Subcellular localization and partial purification of a glucuronyltransferase involved in glucuronoxylan synthesis in pea (*Pisum sativum* var. Alaska) etiolated epicotyls.

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy.

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List of abbreviations

BSA	Bovine serum albumine
CHAPS	3-Cholamidopropyldimethylammonio-1-propane sulphonate
CMC	Critical micelle concentration
CMT	Critical micelle temperature
CTAB	Cetyltrimethylamonium bromide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
FPLC	Fast protein liquid chromatography
GDP'ase	Guanosine diphosphatase
GDP-	Guanosine diphosphate
GS II	Glucan synthase two
HLB	Hydrophile-lipophile balance
IDP'ase	Inosine diphosphatase
KDa	KiloDalton
LDAO	Lauryldimethylamine oxide
Mes	2-[N-morpholino]ethanesulphonic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NP40	Nonidet P40
PM	Plasma membrane
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris[hydroxymethyl] aminomethane
UDP-	Uridine diphosphate
UDP'ase	Uridine diphosphatase

Table of contents

Title page i
Acknowledgements ii
List of abbreviations iii
Table of contents iv
Summary viii

CHAPTER 1. INTRODUCTION. 1

1. 1. THE PLANT CELL WALL. 1
1. 1. 1. General composition and structure. 1
1. 1. 2. Heteroxylans: chemistry and distribution. 3
1. 1. 3. The role of heteroxylans in cell wall architecture. 5
1. 1. 4. Biosynthesis of cell wall polysaccharides. 8
1. 1. 5. Subcellular location of matrix polysaccharide synthases. 10
1. 1. 6. Control of the biosynthesis of cell wall polysaccharides. 13
1. 1. 7. 4-*O*-methyl glucuronoxylan biosynthesis in pea epicotyls. 14

1. 2. THE GOLGI APPARATUS. 16
1. 2. 1. Structure of the Golgi apparatus.. 16
1. 2. 2. Function of the Golgi apparatus. 17

1. 3. PURIFICATION OF MEMBRANE-BOUND PROTEINS. 19
1. 3. 1. Isolation of subcellular organelles. 19
1. 3. 2. Detergents. 24
1. 3. 3. Solubilization of membrane-bound proteins. 26
1. 3. 4. Other techniques used for the purification of glycosyltransferases. 28

1. 4. THE UDP-GLUCURONYLTRANSFERASES IN ANIMAL SYSTEMS. 29

1. 5. INTRODUCTION TO THE EXPERIMENTAL WORK. 31

CHAPTER 2. GENERAL METHODS. 33

2. 1. CHEMICALS. 33

2. 2. PLANT MATERIAL. 33

2. 3. PREPARATION OF PARTICULATE MEMBRANES. 33

2. 4. GLUCURONYLTRANSFERASE ASSAY. 34
2. 4. 1. Enzyme assay. 34
2. 4. 2. Extraction of chelator-soluble material. 35

2. 5. PROTEIN ASSAY. 36

2. 6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS. 37
2. 6. 1. 7.5 -15% polyacrylamide gradient gel. 37
2. 6. 2. 12 % polyacrylamide gel. 38

2. 7. SILVER STAINING. 39
2. 7. 1. Silver staining according to Morrissey (1981). 39
2. 7. 2. Silver staining according to Oakley *et al.* (1980). 39

CHAPTER 3. TIME COURSE EXPERIMENT. 41

3. 1. INTRODUCTION. 41

3. 2. METHOD. 41

3. 3. RESULTS. 42

3. 4. DISCUSSION. 43

CHAPTER 4. GOLGI MEMBRANE ISOLATION. 46

4. 1. INTRODUCTION. 46
4. 2. METHODS. 46
4. 2. 1. Organelle marker enzyme assays. 46
4. 2. 1. 1. Glucan synthase II. 46
4. 2. 1. 2. Latent IDP'ase. 47
4. 2. 1. 3. NADH-cytochrome C reductase. 48
4. 2. 1. 4. Succinate dehydrogenase. 48
4. 2. 2. Golgi subcompartmentation. 49
4. 2. 2. 1. Discontinuous sucrose density gradient. 50
4. 2. 2. 2. Continuous sucrose density gradient. 51
4. 2. 3. Rapid preparation of Golgi membrane enriched fractions. 51
4. 2. 4. Cell fractionation and Golgi subcompartmentation. 50

4. 3. RESULTS. 53
4. 3. 1. Development of a method for the preparation of a membrane fraction enriched with glucuronyltransferase activity. 53
4. 3. 2. Investigation of Golgi apparatus subcompartments. 56
4. 3. 2. 1. Isolation of subcellular organelle membrane fractions on discontinuous gradient. 56
4. 3. 2. 2. Golgi apparatus subfractionation. 57
4. 3. 2. 2. 1. 1.115 to 1.165 $g \cdot ml^{-1}$ (27 to 39% sucrose (w/w)) linear gradient. 57
4. 3. 2. 2. 2. 1.083 to 1.155 $g \cdot ml^{-1}$ (20 to 35% sucrose (w/w)) linear gradient. 58

4. 3. 3. Characterization of membrane fractions from discontinuous sucrose density gradient used for a rapid isolation of Golgi membranes . 58

4. 4. DISCUSSION 61

CHAPTER 5. DETERGENTS AND SOLUBILIZATION. 72

5. 1. SOLUBILIZATION OF PROTEINS FROM WHOLE MEMBRANE PREPARATIONS. 72

5. 1. 1. Introduction. 72

5. 1. 2. Methods. 72

5. 1. 2. 1. Solubilization with a selection of detergents. 72

5. 1. 2. 2. Two step solubilization. 73

5. 1. 3. Results. 74

5. 1. 3. 1. Solubilization of a crude membrane preparation. 74

5. 1. 3. 2. Sequential solubilization. 75

5. 2. SOLUBILIZATION OF PROTEINS FROM GOLGI MEMBRANES. 77

5. 2. 1. Introduction. 77

5. 2. 2. Methods. 77

5. 2. 2. 1. Glucuronyltransferase activity assay in the presence of different detergents. 77

5. 2. 2. 2. Golgi membrane-bound protein solubilization. 78

5. 2. 3. Results. 78

5. 2. 3. 1. Effect of selected detergents on glucuronyltransferase activity from particulate Golgi membranes. 78

5. 2. 3. 2. Effect of a range of Lubrol concentrations on particulate Golgi membranes. 80

5. 2. 3. 3. Effect of LDAO on particulate Golgi membranes. 80

5. 2. 3. 3. 1. Variations of LDAO concentration.. 80

5. 2. 3. 3. 1. Variations of the protein concentration of the particulate Golgi membranes. 81

5. 2. 3. 4. Solubilization of Golgi membranes. 81

5. 2. 3. 4. 1. LDAO solubilization. 82

5. 2. 3. 4. 2. Lubrol PX solubilization. 83

5. 2. 3. 4. 3. Dodecyl maltoside solubilization. 84

5. 3. DISCUSSION. 86

CHAPTER 6. CHROMATOGRAPHY. 91

6. 1. INTRODUCTION. 91

6. 2. METHODS. 91

6. 2. 1. Affinity chromatography. 91
6. 2. 2. Anion exchange chromatography. 92
6. 2. 2. 1. DEAE Sephadex G25 anion exchange chromatography. 92
6. 2. 2. 2. Dowex 1x2 (200) anion exchange chromatography. 92
6. 2. 2. 3. Anion exchange chromatography with the FPLC. 93
6. 2. 2. 3. 1. Anion exchange of LDAO solubilized material. 93
6. 2. 2. 3. 2. Anion exchange of Lubrol PX solubilized material. 94
6. 2. 2. 3. 2. Anion exchange of dodecyl maltoside solubilized material. 94

6. 3. RESULTS. 95
6. 3. 1. Affinity chromatography of NP 40 solubilized material. 95
6. 3. 2. Anion exchange chromatography. 96
6. 3. 2. 1. DEAE Sephadex G25 anion exchange. 96
6. 3. 2. 2. Dowex 1x2 (200) anion exchange. 96
6. 3. 2. 3. Anion exchange with the FPLC. 97
6. 3. 2. 3. 1. Anion exchange of LDAO solubilized material. 97
6. 3. 2. 3. 2. Anion exchange of Lubrol PX solubilized material. 97
6. 3. 2. 3. 2. Anion exchange of dodecyl maltoside solubilized material. 97

6. 4. DISCUSSION. 98

CHAPTER 7. CONCLUSION. 101

REFERENCES 103

SUMMARY

Glucuronyltransferase is an enzyme involved in the synthesis of the hemicellulosic glucuronoxylan. It catalyses the transfer of glucuronic acid from UDP-glucuronic acid onto a xylan backbone. The activity of this enzyme has previously been studied in whole particulate membrane preparation of etiolated pea epicotyls (Waldron & Brett, 1983). It was shown that this transferase is located chiefly within the Golgi apparatus membrane (Waldron & Brett, 1987) and that the activity can be solubilized from a crude membrane fraction with the non-ionic detergent, Triton X-100 (Waldron & Brett, 1983).

Because of the interference of Triton X-100 with the monitoring of proteins at 280 nm, another detergent was needed for the solubilization of the enzyme and its subsequent purification by chromatography. The enzyme activity was solubilized from a whole membrane fraction using different detergents singly and in combination. Some degree of glucuronyltransferase purification was obtained by sequential solubilization of the enzyme with a two step solubilization method involving i) Triton X-100 at low concentration and ii) NP40 at high concentration. In order to improve the purity of the initial membrane fraction used for solubilization, a protocol was designed to obtain a Golgi membrane-enriched fraction. The more precise localization of the glucuronyltransferase was then investigated. Using a discontinuous sucrose density gradient (18%/25%/35% sucrose (w/w)), three membrane fractions were obtained from an initial whole membrane homogenate. These were characterized by their marker enzyme activity and found to be enriched either with endoplasmic reticulum, Golgi apparatus or plasma membranes. The glucuronyltransferase was found chiefly with the Golgi membrane-enriched fraction. This was subsequently fractionated by centrifugation through a continuous sucrose density gradient. Three Golgi apparatus subcompartments (*cis-*, *medial-* and *trans-*) were obtained and characterized by their

latent IDP'ase activity. The glucuronyltransferase activity was found to be mainly associated with the less dense fraction, which corresponds to the *cis*-Golgi cisternae membranes.

The method employed to study of the Golgi apparatus subcompartmentation was later modified in order to provide a rapid protocol for the isolation of a fraction enriched with Golgi membranes and therefore, with glucuronyltransferase. This fraction was used routinely for the solubilization of membrane-bound proteins. Prior to attempting the solubilization, a selection of detergents (LDAO, CHAPS, octyl glucoside, dodecyl maltoside, Lubrol PX and Triton X-100) were tested for their effects on glucuronyltransferase activity from particulate Golgi membranes. They were assayed at concentrations near and above their CMC. Dodecyl maltoside and Lubrol PX were the least detrimental to glucuronyltransferase activity. The solubilization of proteins from the membrane-enriched fraction was carried out, and was efficient only when high concentration of detergent (LDAO, dodecyl maltoside and Lubrol PX) was used. This unfortunately resulted in inhibition of the glucuronyltransferase activity, but could be reversed, to some extent, by dialysis against buffer containing a lower concentration of the detergent. Purification of the solubilized glucuronyltransferase was attempted using different chromatographic techniques such as affinity (agarose:UDP-GlcA) and anion exchange chromatography (DEAE Sephadex G25, Dowex 1x2 (200) and FPLC Pharmacia Mono Q). Although chomatography led to the fractionation of the different proteins solubilized, as shown by SDS-PAGE and/or protein elution profiles, no glucuronyltransferase activity was recovered from any of the protein peaks collected during elutions.

CHAPTER 1: INTRODUCTION

1. 1. THE PLANT CELL WALL.

1. 1. 1. General composition and structure.

Essentially every cell of higher plants is encased in a cellulosic wall that consists of a framework of cellulose microfibrils embedded in a complex matrix of polysaccharides, proteins and glycoproteins. This structure has been compared to a "glass-fibre-reinforced-plastic" because of its relative strength and elasticity (Northcote, 1972). Many reviews and articles are available covering the chemistry, biochemistry and architecture of plant cell walls. (Preston, 1974; Darvill *et al.*, 1980a, 1985; Hori & Elbein, 1985; Bacic *et al.*, 1988; Delmer & Stone, 1988; Roberts, 1989; Brett & Waldron, 1990).

Cellulose is the major component of the microfibrillar phase of the cell wall. The microfibrils, which can be in a crystalline state in areas of the wall, consist of highly polymerised and unbranched β-(1→4)-D-glucans.

The matrix phase is composed of complex polysaccharides, proteins and glycoproteins which are closely associated with the cellulose microfibrils. The matrix polysaccharides were first classified according to their solubility properties during their selective extraction from the wall rather than according to their chemical composition (Preston, 1974). The noncellulosic polysaccharides were classified in two categories.

The pectic substances are a complex mixture of colloidal polysaccharides. They can be extracted from the cell walls with hot water or with hot aqueous solutions of chelating agents and/or dilute acids. The solvent action of the

chelating agents depends chiefly on their ability to complex with Ca^{2+} (and Mg^{2+}). The pectin polysaccharides are rich in galacturonic acid, rhamnose, arabinose and galactose. They are mainly rhamnogalacturonans I and II, arabinan, galactan, arabinogalactan I and homogalacturonan.

The hemicellulosic substances are the polysaccharides extracted from the wall with strong alkaline solutions. The hemicelluloses can then be separated into two fractions. Hemicellulose A is the water insoluble fraction which precipitates when the alkaline extract is neutralised to pH 5 with acetic acid. Hemicellulose B, remaining in the neutralised supernatant, is precipitated by the addition of ethanol. Hemicellulose B is thought to contain a higher proportion of uronic acid than hemicellulose A. The major uronic acid component of hemicellulose B is 4-*O*-methyl-D-glucuronic acid.

Hemicelluloses are mainly composed of D-glucose, D-galactose, D-mannose, D-xylose and L-arabinose joined together in different combinations and by various glycosidic linkages. They also contain glucuronic acid and/or other uronic acids. Hemicelluloses can be separated into different groups of polysaccharides: heteroxylans, xyloglucans, gluco-, galacto- and glucuronomannans.

A more recent classification of cell wall polysaccharides is based on their chemical composition and structure rather than on solubility properties during extraction from the wall (Aspinall, 1980; Bacic *et al.*, 1988). Cell wall polysaccharides are then classified as follows: β-(1→4)-D-glucans (cellulose), β-(1→4)-D-mannans and β-(1→3)-D-glucans (callose) form the homopolymer group. The heteropolymer group contains the β-(1→3)(1→4)-D-glucans, the heteroglucans (xyloglucans), the heteroxylans and also galactomannans, glucomannans, galactoglucomannans, rhamnogalacturonans and

arabinogalactans. Arabinans and galactans are also found in the heteropolymer group as they are thought to substitute the rhamnogalacturonans but are released during the pectin extraction procedure.

1. 1. 2. Heteroxylans: chemistry and distribution.

The glucuronoxylans studied in this project belong to the heteroxylan group of cell wall polysaccharides. The heteroxylans constitute a well-characterised group of polysaccharides which form the major components of the hemicellulosic fractions of terrestrial plants (Wilkie, 1979, 1985). Heteroxylans form the major non-cellulosic polysaccharides of primary walls of grasses and secondary walls of all angiosperms.

The heteroxylans comprise a family of polysaccharides consisting of a linear (1→4) linked β-D-xylopyranosyl backbone that can be substituted by various mono and oligosaccharide side chains as well as by *O*-acetyl groups and phenolic acids. Three main types of substitution of the xylan backbone can occur:

i) (1→3) linked α-L-arabinofuranosyl residues (and (1→2) in highly substituted arabinoxylans)

ii) (1→2) linked α-D-glucopyranosyluronide residues that can be methylated at the C_4 in the case of the 4-*O*-methyl glucuronoxylans (Fig. I. 1).

iii) more extended side chains with arabinose carrying more substitutions (e.g. galactose).

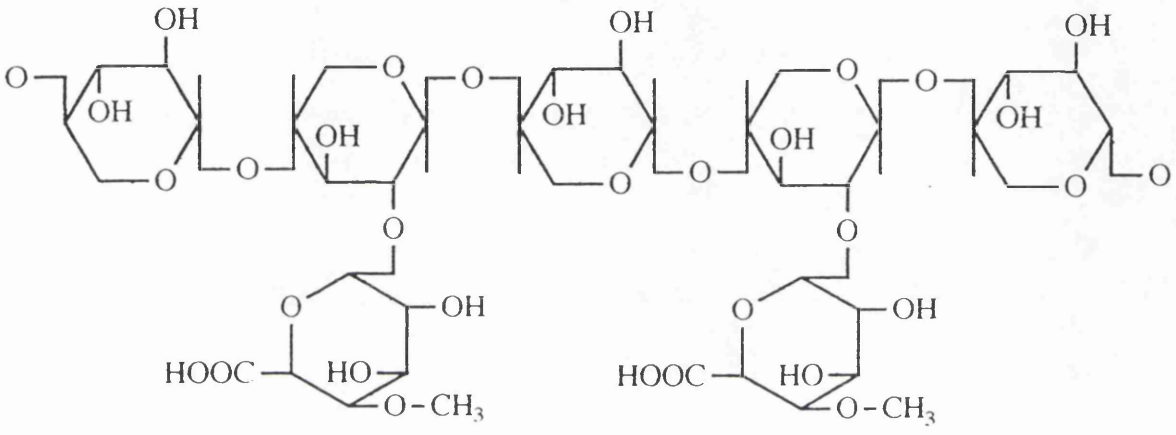


Fig. I.1: Structure of the glucuronoxylan: a (1→4)-linked chain of β-D-xylose residues substituted by a (1→2)-linked 4-*O*-methyl-α-D-glucuronic acid. The backbone can also be acetylated on either the C_2 or C_3.

The xylan backbone can also be substituted by an acetyl group linked to the C_3 or the C_2 of the xylosyl residue through an ester linkage.

Heteroxylans are a minor part of the non-cellulosic polysaccharides of the primary walls of dicotyledons. Cambial walls of different trees were shown to contain 2 to 10% of 4-*O*-methyl glucuronoxylans (Thornber & Northcote, 1961a,b, 1962). Glucuronoarabinoxylans were also found in the walls of suspension-cultured sycamore cells (Darvill *et al.*, 1980; Stevenson *et al.*, 1986).

In the lignified secondary wall of dicotyledons, 4-*O*-methyl glucuronoxylans are the major non cellulosic polysaccharides (Aspinall, 1980). Glucuronoxylans showing a high content of uronic acid groups (1 per 2 xylose residues) were extracted from the mucilage of quince tree seeds (Lindberg *et al.*, 1990).

The composition of the hemicellulosic fraction of the primary wall of monocotyledons has been well reviewed (Wilkie, 1979). The major hemicellulosic polysaccharides of the primary wall of monocotyledons are the heteroxylans and, the most common, is a highly substituted glucuronoarabinoxylan (Wilkie, 1985).

Highly substituted glucuronoarabinoxylans were identified in rice straw (Yoshida *et al.*, 1990) and in maize (Carpita, 1983; Kato & Nevins, 1984; Nevins & Kato, 1985). Further work on maize heteroxylans also showed that they were feruloylated (Nishitani & Nevins, 1989, 1990, 1991).

Highly substituted arabinoxylans were found in rye bran (Aspinall, 1980; Ebringerova *et al.*, 1990), rice bran (Shibuya & Iwasaki, 1985), wheat bran (Aspinall, 1980; Brioullet & Joseleau, 1987), in aleurone cell walls of oat

(Aspinall & Carpenter, 1984) and barley (Bacic & Stone, 1981a,b), in sorghum husks (Woolard *et al.*, 1976) and in corn hulls (Medcalf, 1985).

1. 1. 3. The role of heteroxylans in the cell wall architecture.

The cell wall is composed of cellulose microfibrils forming a frame or network which is embedded in a complex amorphous matrix of polysaccharides, proteins and glycoproteins. Lignin can also be present in secondary walls. The molecular organisation and the interactions of these components are important in defining the structure and architecture of the cell wall (Roberts, 1989; Varner & Lin, 1989). Cross-links between hydrophilic polymers of the matrix are the basis of the cohesiveness and flexibility of the cell wall (Aspinall, 1980; McNeil *et al.*, 1984; Fry, 1986; Fry & Miller, 1989).

There are many cross-links (Fry, 1986), both covalent and noncovalent, that could conceivably hold the wall together, such as coupled phenols (e.g. extensin isodityrosine bridges (Epstein & Lamport, 1982; Fry, 1982, 1984)), glycosidic bonds (e.g. xyloglucan to rhamnogalacturonan I (Keegstra *et al.*, 1973; Hayashi, 1989)), ester bonds (e.g. pectin diferuloyl bridges (Fry, 1983; Selvendran, 1985)), hydrogen bonds (e.g. xyloglucan-cellulose (Hayashi *et al.*, 1987)) and ionic bonds (e.g. extensin-pectin (Smith *et al.*, 1984) and pectin-pectin Ca^{2+} bridges (Jarvis, 1984)). More evidence for the importance of polysaccharide cross-links in holding the cell wall polymers together and determining its architecture comes from the work of McCann *et al.* (1990) on onion primary cell wall. Using shadowed replicas of rapidly frozen deep-etched specimens of primary cell wall, direct visualisation of physical cross-links between cellulose microfibrils in intact cell walls was made possible with high resolution electron microscopy. These cross-links were of hemicellulosic

nature, and more specifically xyloglucans, as revealed using selective extraction and analysis of hemicellulosic polymers from the walls. This extraction resulted in the lateral association of cellulose microfibrils in bundles of 2 to 20 units. Selective removal of pectic fractions did not alter the basic cellulose/hemicellulose architecture. Cellulose microfibrils of the onion primary wall seem therefore to be cross-linked together through xyloglucans with pectin fractions forming a separate coextensive network.

As part of the wall matrix, the heteroxylans interact with the other components of the wall through various kinds of linkages. Unsubstituted xylan chains are capable of crystallisation and this conformation is then stabilised intramolecularly by hydrogen bonding. In green algae, a β-(1→3)-D-xylan, studied by C-13-NMR spectroscopy, was found showing three different conformations: i) single chain, ii) single helix, iii) triple helix (Saito *et al.*, 1991). The water soluble heteroxylans can form viscous solutions but usually do not gel (e.g. aggregation of arabinoxylans from barley aleurone cell walls (Dea *et al.*, 1973)). The extent of intermolecular hydrogen bonding is controlled by the degree of substitution of the xylan backbone and the spatial arrangement of the substitutions along the backbone and this, in turn, controls the solubility properties of the polysaccharide (McNeil *et al.*, 1975).

The extraction of small amounts of hemicellulose from the cell wall using chaotropic agents suggests hydrogen bonding of these polysaccharides to cellulose (Selvendran *et al.*, 1985). The ability of heteroxylans to form hydrogen bonds with the cellulose microfibrils is chiefly determined by the extent of the substitution of the xylan backbone. Studies on the binding of arabinoxylans from barley aleurone cell walls to cellulose showed that xylans containing a high degree of side-chain substitution are more water soluble and bind less tightly to cellulose whereas molecules with less frequent substitutions are less

water soluble and bind more tightly to cellulose (McNeil *et al.*, 1975). It is also very probable that hydrogen bonds may occur between xylans and other hemicellulosic components such as xyloglucans (Hayashi *et al.*, 1987).

Heteroxylans can be cross-linked to other polymers of the wall through covalent cross-links. Some heteroxylans are substituted with feruloyl or *p*-coumaryl residues. The presence of diferuloyl residues found in the walls of monocotyledons and a few dicotyledons (Bacic *et al.*, 1988) suggests that ferulic acids esterified to heteroxylans can participate in a peroxidase-catalysed dimerisation reaction to form intra and intermolecular diferulic acid cross-links between the xylan molecules (Fry, 1986, 1988).

Linking of heteroxylans to lignin through benzyl-ester linkage involving the carboxyl groups of uronic acids and the hydroxyls of lignin monomers was postulated (Morrison, 1974; Aspinall, 1980; Bacic *et al.*, 1988). Phenolic acid bridges (involving feruloyl and *p*-coumaryl residues) between polysaccharide and lignin have been proposed (Scalbert *et al.*, 1985).

It is unlikely that the role of heteroxylans in the cell wall is a uniquely structural one, i.e. one which participates in holding the wall components together. There is some evidence for a biologically active involvement of xylose-containing oligosaccharides in providing signals to the cell during pathogenesis (Ryan & Farmer, 1991). The production of ethylene was induced in tobacco leaf discs when they were treated with fungal wall-degrading enzymes (Anderson, 1982). Further study showed that the protein inducing this ethylene production was an endoxylanase, which suggested a role for xylan fragments in signalling and inducing a response by the cell to the external aggression (Fuchs, 1989). In tobacco callus cultures, the activity of an endoxylanase copurified with a fungal protein shown to be an elicitor of

phytoalexin (Farmer & Helgeson, 1987). Although these are indications for a biological function of xylan fragments as signals for the cell, more work is needed in order to determine the exact structure of the signalling residue and its mode of action.

