of the second epicotyl section. (x 4500)

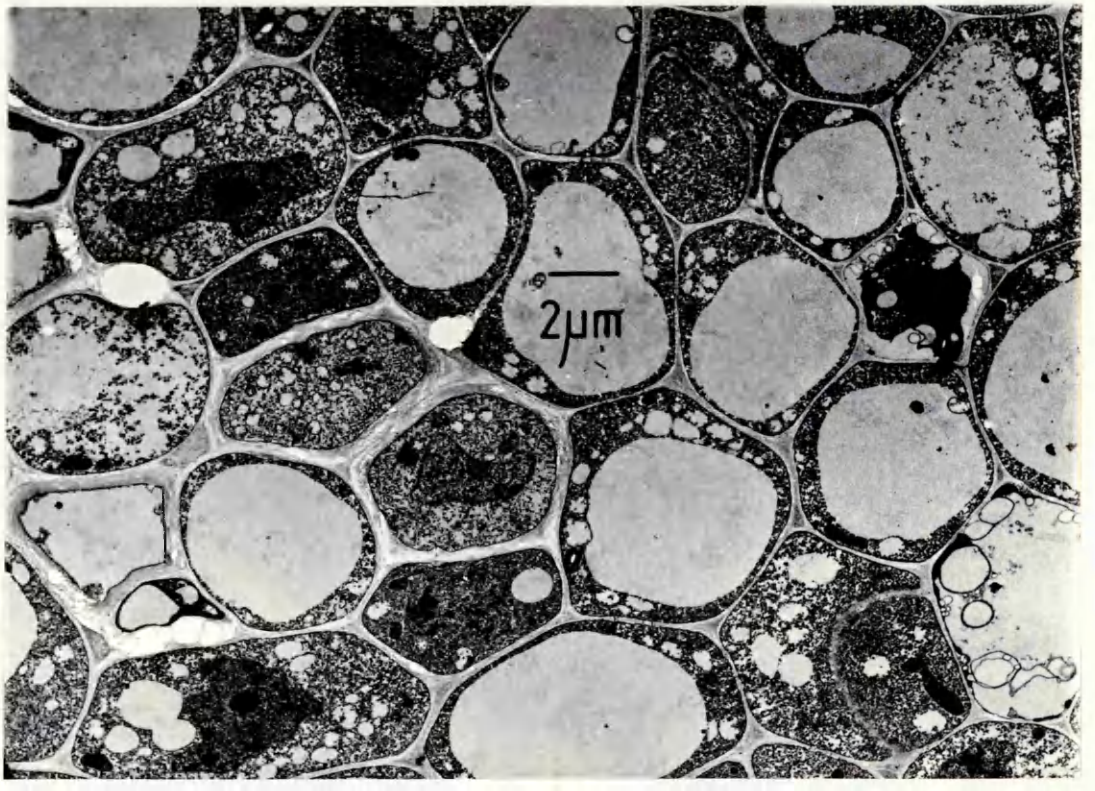


Plate 5

Transverse section of cell walls of parenchymal tissue taken from the bottom of the second epicotyl section. (x 43200)

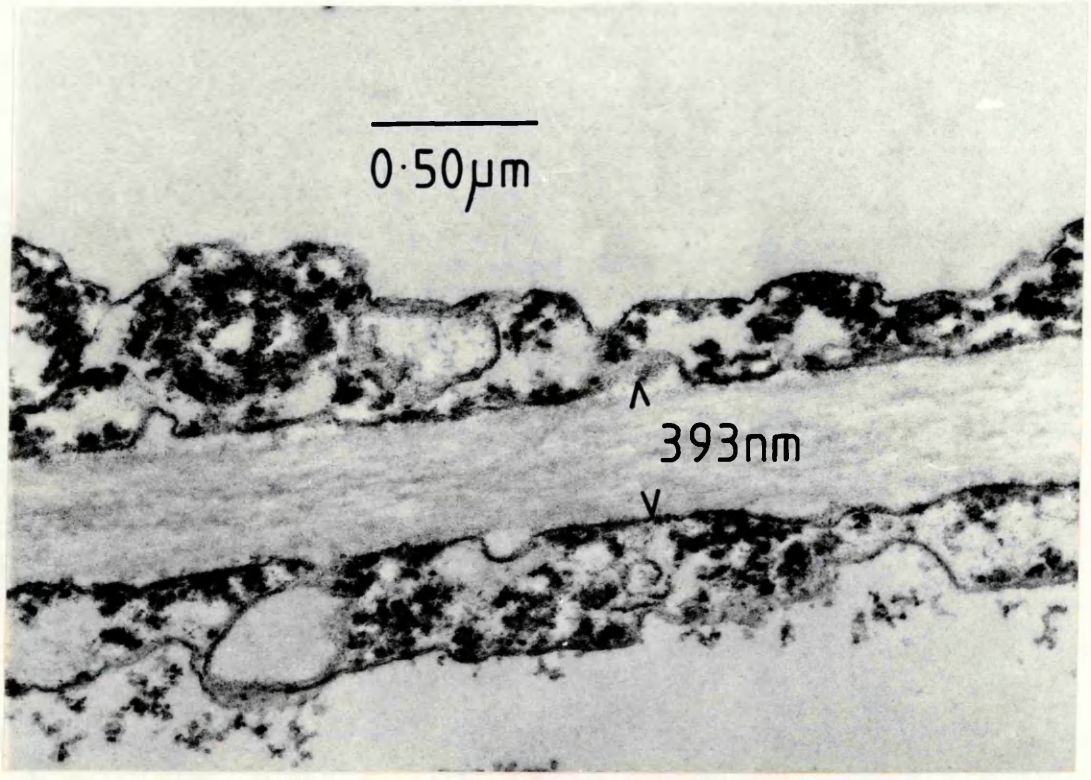


Plate 6

Transverse section of cell walls of vascular tissue taken from the bottom of the second epicotyl section. (x 12000)

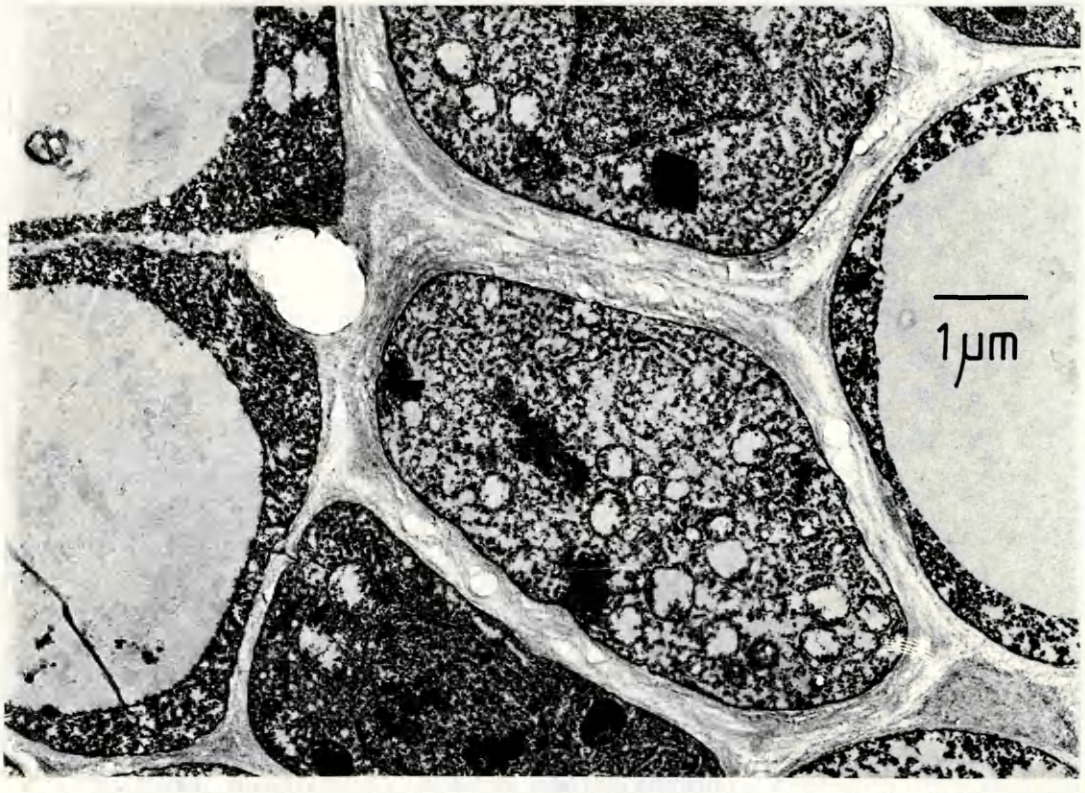


Plate 7

Transverse section of cell walls of parenchymal tissue taken from the sixth epicotyl section. (x 43200)

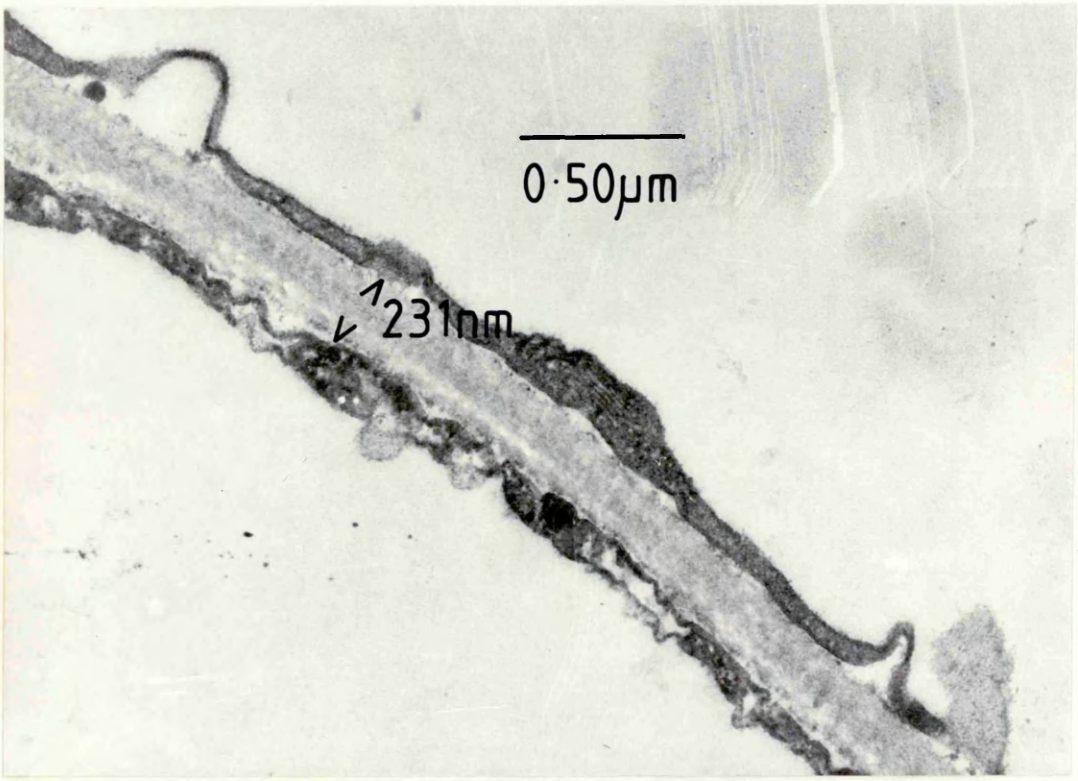
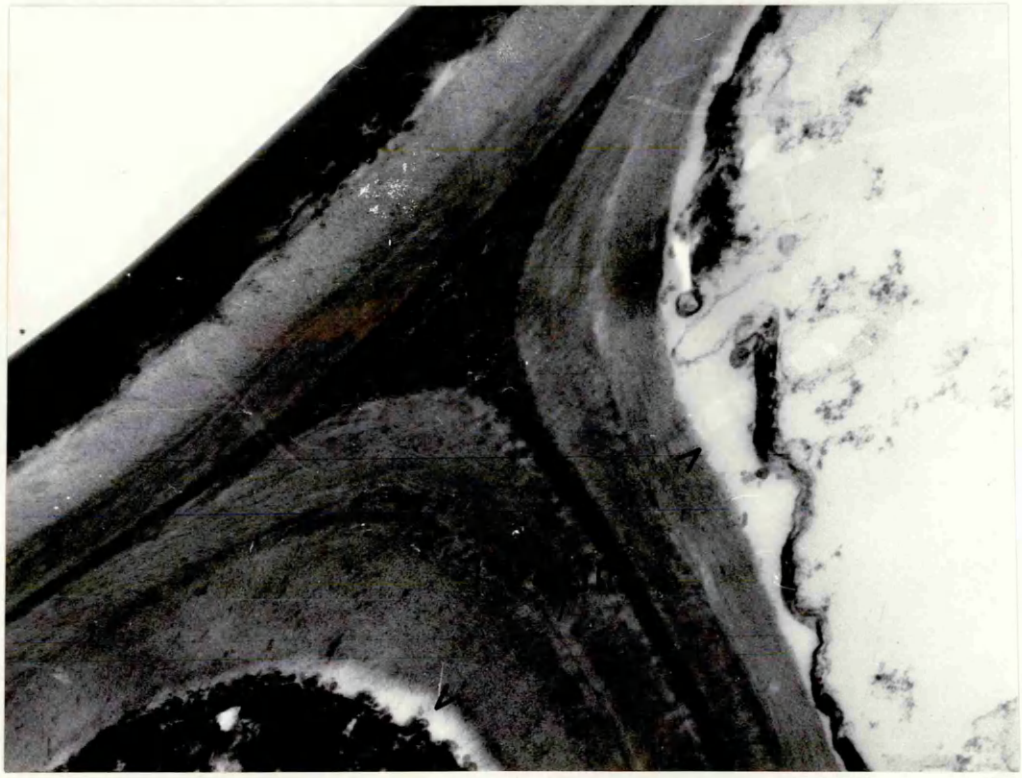


Plate 8

Transverse section of cell walls of vascular tissue taken from the sixth epicotyl section. (x 43200)



Chapter 5.