1. 1. 4. Biosynthesis of cell wall polysaccharides

Prior to polysaccharide (and glycoprotein) synthesis, the monosaccharides, which are generally derived from glucose and fructose *in vivo*, are activated by conversion to nucleoside diphosphate monosaccharides (sugar nucleotides), mainly UDP- and GDP-monosaccharides. These are probably the direct monosaccharide donors for the biosynthesis of wall polysaccharides (Fry, 1988) and are chiefly generated from the cellular pool of hexose phosphates by soluble nucleoside triphosphate pyrophosphorylases, followed by oxidation, decarboxylation and epimerisation (Hori & Elbein, 1985). The known reactions leading to the formation of nucleotide monosaccharides in plants have been reviewed (Feingold & Avigad, 1980; Fry & Northcote, 1983; Fry, 1985, 1988). Sugar nucleotides are synthesized in the cytoplasm and then transferred to the Golgi complex (Fleischer, 1989).

The formation of polysaccharide chains is carried out by membrane-bound enzymes which transfer monosaccharides from sugar nucleotides, usually to the nonreducing end of a growing polysaccharide chain. The enzymes that transfer sugar residues from one molecule to another are called glycosyltransferases and/or polysaccharide synthases when the product of the transfer is a polysaccharide.

There is still much controversy about the mechanism of chain initiation and the priming reactions. The synthesizing activity of isolated membranes studied *in vitro* shows more evidence for a chain elongation than for a *de novo* synthesis of polysaccharides. Monosaccharides are probably transferred to an existing acceptor isolated simultaneously during the membrane preparation. Fragments of pre-existing polysaccharide may act as a primer or acceptor for the elongation of the polymer chain (Delmer & Stone, 1988). The hypothesis of a protein primer or a protein-linked intermediate is supported by the evidence for the presence of a protein attached to *in vitro* synthesized glucuronoxylans which show a significant shift in molecular weight when treated with proteases (Hobbs *et al.*, unpublished results). Although there is evidence for the involvement of protein in polysaccharide chain initiation, the details of the priming mechanism are not yet known.

Cell wall polysaccharides are not synthesized in the wall itself but mainly or entirely by glycosyltransferases located in membranous organelles of the cell. Matrix polysaccharides, except callose, are synthesized in the endomembrane system of the cell.

The biosynthesis of the most abundant stereoregular polysaccharide found in cell walls, cellulose, and its regulation have been well reviewed (Delmer, 1987). The process of cellulose biosynthesis occurs at the plasma membrane, directly at its final wall destination (Shore & Maclachlan, 1975). The formation of cellulose microfibrils is thought to be carried out by cellulose-synthesizing particle complexes located in the plasma membrane as shown by freeze-fracture electron microscopy techniques (Delmer, 1987). Organized protein complexes were found in direct association with the ends of microfibrils or having the frequency and localization of distribution which paralleled the pattern and extent of cellulose deposition and active synthesis.

Callose synthase activity is the other UDP-glucose:β-glucan synthase activity detected in the plasma membrane of plant cells: this is generally latent in intact cells and becomes rapidly activated in response to perturbations (Brett, 1978), while the cellulose synthase is active in intact cells. It is thought that the same enzyme complex could be responsible for the activity of both callose (β-(1→3)-D-glucan) synthase and cellulose (β-(1→4)-D-glucan) synthase (Delmer & Stone, 1988). In response to external stress, the cellulose synthesizing activity of the complex would be switched to callose synthesis.

Complexity is added in the events of the biosynthesis of matrix polysaccharides since the backbone can be composed of more than one monosaccharide (e.g. glucomannans and rhamno-galacturonans) and can be substituted by mono- and/or oligosaccharide side-chains (e.g. xyloglucans, heteroxylans). The action and interactions of several different glycosyltransferases may therefore be involved in the biosynthesis of each polysaccharide. In the case of these heteropolysaccharides, there is evidence for a concerted synthesis of the growing chain by multienzyme complexes (Ray, 1980; Baydoun *et al.*, 1989a; Edwards *et al.*, 1989).

1. 1. 5. Subcellular location of matrix polysaccharide synthases.

The activity of various glycosyltransferases involved in the biosynthesis of cell wall polysaccharides has been investigated. Matrix polysaccharides are synthesized by glycosyltransferases bound to the endomembrane system of the cell and especially to the Golgi apparatus. Since they are membrane-bound, the purification and characterisation of the enzyme molecules in their active form is difficult. However, a number of enzyme preparations capable of synthesizing noncellulosic polysaccharides have been examined for their sugar nucleotide

specificity and structure of the newly synthesized product (Ericson & Elbein, 1980; Fincher & Stone, 1981: Delmer & Stone, 1988).

Particulate membrane fractions were prepared to study the enzymatic activity of galacturonyltransferases (Villemez *et al.*, 1965, 1966; Bolwell *et al.*, 1985), mannan and glucomannan synthases (Ericson & Elbein, 1980; Brett, 1981; Dalessandro *et al.*, 1986), galactosyltransferase involved in β-(1→4)-D-galactan synthesis (Panayotatos & Villemez, 1973), and fucosyltransferases (Green & Northcote, 1979; Camirand & Maclachlan, 1986; Farkas & Maclachlan, 1988). The cooperative action of a Mn^{2+} activated glucosyltransferase and an associated xylosyltransferase is required in the biosynthesis of xyloglucans (Ray, 1980; Hayashi & Matsuda, 1981a,b,c; Campbell *et al.*, 1988; Hayashi, 1989; Brummell & Maclachlan, 1989).

The activity of a xylosyltransferase catalysing the transfer of the xylose residue from UDP-xylose into a 1,4--linked xylan was detected in particulate membrane preparations from immature corn-cobs (Bailey & Hassid, 1966), mung bean (Odzuk & Kauss, 1972), French bean (Bolwell & Northcote, 1983), peas (Baydoun *et al.*, 1989b), sycamore xylem cells (Dalessandro & Northcote, 1981a,b), and from mesophyll cells of *Zinnia elegans* (Suzuki *et al.*, 1991).

The subcellullar location of the sites of matrix polysaccharide biosynthesis was demonstrated by electron microscopy and pulse-chase experiments in tissue fed with ^{3}H-labelled sugars, showing the transit of radioactive newly-synthesised polysaccharides from the Golgi stack to Golgi-derived vesicles and finally to the cell wall (Northcote & Pickett-Heaps, 1966; Bowles & Northcote, 1972, 1974, 1976). The fractionation of plant organelles using density gradient centrifugation and the isolation of Golgi secretory vesicles provided further evidence that the glycosyltransferases are located chiefly within the Golgi

apparatus and that this intracellular membranous system is involved in the synthesis and transport of polysaccharides to the wall (Engles, 1973, 1974; Ray *et al.*, 1976; Robinson *et al.*, 1976; Taiz *et al.*, 1983; Brummell *et al.*, 1990)

In corn roots, the intracellular localization of a fucosyltransferase involved in the biosynthesis of fucose-rich, high molecular weight polysaccharide was examined using density gradient centrifugation. The activity of the GDP-fucose: polysaccharide fucosyltransferase was found to be associated mainly with the Golgi membrane fraction. The results also suggested that the synthesis of the polysaccharide starts in the endoplasmic reticulum and is terminated within the Golgi apparatus (James & Jones, 1979; Green & Northcote, 1979). The same transferase activity was located in dictyosomes from cucumber (Sturm & Kindi, 1983).

The activities of the glucosyl- and xylosyltransferases involved in xyloglucan biosynthesis were found to be associated with membrane fractions corresponding to the Golgi apparatus (Ray, 1980; Hayashi *et al.*, 1988; Gordon & Maclachlan, 1989). Further work on the synthesis of xyloglucans considered the substitution of the polymer side-chains by fucosyl residues (Camirand & Maclachlan, 1986; Farkas & Maclachlan, 1988). The xyloglucan fucosyltransferase was located in dictyosomal membranes (Ray, 1980, Camirand *et al.*, 1987). When the differential distribution of xyloglucan glycosyl transferases was investigated, the fucosyltransferase activity was found to be associated more precisely with Golgi secretory vesicles (Brummell *et al.*, 1990) whereas the glucosyl- and the xylosyltransferase were localized within the Golgi apparatus stack, indicating the probable transfer of fucose residues at a later stage of the synthesis of the xyloglucans.

1. 1. 6. Control of the biosynthesis of cell wall polysaccharides.

The problem of the control of the biosynthesis of the cell wall components has been discussed but is not yet definitively solved (Bolwell, 1988, Northcote, 1989). Since the cell wall is subjected to composition and structural changes - during growth and differentiation and in response to external factors- the control of the biosynthesis of cell wall polysaccharides can be exerted at different levels within the cell. For example, regulation of polysaccharide synthesis may occur by genetic control of the amount of glycosyltransferases synthesized and present in the membranes. The activity of the polysaccharide synthases may also be controlled by the amount of sugar nucleotides available as substrate which depends on the rate of formation of sugar nucleotides in the cytoplasm and their translocation across the endomembrane system. The activity of different epimerases that can catalyse interconversions of sugar nucleotides, also contributes to the control of polysaccharide polymerization (Northcote, 1989). In addition, regulation of the transport, targeting and fusion of secretory vesicles containing the polysaccharides to specific sites of the plasma membrane probably controls the development of the cell wall (Northcote, 1985a,b, 1989).

The fine control of polysaccharide synthesis is probably achieved at the level of interactions between the different transferases of the enzyme complex involved in the process. The degree of precision of synthesis of the cell wall heteropolymers has been discussed (Waldron & Brett, 1985) and two models of heteropolysaccharide synthesis, called "precise" and "imprecise", were proposed. The "imprecise synthesis" describes a model where the backbone of a heteropolysaccharide is synthesized independently of its side-chains, thus resulting in an irregular distribution of the substitution on the backbone. The "precise synthesis" model is one in which the enzymes involved are absolutely

specific in determining the glycosyl residues present, their linkages and the regularity of the primary structure of the polysaccharide, and this can only be achieved if the enzymes involved add glycosyl residues in a precise sequence. The control of the rate of synthesis of the whole polysaccharide in the latter model would be dependent on substrate availability and on inhibition of the enzymes of the complex.

1. 1. 7. 4-*O*-methylglucuronoxylan biosynthesis in pea epicotyls.

The biosynthesis of the 4-*O*-methylglucuronoxylans requires the combined activities of several glycosyltransferases, since, although the xylan backbone is mainly substituted by glucuronic acid, arabinose side-chains can also be present. The glucuronic acid residue is often methylated on the C_4 and feruloylation of the polymer can occur. The biosynthesis of this heteroxylan has been studied in particulate membrane preparations from pea epicotyls. The synthesis of the xylan backbone is catalysed by a xylosyltransferase which transfers the xylose residue from UDP-xylose to the growing polymer. The substitution of the backbone by glucuronic acid is achieved by the action of a glucuronyltransferase which catalyses the transfer of glucuronic acid from UDP-glucuronic acid onto the xylan backbone (Kauss, 1967; Waldron & Brett, 1983). The addition of methyl groups to the side-chain glucuronic acid occurs at a later stage in the synthesis of the 4-*O*-methylglucuronoxylan by transfer from S-adenosyl-methionine (Baydoun *et al.*, 1989a,b). The acetylation of the xylan backbone is thought to occur through a transfer of acetyl residues from acetyl Co-A (Fincher & Stone, 1981). The activity of the glucuronyltransferase in particulate membrane preparations is stimulated in the presence of Mn^{2+} (10 mM) and is optimal between pH 5 and 6. The incorporation of radioactivity from UDP-D-[^{14}C]glucuronic acid into the newly synthesized polysaccharide is

sustained in the presence of UDP-D-xylose during the incubation. This latter contributes to the sustained elongation of the xylan backbone. The glucuronyltransferase activity was shown to be localized within the Golgi apparatus using continuous and discontinuous sucrose density gradient centrifugation (Waldron & Brett, 1987).

The interactions between the xylosyltransferase and the glucuronyltransferase involved in glucuronoxylan synthesis were investigated (Waldron & Brett, 1983). It was shown that the incorporation of glucuronic acid from UDP-glucuronic acid into glucuronoxylan was sustained in the presence of UDP-xylose, suggesting that newly synthesized xylan was acting as an acceptor for the further incorporation of glucuronic acid, but the degree of "precision" of the glucuronoxylan synthesis remained unknown. In more recent work (Baydoun *et al.*, 1989b), particulate membrane preparations were first incubated with radioactive UDP-xylose in order to synthesize the xylan backbone. The second incubation was carried out in the presence of radioactive UDP-glucuronic acid to promote the addition of glucuronic acid onto the preformed xylan backbone. The results of this experiment showed that the ability of a preformed xylan to incorporate glucuronic acid from UDP-glucuronic acid was very limited. This suggested that glucuronic acid would not be added to preformed xylan and that the incorporation of glucuronic acid during glucuronoxylan synthesis seems to match the "precise synthesis" model previously proposed (Waldron & Brett, 1985; Baydoun *et al.*, 1989b).

1. 2. THE GOLGI APPARATUS.

1. 2. 1. Structure of the Golgi apparatus.

The complex Golgi apparatus is a major and highly polarised component of the endomembrane system of both plant and animal cells. This organelle consists of stacks of smooth surfaced, flattened sacs or cisternae with associated small vesicles (Farquhar & Palade, 1981; Robinson & Kristen, 1982; Farquhar, 1985; Morré, 1987). In plant cells, up to several hundred individual dictyosomes (or stacks of cisternae) can be found in the cytoplasm and they are not as closely associated with the endoplasmic reticulum as in animal cells (Robinson & Kristen, 1982). The margins of the cisternae are fenestrated and usually in continuity with a system of tubules and vesicles which may be in connection with other components of the endomembrane system (Morré, 1987).

The entire structure of the Golgi apparatus is a polarised system of cisternae where the membranes at one pole resembles those of the endoplasmic reticulum membranes in their organisation and chemistry, while those of the opposite pole are similar to the plasma membrane. Each Golgi stack contains three types of cisternae. The pole proximal to the ER is called the *cis*- face or forming face. At the opposite pole of the Golgi apparatus, characterized by the presence of mature secretory vesicles, is the distal pole or *trans*-face. This pole of the dictyosome is also known as the mature or maturing face. In between these two poles, there are intermediate cisternae described as intercalary or *medial* cisternae which have characteristics intermediate between those of the *cis*- and the *trans*-face. Within each dictyosome, cisternae are separated from one another by an intercisternal space of 10 to 15 nm. In plant cells, the intercisternal spacing increases from the forming face to the maturing face while the width of the cisternae decreases in the same direction (Robinson &

Kristen, 1982). The increasing presence of intercisternal fibre elements seems to be correlated with the narrowing of the cisternal lumina indicating more evidence for a structural polarity of the dictyosomes from the *cis-* to the *trans-*face. Increase in membrane thickness has also been observed from the forming face to the maturing face (Robinson & Kristen, 1982; Morré, 1987). The changes in membrane characteristics across the polar axis of the Golgi apparatus has been described using free-flow electrophoresis techniques (Morré *et al.*, 1987). Cisternae, enzymically and mechanically unstacked, were separated depending on their surface charges. A survey of cytochemical and biochemical markers was carried out in fractions enriched variously with *cis-*, *medial* and *trans-*cisternae. A gradual appearance of plasma membrane markers from the *cis-* to the *trans*-face was evident: intermediate cisternae possessed intermediate characteristics.

1. 2. 2. Function of the Golgi apparatus.

The major function of the Golgi apparatus in plant and animal cells is to transport, modify and direct proteins and lipids from the endoplasmic reticulum where they are synthesized to their final intra- or extracellular destination. The Golgi apparatus is a transition organelle functioning as intermediary between the endoplasmic reticulum and the cell surface (Morré *et al.*, 1989). The involvement of the Golgi apparatus and its subcompartments in the terminal glycosylation of membrane glycoproteins and glycolipids has been well established (Chrispeels, 1985; Dunphy & Rothman, 1985; Sturm *et al.*, 1987; Trinchera & Ghidoni, 1989; Moore *et al.*, 1991). Thus, acting as the main distribution centre where the majority of the sorting decisions are made, the Golgi apparatus assumes a general role in membrane flux and differentiation associated with the processing of proteins, glycoproteins and glycolipids

(Robinson & Kristen, 1982; Morré, 1987; Rothman & Orci, 1990). Intracellular transport is mediated by carrier vesicles that bud from each compartment of the endomembrane system and then fuse to the next compartment (Farquhar, 1985; Pfeffer & Rothman, 1987; Griffiths *et al.*, 1989, Rothman & Orci, 1990). The vesicle membrane is coated with specific transport proteins which are necessary for the precise targeting and fusion of the vesicles with the target membrane (Rothman & Orci, 1992).

In plant cells, the Golgi apparatus is also involved in the synthesis of the components of the matrix of the cell wall. The dictyosomes of plant cells are the site of assembly of glycoproteins, proteoglycans and complex polysaccharides. Immuno-cytochemical techniques have been used to study the different assembly pathways of glycoproteins and complex matrix polysaccharides of the cell wall (Moore *et al.*, 1991). Within the Golgi stack, a distinct physical separation of the synthesis and secretion pathways of two different matrix polysaccharides was observed. The hemicellulosic xyloglucan seemed to be synthesized in the *trans*- Golgi cisternae and to exit the Golgi apparatus from the *trans*-Golgi network whereas the synthesis of the pectic rhamnogalacturonan I occurred in the *cis*- and *medial* cisternae and departed from the *medial* cisternae.

O-linked arabinosylation of extensin residues was shown to occur in the *cis*-cisternae (Moore *et al.*, 1991). Glycosylated proteins then pass through the dictyosome to be released in secretory vesicles from the *trans*-Golgi network. Compartmental processing of glycoproteins substituted by N-linked oligosaccharide chains from the *cis*- to the *trans*-face of the Golgi apparatus has also been described (Dunphy & Rothman, 1985). Therefore the subcompartmental organization of the Golgi apparatus that permits simultaneous synthesis of complex matrix polysaccharides and glycosylation of

glycoproteins, may also provide a mechanism for the control of the production and content of secretory vesicles and for the accurate targeting of their final destination.

1. 3. PURIFICATION OF MEMBRANE-BOUND PROTEINS.

The first step in the purification of a membrane-bound protein is to obtain a fraction enriched with the membranes containing the protein. This is generally achieved by centrifugation of a whole membrane preparation through a density gradient. The next step is the isolation of the protein from the membranous environment. Purification of the membrane-bound glycosyltransferase from other membrane proteins is essential for the study of its regulation and molecular structure. This is realized by the solubilization of the membranes with a suitable detergent.

1. 3. 1. Isolation of subcellular organelles.

For the successful isolation of an organelle it is necessary for its structure to be retained after homogenization of the cells from the starting material and during subsequent manipulations. The problem with plant cells is the high shear forces required to disrupt the cell wall which is the main barrier to the release of intracellular proteins and organelles. The forces applied to break open the cells are often too high to preserve the structure of the Golgi apparatus and this results in fragmentation of the Golgi stack into single cisternae or vesicles. When the homogenization is too tough on the Golgi apparatus a stabilizing agent such as glutaraldehyde can be added to the homogenization medium, and this has often been used when isolated dictyosomes were to be

studied by electron microscopy. However, for the study of membrane-bound enzyme activity, the addition of glutaraldehyde in the homogenization medium should be avoided since the enzymatic activity of isolated membranes is strongly reduced in the presence of glutaraldehyde (Morré & Buckhout, 1979).

Mechanical methods of cell disruption generate heat. To avoid temperature denaturation of the proteins and membranes, it is therefore essential to pre-chill the equipment to be used for the homogenization of the plant material and ideally pre-cool it to 4°C. All further manipulation of the plant material during the isolation of the organelle should be carried out at 4°C. For the isolation of subcellular organelles the use of a mechanized blender, such as a Polytron blender, generally generates too high shear forces and over-heating of the material to permit the recovery of structurally intact organelles. Lower shear forces are applied when the plant tissue is initially chopped into small pieces with scissors or a razor blade and the homogenization carried out with a chilled pestle and mortar (Green, 1983; Nagahashi, 1985). In order to reduce the forces required to break the wall, plant material can be treated with cell wall degrading enzymes prior to gentle homogenization with pestle and mortar (Brett & Northcote, 1975). Preparation of protoplasts is probably the most extreme answer to the problem as cell walls are totally removed before homogenization. Protoplast formation followed by osmotic shock is a very mild procedure and permits the recovery of organelles with high integrity and purity (Price, 1983; Thayer, 1985; Sturm *et al.*, 1987). However, it is difficult to obtain sufficient material for enzyme studies using this technique.

When designing the homogenization medium one should consider several parameters such as buffering capacity and pH, the ionic and osmotic strength of the medium and the inclusion of some membrane protective agents. The

buffering capacity should be high enough to maintain a constant pH during the manipulation of the extract (Kinne-Saffran & Kinne, 1989). This pH should be the same as the pH in the intact cell. A suitable homogenization medium is buffered between pH 7.0 and 7.4 (Green, 1983). Sucrose is often included to act as an osmolyte in the homogenization medium at a concentration of 8 to 10% (w/w) in order to avoid the bursting of organelles under osmotic stress when the cells are broken open. This also avoids the release of toxins and degrading enzymes from liposomes and peroxysomes. To prevent the oxidation of the sulphydryl groups of proteins, antioxidants such as β-mercaptoethanol or dithiothreitol (DTT) can be added to the homogenization medium (Cleland, 1964). $MgCl_2$ is also added with EDTA which chelates toxic heavy metal ions and prevents the aggregation and flocculation of membranes during subsequent manipulations, especially at the stage of density gradient centrifugation. Finally it may be helpful to include, in the homogenization medium, a protease inhibitor such as phenylmethylsulphonylfluoride (PMSF) at about 50 $\mu g \cdot l^{-1}$ (Green,1983).

Separation of organelles and membranes is based on differences in size and density (or surface properties in the case of free-flow electrophoresis). The most common approach to subcellular fractionation is a combination of differential centrifugation (separation based on molecular weight or size) and density gradient centrifugation (separation based on the relative density of each organelle) (Morré & Buckhout, 1979; Morré *et al.*, 1987). Differential low speed centrifugation is typically used to pellet most of the nuclei, plastids, cell wall fragments and mitochondria. The remaining supernatant can be further fractionated by density gradient centrifugation. Several different materials can be used to make the density gradient such as sucrose, glycerol, dextran, Ficoll, Percoll, caesium chloride, Renografin and Urografin (Nagahashi, 1985). Maximum separation of the subcellular organelles in continuous density

gradients is obtained as membranes reach their characteristic equilibrium density during isopycnic centrifugation. Discontinuous or step gradients are formed by the superposition of several layers of solution of different densities. Material fractionated on such gradients is recovered as bands of membranes of different densities accumulating at the various interfaces.

The nature of the membrane fractions isolated by density gradient centrifugation can be identified by their morphological and cytochemical characteristics and biochemically by their associated markers (Bowles *et al.*, 1979; Quail, 1979; Nagahashi, 1985). A marker is a specific identification label that can be used to distinguish a subcellular organelle from the others after fractionation of the cell. An ideal marker is unique to one subcellular component but such markers are rare (Morré & Buckhout, 1979). Many marker enzymes of plant subcellular organelles have been identified and there are several reviews and books available describing the different organelle markers and the methods of assaying for their presence (Bowles *et al.*, 1979; Quail, 1979; Hall & Moore, 1983; Nagahashi, 1985; Morré *et al.*, 1987).

For the identification of the endomembrane network, various marker enzymes are used. The endoplasmic reticulum is often characterized by the activity of NADPH-cytochrome C reductase. One of the marker enzymes for the plasma membrane is a glucosyltransferase, glucan synthase II, which is activated in the presence of a high concentration of UDP-glucose and in the absence of Mg^{2+}, whereas glucan synthase I, a marker for the Golgi apparatus, is assayed at low concentrations of UDP-glucose and activated by the presence of Mg^{2+} (Bowles *et al.*, 1979; Hall, 1983). The most commonly used marker enzyme for the Golgi apparatus is the inosine diphosphatase (IDP'ase) that was first localized, along with other nucleoside phosphatases such as UDP'ase and GDP'ase, in the Golgi apparatus of plants by histochemical methods

(Dauwalder *et al.*, 1969). Although there are high levels of soluble enzyme activity and although the IDP'ase activity can be associated with other subcellular compartments, the IDP'ase activity of the Golgi apparatus is distinguished from the other activities because of its characteristic latency. The latency of the enzyme activity can be overcome by storage of the membrane fractions for several days in the cold or by assaying the fractions in the presence of a detergent (e.g. Triton X-100) (Green, 1983).

The Golgi apparatus has been isolated by isopycnic centrifugation and it was shown that it forms a band at a density of 29-33% (w/w) sucrose (1.12-1.15 $g \cdot ml^{-1}$) (Shore & Maclachlan, 1975; Green, 1983; Chrispeels, 1985; Waldron and Brett, 1987; Mitsui *et al.*, 1990). When subfractionation of the Golgi stack of plant cells was carried out, only two subfractions of the plant dictyosome were isolated (Ray *et al.*, 1976; Taiz *et al.*, 1983; Ali *et al.*, 1985; Sturm *et al.*, 1987; Brummell *et al.*, 1990), unlike in animal cells where three subfractions (*cis-*, *medial-* and *trans-*) were identified (Rothman & Orci, 1990),.

Using rate-zonal centrifugation in renografin density gradients, Taiz and coworkers (1983) isolated two fractions enriched with IDP'ase and glucan synthase I activities at respective densities of 18-24 and 12-15 eq. % (w/w) sucrose. They were examined by electron microscopy and consist respectively of cisternae and vesicles derived from the Golgi apparatus of cells from pea stem fragments.

From preparations of Golgi membrane-enriched fractions derived from sycamore protoplasts, Ali *et al.* (1985) isolated two subfractions characterised by distinct peaks of IDP'ase activity at respective densities of 1.09 $g \cdot ml^{-1}$ and 1.12 $g \cdot ml^{-1}$. The purity of these fractions was assessed by electron microscopy. The most dense fraction, containing the highest IDP'ase activity, consisted

principally of vesicular Golgi membranes. The other fraction, although showing a high level of IDP'ase activity, seemed to be contaminated by endoplasmic reticulum membranes. It was concluded that these two fractions correspond to the *cis*- and the *trans*-compartment of the dictyosome.

For the study of the differential localization of xyloglucan glycosyltransferases in pea Golgi apparatus, rate zonal centrifugation in renografin gradient was used to separate two distinct peaks of latent UDP'ase activity: a light density (15-20 eq. % (w/w) sucrose), narrow peak corresponding to Golgi secretory vesicles and a denser (28-34 eq. % (w/w) sucrose), broad peak which corresponds to cisternae and whole dictyosomes. When the second peak of membranes was further fractionated on another, shallower, renografin gradient, two peaks of latent UDP'ase were obtained which seemed to correspond to the *cis*- and the *trans*-subcompartments of the dictyosome (Brummell *et al.*, 1990).

1. 3. 2. Detergents.

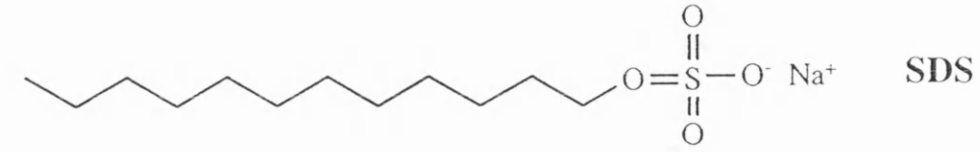
The purification of membrane-bound proteins requires their isolation from the biological membranes. These are formed of phospholipid bilayers in which proteins, glycoproteins, glycolipids and steroids are embedded in relative amounts according to the role and metabolic activity of the membrane. Therefore every approach to the purification of a membrane-bound protein unavoidably deals with solubilization (Hjelmeland, 1990).

Effective solubilization of membrane-bound protein involves both the selection of a detergent and appropriate conditions for solubilization. Although the primary role of detergents in protein purification is to effect solubilization,

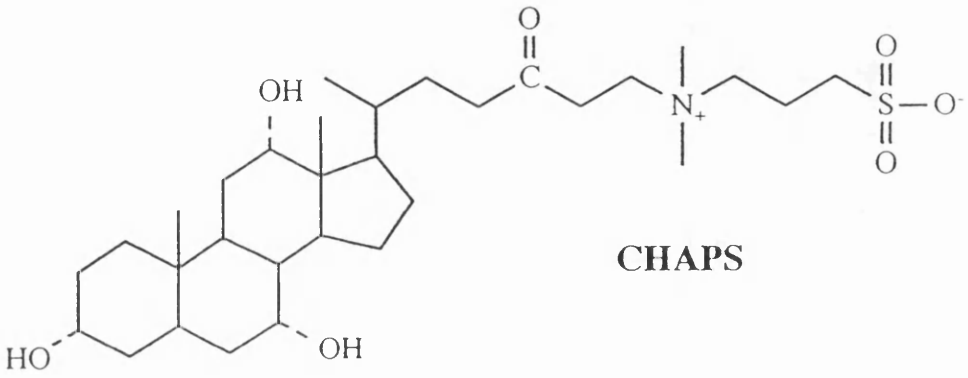
they can also be used as deaggregating agents (e.g. SDS in gel electrophoresis), as aids to membrane-protein reconstitution or recrystallization and as additives to immunoassay or other protein assay mixtures (Neugebauer, 1990).

Detergents belong to the group of chemical components also called amphiphiles since each detergent consists of a hydrophobic portion which is more soluble in oil and other hydrocarbon solvents, and a hydrophilic moiety which is more soluble in water. An example of the structure of some detergents commonly used is shown in Fig. I.2. Detergents are generally classified (i) on the basis of the charge and/or nature of the hydrophilic portion, and (ii) on the flexibility and/or chemical nature of the hydrophobic portion (Neugebauer, 1988). Head groups or hydrophilic portions can be anionic (negatively charged, e.g. SDS), zwitterionic (containing both positive and negative charges, e.g. CHAPS), nonionic (not charged, e.g. Triton X-100) or cationic (positively charged, e.g. CTAB).

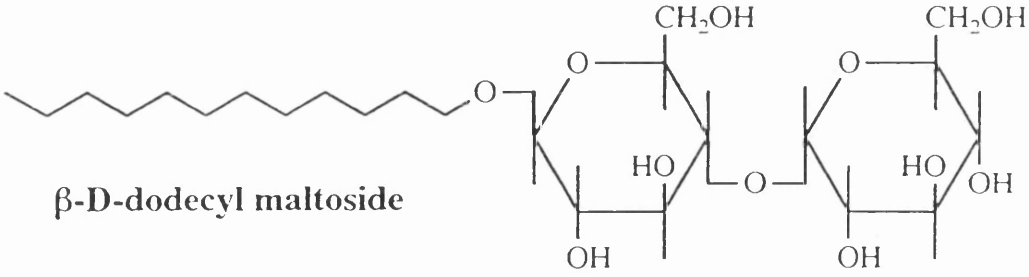
All amphiphiles such as detergents possess a capacity to form structures called micelles. The micelles can be defined as thermodynamically stable colloidal aggregates, spontaneously formed by amphiphiles above a certain concentration range known as the critical micelle concentration (CMC) and at temperatures above the critical micelle temperature (CMT). The CMT is the temperature at which a detergent/solvent system passes from a hydrated crystalline state to an isotropic micellar solution. Below the CMC, the amphiphile is dispersed in its monomeric form (Helenius & Simmons, 1975). Each detergent has a characteristic phase diagram that describes the conditions under which crystalline detergent, micelles or monomers will exist (Fig. I.3). Detergent micelles are also characterised by the aggregation number, which is the average number of monomers per micelle, and by their HLB (hydrophile-



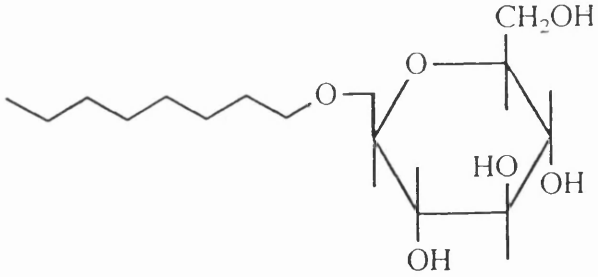
SDS



CHAPS

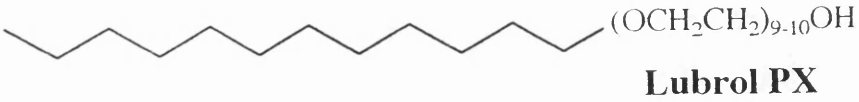


β-D-dodecyl maltoside

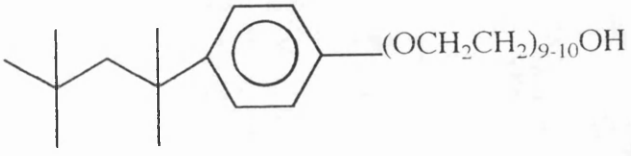


β-D-octyl glucoside

Fig. I.2: Structure of commonly used detergents.



Lubrol PX



Triton X-100

Fig. I.2 (continued): Structure of commonly used detergents.

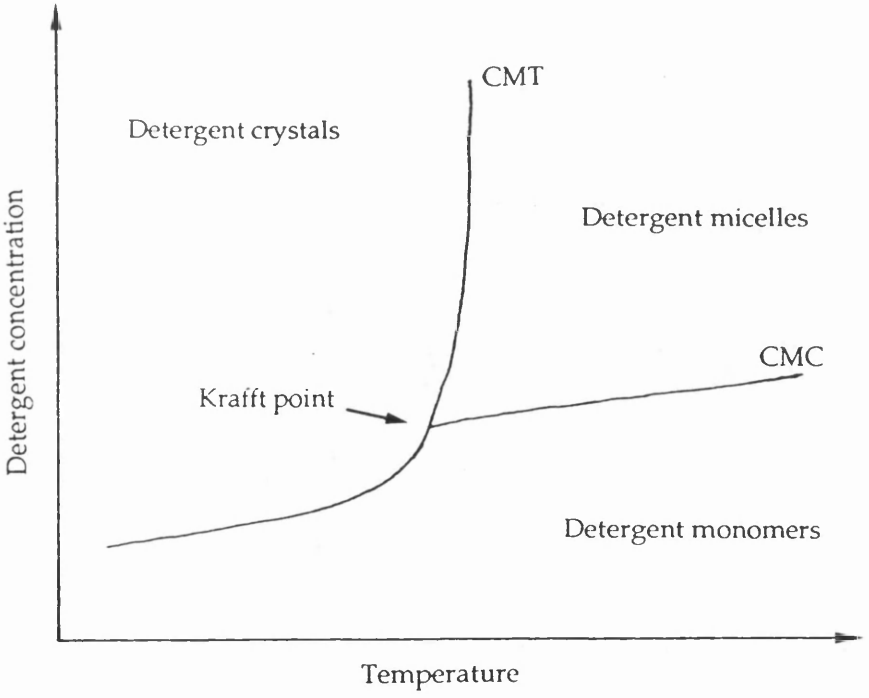


Fig. I.3: Temperature-composition phase diagram for detergent solutions.

lipophile balance number), which provides an indication of the hydrophilicity of the detergent (Neugebauer, 1990).

There are factors that can affect the solubility and/or stability of the detergent, such as temperature, pH, ionic strength, detergent concentration, presence of multivalent ions, purity and the presence of organic additives. In the process of choosing a detergent, it is necessary to consider these parameters in order to preserve the protein to be solubilized in the desired structural and/or functional state. Other criteria can be important in making the choice of a detergent for protein purification, such as its non-denaturing and its spectral properties, since some detergents (e.g. Triton X-100) interfere with the UV monitoring of proteins at 280 nm or can interfere with reagents in protein assays (e.g. LDAO). Effective solubilization of membrane proteins is achieved by the selection of the appropriate detergent and solubilization conditions (Van Reswoude & Kempf, 1984; Jones *et al.*, 1987; Neugebauer, 1988).

1. 3. 3. Solubilization of membrane-bound proteins.

Detergents have been used alone and in combination to solubilize proteins from membrane fractions of animal cells. Glycosyltransferases involved in the glycosylation of glycolipids were solubilized with CHAPS and Zwittergent 3-14 (Durieux *et al.*, 1990). NP40 was used to solubilize protein precursors to glycoproteins (Sharma *et al.*, 1990). Enzymes involved in other metabolic reactions were solubilized with CHAPS (Loe *et al.*, 1989), Zwittergent 3-14 (Barry *et al.*, 1989) and by sequential extraction with CHAPS, Triton N-101 and sodium N-lauroylsarcosinate (Vermillon & Schroepfer, 1990).

The activity of a β-(1→3)-D-glucan synthase has been investigated in both plant and fungal systems and its solubilization was achieved with digitonin (Fèvre, 1979; Henry & Stone, 1982; Eiberger *et al.*, 1985; Thelen & Delmer, 1986; Eiberger, 1987; Kauss & Jeblick, 1987), octyl glucoside (Henry & Stone, 1982), CHAPS (Sloan *et al*, 1987; Lawson *et al*, 1989; Slay & Watada, 1989; Girard & Fèvre, 1989; Wasserman *et al*, 1989; Wu *et al*, 1991), Zwittergent 3-14 (Morrow & Lucas, 1986) and a combination of octyl glucoside and CHAPS (Quigley *et al.*, 1988).

An attempt to purify the glycosyltransferases involved in the synthesis of cell wall heteropolysaccharides was carried out when the glucosyl- and mannosyltransferases involved in the biosynthesis of glucomannan in *Phaseolus aureus* hypocotyls were solubilized using Triton X-100 (Heller & Villemez, 1972a,b). Further attempts to purify these enzymes from the solubilized fractions were unsuccessful as the mannosyltransferase was inactivated when submitted to gel filtration. In addition, the stability of the enzyme activities was rapidly lost upon solubilization.

Triton X-100 was used to solubilize a UDP-glucuronyl- and a UDP-xylosyltransferase, involved in glucuronoxylan biosynthesis, from a particulate membrane preparation from etiolated pea epicotyls (Waldron *et al.*, 1989). Efficient recovery of the enzyme activity in the solubilized fraction was achieved only at relatively high concentrations of the detergent (5% (w/v)) and in this case, the solubilized enzyme could be stored for several weeks at -20°C without losing its activity.

1. 3. 4. Other techniques used for the purification of glycosyltransferases.

A chitin synthetase involved in the biosynthesis of the cell wall was solubilized with digitonin from the membranes of *Saccharomyces cerevisiae* (Kang *et al.*, 1984). The property of the enzyme to catalyse the synthesis of an insoluble product was exploited for its purification using a procedure known as product entrapment. This consists of incubating the solubilized enzyme with substrate (UDP-GlcNAc for chitin synthase) under conditions that allow synthesis of insoluble polymeric product. On centrifugation of the incubation mixture, 50% of the chitin synthase activity cosedimented and was trapped within the network of newly-formed chitin chains. The enzyme was subsequentely recovered by resuspension of the pellet in a large volume of buffer containing digitonin. A similar method of purification of glycosyltransferases by entrapment in the reaction product was used for the isolation of CHAPS solubilized β-(1→3) and β-(1→4) glucan synthases from *Saprolegnia monoica* (Bulone & Fèvre, 1989) and to obtain a preparation enriched with CHAPS solubilized β-(1→3) glucan (callose) synthase from storage tissue of red beet (*Beta vulgaris* L.) (Wu *et al.*, 1991).

Photoaffinity labelling was used to identify polypeptides involved in the biosynthesis of β-(1→3) glucan from membranes of carrot cells. Membrane fractions were incubated with photoaffinity labelled {^{32}P}5N$_3$UDPG and were subsequentely activated by UV-irradiation. Proteins were submitted to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and labelled polypeptides were visualized by autoradiography. Several UDPG-binding proteins were then revealed that had a similar molecular weight to previously identified polypeptide subunits involved in mung bean glucan synthesis (Lawson *et al.*, 1989). Photoaffinity labelling has also been used for the study of animal glucuronyltransferases. When human liver microsomes with known

UDP-glucuronyltransferase activities were photolabelled with [β-^{32}P]5-N_3UDP-glucuronic acid and then submitted to SDS-PAGE, major photolabelled protein bands of 35-37 and 50-54 KDa were detected by autoradiography (Drake *et al.*, 1991). Therefore, photoaffinity analogues used for the labelling of UDP-sugar binding proteins provide specific photoprobes that could be very useful during purification and characterization studies of UDP-glycosyltransferases.

1. 4. THE UDP-GLUCURONYLTRANSFERASES IN ANIMAL SYSTEMS.

In animals, UDP-glucuronyltransferases have an important role in the detoxification process, since glucuronidation of xenobiotics and potentially toxic endogenous substances is a major way of generating water-soluble products that can then be excreted from the animal in the urine or the bile. However, in some cases, potential toxicity may result from high levels of certain glucuronides formed from xenobiotic substances (Tephly & Burchell, 1990).

The UDP-glucuronyltransferases of animals are enzymes bound to the membranes of the endoplasmic reticulum of the cells and highest activity is usually found in the hepatocytes. They catalyse the linkage of glucuronic acid from UDP-glucuronic acid to substances possessing OH, COOH, NH_2 and SH moities (Siest *et al.*, 1989). Within the same tissue several isoenzymes have been characterized which differ from each other by their substrate specificity and differential inducibility. Differences have also been observed between isoenzymes studied in cells derived from various animals (Tephly, 1990).

Glucuronyltransferases have been purified from the hepatic microsomes of several mammalian species. The extreme lability of the enzymes allowed only limited purification since the activity decreased rapidly as resolution was achieved. Various biochemical techniques were used to isolate the enzymes. The main breakthrough was achieved by the utilisation of affinity chromatography of microsomal extracts on UDP-hexanolamine Sepharose 4B or UDP-glucuronosyl-hexanolamine Sepharose 4B (Singh *et al.*, 1981; Tephly, 1990). The discovery that different isoenzymes have different isoelectric points was also exploited to separate these enzymes by isoelectrofocusing. The molecular weight of the different UDP-glucuronyltransferases was determined by SDS-PAGE and was shown to vary from 50 KDa to 60 KDa. Studies on the interactions between the membrane environment and the glucuronyltransferases were carried out by solubilisation, total delipidation and reconstitution of a phospholipid membrane. The results of this experiment indicated that, for full activity, the enzymes depend on their association with phospholipids (Singh *et al.*, 1981; Castuma & Brenner, 1989).

In order to overcome the problem of lability and to obtain preparations of glucuronyltransferases retaining their catalytic functions, immunology and molecular biology have been used to approach the study of these enzymes. Antibodies have been raised against the enzymes and used for further molecular biological studies (Yokota & Yuasa, 1990). Expression of cDNAs coding for isoforms of UDP-glucuronyltransferases in mammalian cell cultures provides a good source of enzymes for metabolic and toxicological studies. This approach was used to demonstrate the substrate specificity of different isoenzymes in rat and human cells (Siest *et al.*, 1989).

1. 5. INTRODUCTION TO THE EXPERIMENTAL WORK.

The initial aim of this project was the purification of the glucuronyltransferase responsible for the transfer of glucuronic acid from UDP-glucuronic acid onto a xylan backbone during the biosynthesis of glucuronoxylans. This glucuronyltransferase is located within the membrane of the Golgi apparatus (Waldron & Brett, 1987) and its activity can be solubilized using the non-ionic detergent, Triton X-100 (Waldron *et al.*, 1989). The results presented here report on the subcellular localization of the enzyme within the subcompartments of the Golgi apparatus and on the solubilization of the glucuronyltransferase activity.

Because of the interference of Triton X-100 with the monitoring of proteins at 280 nm, another detergent was needed for the solubilization of the enzyme and its subsequent purification by chromatography. The enzyme activity was solubilized from a whole membrane fraction using different detergents singly and in combination. Some degree of glucuronyltransferase purification was obtained by sequential solubilization of the enzyme with a two step solubilization method involving i) Triton X-100 at low concentration and ii) NP40 at high concentration. In order to improve the purity of the starting membrane fraction used for solubilization, a protocol was designed to obtain a Golgi membrane-enriched fraction. This protocol was developed initially to investigate the more precise localization of the enzyme within the subcompartments of the Golgi stack and was later modified in order to provide a rapid method of preparing a Golgi membrane-enriched fraction. This fraction was used routinely for the solubilization of the glucuronyltransferase activity. In order to choose a suitable detergent, the effects, on the enzyme activity, of a selection of detergents at different concentrations were tested on particulate Golgi membranes. Selected detergents were assayed, at various concentrations,

for the solubilization of glucuronyltransferase activity from the Golgi membranes. Purification of the solubilized glucuronyltransferase was attempted using various chromatographic techniques such as affinity and anion exchange chromatography.

CHAPTER 2: GENERAL METHODS.

2. 1. CHEMICALS.

All chemicals were obtained from BDH (Poole, Dorset) and Sigma (Poole, Dorset), unless stated otherwise in the text, and were of the highest grade.

2. 2. PLANT MATERIAL.

Peas (*Pisum sativum* var. Alaska) were purchased from Sinclair-McGill (Ayr, UK). They were obtained precoated with fungicide in order to prevent any contamination from fungi during the germination of the seeds. Peas were soaked overnight in tap water (2 volumes of water per volume of peas) and sown on damp vermiculite. They were then left to germinate at 25°C in darkness for up to 7 days.

2. 3. PREPARATION OF PARTICULATE MEMBRANES.

Particulate membranes were prepared as a reference for the degree of purification of the glucuronyltransferase in subsequent manipulations. A fixed weight of etiolated pea epicotyls was harvested and the hook removed. All further manipulations were carried out between 0 and 4°C and the following buffers were used :

Homogenisation buffer		*Resuspension buffer*	
Tris-HCl pH 7.4	10 mM	Mes-NaOH pH 6	1 mM
Potassium chloride	10 mM		
Magnesium chloride	1.5 mM		
DTT	10 mM		

The epicotyls (150 g) were chopped into 2 to 4 mm pieces with a single-edged razor blade on a precooled glass plate. This was then homogenized using a chilled mortar and pestle in homogenization buffer (70 ml). A small amount of acid-washed sand was added to facilitate the grinding. The homogenate was strained through 4 crossed layers of muslin then centrifuged for 10 min at 13,000 $g_{av.}$ at 4°C in a Sigma 3K20 bench centrifuge using a Sigma 12156 fixed-angle rotor in order to precipitate cell wall fragments, nuclei and mitochondria. The supernatant was collected and centrifuged at 150,000 $g_{av.}$ for 1 hour at 4°C in a Sorvall OTD65 ultracentrifuge using a Sorvall T865 fixed-angle rotor. The pelleted membranes were then resuspended in resuspension buffer (1 ml) before being assayed for glucuronyltransferase activity.

2. 4. GLUCURONYLTRANSFERASE ASSAY.

2. 4. 1. Enzyme assay.

The glucuronyltransferase assay was performed as described by Waldron & Brett (1983). The incubation mixture contained 50 µl of membrane sample, UDP-D-[^{14}C]-glucuronic acid (0.14 nmol; 1.67 KBq, New England Nuclear, Dupont, Boston), UDP-D-xylose (1 mM,), manganese chloride (10 mM) in a final volume of 100 µl. When solubilized material was assayed, the concentration of $MnCl_2$ in the assay was 1 mM. The incubation was carried out in 1.5 ml Eppendorf tubes at 25°C for 2 hours in duplicate. Incubations were stopped by the addition of 1 ml of 96% ethanol. For the controls, 1 ml of 96% ethanol was added to the tubes before adding the 50 µl of membranes in order to stop the reaction immediately. A few mg of cellulose powder was added to each tube after termination of the assay with ethanol to act as a carrier for the extraction of the newly synthesized material. The tubes were then vortexed and

left to equilibrate for at least 30 min. The cellulose powder was pelleted by centrifugation at 10,000 $g_{av.}$ for 1 min. The supernatant containing the non-incorporated radioactive material was discarded. The incorporation of radioactive material into newly formed polysaccharides was measured by washing the pellet once with 70% ethanol (1 ml) and three times in distilled water (1 ml). The washed pellet was resuspended in distilled water (1 ml), transferred to a scintillation vial and mixed with scintillation liquid (4 ml, Ecoscint A, National Diagnostics, Manville, New Jersey). The radioactivity of the pellet was measured for 5 min with a liquid scintillation counter (LKB Wallac 1211 Minibeta).

2. 4. 2. Extraction of chelator-soluble material.

The glucuronyltransferase assay was carried out and the incubations were stopped as described in 2. 4. 1. A few mg of cellulose powder was added after termination of the assay with ethanol to act as a carrier for the extraction of the newly synthesized material. The tubes were vortexed and left to equilibrate for at least 30 min. The cellulose powder was pelleted by centrifugation at 10,000$g_{av.}$ for 1 min. The supernatant containing the non-incorporated radioactive material was discarded. The pellet was washed twice with 70% ethanol (1 ml) and twice with distilled water (1 ml). The remaining insoluble material was resuspended in buffer (0.5 ml) containing Na_2HPO_4 (50 mM) and Na_2EDTA (50 mM), pH 6.8. The extraction of chelator soluble material was achieved by incubating the samples for 15 min in a boiling water bath. Insoluble material was precipitated by centrifugation at 10,000 $g_{av.}$ for 1 min. The supernatant was collected and the pellet was resuspended in the same buffer (0.5 ml). The extraction of chelator soluble material was repeated with the resuspended pellet as described above. The chelator insoluble material

was precipitated by centrifugation at 10,000 $g_{av.}$ for 1 min. The supernatant was pooled with the one obtained from the previous extraction of chelator soluble material and transferred to a scintillation vial. The pooled supernatants were mixed with Ecoscint A (4 ml) and the radioactivity was measured for 5 min in a scintillation counter. The pelleted chelator insoluble material was washed twice in the chelator extraction buffer. The washed pellet was resuspended in 1 ml of water, transferred to a scintillation vial and mixed with 4 ml of Ecoscint A. The radioactivity of the chelator insoluble material was measured for 5 min in a scintillation counter.

2. 5. PROTEIN ASSAY.

The Peterson protein assay (Peterson, 1977) derived from the method of Lowry *et al.* (1951) was followed. A small volume (1 to 50 µl) of the sample was taken for protein determination. The final volume was brought to 1 ml with water. To this solution was added 0.15% sodium deoxycholate (0.1 ml). This was vortexed and left to equilibrate for at least 10 min. Proteins were then precipitated by the addition of 72% TCA (0.1 ml). The samples were vortexed and microfuged at 10,000 $g_{av.}$ for 30 min. The resulting supernatant was discarded. Distilled water (0.4 ml), reagent A (0.4 ml of freshly mixed equal volumes of water, CTC [20% sodium carbonate added slowly to an equal volume of 0.2% copper sulphate, 0.4% potassium tartrate solution], 0.8 N NaOH and 10% SDS) were added to the pellet, mixed and left to equilibrate for 10 min. Reagent B (0.2 ml of a mixture of 1 volume of 2 N Folin-Ciocalteu phenol reagent in 5 volumes of water) was then added. The solution was mixed and left to stand for 30 min before reading the optical density at 750 nm. A BSA standard curve was used as reference to calculate protein concentrations.