SOLUBILISATION OF THE GLUCURONYLTRANSFERASE ACTIVITY IN DETERGENTS.

I Introduction

Before any purification of the membrane-bound glucuronyltransferase enzyme(s) could be carried out successfully, the enzymes had to be solubilised. This was achieved with the use of detergents.

Because of the apparent pH optimum of the glucuronyltransferase activity (Ch. 3, p. 86), a new buffer comprising Tris-HCl (10 mmol dm^{-3}) and Mes (10 mmol dm^{-3}) at pH 6.0 was employed for tissue homogenisation and enzyme pellet resuspension. Further details are given in the materials and methods chapter (Ch. 2, p. 52) and the effect of pH on the composition of the hemicellulosic product is discussed later on in this chapter.

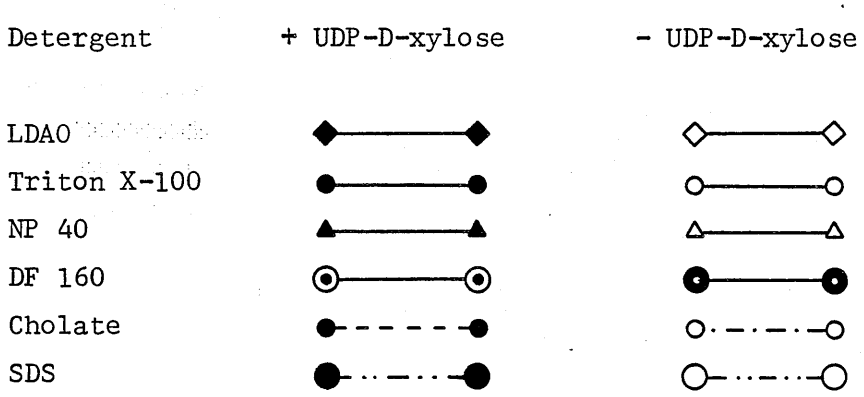
II Solubilisation of the Glucuronyltransferase Activity

The effect of different detergents at varying concentrations on the glucuronyltransferase activity

Particulate enzyme preparation was incubated with different detergents in the presence and absence of UDP-D-xylose (1 mmol dm^{-3}). Because preliminary experiments had indicated a decrease in the Mn^{2+} optimum in the presence of a detergent (see below), the Mn^{2+} concentration used in this experiment was 5 mmol dm^{-3}, instead of the usual 10 mmol dm^{-3}. As shown in Fig. 36, the UDP-D-xylose stimulatory effect is still evident and some detergents caused massive inhibition

Fig. 36

The effect of detergents at different concentrations on the incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in 4h. The detergents used are described below:



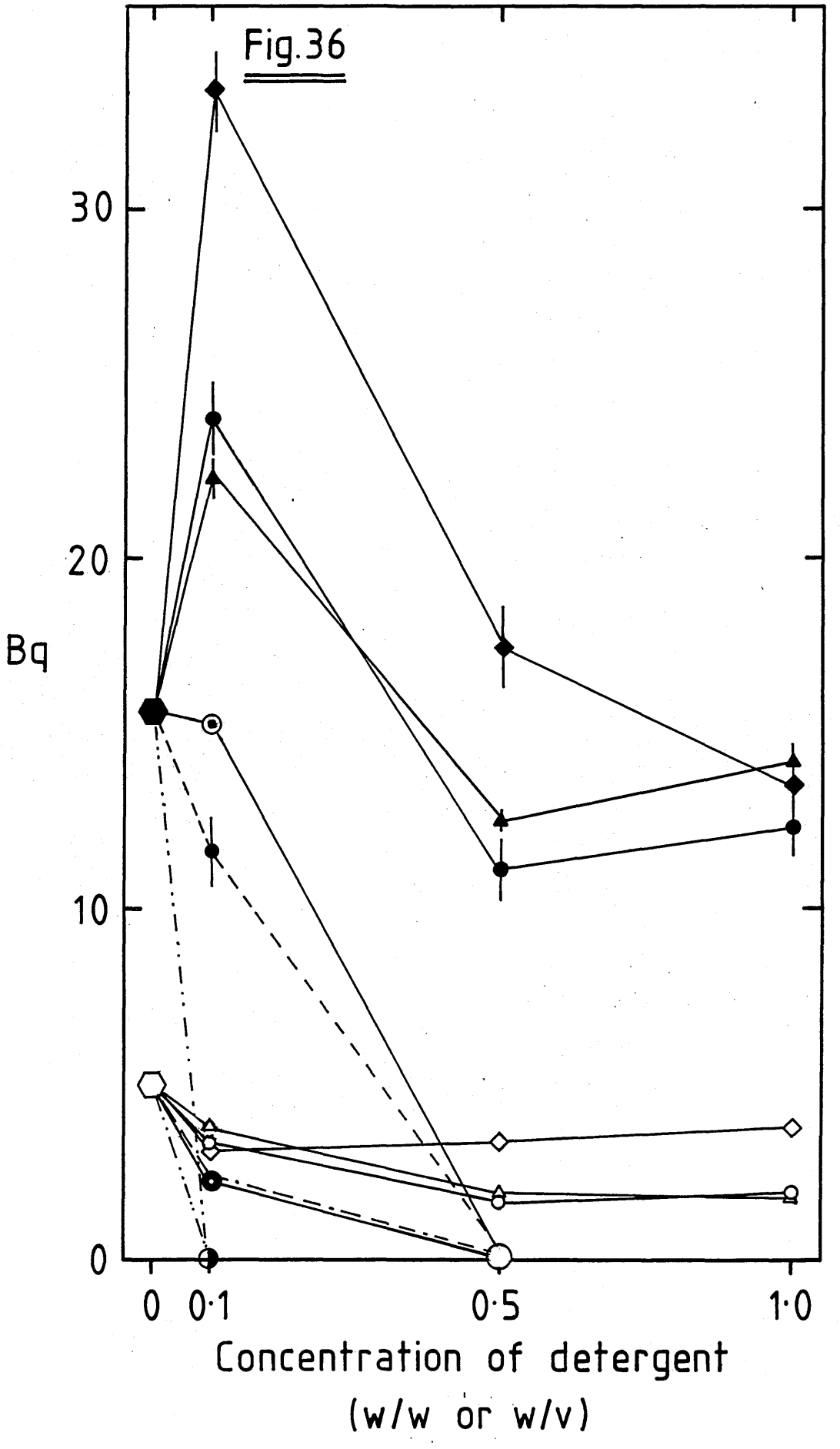


Fig.36
30
20
10
0
Bq
0
0·1
0·5
1·0
Concentration of detergent
(w/w or w/v)

at very low concentrations. Of those that did not, Triton X-100 and LDAO were chosen for attempts to solubilise the glucuronyltransferase activity. Triton X-100 was deemed suitable in view of the successful solubilisation of enzymes involved in gluomannan biosynthesis (Heller and Villemez, 1972a&b). The choice of LDAO was based on its suitability for enzyme purification - it has a low C.M.C. and the micelle size is small.

More detailed effects of TX-100 and LDAO on glucuronyltransferase activity are shown in Fig. 37(a) and 37(b) respectively. Fig. 37(b) shows that the concentration of LDAO is important in that too much or too little will result in low incorporation. Fig. 37(a) however, shows that the concentration of Triton X-100 is not as critical.

Solubilisation of the glucuronyltransferase in Triton X-100.

Solubilisation was performed as described in Ch. 2 (p. 52). The final Triton X-100 concentration in the incubations was 5% (w/v) and was chosen as the basis of the results in Fig. 37(a). Because increases in Triton X-100 concentration above 1% (w/v) had little effect upon enzyme activity, the detergent:protein ratio was not taken into account. Initial solubilisation experiments employed centrifugation at 314,000 g for 2 h to sediment non-dissolved material. Analysis of different regions of the centrifuge tube, however, indicated a slight gradation of active enzyme,

Fig. 37

The effect of (a) Triton X-100 and (b) LDAO at different concentrations on the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose in the presence (●—●) and absence (o—o) of UDP-D-xylose (1mmol.dm^{-3}). Incubations were of 4h duration.

The concentrations of detergents used in the experiments portrayed in Fig. 37 were greater than those used in the experiment shown in Fig. 36. As a result, the stimulatory effect of detergents at very low concentrations is not seen in Fig. 37.

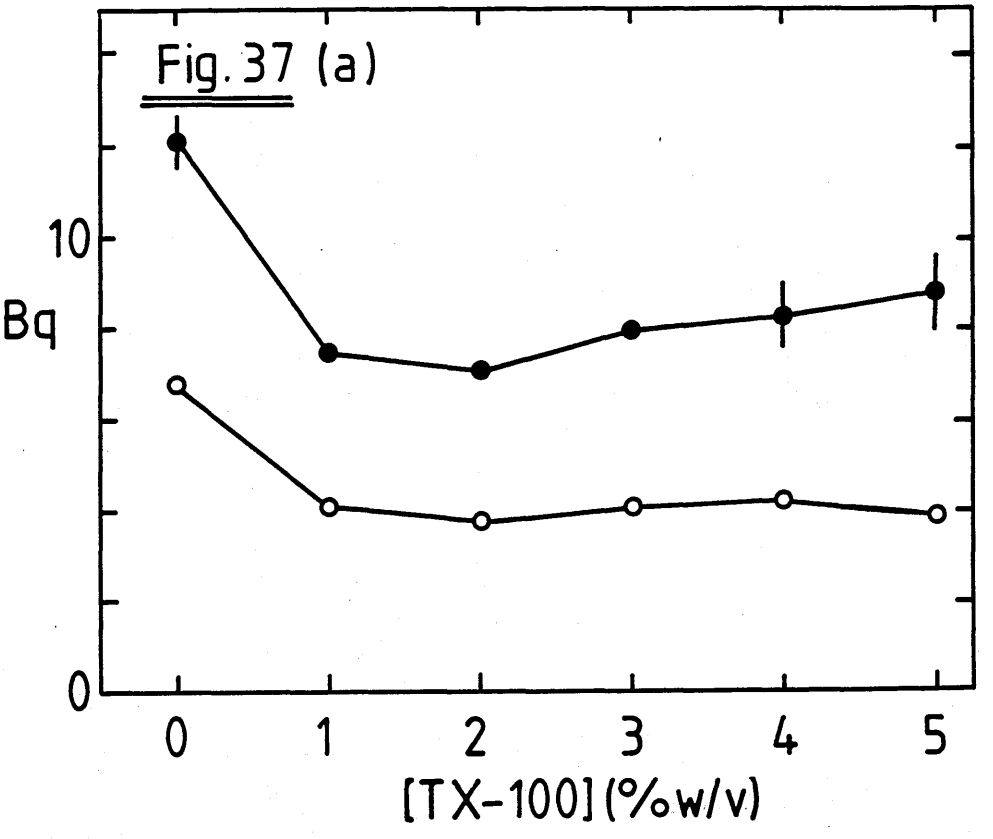


Fig. 37 (a)
10
Bq
0
0
1
2
3
4
5
[TX-100] (%w/v)

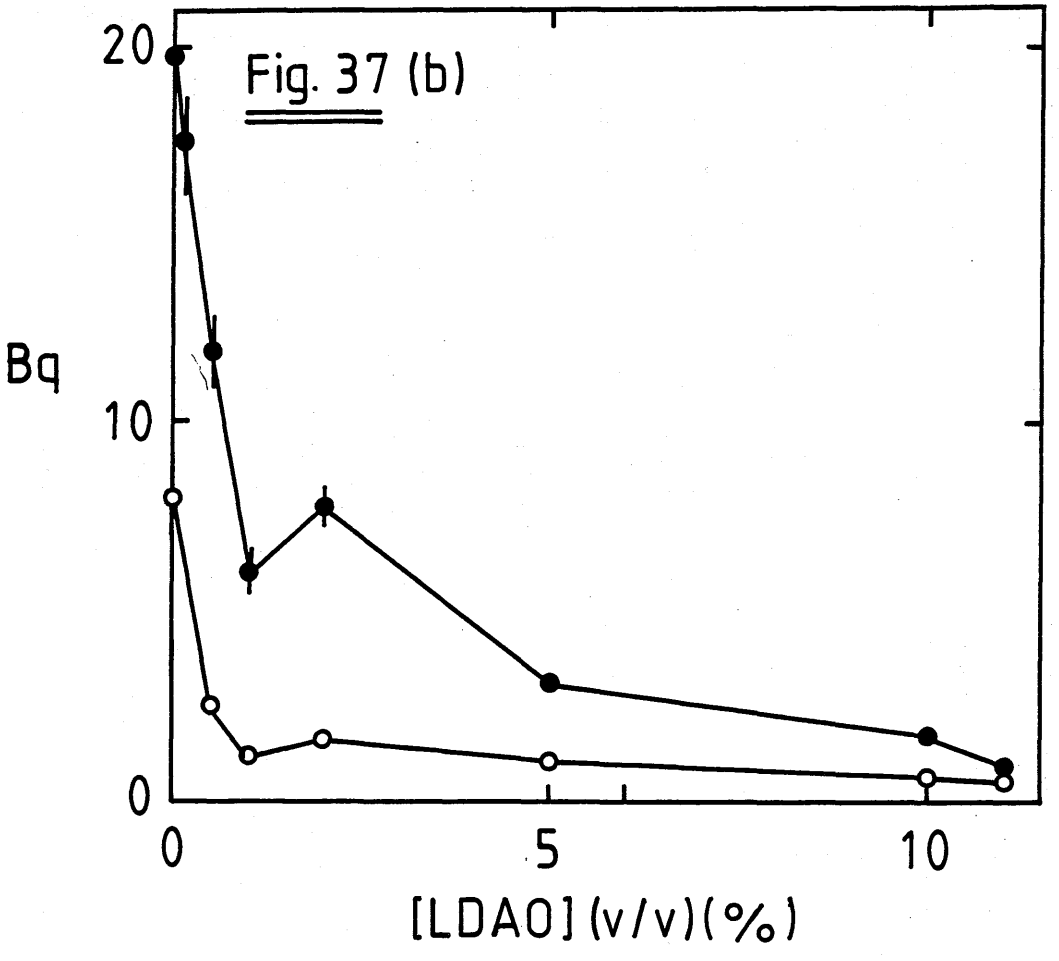


Fig. 37 (b)
20
Bq
10
0
0
5
10
[LDAO] (v/v) (%)

probably as a result of Triton X-100 micelle movement. In later experiments, 225,000 x g was used as the sedimenting force. The resulting supernatant was clear and colourless at both g-values and contained approximately 70% of the enzyme activity (Table IX). All remaining activity was present in the pellet. The solubilised enzyme was fully included by a sepharose CL-2B gel filtration column, and to a lesser extent by a CL-6B column (Fig. 38), indicating good solubility.

The effect of Mn^{2+} divalent cations on the activity of Triton X-100-solubilised glucuronyltransferase

Preliminary experiments had shown that the optimum Mn^{2+} concentration for solubilised enzyme was markedly lower than for ordinary particulate enzyme (Fig.39). Subsequently, the effect of Mn^{2+} concentration was investigated more closely. The effect of Mn^{2+} concentration on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose or high-molecular-weight pectin in the presence of UDP-D-xylose is shown in Fig. 40 for both Triton X-100-solubilised and particulate enzyme preparations. The results show that solubilisation in Triton X-100 causes a drop in the optimum concentration of Mn^{2+} from approximately 10 mmol dm^{-3} to below 2 mmol dm^{-3}. This is probably due to the change in the environment of the glucuronyltransferase upon solubilisation. In its native state, the membrane environment will tend to repel cations, thereby making it difficult for them to gain access to the enzyme. In the solubilised

Table IX

Distribution of enzyme activity between the supernatant and pellet after solubilizing the particulate enzyme in Triton X-100 and centrifuging.

Incubations were performed in the presence and absence of UDP-D-xylose and were of 4h duration. Terminated incubations were extracted for hemicellulose.

	% incorporation between supernatant and pellet.	
	No UDP-D-xylose present	UDP-D-xylose present
Supernatant	75.3	72.6
Pellet	24.6	27.4

Fig. 38

Profile of Triton X-100-solubilised enzyme on (a) Sepharose CL-2B and (b) Sepharose CL-6B gel-filtration columns. The eluted fractions were assayed for the ability to incorporate radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose in the presence (●—●) and absence (o—o) of UDP-D-xylose (1mmol.dm^{-3}).

Fig.38 (a)

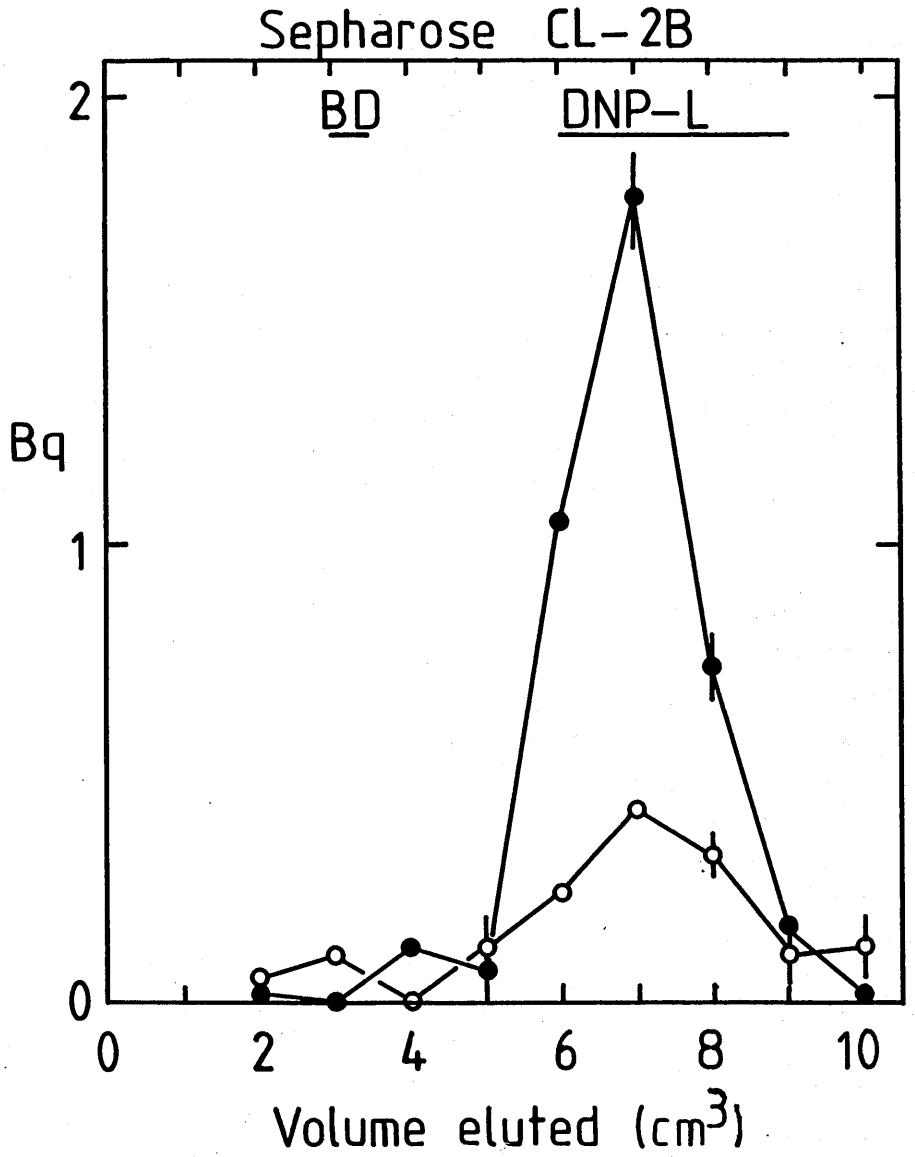


Fig.38 (b)

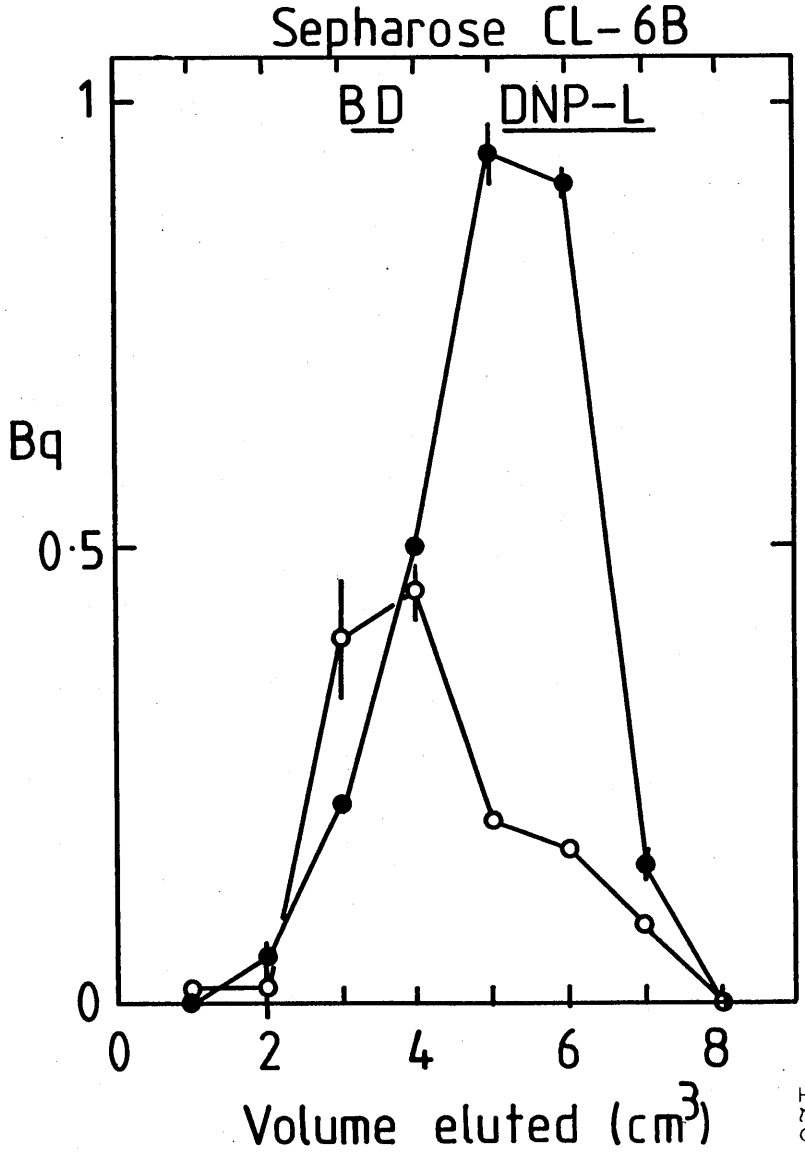


Fig. 39

The result of a preliminary experiment to show the effect of Mn^{2+} cations at different concentrations on the ability of Triton X-100-solubilised enzyme to incorporate radioactivity from UDP-D-[U-^{14}C]glucuronic acid into hemicellulose. Incubations were performed in the presence (●—●) and absence (o—o) of UDP-D-xylose (1mmol.dm^{-3}).

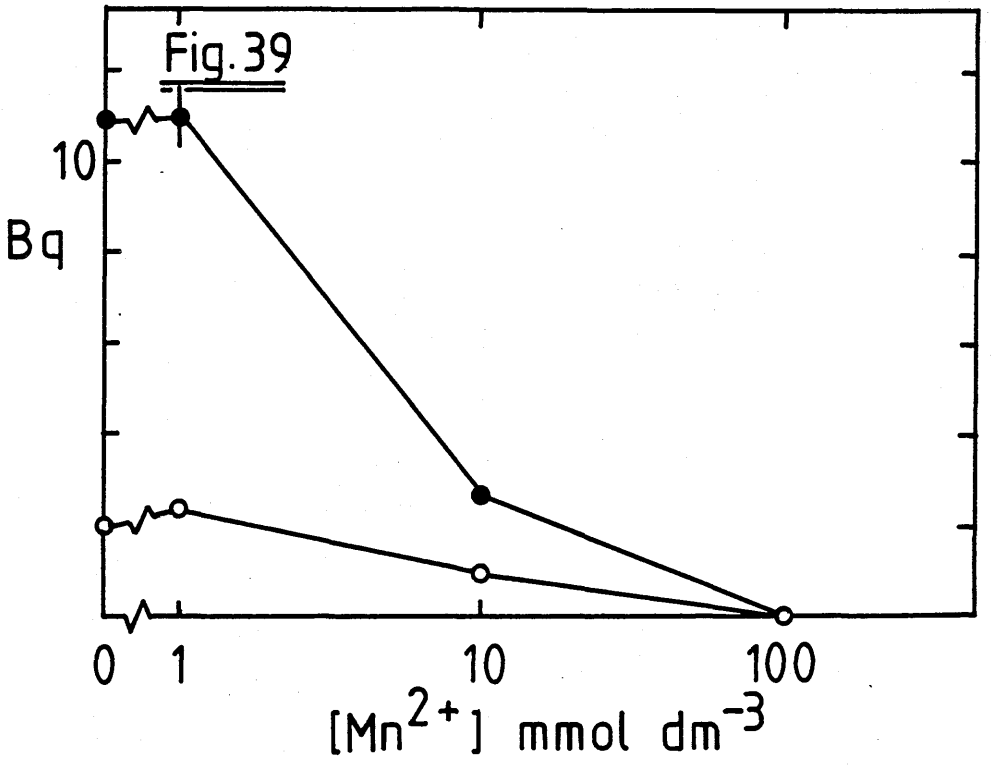


Fig. 40

Effect of Mn^{2+} concentration on the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into (a) hemicellulose and (b) pectin by Triton X-100-solubilised enzyme (solid lines) and particulate enzyme (broken lines). Incubations were performed in the presence of UDP-D-xylose at pH 6.0 and were of 4h duration.