2. 6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

2. 6. 1. 7.5 -15% polyacrylamide gradient gel.

SDS-PAGE was carried out according to Chua (1980) in 7.5 to 15% polyacrylamide gradient gels with 6% stacking gels. The linear acrylamide concentration gradient, established by mixing a 7.5% acrylamide solution (17ml) [5.625 ml of a 30% (w/v) acrylamide and 0.8% (w/v) methylenebisacrylamide solution, 4.5 ml buffer 1 (0.4244 M Tris-HCl, pH 9.18), 1.9 ml of a 60% (w/w) sucrose solution, 225 µl of a 10% (w/v) SDS solution, 10µl TEMED, 150 µl 10% ammonium persulphate and distilled water up to 22.5ml] with a 15% solution (15 ml) [11.25 ml of a 30% (w/v) acrylamide and 0.8% (w/v) methylenebisacrylamide solution, 4.5 ml buffer 1 (0.4244 M Tris-HCl, pH 9.18), 6.45 ml of a 60% (w/w) sucrose solution, 225 µl of a 10% (w/v) SDS solution, 5 µl TEMED, 150 µl 10% ammonium persulphate] using a gradient maker, was poured between glass plates (16 cm x 18 cm) separated by 2 mm spacers. After pouring the gel, approximately 500 µl of distilled water was layered on top to ensure complete polymerization. Once the gel had set (approximately 60 min) a 6% stacking gel [2 ml of a 30% (w/v) acrylamide and 0.8% (w/v) methylenebisacrylamide solution, 2.5 ml buffer 2 (0.0541 M Tris-H_2SO_4, pH 6.1), 100 µl of a 10% (w/v) SDS solution, 10 µl TEMED, 100 µl 10% ammonium persulphate and distilled water up to 10 ml] was poured on top with a sample well former in place and left to set for 1 hour. The gels were run in Genetic Instruments electrophoresis tanks, with buffer 3 [0.04 M Tris, 0.04 M borate, pH 8.64, 1% (w/v) SDS] in the upper reservoir and buffer 1 diluted 5 times in the lower reservoir. The samples were prepared as follows: solution A (0.1 M Na_2CO_3, 0.1 M DTT) was added to an aliquot of the sample to a final volume of 0.3 ml and mixed with solution B (0.2 ml of a 5% (w/v) SDS, 30% (w/v) sucrose and 0.1% (w/v) bromophenol blue solution). The samples were

then boiled for 2 min. After loading the samples (20 to 30µl), electrophoresis was conducted at 65 mA until the dye front reached the bottom of the gel.

2. 6. 2. 12% polyacrylamide gel.

SDS-PAGE was carried out according to Laemmli (1970) in 12% polyacrylamide gels with 3% stacking gels. Resolving gel [9 ml buffer 1 (1.5 M Tris-HCl, 0.04% (w/v) SDS, pH 8.8), 14.4 ml of a 30% (w/v) acrylamide and 0.08% (w/v) methylenebisacrylamide solution, 2.4 ml 50% (v/v) glycerol, 12 µl TEMED, 135 µl 10% ammonium persulphate and distilled water up to 36 mls] was poured between glass plates (16 cm x 18 cm) separated by 2 mm spacers. After pouring the gel, approximately 500 µl of distilled water was layered on top to ensure complete polymerization. Once the gel had set (approximately 60 min) a 3% stacking gel [3.75 ml buffer 2 (0.5 M Tris-HCl, 0.4% (w/v) SDS, pH 6.8), 1.5 ml of a 30% (w/v) acrylamide and 0.08% (w/v) methylenebisacrylamide solution, 8 µl TEMED, 150 µl 10% ammonium persulphate and distilled water up to 15 mls] was poured on top with a sample well former in place and left to set for 1 hour. The gels were run in Genetic Instruments electrophoresis tanks, with running buffer [0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3] in both the upper and lower reservoirs. Samples were added to 2 volumes of loading buffer [50 mM Tris-HCl pH 8, 2% (w/v) SDS, 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue] and boiled for 2 min. After loading the samples (20 to 30l), electrophoresis was conducted at 65 mA until the dye front reached the bottom of the gel.

2. 7. SILVER-STAINING.

2. 7. 1. Silver-staining according to Morrissey (1981).

Gels were silver-stained according to Morrissey (1981) except that 12 ml of 2.3 M citric acid was added per 100 ml developer to stop the staining reaction. Briefly, gels were shaken gently for 30 min in 100 ml of each of the following solutions: (1) methanol : distilled water : acetic acid (5:4:1); (2) distilled water : methanol : acetic acid (83:10:7); (3) 10% (v/v) glutaraldehyde (Aldrich Chemical Company Ltd.). Excess glutaraldehyde was removed and the gels were soaked in several changes of distilled water over a 2 hour period or in a large volume of distilled water overnight. After a final wash, the gels were shaken gently in a solution of DTT (5 $mg \cdot l^{-1}$) for 30 min. The DTT solution was replaced by 0.1% (w/v) silver nitrate (John Matthey Materials Technology) and the gels were shaken for a further 30 min. Following this, the gels were rinsed quickly in a small volume of distilled water, then shaken for 1 min in a small amount of developer [3% sodium carbonate, 50 µl 47% formaldehyde / 100 ml], then shaken in 100 ml of developer until the desired level of staining was achieved. To stop the staining reaction, 12 ml of 2.3 M citric acid was added directly to the developer and the solution agitated for 10 min. After rinsing several times in distilled water, the stained gels could be soaked in a solution of 0.03% (w/v) sodium carbonate for 10 min and stored for several months in distilled water.

2. 7. 2. Silver-staining according to Oakley *et al.* (1980).

Gels were stained according to Oakley *et al.* (1980) except that the staining reaction was stopped by the direct addition of 10% acetic acid to the developer.

Briefly, the gels were fixed with methanol : acetic acid and glutaraldehyde solutions as described in section **2. 7. 1.** The gels were rinsed several times in distilled water (500 ml). After a final wash, the gels were shaken gently for 10 min in the staining solution [21.1 ml buffer I (0.09 M NaOH), 1.4 ml NH_4OH (concentrated), 3.2 ml of 20% (w/v) $AgNO_3$ solution added very slowly while stirring, distilled water to bring the volume to 100 ml]. Following this, the gels were rinsed quickly in small volumes of distilled water, then shaken for 1 min in a small amount of developer [0.05% (w/v) citric acid, 5 µl 47% formaldehyde per ml of 0.05% (w/v) citric acid]. This was discarded and the gel was then shaken in 100 ml of developer until the desired level of staining was achieved. To stop the staining reaction, 15 ml of 10% acetic acid was added directly to the developer and the solution agitated for 10 min. After rinsing several times in distilled water, the stained gels could be soaked in a solution of 0.03% (w/v) sodium carbonate for 10 min and stored for several months in distilled water.

CHAPTER 3: TIME COURSE EXPERIMENT.

3. 1. INTRODUCTION

The aim of this experiment was to investigate the optimal time to harvest pea epicotyls with regard to glucuronyltransferase activity. It also permitted the study of different parameters of pea seedling growth, such as epicotyl length, fresh and dry weights and protein content.

3. 2. METHOD.

Peas were grown as described in section **2. 2.** except that epicotyls were harvested every day after 3, 4, 5, 6 and 7 days from germination. In order to standardise the harvest of the epicotyls on the day of the experiment a 10 x 10 cm metallic square frame was placed in the centre of the tray. Every epicotyl within the frame was harvested, the hook removed and its length measured. The total fresh weight of all the epicotyls harvested was determined. This material was subsequently placed on a petri dish, covered with muslin and put for a week in a oven set at 40°C to allow the determination of the dry weight of the epicotyls.

To assay the glucuronyltransferase activity on each day of the time course experiment, 10 g of epicotyls were harvested to prepare a crude membrane extract. Epicotyls were homogenised in 5 ml of homogenization buffer, strained once through muslin and the homogenate was then centrifuged at 13,000 $g_{av.}$. The supernatant was centrifuged at 150,000 $g_{av.}$ and the pelleted membranes were resuspended in 0.5 ml of resuspension buffer and assayed for glucuronyltransferase activity [section **2. 4. 1**] and for protein [section **2. 5.**].

3. 3. RESULTS.

The results shown in the different figures (Fig. III. 1 to III.3) are means of two similar experiments carried out over two separate weeks. Pea epicotyls were harvested after 3, 4, 5, 6 and 7 days of incubation on vermiculite.

From day 3 to day 6 the mean epicotyl length increases at a steady rate of 15-20 mm per 24 hours, this rate slowing down slightly after the 6^{th} day of growth. The mean epicotyl length varies from 10 mm on day 3 to 75 mm on day 7 (Fig. III.1).

As in the case of length, the mean epicotyl fresh weight increases at a steady rate of about 12 mg per 24 hours from day 3 to day 6, then the growth rate slows down to 8 mg per 24 hours (Fig. III.2). Although the mean epicotyl dry weight increases constantly over the period of growth from 2.2 mg on day 3 to 10.5 mg on day 7, the relative dry weight, expressed as a percentage of the fresh weight, decreases from day 3 to day 7 with an average loss of 0.5 % per 24 hours.

The total amount of membrane-bound protein obtained from the initial 10 g of fresh material decreases over two days from day 3 to day 5 then decreases more slowly till day 7 (Fig. III.3). Along with the rapid decrease in the yield of membrane-bound protein, the total glucuronyltransferase activity decreases rapidly from 620 $Bq \cdot hr^{-1}$ on day 3 to 250 $Bq \cdot hr^{-1}$ on day 7 but the glucuronyltransferase specific activity shows an increase from 40 $Bq \cdot hr^{-1} \cdot mg^{-1}$ on day 3 to 60 $Bq \cdot hr^{-1} \cdot mg^{-1}$ on day 5 when a plateau is reached (Fig. III.3). It then decreases to 50 $Bq \cdot hr^{-1} \cdot mg^{-1}$ on day 7.

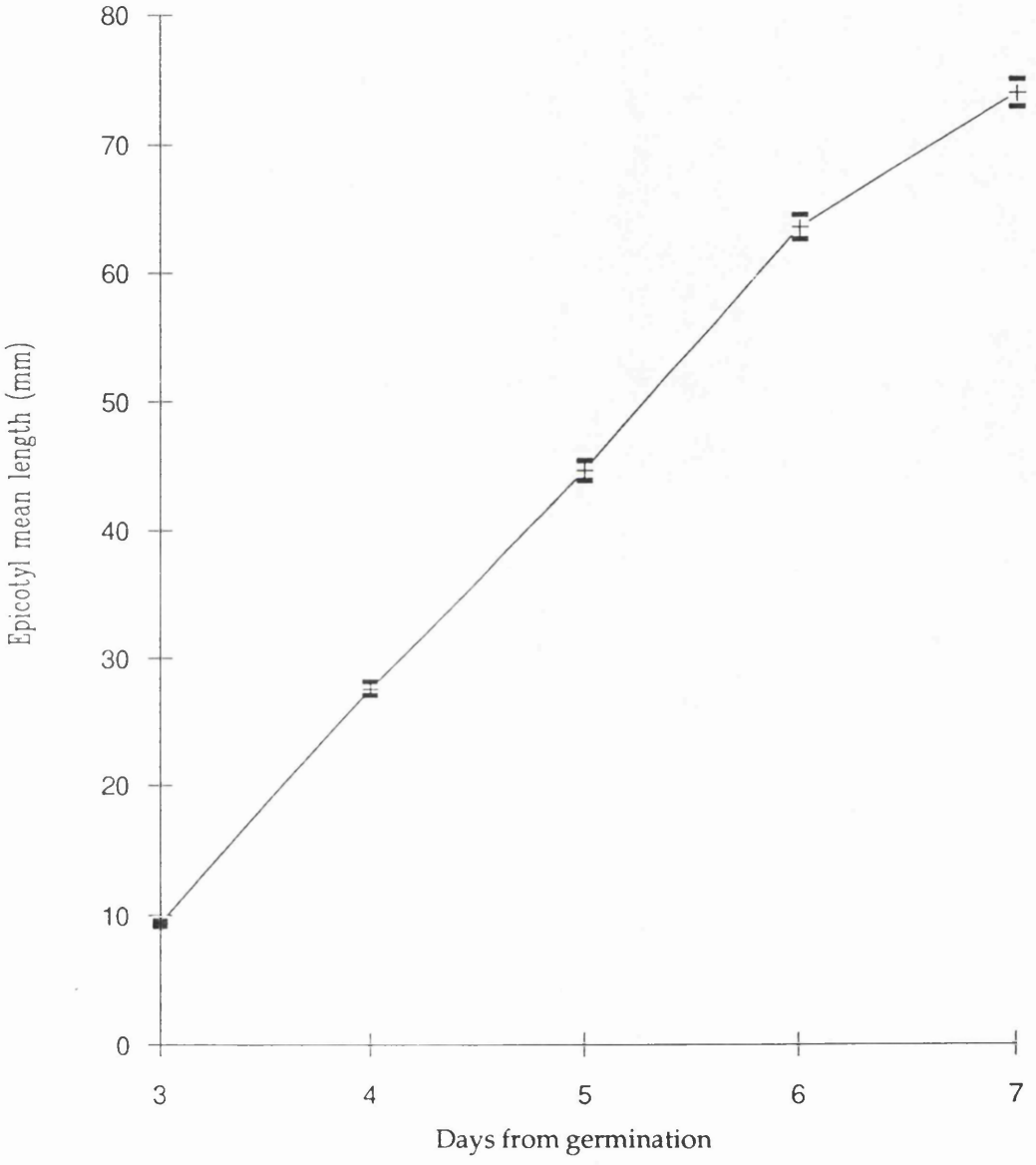


Fig. III.1. Variations in mean epicotyl length during early growth.

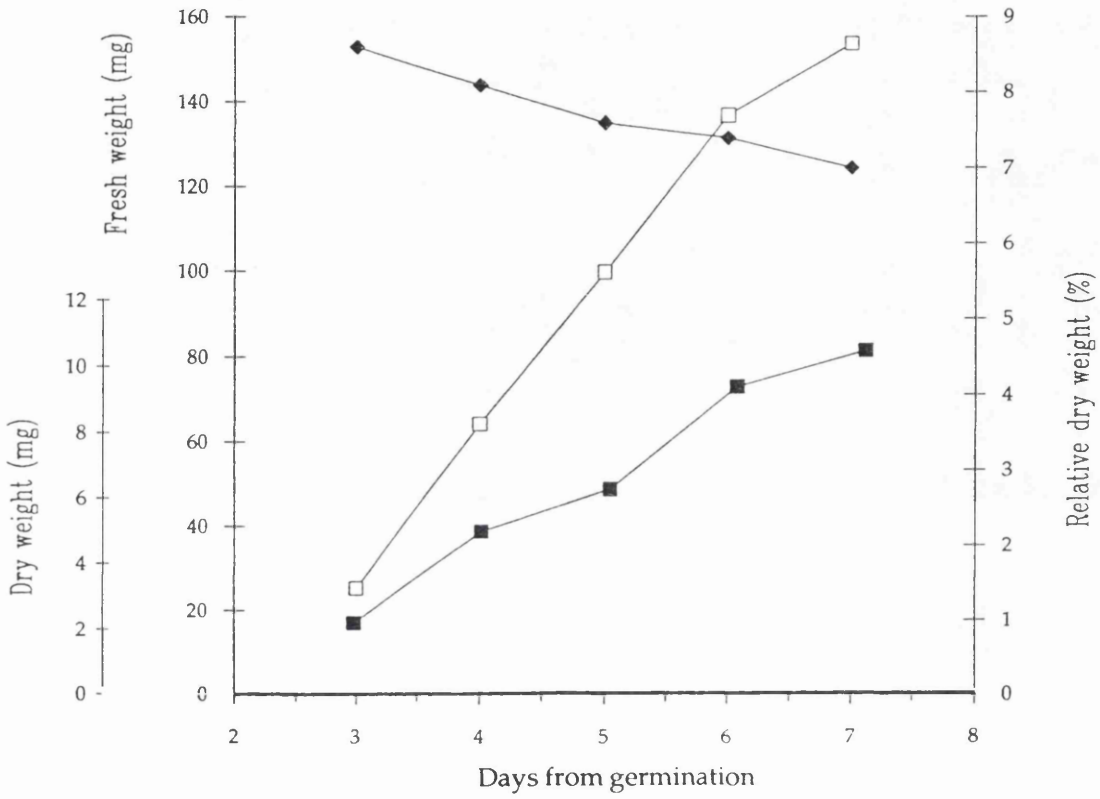


Fig. III.2: Variations in epicotyl fresh (■), dry (□) and relative dry weight (♦) during early growth.

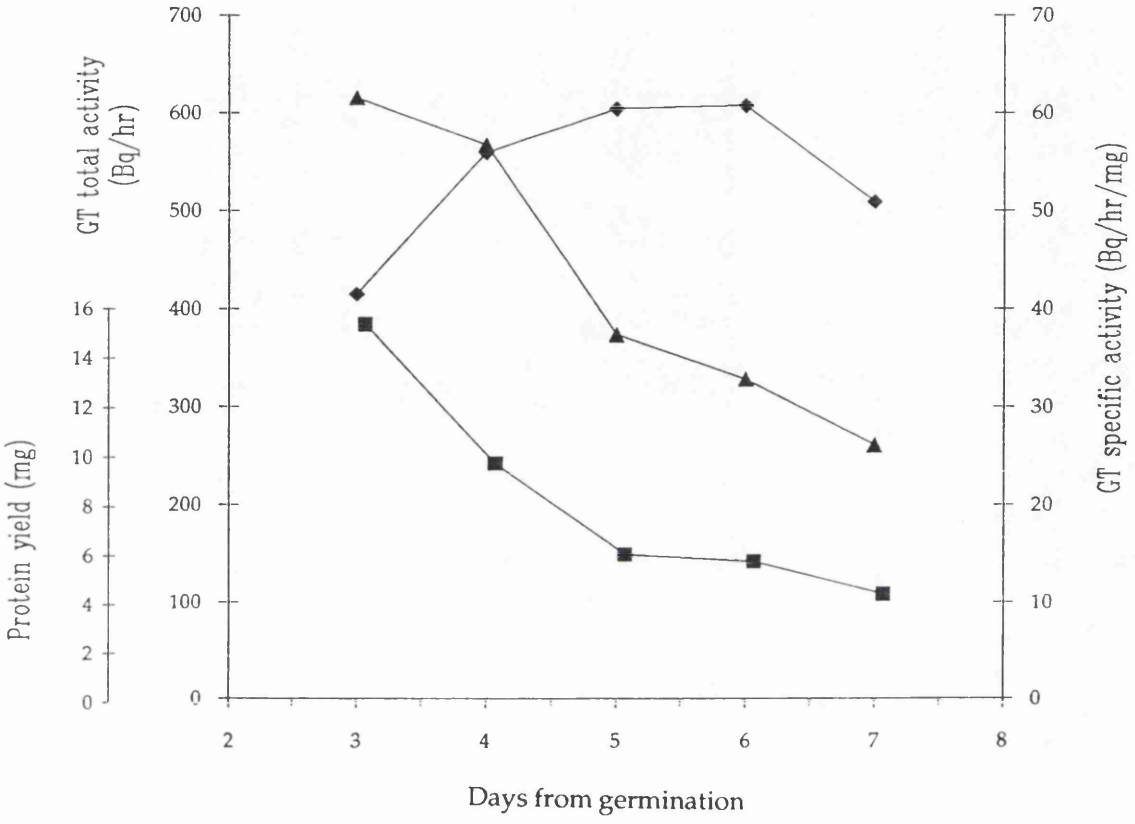


Fig. III.3: Variations in protein yield (■), GT specific (♦) and total activity (▲) in pea epicotyls during early growth.

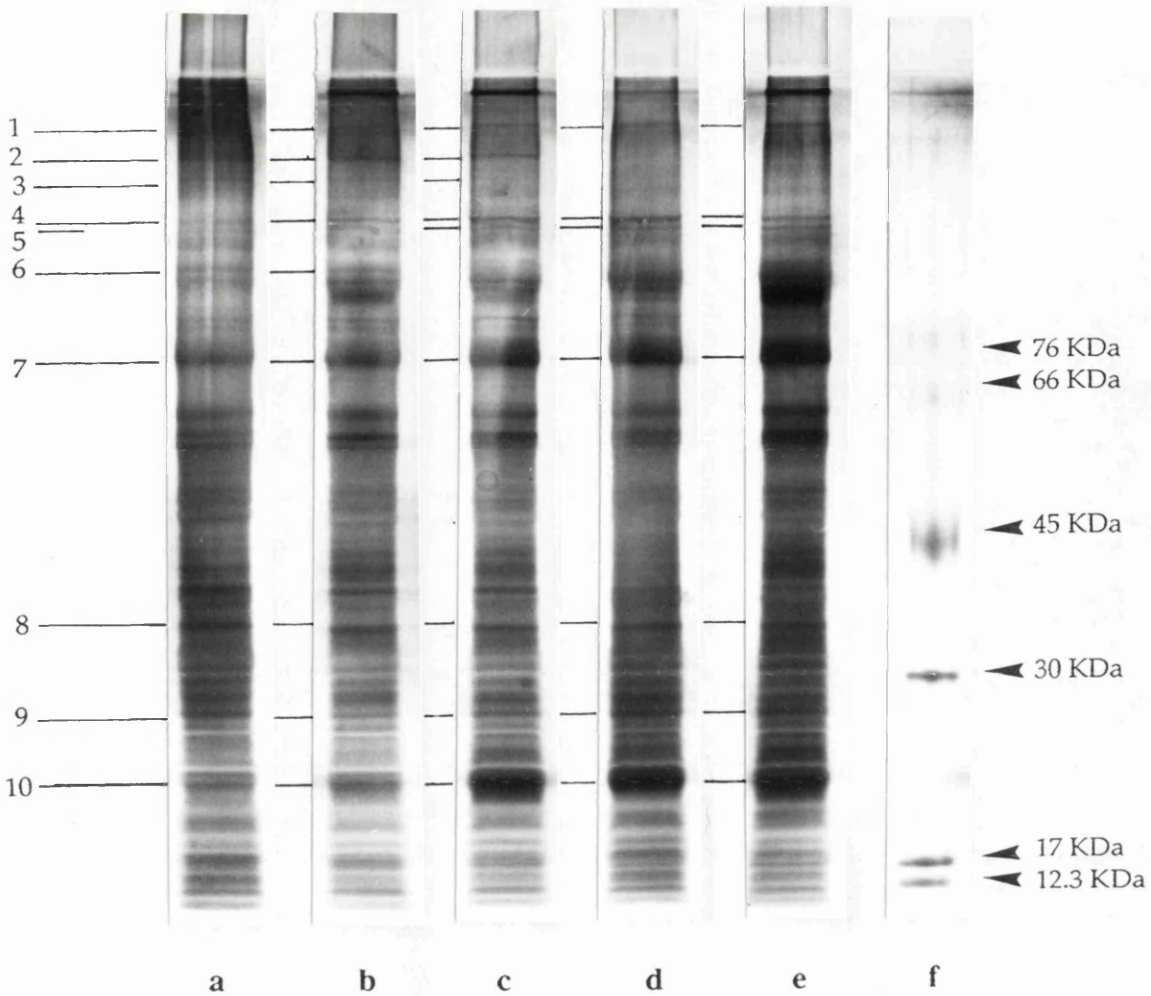


Fig. III. 4: Silver-stained 7.5-15% SDS polyacrylamide electrophoresis gel of whole membrane fraction from each day of the time course experiment.

Lanes: a) 3 days from germination,
b) 4 days from germination,
c) 5 days from germination,
d) 6 days from germination,
e) 7 days from germination,
f) molecular weight markers.

SDS-polyacrylamide gel electrophoresis of membrane fractions was carried out in order to look for changes in protein content during the time course experiment. Samples (20 µl, 50 µg of protein) were loaded onto a 7.5-15% acrylamide gradient gel [section **2. 6. 1**] and, after electrophoresis, the gel was silver-stained as described in **2. 7. 2**. As expected for a crude membrane preparation, a large number of proteins of a wide range of molecular weight were detected in samples taken on each day of the experiment (Fig. III.4). It is nevertheless possible to see changes in protein content during the growth of pea epicotyls. Some proteins (e.g. band 1, 8 and 9) are present at a constant concentration over the five days of the experiment while some others (e.g. band 4, 7 and 10) show a constant increase from day 3 to day 7. Other proteins are only present in epicotyls at the start (e.g. band 2, 3 and 6) or the end (e.g. band 5) of the time course.

3. 4. DISCUSSION.

The aim of this experiment was to determine when, after the sowing of the hydrated pea seeds, the activity of the glucuronyltransferase was greatest. The results of the time course experiment showed that glucuronyltransferase specific activity was at its highest level five days after the pea seeds were sown.