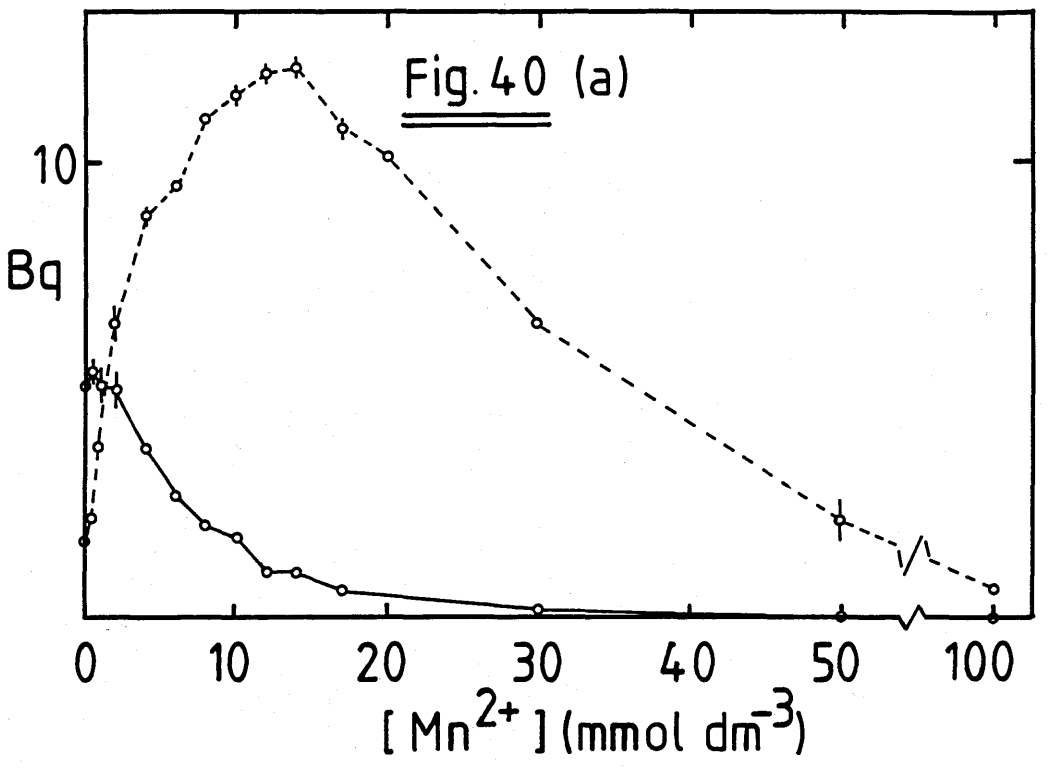


Fig. 40 (a)
10
Bq
0
10
20
30
40
50
100
[Mn2+] (mmol dm-3)

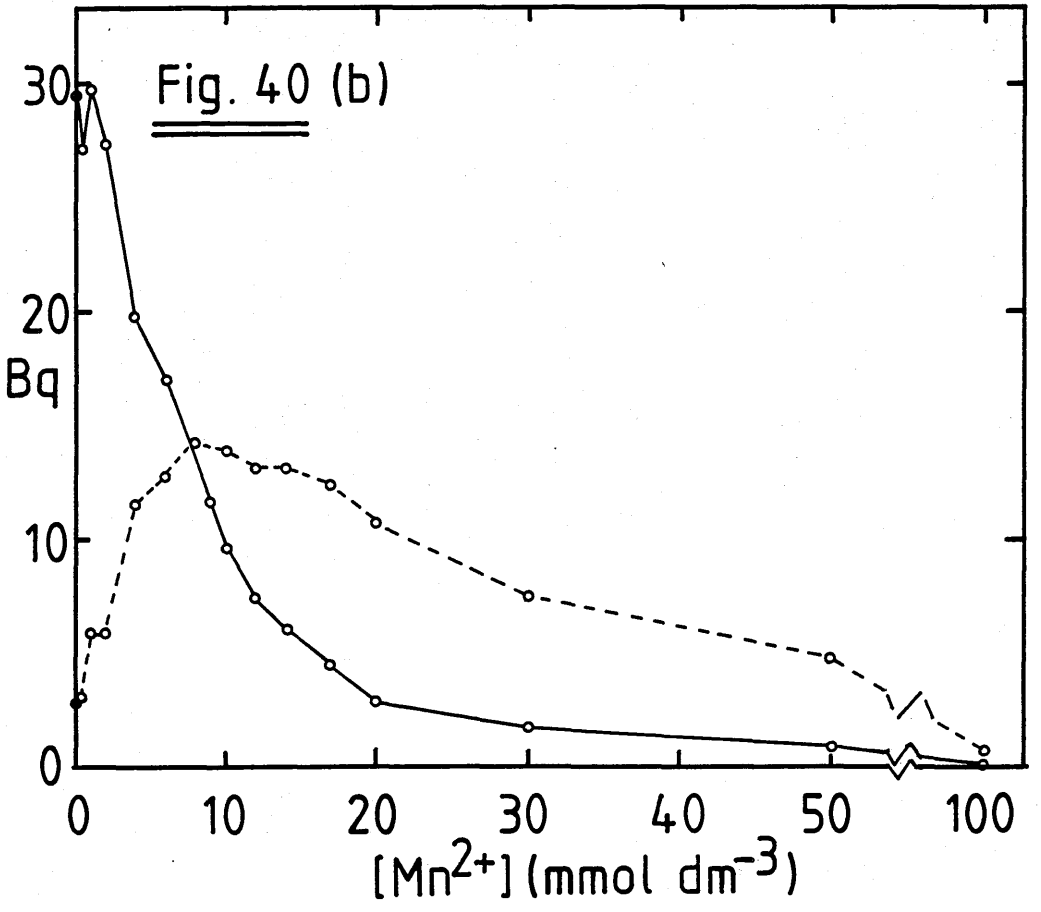


Fig. 40 (b)
30
20
Bq
10
0
0
10
20
30
40
50
100
[Mn2+] (mmol dm-3)

environment, this repulsion will be less, resulting in lower divalent cation concentration necessary for the same stimulatory effect. Further experiments with Triton X-100 -solubilised enzyme used Mn^{2+} at 1 mmol dm^{-3}.

Solubilisation of the glucuronyltransferase also resulted in a large increase in the ratio of high-molecular-weight pectin to hemicellulose. This is probably due to a change in the relative abundances of low-and high-molecular-weight precursors to which the glucuronic acid can be attached. High speed centrifugation during enzyme solubilisation probably resulted in the sedimentation of larger and more insoluble hemicellulosic molecules, thereby reducing their availability as acceptors.

The effect of UDP-D-xylose on the incorporation of radioactivity from UDP-D-[U-^{14}C]-glucuronic acid into hemicellulose and pectin by Triton X-100-solubilised glucuronyltransferase

To confirm that the UDP-D-xylose (1 mmol dm^{-3}) stimulated the incorporation of radioactivity into pectin and hemicellulose by Triton X-100-solubilised enzyme, a time course was performed (Fig. 41). The results are similar to those in Fig.15b (Ch. 3) and strongly indicate that the glucuronyltransferase has been fully solubilised along with the other enzymes responsible for glucuronoxylan biosynthesis. Although the incorporation into high molecular weight pectin follows the same pattern as the incorporation into hemicell-

Fig. 41

Time courses of the incorporation of radioactivity from UDP-D-[U-^{14}C]glucuronic acid into (a) hemicellulose and (b) pectin. Incubations were performed in the presence (●—●) and absence (o—o) of UDP-D- xylose (1mmol.dm^{-3}).

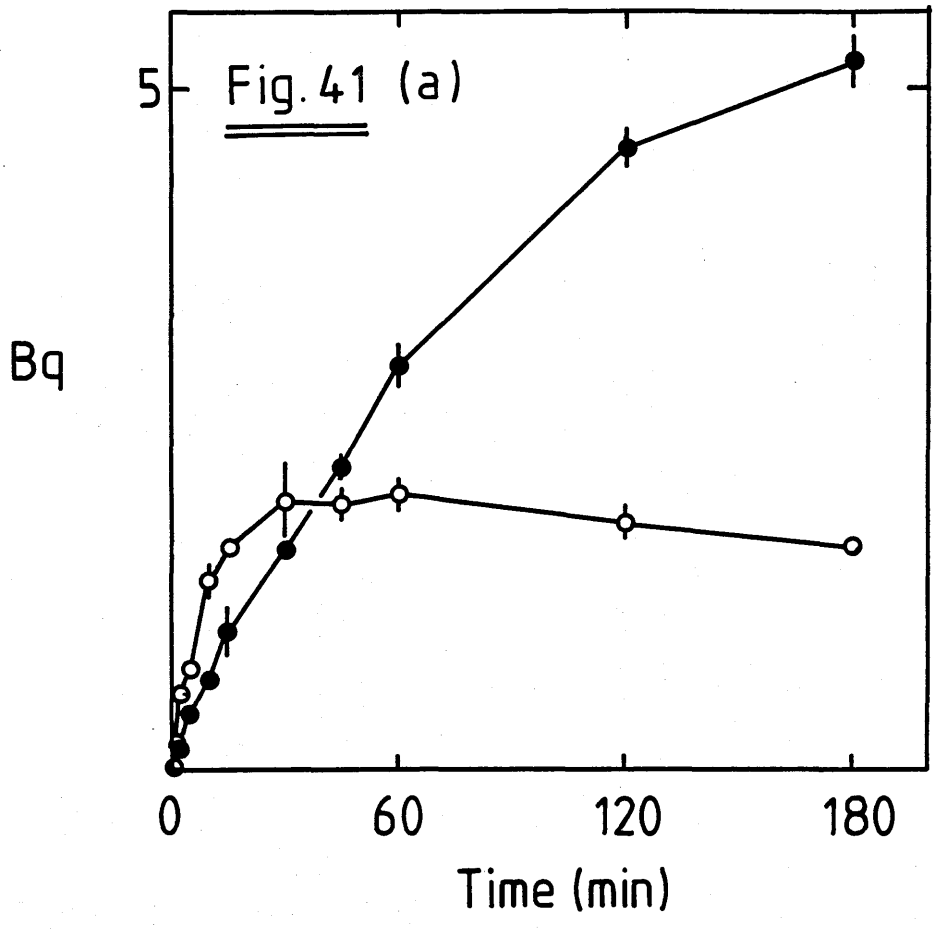


Fig. 41 (a)
5
Bq
0
60
120
180
Time (min)

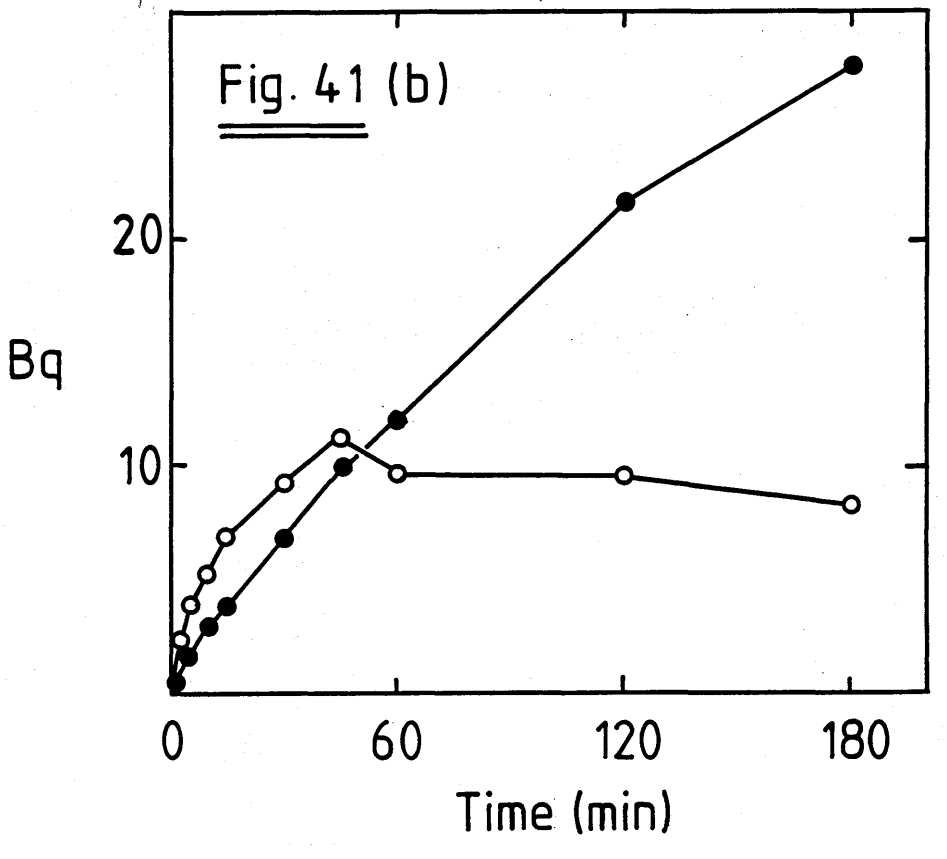


Fig. 41 (b)
20
10
Bq
0
60
120
180
Time (min)

ulose, there is far more incorporation into pectin than into hemicellulose.

The effect of freezing on the activity of Triton X-100-solubilised glucuronyltransferase

Freezing the Triton X-100-solubilised enzyme to -20°C was shown to have little effect on the activity in the presence of UDP-D-xylose, thus allowing large preparations of solubilised enzyme to be stored at -20°C before use. This contrasts with particulate enzyme.

Attempted solubilisation of glucuronyltransferase in LDAO

Following a similar procedure to that used in the case of Triton X-100, solubilisation of the glucuronyltransferase was attempted in LDAO. Because the concentration of LDAO was of importance (Fig. 37(b)), the ratio of protein:LDAO was taken into account. Unfortunately, no activity was found in the supernatant, but some was found in the non-solubilised pellet.

III Analysis of the Pectic and Hemicellulosic Products of Triton X-100-solubilised enzyme

Hemicellulose and pectic polysaccharides radioactively labelled with ^{14}C from UDP-D- [U-^{14}C] glucuronic acid were synthesised in large incubations in the presence of UDP-D-

xylose by enzyme solubilised in Triton X-100 at pH 6.0. These products were then analysed by partial-acid hydrolysis followed by descending-paper chromatography in solvent I. The results of the analyses are given in Fig. 42 and show that the major product is an oligosaccharide which co-chromatographs with that oligosaccharide labelled peak II in Ch. 3, which was then identified as glucuronic acid linked $\alpha 1 \rightarrow 2$ to xylose linked $\beta 1 \rightarrow 4$ to xylose.

At this stage of the research programme, hemicellulose labelled with ^{14}C from UDP-D-[U-^{14}C] glucuronic acid made in the presence of UDP-D-xylose at pH 6.0 with particulate enzyme was analysed in a similar manner. The results of this analysis are illustrated in Fig. 43 and show that six peaks of radioactivity are present. Peaks I, II and III have the same R_{xylose} values as their respective peaks in Fig. 27 (Ch. 3, p. 98), but peaks IV and V are new and have not yet been identified. Thus, hemicellulose labelled with radioactivity from UDP-D- U-^{14}C -glucuronic acid in the presence of UDP-D-xylose (1 mmol dm^{-3}) at pH 6.0 contains not only glucuronoxylan, but other products. This indicates that increasing the incorporation of radioactivity into hemicellulose by lowering the pH from 7.5 to 6.0 (Fig. 21, Ch. 3) is not necessarily due to a change in the rate of synthesis of glucuronoxylan, but may be due to an increase in the activity of other enzymes. Because of this, the solubilisation of the enzyme system was repeated, this time at pH 7.5. Analysis of the hemicellulosic and pectic products made at pH 7.5 in the presence of UDP-D-xylose was

Fig. 42

Paper-chromatographic analysis of partial-acid hydrolysates of (a) pectin and (b) hemicellulose labelled with radioactivity from UDP-D-[U-^{14}C]glucuronic acid. The pectin and hemicellulose were synthesised by Triton X-100 solubilised enzyme at pH 6.0. Incubations were carried out in the presence of UDP-D-xylose and were of 4h duration.