The development of plants is defined by two different processes: growth and differentiation. Differentiation is characterized by qualitative changes involved in the functional specialization of the cell or the organ whereas during growth, quantitative changes occur. The growth of plants is characterized by an increase in cell number, as result of cell division, and by the elongation of the daughter cells (Wareing, 1978).

The rapid growth of the pea seedlings was characterized by the increase in length and fresh weight of the epicotyls although the relative dry weight, determined as a ratio of fresh to dry weight, decreased. In rapidly growing plants, cell division is followed by the formation of vacuoles and by the elongation of the cell. Enlargement of the cell is facilitated by loosening of the cell wall controlled by the action of growth hormones such as auxins. During this period, vacuolation occurs and there is a great uptake of water as a consequence of the increase of osmotic potentiel in the cell. As a result of the increase in turgor pressure applied to the wall, an irreversible stretching of the wall occurs (Taiz, 1984). To compensate for the increase in volume of the cell, new cytoplasmic and wall material are actively synthesized which, in turn, contribute to the increase in fresh and dry weight. The decrease in relative dry weight of the epicotyls may be explained by the increase of the proportion of cells which have elongated since, due to vacuolation, the water content of these cells is increasing over the period studied.

The decrease in the total activity of the glucuronyltransferase detected during the time course experiment corresponds to the decrease in recovery of membrane-bound proteins from the initial 10 g of fresh material. This decrease in protein detection is probably related to the active vacuolation and uptake of water in elongating cells. However, the specific activity of the glucuronyltransferase increased until day 5 and this increase is probably associated with the active synthesis of secondary cell wall material occurring during the differential growth and after elongation of the pea cells.

Waldron & Brett (1984) described the distribution of the glucuronyltransferase activity among 1 cm long segments of epicotyls from pea seedlings which were grown for seven days in darkness. The maximum amount of glucuronyltransferase activity was recovered in the second and third

1 cm sections of the epicotyls below the hook. This peak of enzymatic activity was dissociated from the section displaying the greatest elongating capability, the first 1 cm segment. This result indicated that glucuronoxylans were synthesized and laid down principally as a secondary wall polymer. However some glucuronyltransferase activity was also detected in the first segment which showed maximum cell elongation. This suggested that some glucuronoxylans were also deposited in the growing primary wall of pea epicotyls. The increase of glucuronyltransferase specific activity observed in the tissue harvested on the fourth and fifth days after sowing probably corresponds to the synthesis and deposition of glucuronoxylans occurring as a secondary thickening of the wall in epicotyl cells which have stopped elongating.

CHAPTER 4: GOLGI MEMBRANE ISOLATION.

4. 1. INTRODUCTION.

As a first stage in the glucuronyltransferase purification it was necessary to obtain a fraction enriched with Golgi membranes, the subcellular organelle where the glucuronyltransferase is located (Waldron & Brett, 1987). A series of experiments [described in section **4. 2.**] involving sucrose density gradients was designed for the routine extraction of a membrane fraction enriched in glucuronyltransferase activity, and therefore in Golgi membranes, from etiolated pea epicotyls. In addition, the subcellular location of the glucuronyltransferase within the Golgi stack was investigated in more detail. In order to characterise the different membrane fractions obtained with the density gradients, the activity of organelle marker enzymes was determined.

4. 2. METHODS.

4. 2. 1. Organelle marker enzyme assays.

4. 2. 1. 1. Glucan synthase II.

Glucan synthase II was assayed as a marker enzyme for the plasma membrane. This assay was based on that of Ray *et al.* (1976). Incubations contained 50 µl of membrane, Tris-HCl pH 8 (30 mM), UDP-D-glucose (0.5 mM), UDP-D-[^{14}C]-glucose (0.435 µM, 0.522 kBq, Amersham) in a final volume of 100 µl. The assay was performed at 25°C for 10 min and terminated by the addition of 96% ethanol (1 ml) and a small amount of cellulose powder. The terminated incubations were left to equilibrate for at least 30 min before

being microfuged at 10,000 $g_{av.}$ for 2 min. The resulting pellet was washed 3 times with 70% ethanol (1 ml). To measure the incorporation of radioactivity into the 70% ethanol insoluble material, the pellet was resuspended in water (1 ml) and mixed with Ecoscint (4 ml) before scintillation counting.

4. 2. 1. 2. Latent IDP'ase.

The activity of latent IDP'ase was assayed as a marker for the Golgi apparatus. This assay was based on the methods used by Ray *et al.* (1969) and Bowles *&* Kauss (1976). The latent IDP'ase activity was measured as the difference between IDP'ase activity in the presence (total IDP'ase) and in the absence of Triton X-100.

Incubations contained 50 µl of membranes, IDP (3 mM), magnesium chloride (2.5 mM), Tris-HCl pH 7.5 (28 mM) with or without 1% (w/w) Triton X-100 in a final volume of 200 µl. Incubations were carried out at 25°C for 15 min in 1.5 ml Eppendorf tubes and terminated by the addition of 11.5% (w/w) TCA (1.3 ml). This was then centrifuged for 10 min at 10,000 $g_{av.}$ and the concentration of phosphate released in the supernatant was determined. To measure its phosphate concentration the supernatant was transferred to a test tube and distilled water (0.5 ml) was added. Ferrous sulphate-ammonium molybdate reagent (1 ml) was added and the coloured reaction was allow to develop for 3 min at room temperature. The absorbance at 720 nm was measured. A standard curve was made with potassium dihydrogen phosphate in distilled water.

The ferrous sulphate-ammonium molybdate reagent was made up fresh by mixing 10 ml of ammonium molybdate reagent (50 g ammonium

molybdate, 500 ml 10 N sulphuric acid), distilled water (60 ml) and ferrous sulphate (5 g). When dissolved, the final volume was brought to 100 ml.

4. 2. 1. 3. NADH-cytochrome C reductase.

NADH-cytochrome C reductase was assayed as a marker enzyme for the endoplasmic reticulum (Hall & Moore, 1983). Incubations contained membrane (50 µl), sodium phosphate (300 µl, 0.2 M, pH 7.4), cytochrome C (150 µl, 5 $mg \cdot ml^{-1}$), potassium cyanide (15 µl, 10 mM) and antimycin A (7.5 µl, 2 $mg \cdot ml^{-1}$) in a total volume of 852.5 µl. The absorbance at 550 nm was recorded for 2 min in order to obtain a steady base line. The assay was initiated by the addition of NADH (150 µl, 3 $mg \cdot ml^{-1}$) and the rate of increase in absorbance at 550 nm measured over 2 min. The enzymatic activity of cytochrome C reductase was estimated using the extinction coefficient for cytochrome C of 18.5 $mM^{-1} \cdot cm^{-1}$.

4. 2. 1. 4. Succinate dehydrogenase.

Succinate dehydrogenase was assayed as a marker enzyme for mitochondria. This assay was based on that of Maeshima *et al.* (1987). Incubations contained membrane (50 µl), sodium phosphate (1.2 ml, 0.1 M, pH 7.5) containing potassium cyanide (1.7 mM), phenazine methyl sulphate (50 µl, 1% w/v) and 2,6-dichlorophenol-indophenol (50 µl, 50 $\mu g \cdot ml^{-1}$). The absorbance at 600 nm was recorded for 2 min in order to obtain a steady base line prior to the initiation of the assay by the addition of sodium succinate (150 µl, 0.4 M). The rate of decrease in absorbance at 600 nm was then measured over 2 min. The enzymatic activity of succinate dehydrogenase was

Etiolated epicotyls

↓ Chop in 2-3 mm pieces, homogenise with chilled mortar and pestle in homogenisation buffer, Strain through 4 crossed-layers of muslin. Bring homogenate to 8% (w/w) sucrose with compensation buffer. Regrind solid residue in homogenisation buffer and restrain through muslin. Pool homogenates. Bring homogenate to 8% (w/w) sucrose.

Homogenate

↓ Centrifuge at 13,000 g_{av} for 10 min.

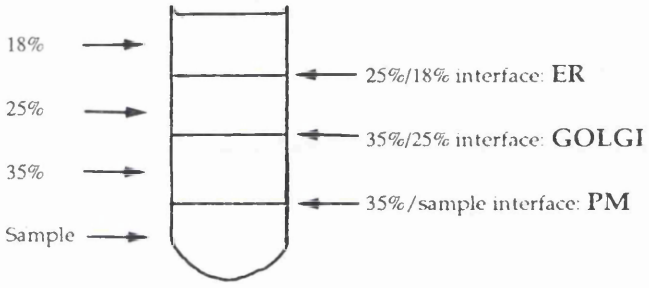
→ **Pellet** = cell wall debris and mitochondria.

Supernatant = whole membrane fraction

↓ Load on top of a 40% (w/w) sucrose cushion (5 ml)
Centrifuge at 150,000 g_{av} for 30 min.

40%/Sample interface = concentrated membranes

↓ Adjust to 40% (w/w) sucrose with 60% (w/w) sucrose buffer.
Form a discontinuous sucrose gradient on top of the membrane fraction.
Centrifuge at 150,000 g_{av} for 3 hrs.



Membrane enriched interfaces → Dilute an aliquot of the Golgi fraction and the total volume of the other organelle fractions with resuspension buffer
Centrifuge at 150,000 g_{av} for 30 min.

↓ **Pellet:** particulate membranes
Resuspend in resuspension buffer (1 ml).

↓ **Continuous sucrose gradients**

Adjust Golgi membrane enriched fraction to 45% (w/w) sucrose with 60% (w/w) sucrose buffer and form a linear sucrose gradient on top of the Golgi fraction.
Centrifuge at 97,000 g_{av} for 16 hrs.
Collect 2 ml fractions and determine their density.
Dilute to 30 ml with NaCl-buffer and centrifuge at 150,000 g_{av} for 30 min.
Resuspend resulting pellets in resuspension buffer (0.5 ml).

Fig. IV.1: Protocol for the fractionation of the Golgi apparatus.

estimated using 21 $mM^{-1} \cdot cm^{-1}$ as extinction coefficient for 2,6-dichlorophenol-indophenol

4. 2. 2. Golgi subcompartmentation.

The localisation of the glucuronyltransferase within the Golgi compartments was investigated in more detail using a sequence of discontinuous and continuous sucrose density gradients (Fig. IV.1). The following buffers were used during the preparation of the different membrane fractions.

Compensation buffer	
sucrose	2 M
Tris-HCl pH 7.4	10 mM
Magnesium chloride	28.5 mM
Potassium chloride	0.72 M
DTT	10 mM

Sodium chloride buffer	
Mes-NaOH pH 6	1 mM
sodium chloride	1 M

Sucrose gradient buffers	
Tris-HCl pH 7.4	40 mM
Magnesium chloride	0.1 mM
EDTA	1 mM
DTT	10 mM
Sucrose to make (w/w) solutions	18, 25, 35, 40, 60%

Peas were grown for 7 days in darkness at 25°C. Etiolated epicotyls (100 g) were chopped with a single-edged razor blade and homogenised with homogenisation buffer (35 ml) using a chilled mortar and pestle. The homogenate was strained through 4 crossed layers of muslin and compensation buffer was added to give a sucrose density of 8% (w/w) using an Atago refractometer. The solid residue was rehomogenised in homogenisation buffer (50 ml) and strained again through muslin. The homogenate was brought to 8% (w/w) sucrose with compensation buffer. It was subsequently centrifuged at 13,000 $g_{av.}$ for 10 min in a Sigma 3K20 bench centrifuge with a Sigma 12156

fixed angle rotor. The resulting pellet was resuspended in 5 ml of resuspension buffer (1 mM Mes-HCl, pH 6) using a glass-on-glass homogeniser. The supernatant was concentrated by being loaded onto a 40% (w/w) sucrose cushion and centrifuged for 30 min at 150,000 $g_{av.}$ in a Sorvall OTD-55B ultracentrifuge with a Sorvall T865 fixed angle rotor. The membrane fractions on top of the sucrose cushion were collected, pooled and adjusted to a density of 40% (w/w) sucrose with a 60% (w/w) sucrose buffer. The remaining solutions were pooled and an aliquot was diluted with an equal volume of resuspension buffer before being centrifuged at 150,000 $g_{av.}$ in a Sorvall OTD-55B ultracentrifuge with a Sorvall T865 fixed angle rotor for 30 min. The resulting pellet was resuspended in resuspension buffer (2 ml) and called R_1.

4. 2. 2. 1. Discontinuous sucrose density gradient.

Sucrose step gradients were formed on top of the 40% sucrose membrane suspension (10 ml) and consisted of 35% (10 ml), 25% (7 ml) and 18% (3 ml) (w/w) sucrose buffer. These discontinuous gradients were centrifuged at 150,000 $g_{av.}$ in a Sorvall OTD-55B ultracentrifuge with a Sorvall T865 fixed angle rotor for 3 hours. Membranes from each interface were collected and pooled. The 18/25% (w/w) sucrose interface was called ER (endoplasmic reticulum), the 25/35% Golgi and the 35/40% PM (plasma membrane). The remaining liquid was termed R_2. An aliquot of the Golgi membrane-enriched fraction and the total volume of the other fractions were diluted with an equal volume of resuspension buffer and pelleted at 150,000 $g_{av.}$ for 30 min in a Sorvall OTD-55B ultracentrifuge with a Sorvall T865 fixed angle rotor. Pellets were resuspended in resuspension buffer (1 ml) with a glass-on-glass homogenizer.

Etiolated epicotyls

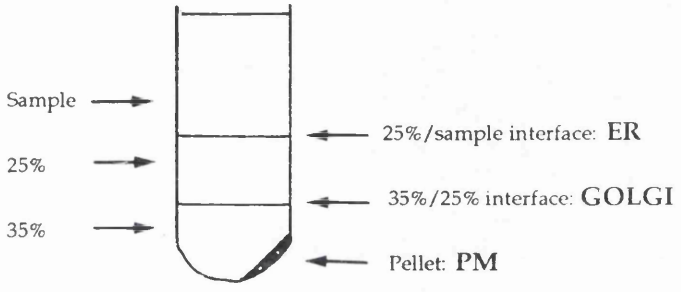
| Chop in 2-3 mm pieces, homogenise with chilled mortar and pestle in homogenisation buffer,
| strain through 4 crossed-layers of muslin.
| Bring to 8% (w/w) sucrose with compensation buffer.
↓

Homogenate

| Centrifuge at 13,000 g_{av} for 10 min.
|
|→ **Pellet** = cell wall debris and mitochondria.
↓

Supernatant = whole membrane fraction.

| Load on top of a discontinuous sucrose gradient made of 35% (w/w) sucrose (5 ml) and 25%
| (w/w) sucrose (8 ml) cushions.
| Centrifuge at 150,000 g_{av} for 3 hrs.
↓



Golgi membrane-enriched interface

| Dilute with NaCl-buffer. Centrifuge at 150,000 g_{av} for 30 min.
↓

Pellet: particulate Golgi membranes.

| Resuspend in resuspension buffer (1 ml). Protein assay. Adjust to 2 $mg \cdot ml^{-1}$ protein with
| resuspension buffer. Add detergent at twice the concentration required and homogenise
| in a glass-on-glass homogeniser (10 strokes).
↓

Homogenate: Golgi membranes (1 $mg \cdot ml^{-1}$ protein) + detergent.

| Incubate for 15 min at room temperature.
| Centrifuge at 230,000 g_{av} for 1 hr.
|
|→ **Supernatant:** solubilized material.
↓

Pellet: non-solubilized material.

| Resuspend in resuspension buffer (0.5 ml).

Fig. IV.2: Protocol for rapid preparation and solubilization of Golgi membranes.

4. 2. 2. 2. Continuous sucrose density gradient.

The remainder of the Golgi fraction was adjusted to 45% (w/w) sucrose with 60% (w/w) sucrose buffer. Two linear sucrose gradients (24 ml) [see section **4. 3. 2.** for detail] were formed on top of the Golgi membranes (14 ml). The gradients were centrifuged at 97,000 $g_{av.}$ for 16 hours in a Sorvall AH629 swing-out rotor. An ISCO model 185 gradient fractionator was used to collect 2 ml fractions from the top of the gradient. Their density was determined with an Atago refractometer. Fractions corresponding to the membrane sample were pooled and termed R_3. Each fraction was diluted to 30 ml with sodium chloride buffer and centrifuged for 30 min at 150,000 $g_{av.}$ in a Sorvall OTD-55B ultracentrifuge with a Sorvall T865 fixed angle rotor. Pellets were resuspended in resuspension buffer (0.5 ml).

4. 2. 3. **Rapid preparation of Golgi membrane-enriched fractions.**

A procedure more suitable for a routine preparation of a Golgi membrane-enriched fraction, and involving just one discontinuous sucrose density gradient, was derived from the method used for the localisation of the glucuronyltransferase within the compartments of the Golgi stack [section **4. 1.** 3.]. The protocol is summarized in figure IV.2.

To prepare the Golgi membrane-enriched fraction, etiolated pea epicotyls (150 g) were harvested. Epicotyls, chopped into small pieces using a razor blade, were homogenised in homogenisation buffer (70 ml). The homogenate was strained through 4 crossed layers of muslin. Before being submitted to the 13,000 $g_{av.}$ centrifugation the muslin-filtered homogenate was brought to 8% (w/w) sucrose with compensation buffer. The density was

adjusted using a refractometer. This was then centrifuged at 13,000 $g_{av.}$ as described before [section **4. 1. 3.**].

The supernatant was loaded onto a discontinuous sucrose density gradient formed of 8 ml of 25% (w/w) sucrose buffer on top of 5 ml of 35% (w/w) sucrose buffer. This gradient was then centrifuged for 3 hours at 150,000 $g_{av.}$ in a Sorvall OTD65 ultracentrifuge with a Sorvall T865 fixed-angle rotor.

Golgi membrane-enriched fractions were collected carefully with a Pasteur pipette at the interface between the 25% and 35% sucrose cushions. Fractions from the different tubes were pooled and diluted with an equal volume of NaCl-buffer. They were then centrifuged for 30 min at 150,000 $g_{av.}$ in a Sorvall OTD65 ultracentrifuge with a Sorvall T865 fixed-angle rotor. Pelleted Golgi membranes were resuspended in resuspension buffer (1 ml).

In order to characterize the different organelle fractions isolated on the discontinuous sucrose density gradients, material equilibrating at the sample / 25% sucrose cushion interface was collected and called endoplasmic reticulum membrane fraction. The pooled ER fractions were diluted with an equal volume of resuspension buffer. They were centrifuged at 150,000 $g_{av.}$ in a Sorvall OTD65 ultracentrifuge with a Sorvall T865 fixed-angle rotor for 30 min. The pelleted membranes were resuspended in resuspension buffer (1 ml). Material pelleted during the step-gradient centrifugation was resuspended in resuspension buffer (1 ml). This fraction was called the plasma membrane fraction. The different membrane fractions obtained were then assayed for organelle marker enzyme activities.

4. 3. RESULTS.

4. 3. 1. Development of a method for the preparation of a membrane fraction enriched with glucuronyltransferase activity.

A series of experiments was carried out in order to design a protocol for the preparation of a membrane fraction enriched with glucuronyltransferase activity using discontinuous and continuous sucrose density gradients.

All manipulations described below were carried out at 4°C. Particulate membranes were prepared as described in section **2. 3.** except that 50 g of etiolated epicotyls were harvested and homogenized in homogenization buffer (35 ml). The muslin strained homogenate was centrifuged at 97,000 $g_{av.}$ for 30 min in a Sorvall OTD65 ultracentrifuge with a AH629 swing-out rotor. The pelleted membranes were resuspended in resuspension buffer (2 ml) and assayed for glucuronyltransferase activity. This membrane preparation was used as a control for experiment 1.

For experiment 1, the muslin strained homogenate was centrifuged at 13,000 $g_{av.}$ for 10 min at 4°C in a Sigma 3K20 bench centrifuge with a Sigma 12156 fixed angle rotor. The resulting supernatant was collected and the pellet was resuspended in homogenization buffer (10 ml). The resuspension was centrifuged at 13,000 $g_{av.}$ for 10 min at 4°C in a Sigma 3K20 bench centrifuge with a Sigma 12156 fixed angle rotor. The pooled supernatant was collected and pooled with the first one. This solution was then centrifuged at 97,000 $g_{av.}$ for 30 min in a Sorvall OTD65 ultracentrifuge with a AH629 swing-out rotor. The pelleted membranes were resuspended in resuspension buffer (2 ml) before assaying for protein concentration and glucuronyltransferase activity.

As shown in table IV.1, the total activity of glucuronyltransferase in the 13,000 $g_{av.}$ supernatant was at the same level as that found in the control. However, with 60 $Bq \cdot hr^{-1} \cdot mg^{-1}$, the glucuronyltransferase specific activity in the 13,000 $g_{av.}$ centrifugation supernatant (Exp.1) was almost twice the specific activity of the control, therefore showing a two fold purification.

The control of the second experiment (Exp.2) was a particulate membrane preparation obtained as in Exp.1, i.e. a 13,000 $g_{av.}$ supernatant was prepared as in Exp.1. Exp.2 differed from Exp.1 as the pooled 13,000 g_{av} supernatant was loaded onto a 3 ml 40% sucrose cushion and centrifuged at 97,000 $g_{av.}$ for 30 min in a Sorvall OTD65 ultracentrifuge with a AH629 swing-out rotor. The interface between sucrose cushion and sample was carefully collected and diluted with an equal volume of resuspension buffer. The diluted interface was centrifuged at 97,000 $g_{av.}$ for 30 min in a Sorvall OTD65 ultracentrifuge with a AH629 swing-out rotor to precipitate the membranes. The pelleted membranes were resuspended in resuspension buffer (2 ml) before assaying for protein concentration and glucuronyltransferase activity.

As shown in table IV.2, there was a loss in total glucuronyltransferase activity after the centrifugation of the membranes on the 40% sucrose cushion (Exp.2) relative to the control (Exp.1) as only 83% of glucuronyl transferase total activity was recovered. However, a 4 fold increase in specific activity was obtained after the centrifugation on a 40% (w/w) sucrose cushion.

The control for Exp.3 was a repeat of Exp.2. Exp.3 was carried out as Exp.2 except that the sucrose density of the interface collected on top of the 40% sucrose cushion was brought to 45% sucrose with a 60% sucrose buffer. Discontinuous sucrose density gradients were formed on top of the sample (3 ml) and consisted of 3 ml of 35%, 3 ml of 25%, 3 ml of 18% sucrose buffers.

EXP. 1.	Protein	GT	GT S.A.
	$mg \cdot ml^{-1}$	$Bq \cdot hr^{-1} \cdot ml^{-1}$	$Bq \cdot hr^{-1} \cdot mg^{-1}$
Particulate enzyme	19	626	33
13,000 $g_{av.}$ SN	11	658	60

Table IV.1: Experiment 1. 13,000 $g_{av.}$ centrifugation.

Glucuronyltransferase (GT) specific activity (S.A.) in whole membrane fraction (particulate enzyme) and in 13,000 $g_{av.}$ supernatant (SN).

EXP. 2.	Protein	GT	GT S.A.
	$mg \cdot ml^{-1}$	$Bq \cdot hr^{-1} \cdot ml^{-1}$	$Bq \cdot hr^{-1} \cdot mg^{-1}$
13,000 $g_{av.}$ SN	13	647	50
Concentrated membranes	3	536	179

Table IV.2: Experiment 2. Membrane concentration by centrifugation on a 40% sucrose cushion.

Glucuronyltransferase (GT) specific activity (S.A.) in 13,000 $g_{av.}$ supernatant (SN) and in membranes concentrated by centrifugation onto a 40% (w/w) sucrose cushion.

EXP. 3.	Protein	GT	GT S.A.
	$mg \cdot ml^{-1}$	$Bq \cdot hr^{-1} \cdot ml^{-1}$	$Bq \cdot hr^{-1} \cdot mg^{-1}$
Concentrated membranes	0.85	56	66
Sample/35% interface	2.2	341	155
25/35% interface	3.3	503	151

Table IV.3: Experiment 3: Discontinuous sucrose density gradient.

Glucuronyltransferase (GT) specific activity (S.A.) in membranes concentrated by centrifugation onto a 40% (w/w) sucrose cushion and membrane fraction isolated by sucrose density step gradient centrifugation.

These gradients were centrifuged for 90 min at 97,000 g_{av} in a Sorvall OTD65 ultracentrifuge with a Sorvall AH629 swing-out rotor. The interfaces sample/35% and 35/25% were collected and resuspended in resuspension buffer (35 ml). They were then centrifuged at 97,000 g_{av} for 30 min in a Sorvall OTD65 ultracentrifuge with a Sorvall AH629 swing-out rotor and the pelleted material resuspended in resuspension buffer (2 ml) prior to protein and glucuronyltransferase assays. Glucuronyltransferase activity was found at the interfaces between the different sucrose cushions with similar levels of specific activity (table IV.3).

Exp.4 was carried out as Exp.3 except that the 25/35% sucrose interface was brought to a higher sucrose density (45% sucrose w/w) with 60% sucrose solution. A 35 to 25% continuous sucrose gradient (12 ml) was loaded on top of the sample (3 ml). It was centrifuged at 97,000 g_{av} for 16 hours in a Sorvall OTD65 ultracentrifuge with a Sorvall AH629 swing-out rotor and 1 ml fractions were collected and diluted to 12 ml with resuspension buffer before centrifugation at 97,000 g_{av} for 30 min in a Sorvall OTD65 ultracentrifuge with a Sorvall AH629 swing-out rotor. Pelleted material was resuspended in resuspension buffer (1 ml) prior to protein and glucuronyltransferase assays. As seen in fig. IV.3, there are 2 peaks of glucuronyltransferase activity within the gradient but the gradient was not absolutely uniform. However, the main peaks of glucuronyltransferase activity were found to be associated with the main peaks of protein recovery from the gradient, and the glucuronyltransferase activity was found mainly at a density of about 1.130 $g \cdot ml^{-1}$ (30% sucrose (w/w)).

The protocol was subsequently revised and adapted for a better and quicker separation of the membranes more suitable for the study of the Golgi

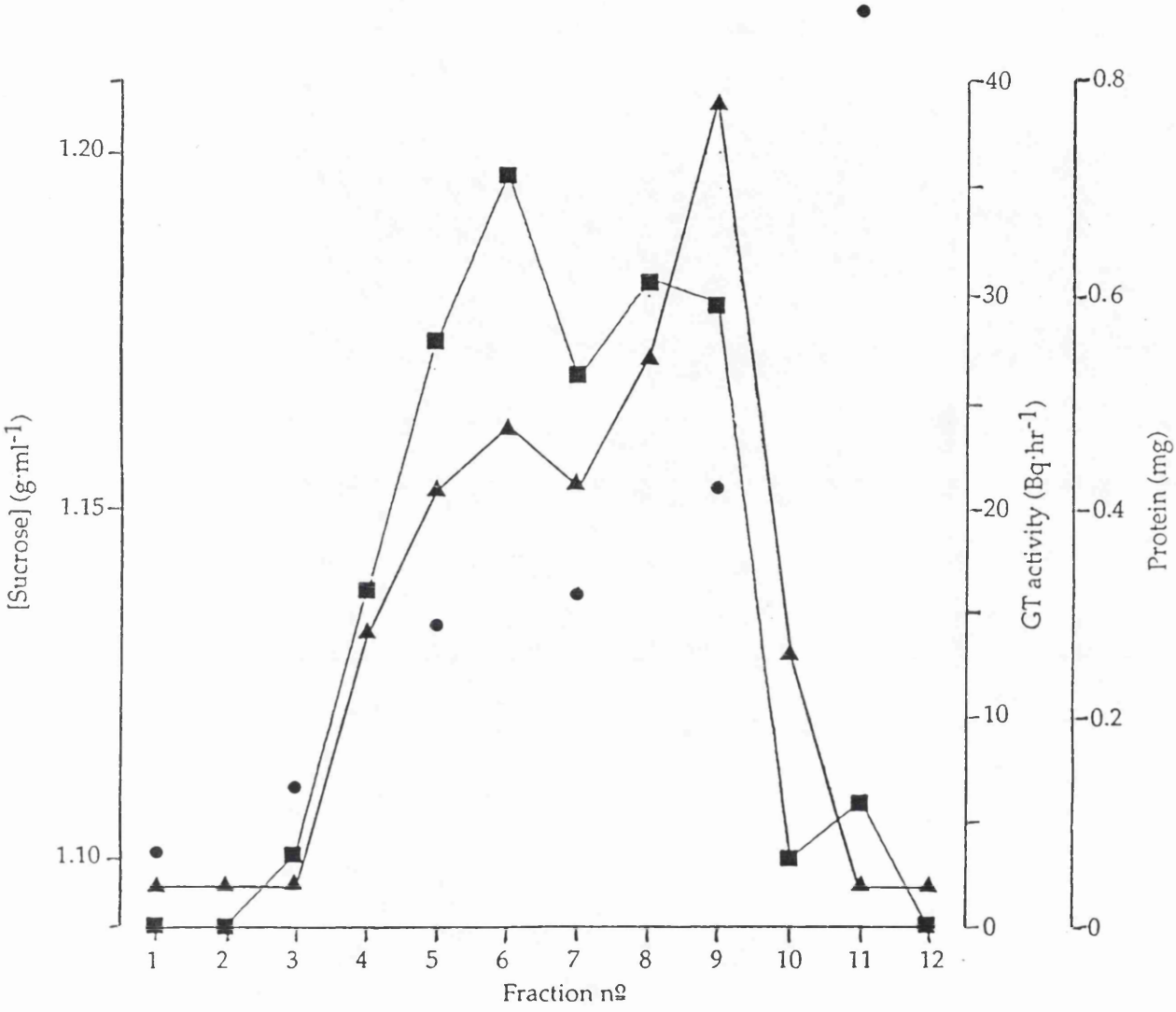


Fig. IV.3: Experiment 4. Fractionation of Golgi membranes on a continuous sucrose density gradient. Profiles of protein recovery and glucuronyltransferase activity.

■ GT activity; ▲ Protein; ● Sucrose concentration in fractions.

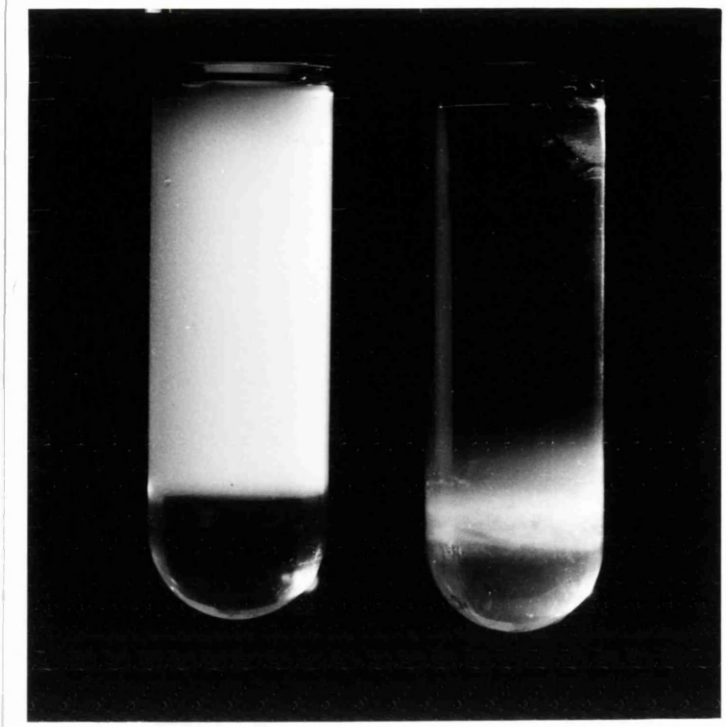


Fig. IV.4: Concentration of whole membranes on a 60% sucrose density cushion.

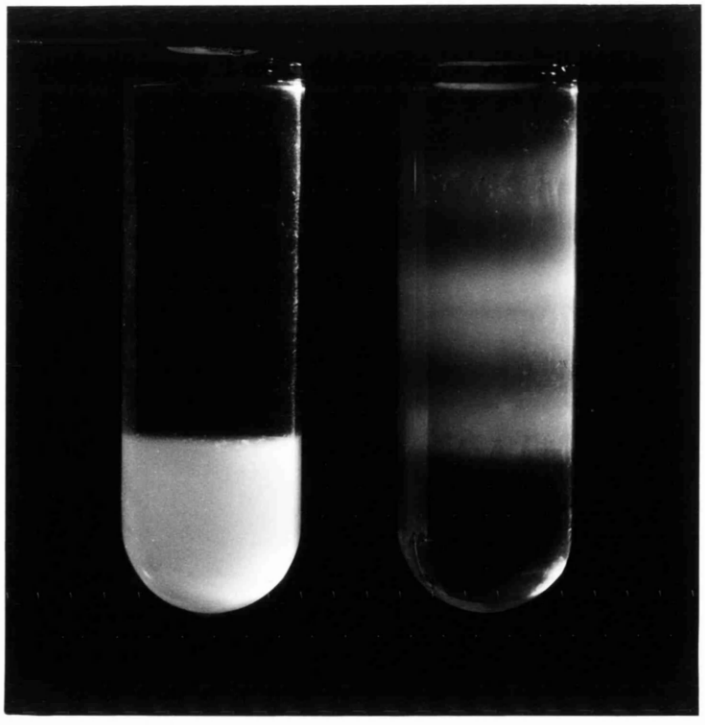


Fig. IV.5: Membrane fractionation on a discontinuous sucrose density gradient.

apparatus subcompartments and for the purification of the glucuronyltransferase.

4. 3. 2. Investigation of Golgi apparatus subcompartments.

4. 3. 2. 1. Isolation of subcellular organelle membrane fractions on discontinuous sucrose gradient.

Organelles were first fractionated on a discontinuous sucrose density gradient [section **4. 1. 3. 1.**] (Fig. IV.4 & IV.5) and each membrane fraction was assayed for marker enzyme [section **4. 1. 2.**] and glucuronyltransferase total and specific activities (Table IV.4).

The 13,000 $g_{av.}$ centrifugation of the homogenate resulted in 46% of protein recovered within the pellet. Among the membrane-enriched fractions, the Golgi fraction was the one with the highest protein content with a yield of 5.7 mg and a 6% recovery of protein over the homogenate, while the ER showed a 2% recovery and the PM 3%.

The activity of the succinate dehydrogenase was only detected in the homogenate and in the 13,000 $g_{av.}$ pellet, indicating that mitochondria were successfully removed during the 13,000 $g_{av.}$ centrifugation.

Most of the NADH-cytochrome C reductase activity was found in the 13,000 $g_{av.}$ pellet where 48% of the total activity of the homogenate was recovered. Among the membrane fractions the ER was the one with the highest total activity, having 1.9% of recovery while the Golgi and PM fractions

Fraction	Protein			GT			Latent IDP'ase			GS II			Cytochrome C Reductase		
	Concent. ($mg \cdot ml^{-1}$)	Yield (mg)	Recov. (%)	S.A. ($Bq \cdot hr^{-1} mg^{-1}$)	Yield ($Bq \cdot hr^{-1}$)	Recov. (%)	S.A. ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Yield ($\mu mol \cdot min^{-1}$)	Recov. (%)	S.A. ($Bq \cdot min^{-1} mg^{-1}$)	Yield ($Bq \cdot min^{-1}$)	Recov. (%)	S.A. ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Yield ($\mu mol \cdot min^{-1}$)	Recov. (%)
Homog.	8.26	101	100	21.8	2206	100	0	0	-	0.82	82.3	100	198	20×10^3	100
Pellet	9.26	46	46	15.3	704	32	0	0	-	4	182.9	222	207	9.5×10^3	48
ER	2.20	2.2	2	284	624	28	8.5	18.6	-	6.5	14.3	17	172	378	1.9
Golgi	0.68	5.7	6	416	2370	107	19.5	111	-	0.15	0.87	1	25	144	0.7
PM	2.96	3.0	3	92	276	13	0.43	1.3	-	39.4	118.1	144	34	102	0.5
R_1	3.06	13	13	21.3	277	13	0.07	0.87	-	1.3	17.9	22	32	411	2.1
R_2	2.66	5.3	5	69.8	370	17	0.13	0.67	-	16.8	88.9	108	51	270	1.4

Table IV.4: Specific activity (S.A.), yield and recovery (recov.) of GT and marker enzymes during the fractionation of membranes on a discontinuous sucrose-density gradient.

Membranes were fractionated by flotation through a 18/25/35% (w/w) sucrose discontinuous density gradient.
Homog. = Muslin filtrate; Pellet = Pellet 13,000 $g_{av.}$; ER (endoplasmic reticulum) = 18/25% interface; Golgi = 25/35% interface;
PM (plasma membrane) = 35%/Sample interface; R_1 = Remainder 1; R_2 = Remainder 2.

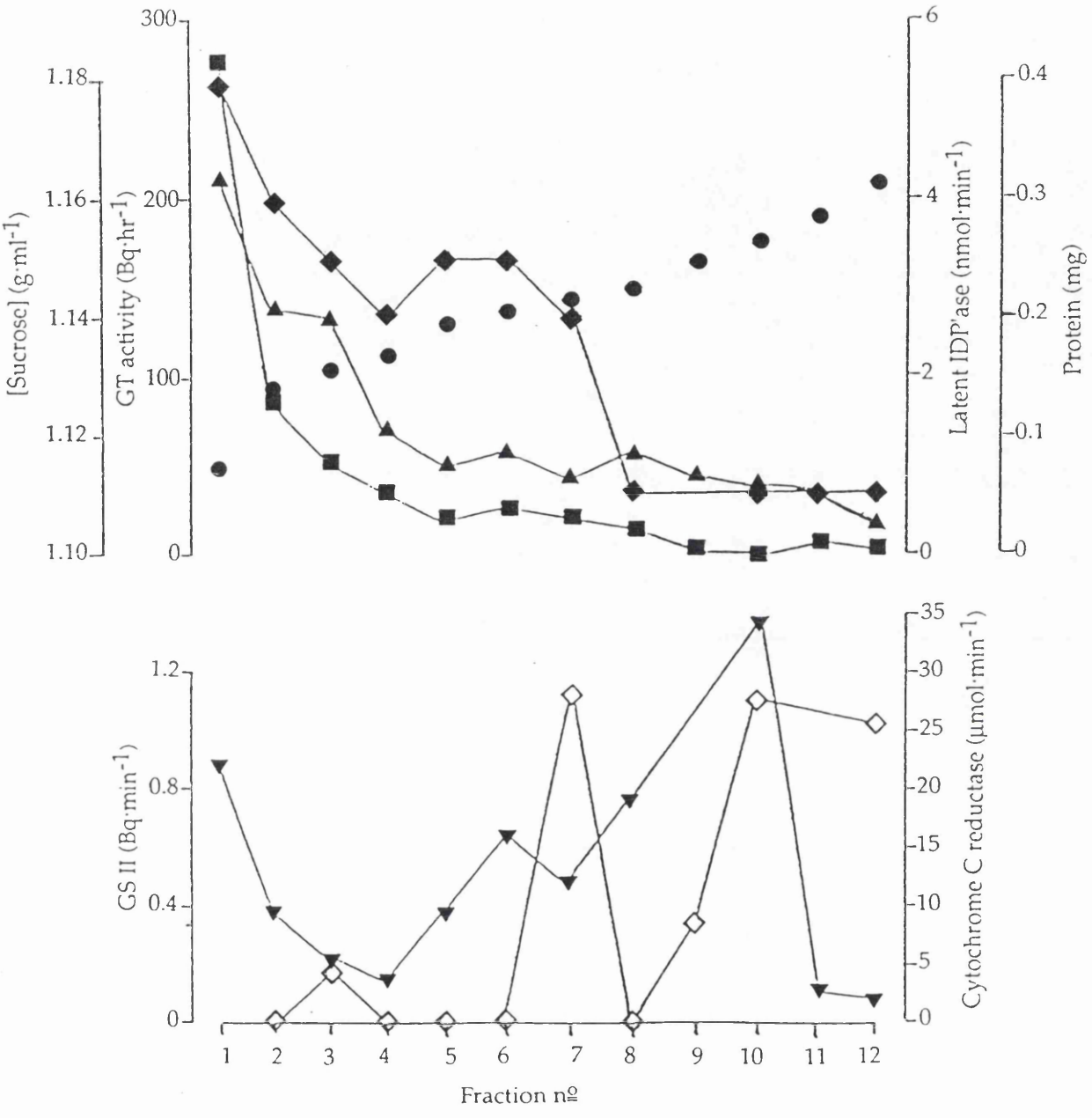


Fig. IV.6: Fractionation of Golgi membranes on a continuous 1.115 to 1.165 g·ml^{-1} sucrose density gradient. Profiles of GT and marker enzyme activities.

■ GT activity; ◆ Latent IDP'ase activity; ◇ GS II activity; ▲ Protein;
▼ NADH cytochrome C activity; ● Sucrose concentration in fractions;

showed respectively 0.7% and 0.5%. The ER fraction also contained the highest specific activity of NADH-Cytochrome C reductase with 172 mmole·min^{-1}·mg^{-1}.

The activity of GS II was found in all the fractions but it was mainly located in the PM fraction where its total activity was 118.1 Bq·min^{-1} and its specific activity 39.4 Bq·min^{-1}·mg^{-1}. The recovery of GS II activity in this fraction was of 144%, while 1% was found within the Golgi and 17% within the ER.

Latent IDP'ase activity was not detected in the homogenate nor in 13,000 $g_{av.}$ pellet. Among the membrane fractions the Golgi interface contained the highest total activity with a yield of 111 mmole·min^{-1} and the highest specific activity with 19.5 mmole·min^{-1} ·mg^{-1}.

Glucuronyltransferase activity was mainly located in the Golgi fraction associated with the main peak of latent IDP'ase activity and with the main peak of protein recovery. In this fraction the recovery of total glucuronyltransferase activity was 107% and its specific activity was 416 Bq· hr^{-1}·mg^{-1}, resulting in a 19-fold purification over the homogenate.

4. 3. 2. 2. Golgi apparatus subfractionation.

4. 3. 2. 2. 1. 1.115 to 1.165 g·ml^{-1} (27 to 39% sucrose (w/w)) linear gradient.

Golgi membranes obtained from the discontinuous sucrose-density gradient were subfractionated on a 1.115 to 1.165 g·ml^{-1} (27 to 37% sucrose (w/w)) linear density gradient (Fig. IV.6). The main peak of protein recovery was found at the top of the gradient at densities between 1.115 and 1.134 g·ml^{-1}

(27 -32% sucrose (w/w)). This protein peak was associated with the majority of latent IDP'ase and glucuronyltransferase activities. A second peak of latent IDP'ase activity was detected at a density between 1.134 $g \cdot ml^{-1}$ and 1.146 $g \cdot ml^{-1}$ (32-34% sucrose (w/w)). GS II activity was mainly recovered at the bottom of the gradient at densities greater than 1.153 $g \cdot ml^{-1}$ (35% sucrose (w/w)). The NADH-cytochrome C reductase activity was recovered in 2 main peaks located at densities of 1.131 $g \cdot ml^{-1}$ and 1.153 $g \cdot ml^{-1}$ (32% and 35% sucrose (w/w)).

The recovery of glucuronyltransferase activity and protein from the linear gradient was approximately 48% with respect to the Golgi membrane fraction applied to the gradient. With a yield of 1.37 mg the recovery of protein from the gradient relative to the homogenate was 1.4%. The recovery of glucuronyl transferase activity from the gradient was 26% of the homogenate with a total yield of 567.6 $Bq \cdot hr^{-1}$. The most active fraction of the gradient showed a yield of 275 $Bq \cdot hr^{-1}$ and was found at the top of the gradient. In this fraction glucuronyltransferase specific activity was twice as great as in the Golgi membranes applied to the gradient and this represented a total purification of 40 fold over the homogenate. The highest yield of activity of the latent IDP'ase was found at the top of the gradient with 5.3 nmole of Pi released per min. The highest peak of specific latent IDP'ase activity was of 41.3 nmole of Pi released per min and per mg of protein and was found at a density of 1.1398 $g \cdot ml^{-1}$ (33% sucrose (w/w)). This was twice as great as in the Golgi fraction but the recovery from the homogenate was only 0.5%.

4. 3. 2. 2. 1.083 to 1.155 $g \cdot ml^{-1}$ (20 to 35% sucrose (w/w)) linear gradient.

In order to study the distribution of enzymes activities found at the top of the previous gradient, another linear sucrose gradient, covering lower density,

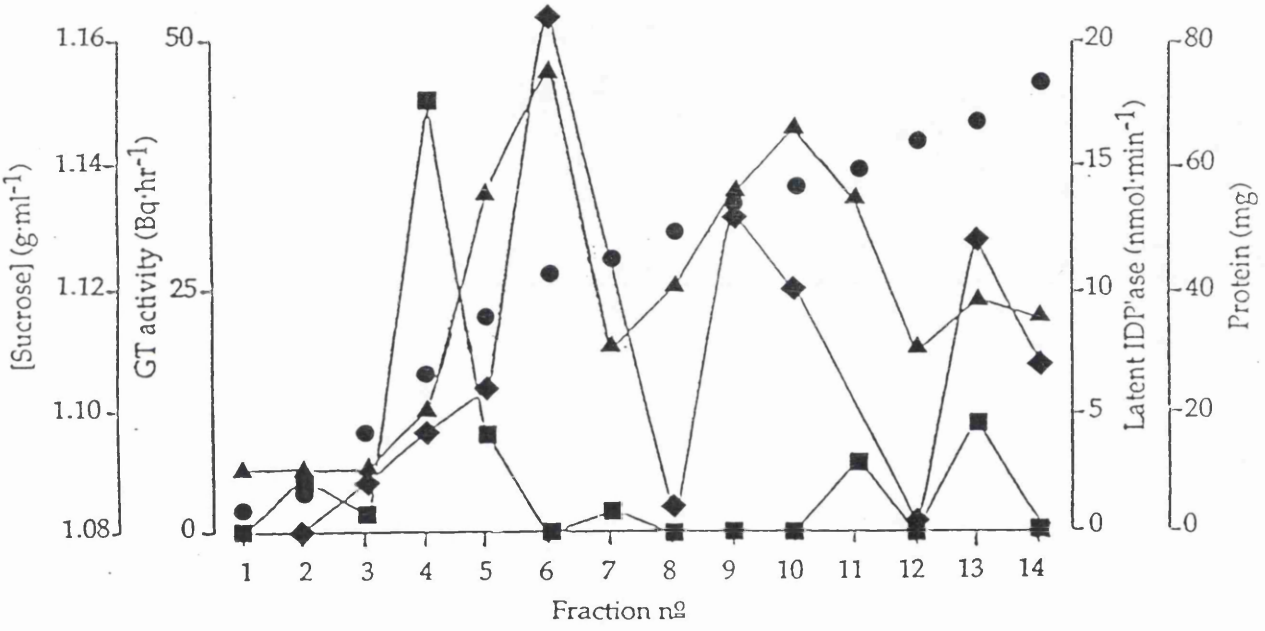


Fig. IV.7: Fractionation of Golgi membranes on a continuous 1.083 to 1.155 g·ml^{-1} sucrose density gradient. Profiles of GT and marker enzyme activities.

■ GT activity; ◆ Latent IDP'ase activity; ▲ Protein;
● Sucrose concentration in fractions.

was performed. The results are shown in figure IV.7. Protein and latent IDP'ase were associated in 3 peaks that fractionated at densities of 1.122, 1.135 and 1.146 $g \cdot ml^{-1}$ (28, 32 and 34% sucrose (w/w)). Glucuronyltransferase activity was mainly located at a density of 1.106 $g \cdot ml^{-1}$ above the first peak of latent IDP'ase activity. A small amount of glucuronyltransferase activity was found at densities between 1.136 and 1.155 $g \cdot ml^{-1}$. The most active glucuronyltransferase fraction showed a recovery of 3.2% of activity resulting in a purification of 345 fold over the homogenate.

4. 3. 3. Characterization of membrane fractions from discontinuous sucrose density gradient used for a rapid isolation of Golgi membranes.

This rapid method of Golgi membrane isolation by discontinuous sucrose density gradient centrifugation was developed in order to facilitate routine extraction of glucuronyltransferase activity. This modification of the method described for the Golgi subcompartmentation allows a gain of a few hours on the previous schedule. Organelle marker enzymes and glucuronyltransferase activities were determined for crude homogenate (H), the 13,000 $g_{av.}$ supernatant (S_1) and for the different membrane fractions obtained from the discontinuous sucrose density gradient (Fig.IV.8).

Succinate dehydrogenase activity was only detected in the homogenate (H) indicating that mitochondria were removed during the 13,000 $g_{av.}$ centrifugation. The results for the other marker enzymes and glucuronyltransferase activities are shown in table IV.5. All percentages of enzyme activity recovery are expressed relative to their total activity within the homogenate.

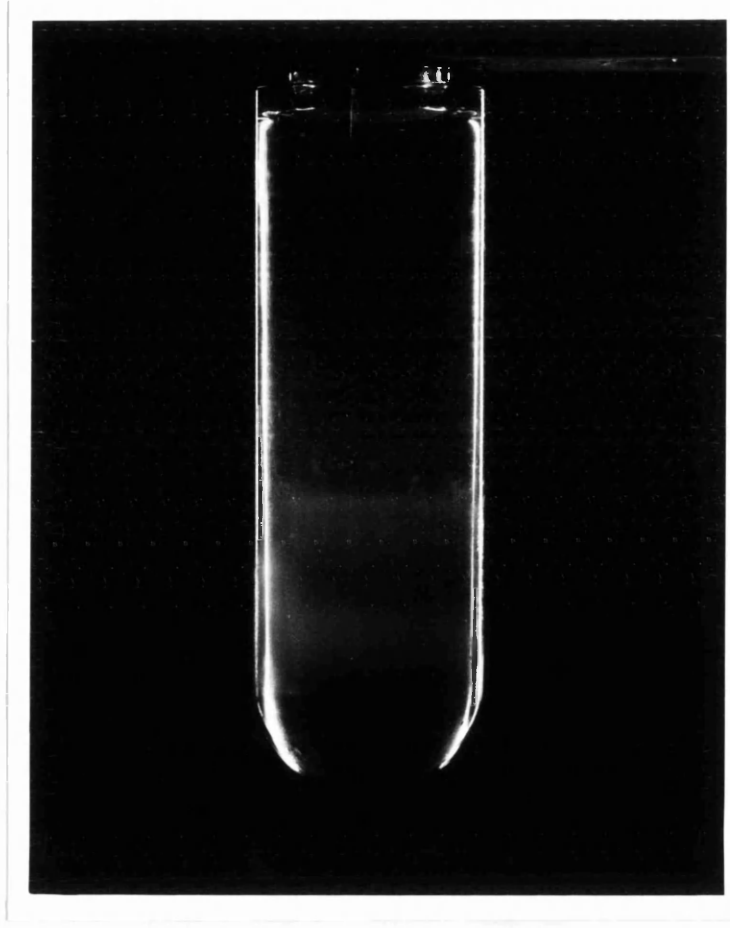


Fig. IV.8: Rapid isolation of a Golgi membrane enriched fraction on a discontinuous sucrose density gradient.