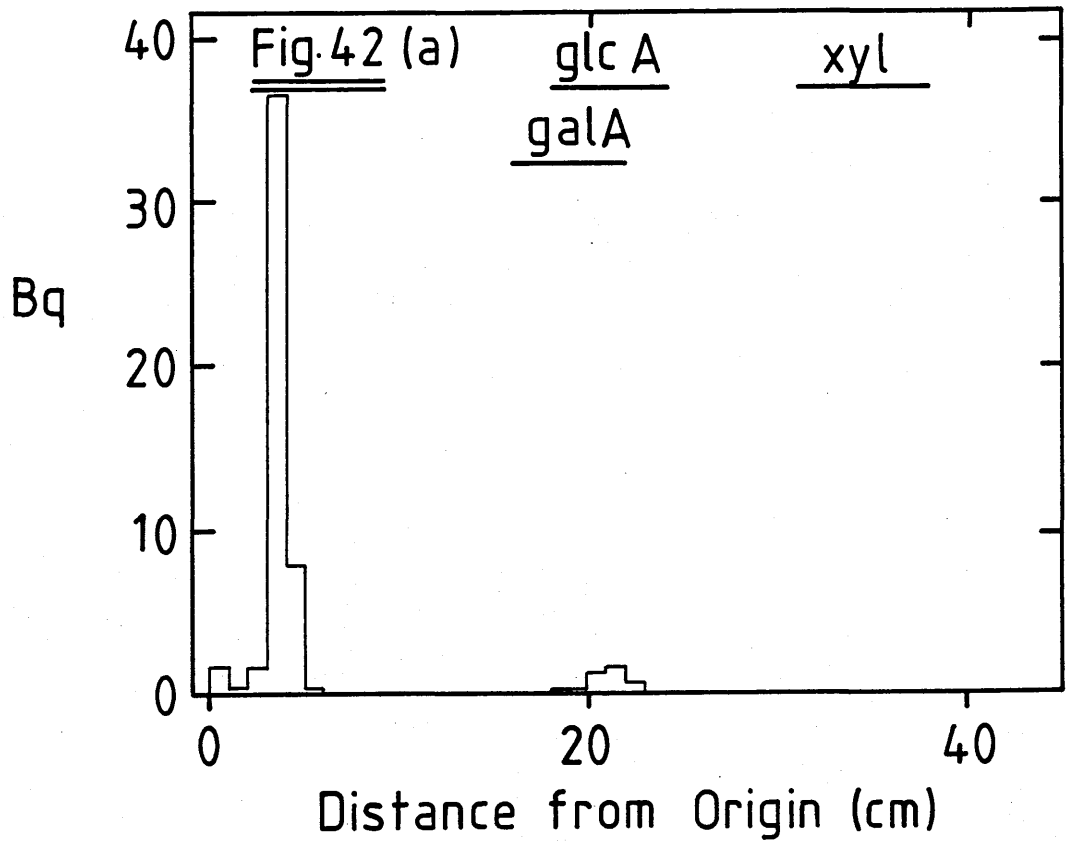


Fig. 42 (a)
glc A
xyl
galA
Bq
40
30
20
10
0
0
20
40
Distance from Origin (cm)

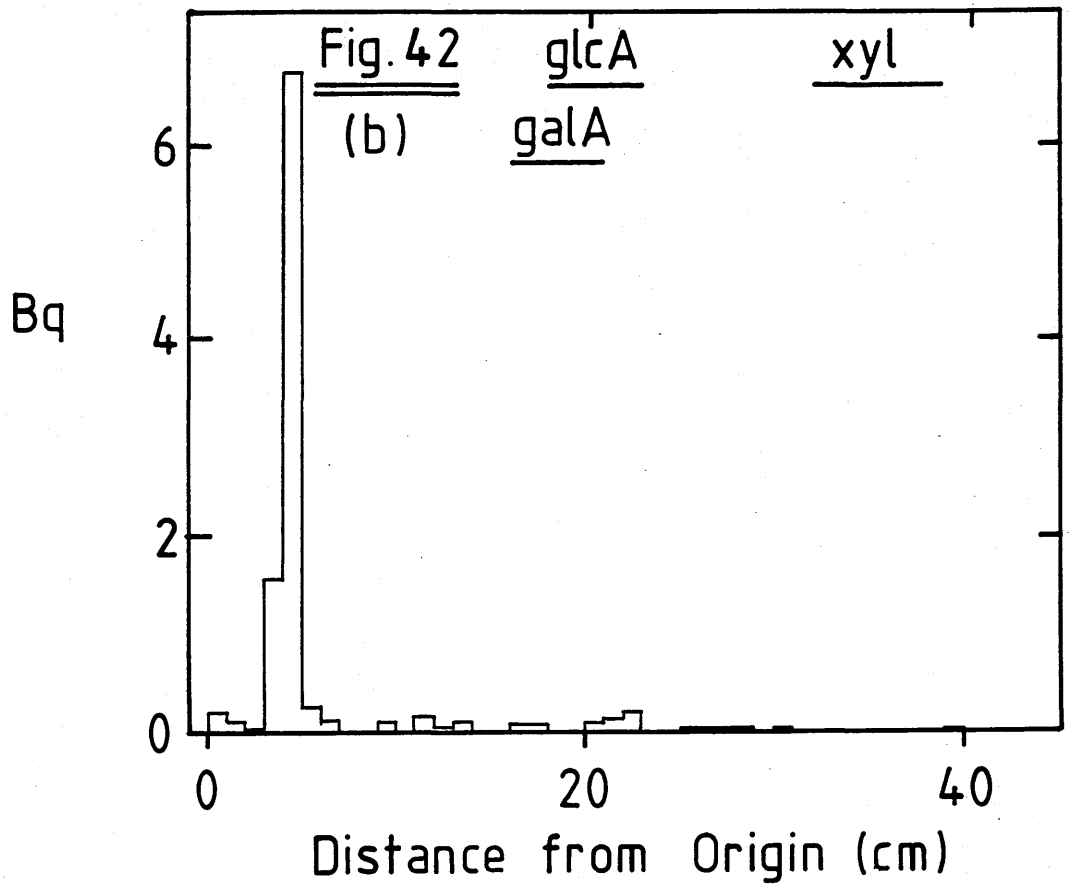
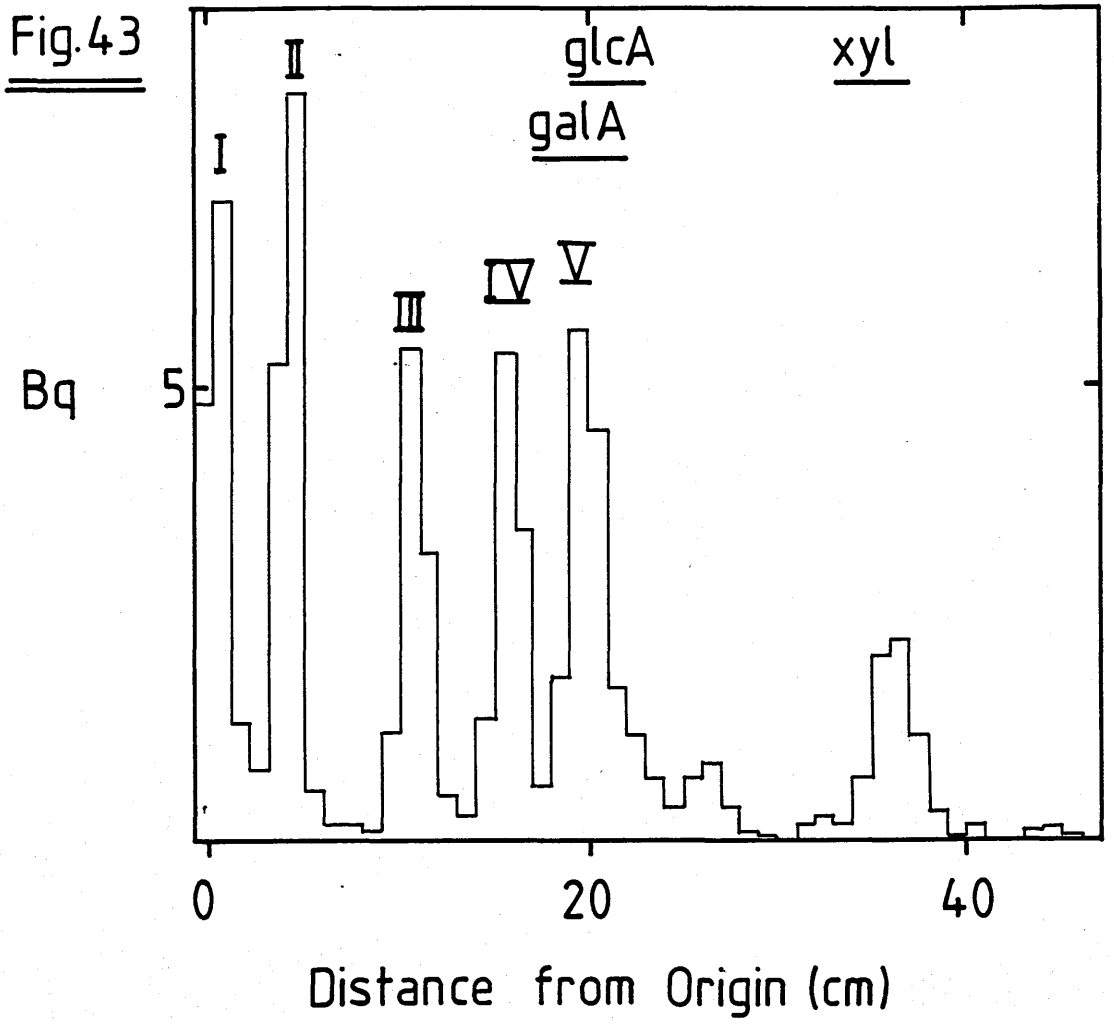


Fig. 42
(b)
glcA
xyl
galA
Bq
6
4
2
0
0
20
40
Distance from Origin (cm)

Fig. 43

Paper-chromatographic analysis of a partial acid hydrolysate of hemicellulose labelled with radioactivity from UDP-D-[U-^{14}C] glucuronic acid. The hemicellulose was synthesised by particulate enzyme at pH 6.0. Incubations were carried out in the presence of UDP-D-xylose and were 4h duration.

Fig.43



performed as before and the results (Fig. 44) show that in the case of solubilised enzyme, pH has no effect on the product. Further experiments with enzyme solubilised at pH 7.5 in Triton X-100 also showed that Mn^{2+} had the same effect as at pH 6.0.

IV Conclusions

From the results described in this chapter, it is likely that enzymes involved in glucuronoxylan biosynthesis have been solubilised as shown by the UDP-D-xylose stimulatory effect seen in Fig. 41. The pectic and hemicellulosic products of the solubilised enzyme system, however, are different to those produced by particulate enzyme at pH 7.5. The exact composition of the oligosaccharide shown in Fig. 42 and 44 is not known. It is unlikely to be the trisaccharide glucuronic acid $\alpha 1 \rightarrow 2$ linked to xylose $\beta 1 \rightarrow 4$ linked to xylose because it would be expected to break down into the disaccharide glucuronic acid $\alpha 1 \rightarrow 2$ linked to xylose during hydrolysis, and this does not occur. Further experiments will be necessary, including the chromatography of partial acid hydrolysates on different solvent systems, if the product is to be identified.

The product of Triton X-100-solubilised enzyme is not affected by changes in pH and this may be due to the selective solubilisation of enzymes by the detergent. Those enzymes whose activity increases as the pH drops from 7.5 to 6.0 probably remain membrane-bound or are inactivated by

Fig. 44

Paper-chromatographic analysis of partial acid hydrolysates of (a) pectin and (b) hemicellulose labelled with radioactivity from UDP-D-[U-^{14}C]glucuronic acid. The pectin and hemicellulose were synthesised by Triton X-100 solubilised enzyme at pH 7.5. Incubations were carried out in the presence of UDP-D-xylose and were of 2h duration.