	Protein		GT			Latent IDP'ase			GS II			NADH Cyt. C reductase		
	Yield (mg)	Recov. (%)	S.A. (Bqhr^{-1}· ·mg^{-1})	Yield (Bq·hr^{-1})	Recov. (%)	S.A. (µmole· min^{-1})	Yield (µmole· min^{-1})	Recov. (%)	S.A. (Bq· min^{-1}· mg^{-1})	Yield (Bq· min^{-1})	Recov. (%)	S.A. (µmole· min^{-1}· mg^{-1})	Yield (µmole· min^{-1})	Recov. (%)
H	24	100	114.7	2752	100	0.35	8.4	100	56.8	1363	100	1.1	26	100
S1	10	41.7	175.5	1755	63.8	0.55	5.5	65.5	32.3	323	23.7	2	20	76.9
ER	4.8	20	52.9	254	9.2	0.17	0.8	9.5	0	0	0	4.6	22	84.6
G	7.3	30.4	427.7	3122	113.5	0.16	1.2	14.3	9.7	71	5.2	0.7	5.2	20
PM	2.0	8.4	14	28	1	0.45	0.9	10.7	60.5	121	8.9	0.9	1.9	6.6

Table IV.5: Characterization of membrane fractions obtained by two step discontinuous sucrose density gradient centrifugation. Protein yield and recovery (Recov.), glucuronyltransferase (GT) and marker enzymes specific activity (S.A.), yield and recovery.

H = crude homogenate; S1 = 13,000 $g_{av.}$ supernatant; ER (endoplasmic reticulum) = sample/25% interface; G (Golgi membranes) = 25/35% interface; PM (plasma membrane) = pellet.

All the fractions showed some NADH-Cytochrome C reductase activity but the ER interface (sample/25% sucrose interface) had the highest recovery of activity. At this interface 87% of the enzyme activity was recovered while the Golgi fraction (25/35% sucrose interface) showed 20% and the PM (pellet) 7.3% of enzyme activity recovery.

Much of glucan synthase II activity was lost during the 13,000 $g_{av.}$ centrifugation, as only 24.7% of enzyme activity was found in the resulting supernatant. Some activity was found within the PM fraction where 8.9% of activity was recovered. GS II activity was also detected in the Golgi membrane fraction with 5.2% of recovery, but no activity was found in the ER fraction.

The activity of the latent IDP'ase, marker enzyme for the Golgi apparatus, appeared in all fractions but was mainly recovered within the 25/35% sucrose interface, although much of IDP'ase activity was not recovered from the 13,000 $g_{av.}$ centrifugation. At this interface 14.3% of the enzyme activity was recovered, while 10.7% and 9.5% were found in the PM and ER fractions respectively.

A high peak of glucuronyltransferase activity was found, at the 25/35% sucrose interface, to be associated with the main peak of latent IDP'ase activity and with the highest recovery of membrane-bound protein. In this fraction 113.5% of glucuronyltransferase total activity was recovered relative to the homogenate, and 178% relative to the 13,000 $g_{av.}$ supernatant. In the other fractions no significant glucuronyltransferase activity was recovered, as only 9.2% was found in the ER fraction and 1% in the PM fraction. The glucuronyltransferase specific activity was very high in the Golgi fraction, with 428 $Bq \cdot hr^{-1} \cdot mg^{-1}$ resulting in almost a 4 fold purification relative to the homogenate.

Much protein was lost during the 13,000 $g_{av.}$ centrifugation, as 41.7% was found in the resulting supernatant. In the different fractions obtained from the gradient the main peak of protein was found at the 25/35% sucrose interface with 30.4% recovered. The other fractions showed 2% and 8.4%, for the ER and for the PM respectively .

4. 4. DISCUSSION.

The subcellular organelles were first separated by centrifugation on a discontinuous sucrose density gradient. The different fractions obtained were characterized by their respective enrichment in marker enzyme activity (NADH Cytochrome C reductase for the endoplasmic reticulum; latent IDP'ase for the Golgi apparatus; glucan synthase II for the plasma membrane).

The 13,000 $g_{av.}$ centrifugation of the homogenate resulted in the precipitation of the mitochondria, since no succinate dehydrogenase activity was detected in the subsequent fractions. There was also a loss of membranes and proteins as shown by both glucan synthase II and NADH cytochrome C reductase activity within the pellet. The loss of GSII activity may be due to the association of the plasma membranes with pelleting wall debris as previously observed (Waldron *&* Brett, 1987).

In the 13,000 $g_{av.}$ pellet and in the endoplasmic reticulum fraction the recovery of glucan synthase II activity was greater than in the initial homogenate. This may be due to the involvement of this enzyme in stress and wound response in plants (Brett, 1978; Delmer, 1987). The increase in enzymatic activity may appear as a response to the various treatments and manipulations to which the membranes were submitted. In the homogenate,

the lower activity of this enzyme, and also of the glucuronyltransferase, may have been caused by competition for the substrate, the action of proteinases released during the homogenization, or by the effect of soluble factors present in the cytoplasm.

The highest level of IDP'ase activity was detected at the interface between the 35 and 25% sucrose cushions and this fraction was therefore called the Golgi membrane fraction. The glucuronyltransferase activity was also mainly found in this fraction. These results match those previously reported by Waldron and Brett (1987) on the subcellular location of the glucuronyltransferase. They also corroborate reports on the location of several glycosyltransferases involved in the biosynthesis of cell wall matrix polysaccharides. These enzymes were previously localized within the membrane system of the Golgi apparatus (enzymes involved in the biosynthesis of xyloglucan (Ray, 1980; Hayashi *et al.*, 1988; Gordon & Maclachlan, 1989; Brummel *et al.*, 1990; Moore *et al.*,1991) and xylan (Waldron & Brett, 1987; Gibeaut & Carpita, 1990)).

Some glucuronyltransferase activity was also found to be associated with the endoplasmic reticulum and plasma membrane fractions. The activity detected in the endoplasmic reticulum fraction was initially assumed to be due to cross contamination of the membranes during the discontinuous sucrose density gradient centrifugation and/or the pipetting of the fractions. In order to assess this hypothesis, the ratios of total glucuronyltransferase and latent IDP'ase activities in the Golgi to that in the endoplasmic reticulum were determined. Two very similar ratios (0.15 and 0.21) of the relative activity of these two enzymes were obtained. Since the main peak of glucuronyltransferase activity was associated with the IDP'ase activity in the Golgi fraction, the similar levels of enzyme activities suggested that the

glucuronyltransferase activity found in the endoplasmic reticulum fraction was due to a cross contamination between the two membrane fractions. Furthermore, when the Golgi fraction was later subjected to centrifugation on a continuous sucrose density gradient, a Golgi membrane subcompartment showing a high level of glucuronyltransferase activity equilibrated at the same density, 1.12 $g \cdot ml^{-1}$. This was previously reported for an endoplasmic reticulum membrane fraction isolated from etiolated leek seedlings (Bertho *et al.*, 1989). However, a recent study of the interactions between the xylosyl- and the glucuronyltransferase involved in glucuronoxylan synthesis (Hobbs *et al.*, 1991) has revealed that the glucuronyltransferase activity found in the endoplasmic reticulum fraction is different from that detected in the Golgi and plasma membrane fractions, as the degree of precision of the polysaccharide biosynthesis appears to be different in the endoplasmic reticulum from that in the other membrane fractions. These findings support the possibility that the endoplasmic reticulum is the site for the initiation of glucuronoxylan biosynthesis. It is possible that the enzymatic activity detected in the endoplasmic reticulum fraction is involved in the biosynthesis of a priming molecule which would later be exported from the endoplasmic reticulum to the *cis*-Golgi cisternae for elongation of the glucuronoxylan backbone.

The level of glucuronyltransferase activity detected in the plasma membrane fraction was investigated in the same way. The ratios of total activity in the Golgi fraction to that in the plasma membrane, of this enzyme and of the Golgi latent IDP'ase, were evaluated. The relative level of glucuronyltransferase activity (0.10) found in the plasma membrane fraction was ten times greater than that of latent IDP'ase (0.01) in the same fraction. Therefore it was concluded that the presence of the glucuronyltransferase activity in the plasma membrane could not only be caused by cross contamination of the membrane fractions, since the level of latent IDP'ase

activity found in the plasma membrane was much lower than what would be expected if all associated glucuronyltransferase activity originated from the Golgi apparatus membranes. In rapidly growing cells there is a high rate of vesicle formation and re-formation as material passes rapidly from the endoplasmic reticulum to the plasma membrane through the Golgi apparatus membrane system. Therefore, the presence of the glycosyltransferase activity in the plasma membrane may well be a consequence of intensive membrane flow occurring during the growth of the cells (Karrenbauer *et al.*, 1990). The process of membrane flow through the Golgi apparatus in animal and plant cells has been reviewed by different authors (Robinson & Kristen, 1982; Farquhar, 1985; Pfeffer & Rothman, 1987; Della-Cioppa *et al.*, 1987; Karrenbauer *et al.*, 1990). Membrane flow in the Golgi apparatus of animal cells was shown to occur via repeated rounds of budding and fusion of transport vesicles from one cisterna to another so that the cisternae do not turn-over in their entirety (Pfeffer & Rothman, 1987; Morré, 1987). This process of vesicle formation and transport of material from one cisterna to another, coupled with the formation of vesicles destined for different subcellular compartments -and some more especially targetted at the plasma membrane- may have resulted, in a rapidly growing cell, in the exit and co-transport of a small amount of glucuronyltransferase. The enzyme would finally accumulate in the plasma membrane since this organelle may be regarded as an end point of intracellular membrane flow. The flow of membrane material through the Golgi apparatus to the plasma membrane in developing pea cells may then be considered as slighty "leaky", especially in a rapidly growing system.

The Golgi membranes were subfractionated on a continuous sucrose density gradient. The method involved loading the sucrose gradient on top of the membrane cushion -the sucrose concentration of which had been increased- in order to allow the membranes to fractionate in an upward direction by

flotation through the gradient rather than by a downward centrifugation. Gibeaut and Carpita (1990), using this method, reported an improvement in the purity of membrane fractions obtained from etiolated leek seedlings.

Using a continuous sucrose density gradient, Waldron and Brett (1987) described the separation of the Golgi latent IDP'ase in two peaks with densities of 1.06 $g \cdot ml^{-1}$ and between 1.12 and 1.17 $g \cdot ml^{-1}$, the latter containing the glucuronyltransferase activity. Subfractionation of the Golgi membrane-enriched fraction obtained by discontinuous density gradient centrifugation was first achieved on a shallow continuous sucrose gradient of 1.115 to 1.165 $g \cdot ml^{-1}$. This resulted in the separation of the previously reported glucuronyltransferase - latent IDP'ase associated peak into two peaks of latent IDP'ase, with the peak of lower density containing the transferase activity. Some NADH cytochrome C reductase activity was also detected alongside with the less dense peak of IDP'ase activity. The presence of this enzyme at the same density as the Golgi marker is certainly due to the smooth endoplasmic reticulum which has a density of 1.11 to 1.12 $g \cdot ml^{-1}$ (Quail, 1979; Bertho *et al.*, 1989). The more dense peak of NADH cytochrome C reductase activity may correspond to rough endoplasmic reticulum. Although EDTA was present in the sucrose gradient media, it is possible that this did not lead to the dissociation of all ribosomes from the endoplasmic reticulum (Sabatini *et al.*, 1966). However, the activity of NADH cytochrome C reductase has also been reported in association with Golgi membranes (Hino *et al.*, 1978a,b,c; Bergeron, 1979). Therefore it is unlikely that the small peak of this enzyme activity found to be associated with the more dense peak of latent IDP'ase was due to endoplasmic reticulum contamination.

In order to study in more detail the peak of glucuronyltransferase found at the top of the continuous sucrose density gradient, the Golgi membranes

were flotated in a linear density gradient of lower density (1.083 to 1.155g·ml^{-1}). The Golgi apparatus membranes fractionated into three subfractions identified by their latent IDP'ase activity. These three subfractions also corresponded to three peaks of proteins. The least dense of the subfractions contained most of the glucuronyltransferase activity.

In animal cells, considering the process of post-translational glycosylation and sorting of proteins, at least three compartments of distinct enzymatic and functional characteristics have been recognised and called *cis-*, *medial-* and *trans*-Golgi (Dunphy & Rothman, 1985; Farquhar, 1985; Tartakoff, 1987; Rothman & Orci, 1990). Proteins synthesized in the endoplasmic reticulum are modified during their transit through the different compartments of the Golgi dictyosome and transported to their final destination in specific coated vesicles budding mainly from the *trans*-face of the Golgi apparatus.

Studies of the subfractionation of plant cell Golgi apparatus led to the isolation of two subcompartments (Ali *et al.*, 1986; Sturm *et al.*, 1987; Brummel *et al.*, 1990). Some evidence was found for a differential distribution of glycosyltransferases within the Golgi apparatus of suspension-cultured cells of sycamore, but it was not clear whether the glycosyltransferase activities measured related to the oligosaccharide processing enzymes or whether they representated activities involved in wall polysaccharide biosynthesis as well as glycan processing (Ali *et al.*, 1986).

Sturm *et al.* (1987) isolated two peaks of latent IDP'ase while subfractionating Golgi membranes from French bean on a Percoll density gradient. They found no evidence that the enzymes involved in the processing of *N*-linked oligosaccharides were associated with Golgi cisternae of different densities.

Two subfractions were obtained from dictyosomes from pea Golgi when the membranes were fractionated on a Renographin gradient (Brummel *et al.*, 1990). The lighter Golgi subfraction, thought to correspond to the *cis*-Golgi cisternae, contained xylosyltransferase activity but no associated fucosyltransferase activity, whereas the second, denser peak of Golgi membrane, probably composed of *medial* and *trans*-Golgi cisternae, contained the two transferase activities. From these results, it was concluded that the synthesis of the glucose-xylose backbone of xyloglucans was initiated in a light density subcompartment (*cis*-Golgi) and completed in heavier cisternae (*trans*-Golgi) with further substitution, such as the fucosylation of the polysaccharide, taking place in the *trans*-Golgi and in secretory vesicles. It was then suggested that, in the case of xyloglucans, the glycosyltransferases involved in the biosynthesis of the glucan backbone, and in its substitution by xylosyl side-chains, were mainly located at the *cis*-end of the Golgi apparatus, whereas the enzymes involved in the subsequent elaboration of the basic xyloglucan polymer were located at the *trans*-face of the Golgi stack and in the *trans*-Golgi network (Brummel *et al.*, 1990). However, immunocytochemical techniques have also been used to study the assembly of glycoproteins and complex polysaccharides in the Golgi apparatus in carrot cells (Moore *et al.*, 1991). Using electron microscopy and immunogold labelling of antibodies raised against an acidic pectin, rhamnogalacturonan I, and against hemicellulosic xyloglucan, the presumed sites of synthesis of these polysaccharides within the different compartments of the dictyosome have been identified. The results obtained showed that the pectic rhamnogalacturonan I was mainly found in the *cis*- and *medial*- Golgi cisternae whereas the xyloglucans were located within the *trans*-Golgi. The possibility that the antibodies would not recognize precursor forms of the xyloglucan present in the *cis*-Golgi, and therefore prevent their detection on the micrographs, was not ruled out. Within the Golgi subcompartments, a spatial organization of the assembly pathways of complex polysaccharides

-where different polymers would be assembled in specific compartments of the Golgi stack- was suggested (Moore *et al.*, 1991).

Since the glucuronyltransferase activity was localized in a Golgi fraction of a density very similar to that of the endoplasmic reticulum, it is also very probable that this transferase is located in the *cis*-Golgi membranes which have been reported to be in close relation with the endoplasmic reticulum (Mollenhauer & Morré, 1980). Previous studies of the glucuronyltransferase have shown that this enzyme glucuronidates a β-(1→4)-xylose backbone (Waldron & Brett, 1983). The activity is stimulated by the presence of UDP-xylose and is therefore influenced by the xylosyltransferase. The xylosyltransferase synthesizes the xylan backbone onto which glucuronic acid is added by transfer from UDP-glucuronic acid (Baydoun *et al.*, 1989b). The subcellular location of the xylosyltransferase is therefore now in question.

Odzuck and Kauss (1972) have shown that crude membrane preparations from mung bean can synthesize xylan from UDP-[^{14}C]-xylose, and a similar activity has been localized in the Golgi apparatus and endoplasmic reticulum of beans (Bolwell & Northcote, 1983). It has been suggested that the synthesis of xylans may begin in the endoplasmic reticulum but that the polymerization to a high molecular weight polymer only occurs in the Golgi apparatus (Bolwell & Northcote, 1983). Autoradiography of secretory polysaccharides (Pickett-Heaps, 1967) generally showed little accumulation of polysaccharides in the endoplasmic reticulum. Thus it is probable that only small precursors of the polysaccharides are processed in the endoplasmic reticulum before being rapidly transported to the Golgi apparatus.

The presence of the glucuronyltransferase -sensitive to activation by xylosyltransferase- in the *cis*-Golgi suggests that the xylosyltransferase is also

located in the same subcompartment of the Golgi and, to a lesser extent, in the endoplasmic reticulum. More evidence for the specific location of the xylan synthase within the membranes of the Golgi was obtained from the isolation of organelles from cells of etiolated maize seedlings (Gibeaut & Carpita, 1990). During the fractionation of subcellular membranes a xylan synthase activity was identified, which copurified with the Golgi apparatus. However, from the study of the radioactive product formed with UDP-[^{14}C]-xylose as substrate, it was not clear whether this xylosyltransferase activity was involved in the formation of hemicellulosic xylans or whether it was involved in the biosynthesis of xyloglucans, as previously reported by Ray in pea (1980) and in suspension-cultured soybean cells (Hayashi & Matsuda, 1981b).

It is therefore highly probable that the xylosyltransferase involved in the biosynthesis of glucuronoxylans in peas is located, with the glucuronyltransferase, in the same Golgi subcompartment, the *cis*-Golgi. Further study on the interactions of the xylosyl- and the glucuronyltransferase showed that the formation of a preformed xylan backbone in a preincubation was not sufficient to sustain the glucuronyltransferase activity (Baydoun *et al.*, 1989b) and that there is a high degree of coordination between the formation of the xylan backbone and the addition of glucuronic acid side-chains (Waldron & Brett, 1985; Baydoun *et al.*, 1989b). If the synthesis of xyloglucans and glucuronoxylans is comparable, it is possible that the basic polysaccharide structure of the glucuronoxylan is synthesized in the *cis*-Golgi and further elaborated, via methylations, acetylations and feruloylation, in the *medial*- or *trans*-Golgi before the completed polymer is packaged and transported in secretion vesicles from the *trans*-Golgi network to the plasma membrane to be deposited in the cell wall. The synthesis of a basic core polymer which is then modified during its transit through the subcompartments of the Golgi apparatus, would be similar to the process of protein glycosylation in the Golgi

(Tartakoff, 1987). More studies are needed in order to assess the precise location of the xylosyltransferase, methyltransferase and acetyltransferase involved in the biosynthesis of glucuronoxylans in peas so that the differential distribution of these enzymes within the Golgi apparatus can be confirmed. Ultimately the isolation of each transferase would permit a detailed study of their interactions. Studies of the biosynthesis of other heteropolysaccharides of the wall matrix are also essential in order to obtain a better understanding of the role of the endoplasmic reticulum and the different subcompartments of Golgi apparatus in cell wall biosynthesis.

The glucuronyltransferase activity was maximal in the *cis*-Golgi fraction isolated from the linear sucrose density gradient [section **4. 1. 3. 2.**] In this fraction, a 345-fold purification was achieved. For the purification and characterization of a membrane-bound enzyme, a membrane fraction containing a high specific activity of the enzyme to be purified is considered to be an ideal starting point. From this point, solubilization and further purification can be carried out (Van Reswoude & Kempf, 1984). However, although a significant purification of the glucuronyltransferase was achieved by the isolation of the *cis*-Golgi membranes, the recovery of the enzyme activity in this fraction was only 6.4%. Therefore, the initial Golgi membrane-enriched fraction -in which the total enzyme activity was slightly greater than in the homogenate- was selected as a starting point for the solubilization of the glucuronyltransferase.

A more practical and rapid method of Golgi membrane isolation was derived from the protocol used for the study of the subcompartmentation of the Golgi stack in order to facilitate routine purification of the enzyme. This new protocol involved loading the 13,000 $g_{av.}$ supernatant on top of a two step sucrose density gradient. Although a certain level of cross-contamination of the

membrane fractions collected was detected, activities from the different membrane marker enzymes showed that separation was achieved to give three membrane fractions enriched with either endoplasmic reticulum, Golgi apparatus or plasma membranes. The higher recovery of the glucuronyltransferase activity in the Golgi membrane fraction relative to the starting homogenate and to the 13,000 $g_{av.}$ supernatant is probably caused by a reduced apparent activity of the enzyme in the latter fractions where substrate competition, protease action or other soluble factors could affect or mask the enzyme activity. The protocol used previously for the preparation of Golgi membranes involved the centrifugation of the 13,000 $g_{av.}$ supernatant onto a 40% (w/w) sucrose cushion followed by the loading of a three step gradient on top of the sample and the subsequent collection of membranes at each interfaces. In comparison, the new method allowed a gain of about three hours on the previous schedule while leading to a very high recovery of the glucuronyltransferase activity at the Golgi membrane interface. It also limited the manipulations and the risks of inactivation of the particulate enzyme, which is unstable (Waldron & Brett, 1983). The modified method was used routinely to obtain a glucuronyltransferase-enriched fraction. This Golgi membrane-enriched fraction was used for the solubilization of the glucuronyltransferase from its membranous environment.

CHAPTER 5: DETERGENTS AND SOLUBILIZATION.

5. 1. SOLUBILIZATION OF PROTEINS FROM WHOLE MEMBRANE PREPARATIONS.

5. 1. 1. Introduction.

Triton X-100 has previously been used at high concentration to solubilize the glucuronyltransferase from a crude membrane preparation (Waldron *et al.*, 1989). Because of the interference of this detergent with the monitoring of proteins at 280 nm during chromatography, another detergent was needed. Thus, various detergents were tested for their solubilization properties and for their effects on glucuronyltransferase activity.

5. 1. 2. Methods.

5. 1. 2. 1. Solubilization with a selection of detergents.

A whole particulate membrane preparation was obtained by homogenization of etiolated pea epicotyls (90 g) in pre-chilled buffer (200 ml) containing Tris (10 mM), Mes (10 mM) brought to pH 6 with HCl. The homogenization was carried out using a Polytron blender for 2 periods of 10 seconds at speed 5. The resulting homogenate was strained through 4 crossed layers of muslin. The filtrate was then centrifuged for 30 min at 97,000 $g_{av.}$ in a Sorvall OTD65 ultracentrifuge with a Sorvall AH629 swing-out rotor. The supernatant was discarded and the pellet resuspended, using a glass-on-glass homogenizer, in Tris-Mes buffer (4.5 ml) containing the selected detergent at the concentration chosen for solubilization. The membrane-detergent solution

was centrifuged for 2 hours at 230,000 $g_{av.}$ in a Sorvall OTD65 ultracentrifuge with a Sorvall T865.1 fixed-angle rotor. The resulting supernatant was collected and the pellet was resuspended in Tris-Mes buffer (1 ml) containing 1% (w/w) detergent. Soluble and insoluble material were assayed for protein concentration. When solubilized material was assayed for glucuronyltransferase activity, 1 mM $MnCl_2$ was included. When insoluble material was assayed, 10 mM $MnCl_2$ was included.

5. 1. 1. 2. Two step solubilization.

Solubilization of the glucuronyltransferase activity was achieved in 2 steps. The first involved solubilization of proteins from a crude membrane preparation with Triton X-100 at a low concentration in order to remove some of the proteins loosely associated with the membranes. In the second step, material not solubilized during the Triton X-100 solubilization was solubilized in buffer containing NP40 at a high concentration.

The preparation of particulate membranes was carried out as described in section **5. 2. 1. 1.** The first solubilization was achieved with 1% (w/w) Triton X-100. The membrane-detergent solution was centrifuged for 2 hours at 230,000 $g_{av.}$ in a Sorvall OTD65 ultracentrifuge with a Sorvall T865.1 fixed-angle rotor. The resulting supernatant was collected and the pellet was re-homogenised in Tris-Mes buffer (4.5 ml) containing 5% (w/w) NP40. This was centrifuged for 2 hours at 230,000 $g_{av.}$. The supernatant was collected and the pellet resuspended in Tris-Mes buffer containing 1% Triton X-100 (1 ml). Fractions were assayed for protein concentration and for glucuronyltransferase activity.