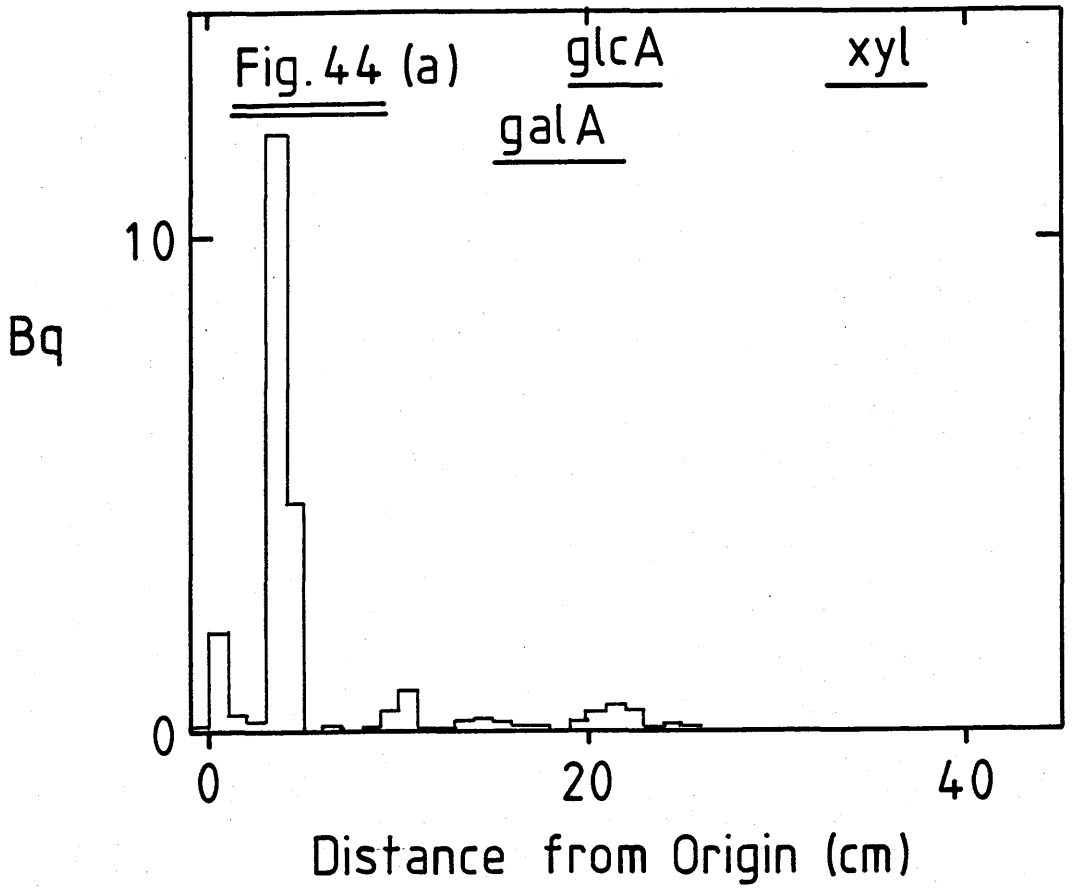


Fig. 44 (a)
glcA
xyl
galA
10
Bq
0
0
20
40
Distance from Origin (cm)

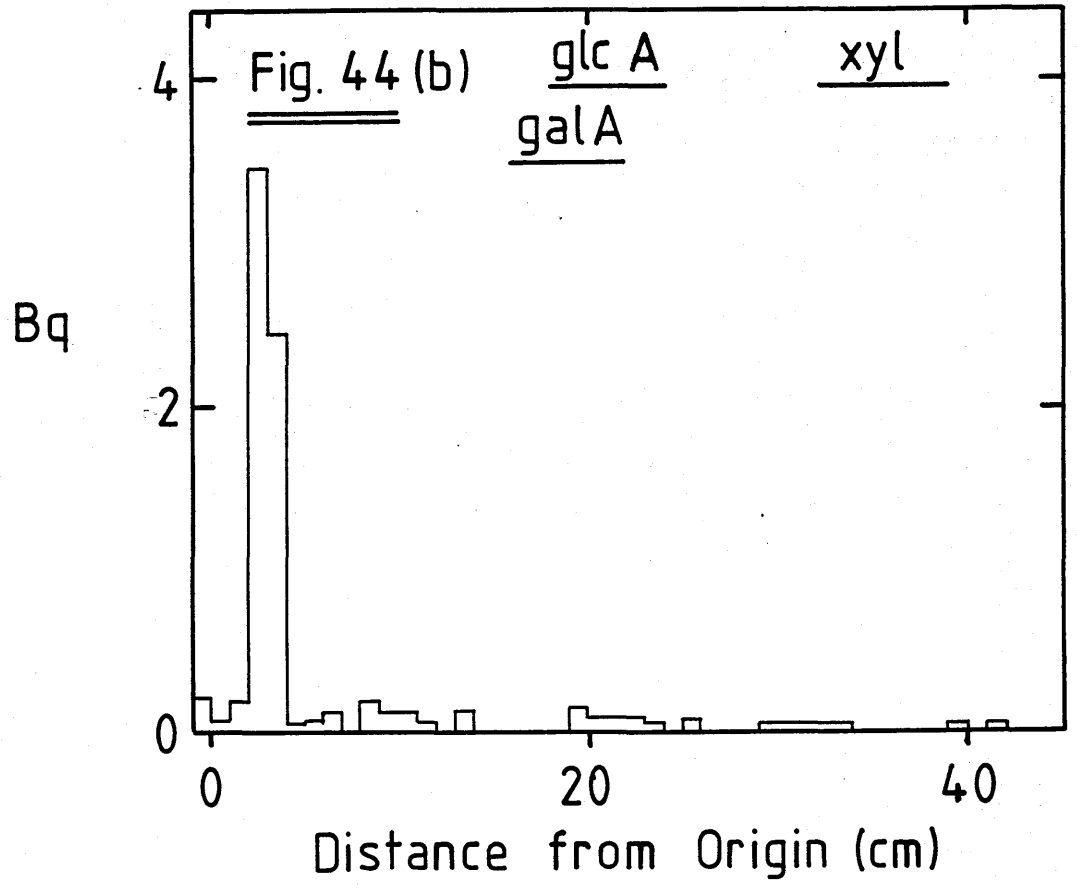


Fig. 44 (b)
glc A
xyl
galA
4
Bq
2
0
0
20
40
Distance from Origin (cm)

the Triton X-100.

The inability of LDAO to solubilise the enzyme activity may be due to partial solubilisation only of the enzyme system. LDAO has a small micelle size and it may not be possible to solubilise all the enzymes necessary for glucuronyltransferase activity in adjacent positions in a single micelle. For instance, if xylan synthesis is required prior to glucuronic acid transfer, and the glucuronyl transferase enzymes are separated from the xylosyl transferase and xylan chain, then no transfer will occur. Alternatively, precursors or other factors which may be necessary for enzyme activity may not have been solubilised by the LDAO, again inhibiting activity. The LDAO does not inhibit the enzyme totally as shown by the results in Fig. 37(b), and it may be possible to increase activity by mixing the non-solubilised pellet with the LDAO-solubilised supernatant.

Chapter 6.

THE SUBCELLULAR LOCATION OF THE GLUCURONYLTRANSFERASE.

I Introduction

The purpose of the experiments described in this chapter was to identify the organelle(s) containing the glucuronyltransferase involved in glucuronoxylan biosynthesis. All the early experiments involved the use of sucrose density gradients at pH 6.0. Later experiments involved gradients at pH 7.5. Unless otherwise indicated, homogenisation of tissue was performed by chopping with razor blades.

II Sucrose-Gradient Centrifugation at pH 6.0

Sucrose density gradients were formed by the methods described in Ch. 2 (p. 54).

Preliminary experiments were designed to test the effect of homogenisation and gradient media on the sedimentation characteristics of membranes containing glucuronyltransferase activity. Fig 45 shows the profile of the transferase assayed in the presence and absence of UDP-D-xylose at pH 6.0 and the distribution of protein on a 10% - 50% (w/w) sucrose density gradient. In this case, both the homogenisation medium and density gradient contained only sucrose, Tris-HCl (10 mmol dm^{-3}) and Mes (10 mmol dm^{-3}) at pH 6.0. (N.B. the membraneous material of the muslin filtrate was concentrated by centrifuging over a cushion of 40% (w/w) sucrose medium prior to its application to the gradient). Although the protein peak was at a density of 1.18

Fig. 45

Enzyme activities of membranes separated by continuous sucrose gradient centrifugation.

Symbols:

◇ ◇ ....Sucrose % (w/w);

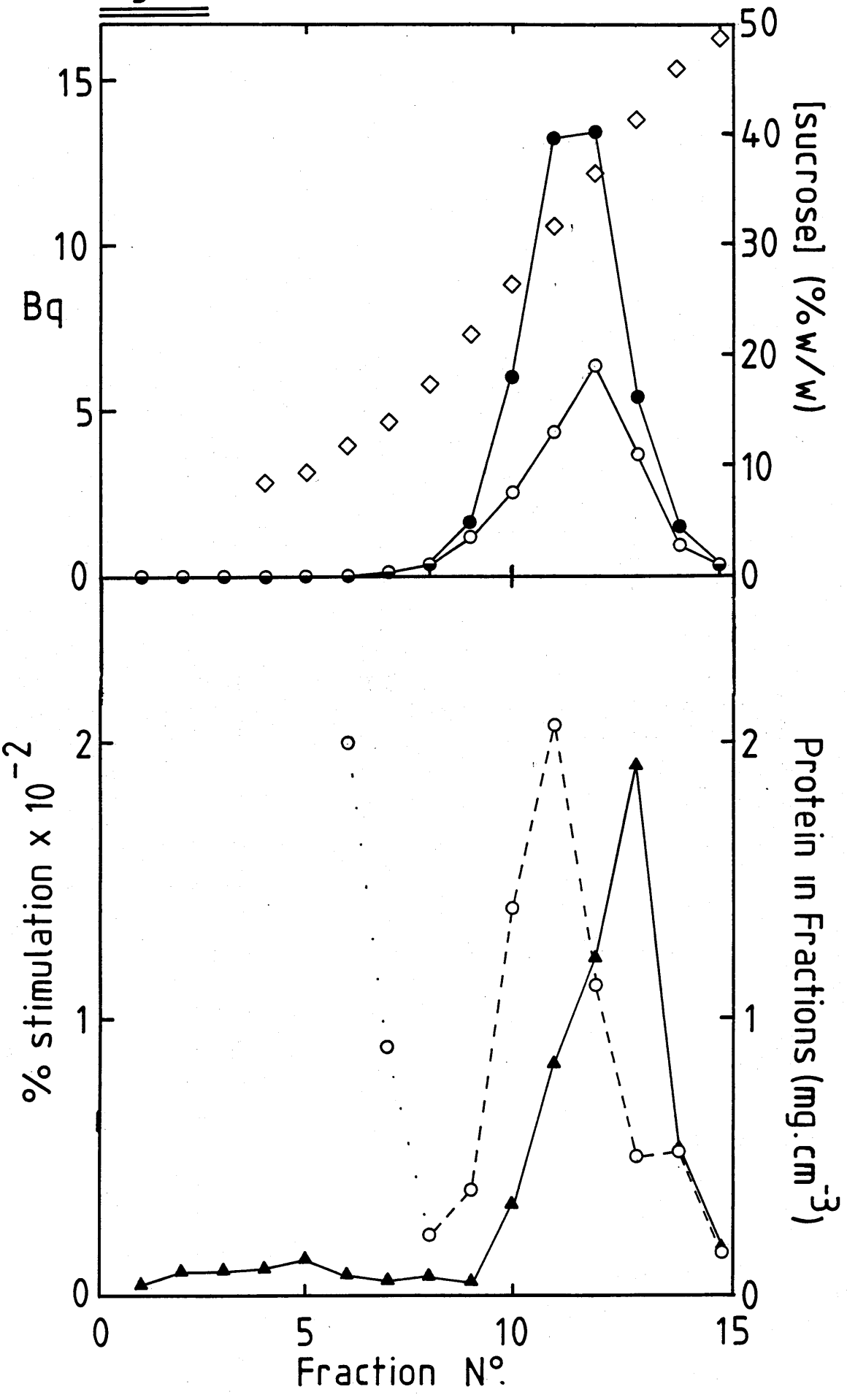
●——● ....Incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in the presence of UDP-D-xylose (1mmol.dm^{-3});

○——○ ....Incorporation ofradioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in the absence of UDP-D-xylose;

○- - -○ ....% stimulation by UDP-D-xylose on the incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose;

▲——▲ ....Protein.

Fig. 45


Bq
[sucrose] (% w/w)
% stimulation x 10^{-2}
Protein in Fractions (mg.cm^{-3})
Fraction N°.
0
5
10
15
20
30
40
50
1
2

g cm^{-3} (40% (w/w) sucrose), the glucuronyltransferase activity reached a maximum at densities between 1.13 and 1.16 g cm^{-3} (31 and 36% (w/w) sucrose). Stimulation of the transferase activities by UDP-D-xylose was greatest at 1.13 g cm^{-3} (31% (w/w) sucrose, Fig. 45).

The effect of using a different homogenisation medium and gradient medium is shown in Fig. 46. The extra constituents present in the new media were added to preserve organelle integrity (Ch. 1, p. 36) and the cushioning of membranes over 40% (w/w) sucrose medium was eliminated. It can be seen that the protein profile was quite different to that shown in Fig. 45 . The glucuronyltransferase peak, however, remained in the same position, slightly overlapping the major peak of protein. In this experiment, the transferase activity was not assayed in the absence of UDP-D-xylose, but the analysis of other gradients of similar composition showed that the stimulatory effect of UDP-D-xylose was maximum at a density equivalent to 29% (w/w) sucrose (1.122 g cm^{-3}). The distribution of β glucan synthetases I and II was investigated and gave the results shown in Fig. 46. In each case both of the peaks equilibrated at the same density as the glucuronyltransferase, and did not allow any discrimination to be made between the plasma membrane (GS II) and golgi (GSI). The high level of GSI at the top of the gradient was surprising and cannot be explained readily.

Total IDPase activity was measured on a similar gradient,

Fig. 46

Enzyme activities of membranes separated by continuous sucrose gradient centrifugation.

Symbols:

◇ ◇ ....Sucrose % (w/w);

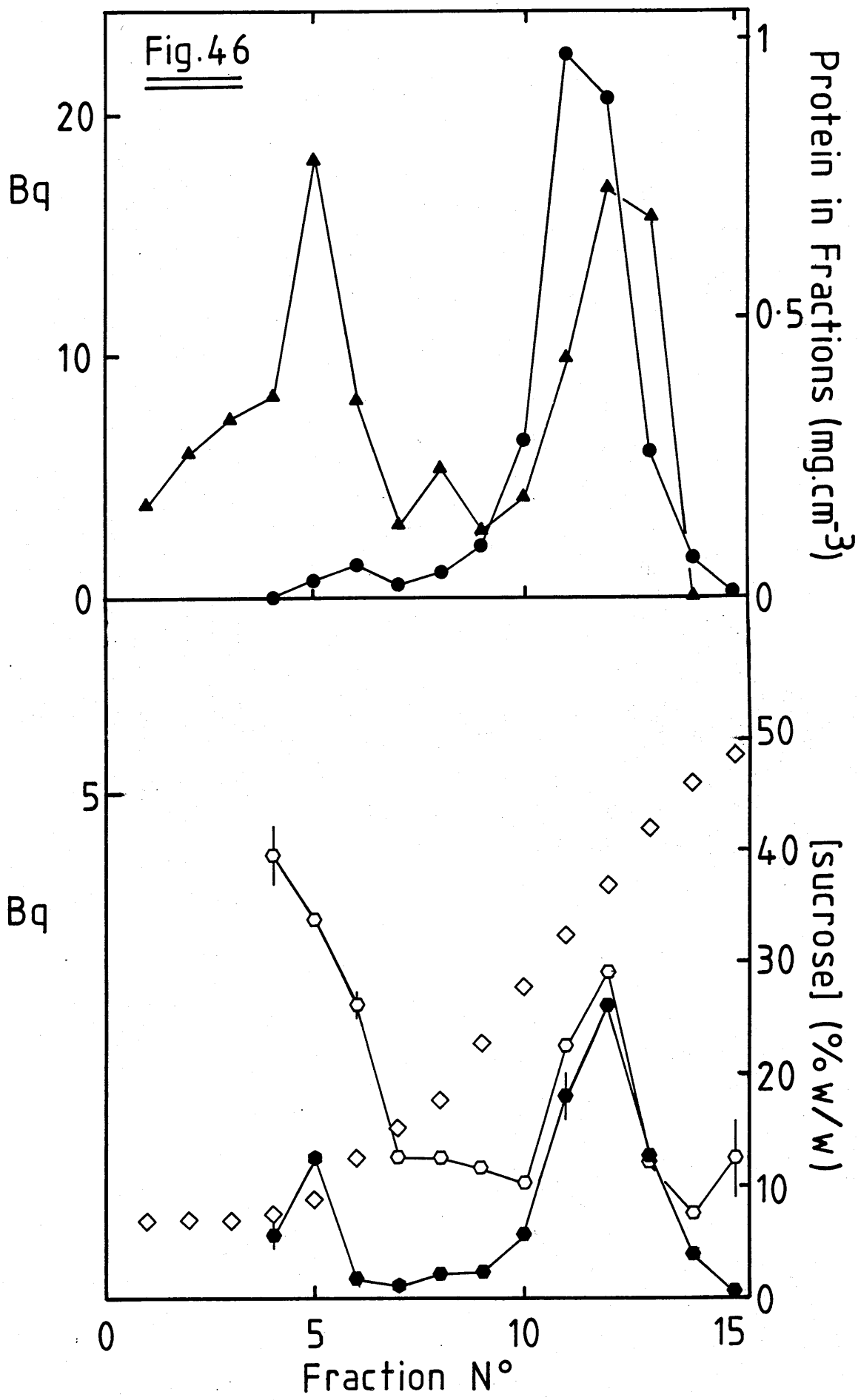
●——● ....Incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in the presence of UDP-D-xylose (1mmol.dm^{-3});

○——○ ....Glucan Synthetase I;

●——● ....Glucan Synthetase II;

▲——▲ ....Protein.

Fig.46


20
Bq
10
0
1
0·5
0
Protein in Fractions (mg.cm-3)
5
Bq
50
40
30
20
10
0
[sucrose] (% w/w)
0
5
10
15
Fraction N°

although this time, KCl was omitted from the gradient and homogenisation media. The results (Fig. 47) show that a peak of IDPase activity co-sedimented with that of glucuronyltransferase which, as usual, was at a slightly lower density than the major protein peak. However, the majority of total IDPase activity was present at the top end of the gradient mainly in the supernatant.

Grinding the pea epicotyls rather than chopping with razor blades had the effect of increasing slightly the density to which membrane-bound glucuronyltransferase sedimented. (Again, KCl was omitted from the gradient and homogenisation media). However, the two glucan synthetases (I and II) still co-sedimented with the glucuronyltransferase and the usual profile of membrane-bound protein was recognised (Fig. 48).

III Sucrose-gradient Centrifugation at pH 7.5

Both continuous and discontinuous sucrose-density gradients were used in the experiments described in this section. In all cases, the composition of the media was identical except for the levels of sucrose and Mg^{2+} (see Ch. 2, p. 54).

Continuous density gradients were used to identify the density of membranes containing Triton X-100-activated IDPase.

Fig. 47

Enzyme activities of membranes separated by continuous sucrose gradient centrifugation.

Symbols:

◇ ◇ ....Sucrose % (w/w);

● ● ....Incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in the presence of UDP-D-xylose (1mmol.dm^{-3});

△——△ ....IDPase; incubations were performed for 2,5,and 10 min.

▲——▲ ....Protein.

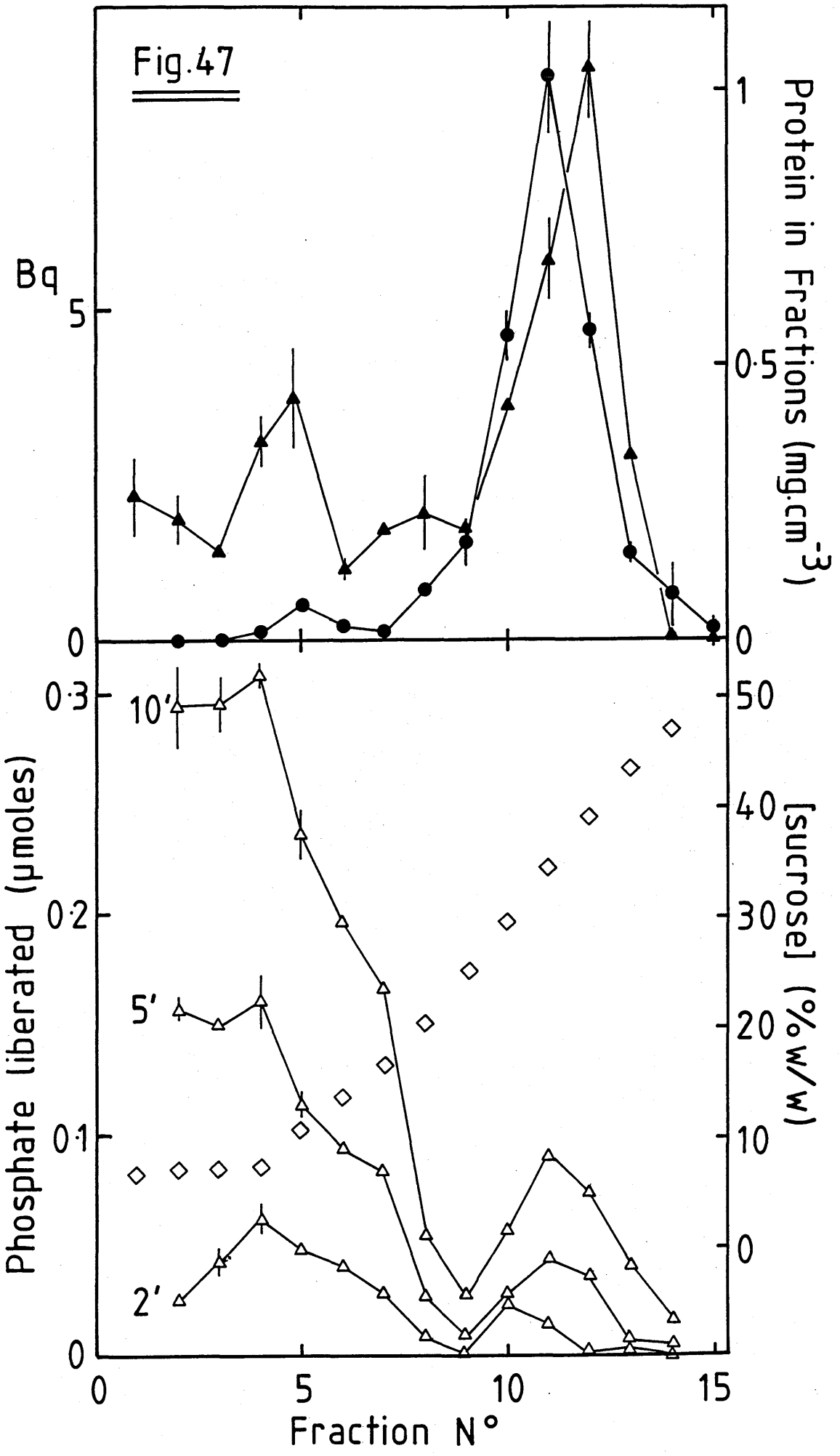


Fig.47
Bq
5
0
1
0·5
0
Protein in Fractions (mg.cm-3)
0·3
0·2
0·1
0
10'
5'
2'
50
40
30
20
10
0
[sucrose] (% w/w)
Phosphate liberated (µmoles)
0
5
10
15
Fraction N°

Fig. 48

Enzyme activities of membranes separated by continuous sucrose gradient centrifugation.

Symbols:

◇ ◇....Sucrose % (w/w);

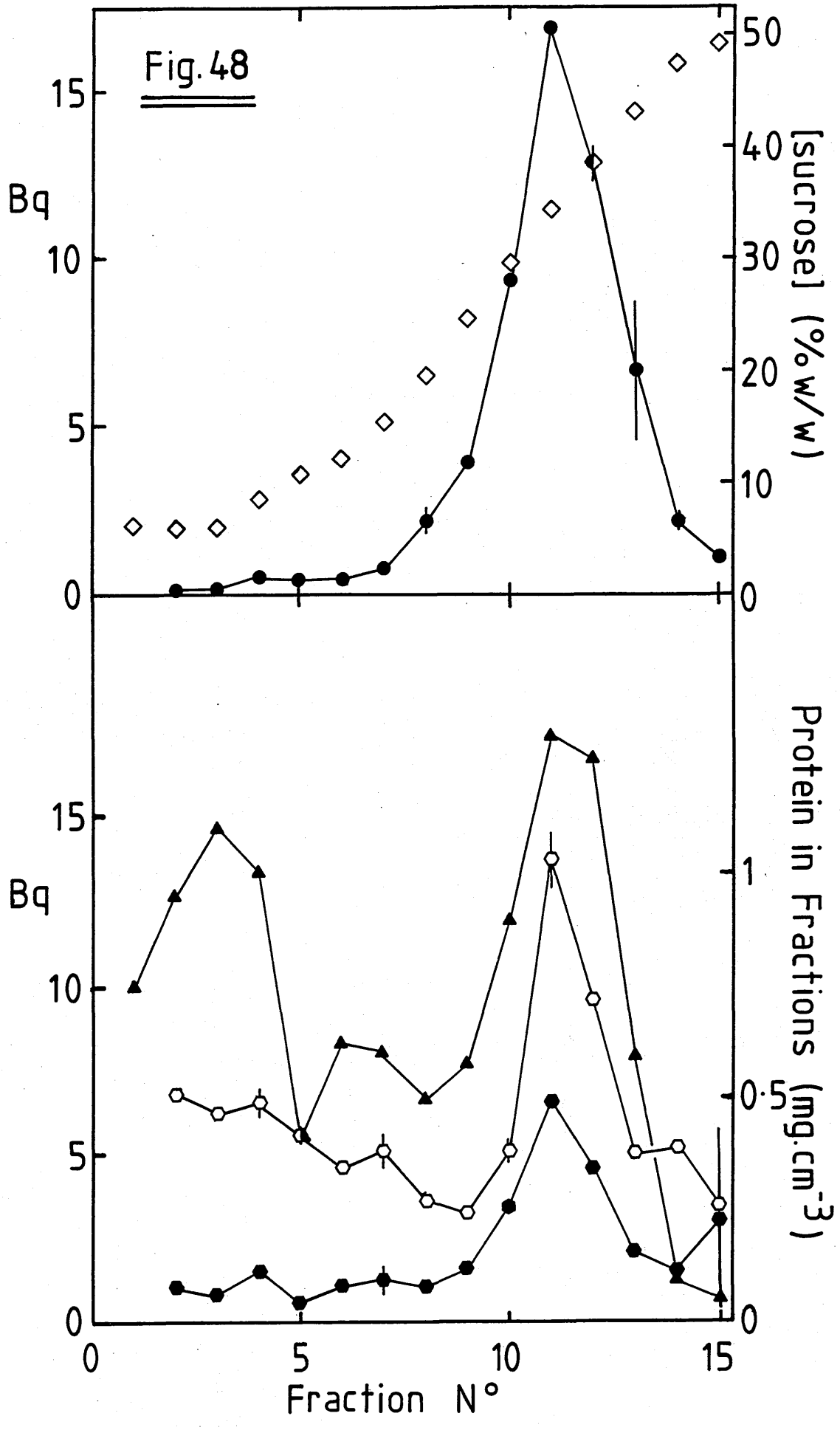
●——● ....Incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in the presence of UDP-D-xylose (1mmol.dm^{-3});

○——○....Glucan Synthetase I;

●——●....Glucan Synthetase II;

▲——▲....Protein.

Fig. 48


Bq
15
10
5
0
50
40
30
20
10
0
[sucrose] (% w/w)
Bq
15
10
5
0
1
0·5
0
Protein in Fractions (mg.cm-3)
0
5
10
15
Fraction N°

IDPase activation was performed as described in Ch. 2 (p. 60) and the results of the analysis are shown in Fig. 49. It can be seen that there are two peaks of activated IDPase. One co-sedimented with glucuronyltransferase while the other, much smaller peak, remained at the top end of the gradient. (The profile of total IDPase activity was similar to that shown in Fig. 47 with the majority of activity present in the soluble fractions at the top of the gradient). The protein profile gives the two main peaks as expected and the density at which UDP-D-xylose gave maximum stimulation was 1.122 g cm^{-3}.

Discontinuous sucrose density gradients were used to determine whether or not the glucuronyltransferase was present in the endoplasmic reticulum. Membranes were separated on gradients with or without Mg^{2+} at 2 mmol dm^{-3} and were collected from the 10-30% and 30-50% (w/w) sucrose interfaces. They were then assayed for glucuronyltransferase, NADH-cytochrome c-reductase and protein. The results (Table X) show that although the removal of Mg^{2+} resulted in a large shift in the position of NADH-cytochrome c-reductase, very little change was noticed in the distribution of glucuronyltransferase activity. This indicates that very little glucuronyltransferase is present in the endoplasmic reticulum.

IV Conclusions

The experiments involving gradients at pH 6.0 were performed before the effect of pH on glucuronyltransferase was recognised. This means that the radioactivity incorpor-

Fig. 49

Enzyme activities of membranes separated by continuous sucrose gradient centrifugation.

Symbols:

◇ ◇ ....Sucrose % (w/w);

●——● ....Incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose in the presence of UDP-D-xylose (1mmol.dm^{-3});

△——△ ....Latent IDPase;

▲——▲ ....Protein.

Total IDPase activity (not shown) gave a profile similar to that in Fig. 47,

Stimulation of incorporation of radioactivity from UDP-D-[U-^{14}C] glucuronic acid into hemicellulose by UDP-D-xylose was maximum at a density of 1.122 g cm^{-3}. (Profile not shown)

The protein concentration in the first gradient fraction is due to the background protein.

Fig. 49

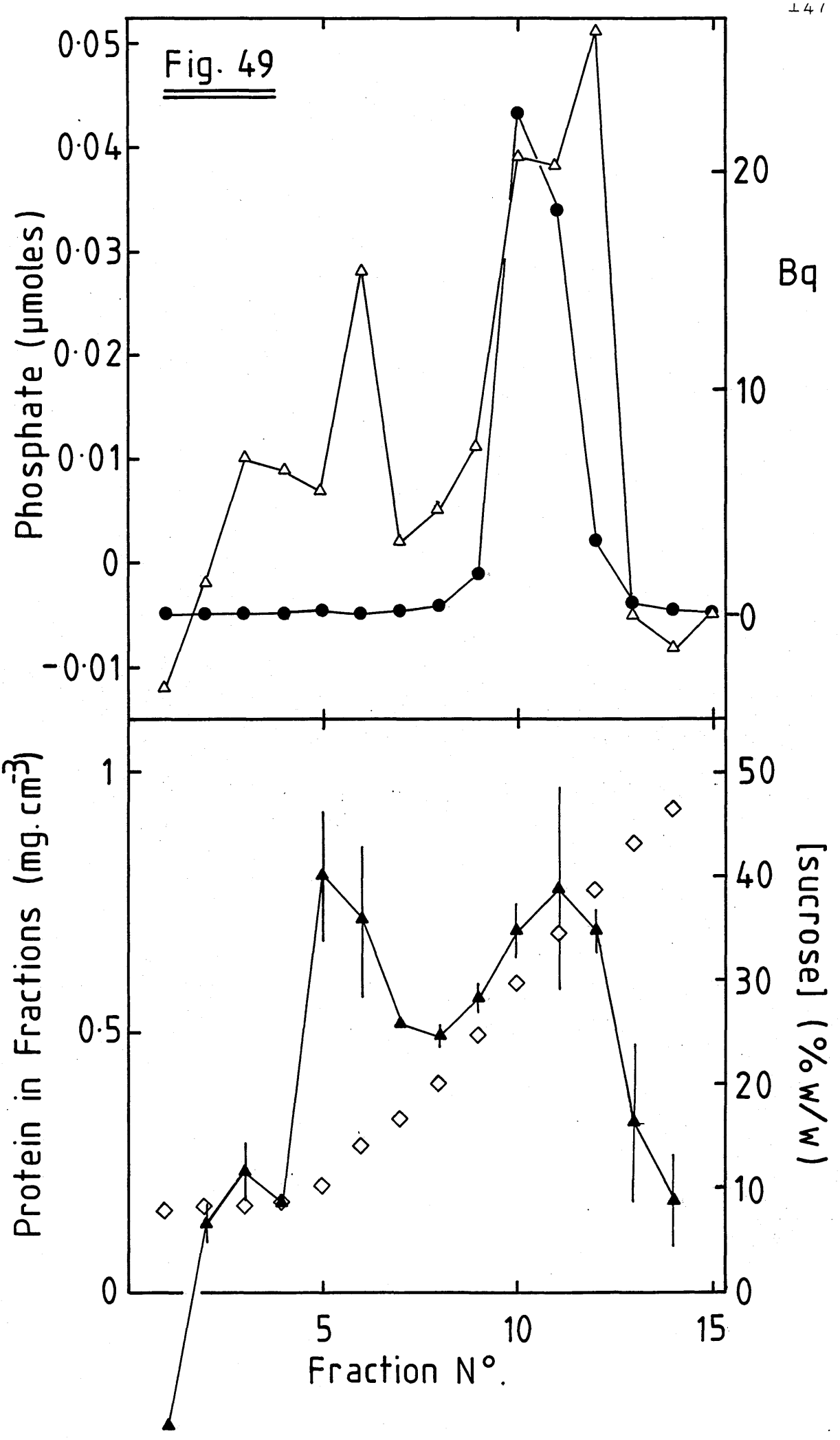

0·05
0·04
0·03
0·02
0·01
0
-0·01
Phosphate (µmoles)
20
10
0
Bq
1
0·5
0
Protein in Fractions (mg.cm⁻³)
50
40
30
20
10
0
[sucrose] (%w/w)
5
10
15
Fraction N°.

Table X

Discontinuous sucrose density gradient analysis of glucuronyltransferase at pH 7.5 in the presence of Mg^{2+} ions at high (H) and low (L) concentrations. The distributions of NADH-cytochrome c-reductase and protein are also shown.(See Ch.2 p 54 for the descriptions of the top (T) and bottom (B) interfaces.

Interface	NADH-cytochrome c-reductase. (OD_{550} min^{-1})	% NADH-cytochrome c-reductase in the T and B interfaces	Protein ($mg.cm^{-3}$)	%protein in both T and B interfaces	Incorporation of ra UDP-D- $U^{14}C$ glucuro hemicellulose in th UDP-D-xylose (Bq).
H T	0.08 +/- 0.01	20.1	0.552 +/- 0.062	36.0	1.03 +/- (
H B	0.33 +/- 0.03	79.9	0.926 +/- 0.070	64.0	6.09 +/- (
L T	0.36 +/- 0.01	74.4	0.507 +/- 0.044	44.0	1.71 +/-
L B	0.12 +/- 0.01	25.6	0.647 +/- 0.037	56.0	5.65 +/-

ated into hemicellulose during a glucuronyltransferase assay is not due purely to glucuronoxylan biosynthesis. However the UDP-D-xylose stimulatory effect is still evident, and because only one peak of activity is present, it is likely that this peak represents the position of glucuronyltransferase activity, but not its precise magnitude. It is interesting to note that the sucrose density at which UDP-D-xylose has its major effect at pH 6.0 (1.122 g cm^{-3}) is similar to the density at which glucuronyltransferase is located in the gradients performed at pH 7.5 (1.12-1.15 g cm^{-3}).

The particulate portion of total IDPase activity is known to be a marker for the golgi apparatus (Quail, 1979) and equilibrates at a different density to that of endoplasmic reticulum (rough and smooth) and mitochondria (Table III). However, the majority of the total IDPase activity is found in soluble fractions at the top of gradients (Fig. 47) as reported by other workers (e.g. Galbraith and Northcote, 1977; Hodges and Leonard, 1974; Morre *et al*, 1977). It is possible that this soluble activity is due to the action of non-specific phosphatases (Hodges and Leonard, 1974) but it may be due to the release of soluble IDPase enzymes from the golgi apparatus during homogenisation.

The IDPase activity that is normally used as a marker for the golgi apparatus is latent and develops to a maximum if left for several days at 4°C or if treated with a detergent (Ray *et al*, 1969). This extra activity (i.e. the

activity after activation minus the activity at extraction) is totally particulate (Morre *et al*, 1977).

In Fig.49 , it can be seen that latent IDPase activity has two peaks, one with a density of 1.06 g cm^{-3} (14% (w/w) sucrose) and the other with a density of between 1.12 and 1.17 g cm^{-3} (29 to 38% (w/w) sucrose). It is likely that the larger, more dense peak of activity represents the position of the golgi apparatus, because its denisty corresponds to that shown by the golgi apparatus in both pea and other systems (Table III,p38). The smaller peak is not as easily explained. A similar result was reported by Bowles and Kauss (1976). They explained the presence of the less dense peak in terms of latent IDPase activity located in transitional membrane elements that exhibited enzyme activities typical of both the endoplasmic reticulum and golgi apparatus. In their case, however, the less dense peak was larger than the more dense peak, and had a density of 1.09 g cm^{-3} (22% (w/w) sucrose). They were also measuring total latent IDPase activity as opposed to the activated increment.

As mentioned above, the larger peak of activated IDPase in the present system has a range in densities of between 1.12 and 1.166 g cm^{-3}. This totally overlaps the range of densities at which glucuronyltransferase is found (1.13 to 1.16 g cm^{-3} at pH 6.0 and 1.12 to 1.15 g cm^{-3} at pH 7.5).

The profile of glucan synthetase I (Fig. 46 and 48) also shows that the glucuronyltransferase equilibrates at a

density similar to that of the golgi apparatus, but because glucan synthetase II also equilibrates at this density, the possibility that the glucuronyltransferase is present in the plasma membrane cannot be ruled out. Glucan synthetase II activity has also been shown to overlap with the golgi in some other systems (Wienecke *et al*, 1982).

From the discontinuous gradients performed at pH 7.5 the results of the NADH-cytochrome c-reductase assays at different Mg^{2+} concentrations suggest that very little glucuronyltransferase is present in the endoplasmic reticulum. The change in density of the endoplasmic reticulum brought about by alterations in the concentration of Mg^{2+} ions is due to the differential binding of ribosomes. In the absence of Mg^{2+} ions, the ribosomes fail to bind to the endoplasmic reticulum, leaving it in the smooth form with a density of between 1.11 and 1.12 g cm^{-3} (26-29% (w/w) sucrose). In the presence of Mg^{2+} ions, the ribosomes bind tightly, producing the rough forms of the endoplasmic reticulum with a density of between 1.15 and 1.18 g cm^{-3} (35-41% (w/w) sucrose). From the results in Table X , it can be seen that alterations in the concentration of Mg^{2+} ions alter considerably the profile of NADH-cytochrome c-reductase without any significant effect on the glucuronyltransferase.

The results shown in this chapter indicate that the majority of glucuronyltransferase activity is present in either the golgi apparatus or the plasma membrane. It does

not appear to be present in the endoplasmic reticulum in any significant quantity. As stated earlier, the results have not allowed any distinction to be made between the golgi apparatus and plasma membrane. In order to do this, the sucrose density gradients will have to be expanded or rate zonal separation of organelles will have to be performed. On the basis of previous work (Ch. 1, p. 31) it is unlikely that the plasma membrane contains any glucuronyltransferase activity. Glucuronoxylan biosynthesis probably occurs in the golgi where the majority of matrix polysaccharides are synthesised.

Chapter 7.

DISCUSSION

The results that have been presented in this thesis have allowed the identification and characterisation of a glucuronyltransferase which is involved in the biosynthesis of glucuronoxylan hemicellulose in dark-grown pea (*Pisum sativum*) seedlings. The activity has been solubilised in Triton X-100 (a non-ionic detergent) and appears to be located in the golgi apparatus. In the particulate form, the glucuronyltransferase has been shown to transfer D-[U-^{14}C]-glucuronic acid from UDP-D-[U-^{14}C]-glucuronic acid to a xylan backbone. The activity is prolonged by the addition of UDP-D-xylose which is used to synthesise more xylan. This effect has not been demonstrated by Kauss (1967) who investigated the transfer of glucuronic acid into glucuronogalactan and glucuronoxylan in corn cobs. However, Kauss carried out incubations of only 15 min duration and it may be that these were not long enough to show an overall increase in activity (as shown in Fig. 15b, p. 82).

In dicotyledonous plants, the structure of glucuronoxylan molecules is quite complex (Ch. 1, p. 11). It is likely that the glucuronyltransferase activity is one of several which may act either in a co-ordinated fashion, or independantly, to produce the final product. Associated enzyme activities will include a xylosyltransferase and enzymes that are involved in the acetylation of xylose residues and the methylation of glucuronic acid residues. It is also likely that an arabinosyl transferase is present, for in dicots, the xylans to which glucuronic acid is attached often contain arabinose and are often called Arabino-(4-O-methyl-glucurono)-

xylans. Because glucuronic acid side chains are always methylated in dicot glucuronoxylans, the methylation reaction and its control may influence the transfer of glucuronic acid to the xylan backbone. It is possible that the pH and Mn^{2+} concentration (Ch. 3, p. 78) are important parameters. The optimum Mn^{2+} concentration involved in methylation is 10 mmol dm^{-3} (Kauss and Hassid, 1967(a)), as for the glucuronyltransferase activity, and the optimum pH for methylation is pH 8.1 (Kauss and Hassid, 1967a), a value which may be required to optimise the incorporation of glucuronic acid into any glucuronoxylan polysaccharides. Further work will have to be undertaken to see if SAM has any stimulatory effect on the transferase.

It is hoped that further research on this enzyme system will be undertaken as part of a broader investigation of polysaccharide synthesis during differentiation, upon which the author is about to embark. The radioactively labelled product of Triton X-100-solubilised enzyme still requires further analysis, and once its identity has been established, purification of the enzyme system will be performed. Gel filtration and ion-exchange chromatography, followed by glucuronyltransferase assays and polyacrylamide gel electrophoresis in SDS, will be undertaken. If possible, electrophoresis in a gel containing Triton X-100 instead of SDS will be carried out, followed by activity staining. Having achieved purification, an antisera will be made against the enzyme(s). Such antisera will allow the identification of the glucuronyltransferase, even when inactive, and will be

useful when investigating regulation of glucuronoxylan biosynthesis.

Other work to be carried out will involve sucrose density gradient analysis of the particulate enzyme at pH 7.5. It is hoped that the plasma-membrane and golgi apparatus can be separated so as to identify which of the two carries the glucuronyltransferase.

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