Detergent	Detergent concent. (% w/w)	Fraction	[^{14}C]-GlcA incorporation (pmoles)	Protein concentration (mg·ml^{-1})	Protein yield (mg)	Protein solubilized (%)
Tween 20	0.1%	SN	0	0.45	1.7	3
		P_2	14	47.5	33	
		P_1	10	20	60	
	1%	SN	0	0.5	1.9	3.5
		P_2	12	68	51	
		P_1	6	26	75	
	5%	SN	0.1	2.7	10	19
		P_2	1.3	56	42	
		P_1	0.8	23	69	
Brij	0.1%	SN	0	-	-	
		P_2	12	-	-	
		P_1	10	-	-	
	1%	SN	0	-	-	
		P_2	12	-	-	
		P_1	6	-	-	
	5%	SN	0	-	-	
		P_2	0	-	-	
		P_1	10	-	-	
NP40	0.1%	SN	0	0.3	0.9	3
		P_2	32	40	30	
		P_1	16	21	63	
	1%	SN	4	1	3.7	9
		P_2	8	51.3	38.5	
		P_1	10	26	75	
	5%	SN	10	3	10	27
		P_2	14	41	31	
		P_1	46	20	59	

Table V.1: Solubilization of whole particulate membranes with Tween 20, Brij and NP40 at concentrations of 0.1, 1 and 5%.

The activity of the glucuronyltransferase is estimated by the determination of the level of [^{14}C]-GlcA incorporation into chelator insoluble material, in pmoles·4hr·ml^{-1} [section II. 4. 2].

No protein assay was performed on material solubilized with Brij.

SN = solubilized material;

P_2 = non-solubilized material;

P_1 = whole particulate membrane fraction.

5. 1. 3. Results.

5. 1. 3. 1. Solubilization of a crude membrane preparation.

The solubilization of the glucuronyltransferase from a whole membrane preparation has previously been carried out using a high concentration (10% w/w) of Triton X-100 (Waldron *et al.*, 1989). Because of its chemical structure, Triton X-100 interferes with the UV monitoring of protein at 280 nm during chromatography. Thus, another detergent was required. Three other non-ionic detergents, Tween 20, Brij and NP 40 were assayed for their ability to solubilize the enzyme. The solubilization of proteins from the particulate membrane preparation was carried out at various detergent concentrations (0.1, 1 and 5% (w/w)). The activity of the glucuronyltransferase in the solubilized material, the remaining non-solubilized material and in the 97,000 $g_{av.}$ pellet was estimated by measuring the incorporation of [^{14}C]-GlcA from UDP-[^{14}C]-GlcA into chelator-insoluble product [see section **2. 4. 2.**]. No glucuronyltransferase activity was recovered in the material solubilized with Brij (Table V.1.). Solubilization of the whole membrane preparation with Tween 20 did not lead to the recovery of the enzyme activity. However, there was an increase in the amount of protein solubilized from 3 to 19% when the concentration of detergent used for solubilization was increased from 0.1 to 5% (w/w). Among the detergents assayed, only NP40 allowed the recovery of the enzyme activity in the solubilized material as well as an increase in the amount of protein solubilized when used as a concentration of 1 and 5 % (w/w). The recovery of glucuronyltransferase activity in the solubilized material increased when the concentration of NP40 used for solubilization was increased from 0.1 to 5% (w/w). This was associated with an increase in the amount of protein solubilized from 3 to 27%.

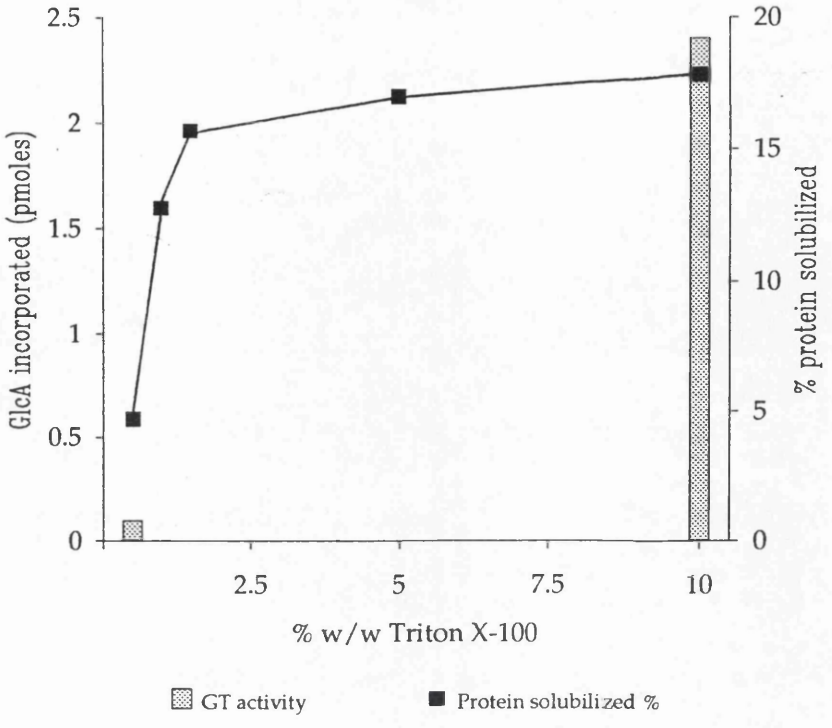


Fig. V.1: Solubilization of whole membranes by different concentrations of Triton X-100.

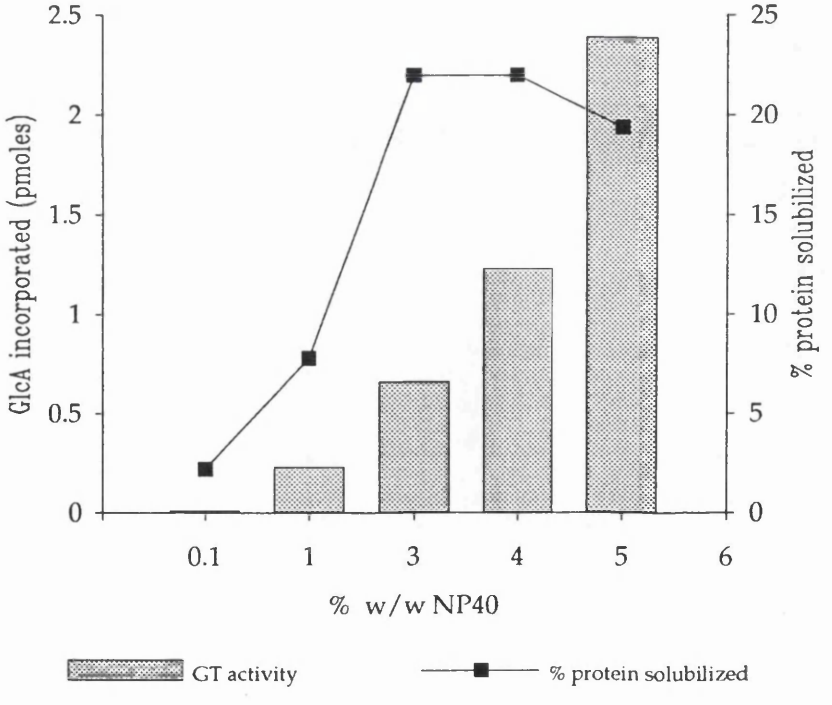


Fig. V.2: Solubilization of whole membranes by different concentrations of NP 40.

5. 1. 3. 2. Sequential solubilization.

The amount of protein solubilized by changing the concentration of Triton X-100 from 0.5 to 1.5% (w/w) increased rapidly, and then reached a plateau where a further increase of the detergent concentration did not lead to greater efficiency in solubilization (Fig. V.1). The glucuronyltransferase activity was assayed in the material solubilized with the lowest and highest concentration of the detergent. Little activity was found to be associated with the material solubilized at low detergent concentration whereas, as shown previously (Waldron *et al.*, 1989), a high concentration resulted in the solubilization of the enzyme activity. Considering the results described in section **5. 3. 1. 1.**, the effect of NP40 on solubilization was assayed at increasing concentrations from 0.1 to 5% (w/w) (Fig V.2). The amount of protein solubilized was greater at high concentration of the detergent and this was associated with the highest recovery of the glucuronyltransferase activity.

Considering the relative amount of solubilized glucuronyltransferase activity and protein recovered using the two detergents, solubilization of the whole membrane fraction was carried out with a two step method involving two successive solubilizations of the membrane proteins (Table V.2). The first solubilization was carried out with a low concentration of detergent. The remaining insoluble material was then solubilized with a higher detergent concentration. Two step solubilization of membrane material was achieved with 1 and 10% (w/w) Triton X-100. Recovery of glucuronyltransferase activity in the solubilized material was very low in the first solubilization with 1% Triton X-100 although 13% of protein was solubilized. In the second solubilization, with 10% Triton X-100, enzyme activity was recovered but there was no increase in the amount of protein solubilized relative to solubilization with 1% (w/w) Triton X-100. NP40 was tested in the two step solubilization

Fraction	UDP GlcA incorporation (pmoles) P	H	Protein ($mg \cdot ml^{-1}$)	Solubilization (%)
S/N 1 Triton X-100 1%	0.11	0.04	2.1	13
S/N 2 Triton X-100 10%	4.68	2.53	2.1	13
Pellet	2.47	1.09	47.5	
S/N 1 NP40 1%	1.75	0.41	3.15	17.1
S/N 2 NP40 5%	1.45	1.81	2.55	13.8
Pellet	2.60	1.35	51	
S/N 1 Triton X-100 1%	0.07	0.02	2.65	15.6
S/N 2 NP40 5%	2.98	1.78	3.3	19.5
Pellet	4.07	1.22	50	

Table V.2: Two step solubilization of whole membranes.

S/N 1 = Solubilized material first solubilization.

S/N 2 = Solubilized material second solubilization.

Pellet = Remaining insoluble material after both solubilizations.

P = Chelator soluble polysaccharides.

H = Chelator insoluble polysaccharides.

Fraction	Protein			Glucuronyltransferase			
	Yield (mg)	Recov. ① (%)	Recov. ② (%)	Total act. ($Bq \cdot hr^{-1}$)	Recov. ① (%)	Recov. ② (%)	S.A. ($Bq \cdot hr^{-1} \cdot mg^{-1}$)
Homogenate	260	100	-	600	100	-	2.3
SN 97,000 g	170	65	-	120	20	-	0.7
Pellet 97,000 g	74	28	100	221	37	100	3
SN Sol. Triton	7.3	3	10	9	2	4	1.2
SN Sol. NP 40	11	4	14	339	57	153	32
Pellet Sol. NP 40	49	18	68	81	14	37	2

Table V.3: Two step solubilization of whole membranes.
Incorporation of glucuronic acid into chelator insoluble material. Protein yield and recovery (Recov.), glucuronyltransferase total and specific activity (S.A) and recovery.
Recov. ① = recovery relative to the homogenate.
Recov. ② = recovery relative to the 97,000 g pellet.
Homogenate = crude homogenate (muslin filtrate); Pellet 97,000 g = whole particulate membranes; SN Sol. Triton = material solubilized in 1% Triton; SN Sol. NP 40 = material solubilized in 5% NP 40; Pellet Sol. NP 40 = material remaining non-solubilized after the two solubilizations.

method. Glucuronyltransferase activity was recovered in material solubilized first with 1% and then with 5% NP40 and the amount of protein solubilized was high after both solubilizations. Comparison of the results obtained with the two detergents shows that at low concentration they are efficient in solubilizing membrane proteins but only NP40 allows recovery of the enzyme activity. At high concentration, Triton X-100 and NP40 show a marked ability to solubilize membrane proteins and to allow the recovery of the glucuronyltransferase activity.

Another experiment was carried out in which membrane proteins were solubilized first with Triton X-100 (1% w/w), then with NP40 (5% w/w). The results show that it is possible to solubilize proteins from the membrane preparation in two sequences, first by solubilization with Triton X-100 (1% w/w), second with NP40 (5% w/w). In the first solubilization of the membranes Triton X-100 was used at a low concentration (1% w/w). Solubilized material was separated from non-solubilized material by a 230,000 $g_{av.}$ centrifugation. In the resulting supernatant, the recovery of protein was 10% of the particulate membrane preparation (97,000 $g_{av.}$ pellet) with a yield of 7.3 mg of protein (Table V.3). The amount of solubilized glucuronyltransferase activity in this fraction was only 4% of the total activity found in the particulate membrane preparation. The specific activity of glucuronyltransferase (1.2 $Bq \cdot hr^{-1} \cdot mg^{-1}$) in this fraction was also lower than the one found in the 97,000 $g_{av.}$ pellet where it was 3 $Bq \cdot hr^{-1} \cdot mg^{-1}$.

The material insoluble in 1% Triton X-100 was subjected to solubilization with 5% (w/w) NP40. The recovery of protein in the material non-solubilized with NP 40 was 68% relative to the 97,000g pellet. This was much greater than in the supernatant where only 14% of protein recovery was achieved. The glucuronyltransferase activity found in the non-solubilized material was 37% of

the 97,000g pellet while in the solubilized fraction, 153% of glucuronyltransferase total activity was recovered. With a specific activity of 32 $Bq \cdot hr^{-1} \cdot mg^{-1}$ for the solubilized glucuronyltransferase, a 10 fold purification was achieved over the particulate membrane preparation (97,000 $g_{av.}$ pellet) and 14 fold over the crude homogenate (muslin filtrate).

5. 2. SOLUBILIZATION OF PROTEINS FROM GOLGI MEMBRANES.

5. 2. 1. Introduction.

The effects of a selection of detergents on the glucuronyltransferase activity from particulate Golgi membranes were investigated. Suitable detergents were subsequently used for the solubilization of Golgi membrane proteins.

5. 2. 2. Methods.

5. 2. 2. 1. Glucuronyltransferase activity assay in the presence of different detergents .

Golgi membrane-enriched fractions were prepared as described previously [section **4. 2. 4**]. Unless stated otherwise, the protein concentration of the sample was brought to 1 $mg \cdot ml^{-1}$ with resuspension buffer in order to have a final protein concentration of 0.5 $mg \cdot ml^{-1}$ in the glucuronyltransferase assay. Detergents were added to the assay to obtain the concentration required in each experiment. The glucuronyltransferase activity was assayed for 2 hours

in the presence of 10 mM $MnCl_2$. Particulate Golgi membranes were also assayed in the absence of detergent.

5. 2. 2. 2. Golgi membrane-bound protein solubilization.

The Golgi membrane-enriched fraction was prepared as previously described [section **4. 2. 4**]. The protein concentration of the resuspended Golgi membranes was adjusted with resuspension buffer to double the concentration required in the solubilization process. Detergent was added to obtain the final protein and detergent concentrations desired in the experiment. The solution of membranes and detergent was homogenized with several strokes of a glass-on-glass homogenizer. The solution was incubated for 15 min at room temperature and then centrifuged at 230,000 $g_{av.}$ for 1 hour at 4°C in a Sorvall OTD65 ultracentrifuge with a Sorvall T865.1 fixed-angle rotor. The resulting supernatant was assayed for solubilized glucuronyltransferase activity.

5. 2. 3. Results.

5. 2. 3. 1. Effect of selected detergents on glucuronyltransferase activity from particulate Golgi membranes.

A range of detergents were assayed for their effect on Golgi membrane-bound glucuronyltransferase activity prior to solubilization in order to select one that would retain the activity of the enzyme during further manipulations of glucuronyltransferase. Protein concentration in the glucuronyltransferase assay was brought to 0.5 $mg \cdot ml^{-1}$. Six detergents were assayed at concentrations near and above their critical micelle concentration (CMC). The detergents used

Detergents	Category	CMC $mg \cdot ml^{-1}$
n-Dodecyl-β-D-maltoside	non-ionic	0.051
n-Octyl-β-D-Glucopyranoside (octyl glucoside, OG)	non-ionic	7.5
Lauryldimethylamine oxide (LDAO)	non-ionic	0.5
Polyoxyethylene lauryl ether (Lubrol PX)	non-ionic	0.058
Polyethylene glycol-*p*-isooctylphenyl ether (Triton X100)	non-ionic	0.18
3-Cholamidopropyldimethylammonio-1-propane sulphonate (CHAPS)	zwitterionic	5

Table V.4: Detergents assayed for their effect on glucuronyltransferase (GT) activity from a particulate Golgi membrane-enriched fraction.

CMC = critical micelle concentration.

Detergent	CMC $mg \cdot ml^{-1}$	Conc. in assay $mg \cdot ml^{-1}$	GT $Bq \cdot hr^{-1}$	Recovery %
Dodecyl maltoside	0.051	0.056	34.4	97.2
		0.1	33.8	95.5
Octyl glucoside	7.5	7.5	3.8	10.7
		10	3.4	9.6
LDAO (protein 1 $mg \cdot ml^{-1}$)	0.5	0.4	13.4	37.8
		0.8	2.3	6.6
Lubrol PX	0.058	0.05	40.2	113.5
		0.1	38.2	107.9
Triton X100	0.18	0.2	27	76.3
		5	10.2	28.8
CHAPS	5	5	1.9	5.4
		10	0.06	0.2

Table V.5: Effect of detergents on glucuronyltransferase (GT) activity from particulate Golgi membranes.

were non-ionic with the exception of the zwitterionic CHAPS. They also provided a range of CMC from 0.05 $mg \cdot ml^{-1}$ to 7.5 $mg \cdot ml^{-1}$ (Table V.4).

To determine the effects of the detergent on glucuronyltransferase activity, particulate Golgi membranes were assayed in the absence and presence of detergent [section **5. 2. 1. 1.**] (Table V.5). Among those assayed, CHAPS was the detergent showing the highest inhibitory effect on the glucuronyltransferase activity, with only 5.4% of the activity recovered at its CMC and 0.2% at a concentration twice its CMC. The recovery of glucuronyltransferase activity in the presence of octyl glucoside was very low at both concentrations assayed. At its CMC (7.5 $mg \cdot ml^{-1}$) a 10.7% recovery was achieved, and increasing the detergent concentration to 10 $mg \cdot ml^{-1}$ resulted in 9.6% of glucuronyltransferase activity recovery. LDAO assayed at 0.4 $mg \cdot ml^{-1}$, a concentration just below its CMC, showed a 62.2% inhibition of glucuronyltransferase activity, and when assayed at 0.8 $mg \cdot ml^{-1}$ only 6.6% of the activity was recovered. The effect of Triton X-100 was relatively low at its CMC with 76.3% of glucuronyltransferase activity recovered, but at a concentration 25 times higher, a 28.8% recovery was obtained. The best results were obtained with dodecyl maltoside and Lubrol PX. Both of these detergents had a very low CMC, around 0.05 $mg \cdot ml^{-1}$. At a concentration near its CMC, dodecyl maltoside had very little effect on glucuronyltransferase activity, as it showed a 97.2% recovery of glucuronyltransferase activity and a 95.5% recovery at twice the CMC. There was a 113.5% recovery of glucuronyltransferase activity with Lubrol PX at the CMC and 107.9% at twice the CMC.

5. 2. 3. 2. Effect of a range of Lubrol concentrations on particulate Golgi membranes.

The glucuronyltransferase activity was assayed in particulate Golgi membranes in the presence of different concentrations of Lubrol PX. The aim of this experiment was to investigate the effect of high levels of the detergent on the enzyme activity. This was carried out in order to determine a concentration of the detergent suitable for solubilization and at which activity would still be detectable.

The result of this experiment is shown in Fig. V.3. Particulate Golgi membranes (1 $mg \cdot ml^{-1}$ protein) were assayed for glucuronyltransferase activity in the presence of Lubrol PX at concentrations ranging from 0.5 $mg \cdot ml^{-1}$ to 10 $mg \cdot ml^{-1}$. Increasing the concentration of Lubrol PX in the assay resulted in a loss of glucuronyltransferase activity relative to the control. There was a rapid decrease in recovery of enzyme activity with an increase of Lubrol PX concentration from 0.5 $mg \cdot ml^{-1}$ to 3 $mg \cdot ml^{-1}$, after which a plateau was reached where most glucuronyltransferase activity was inhibited in the presence of the detergent.

5. 2. 3. 3. Effect of LDAO on particulate Golgi membranes.

5. 2. 3. 3. 1. Variations of LDAO concentration.

Particulate Golgi membranes brought to a final protein concentration of 2 $mg \cdot ml^{-1}$ were assayed for glucuronyltransferase activity in the presence of a range of LDAO concentrations from 0.5 to 10 $mg \cdot ml^{-1}$. As shown in Fig. V.4, the recovery of the enzyme activity relative to the control (Golgi membranes only)

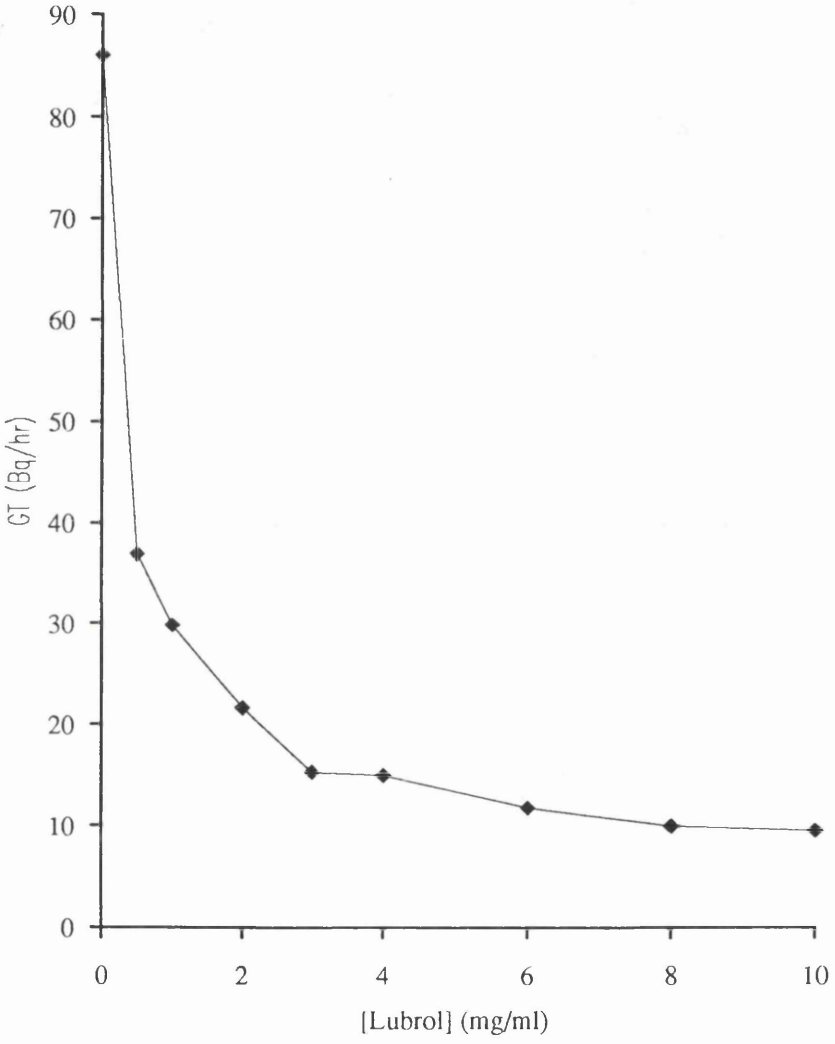


Fig. V.3: Effect of different concentrations of Lubrol PX on particulate Golgi membrane-bound glucuronyltransferase activity.

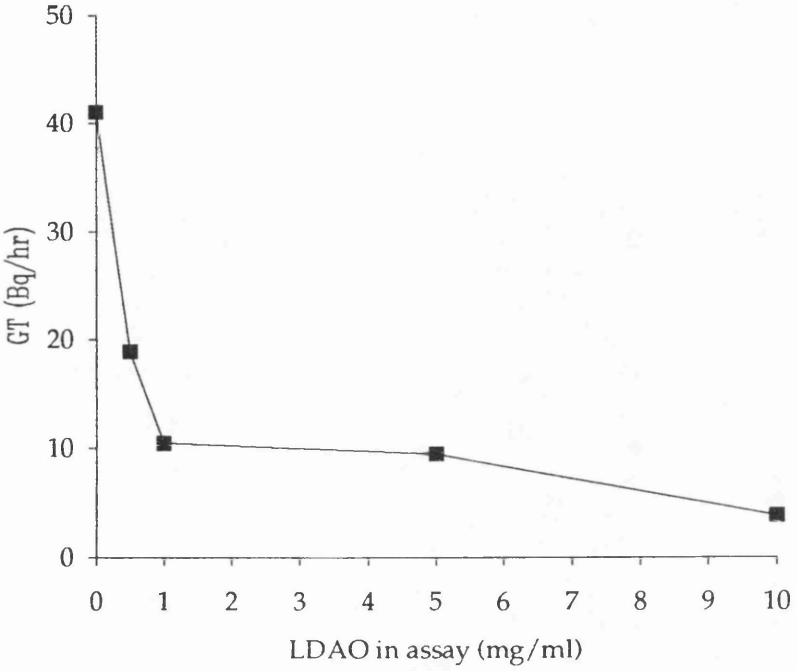


Fig. V.4: Effect of increasing concentrations of LDAO on particulate glucuronyltransferase activity.

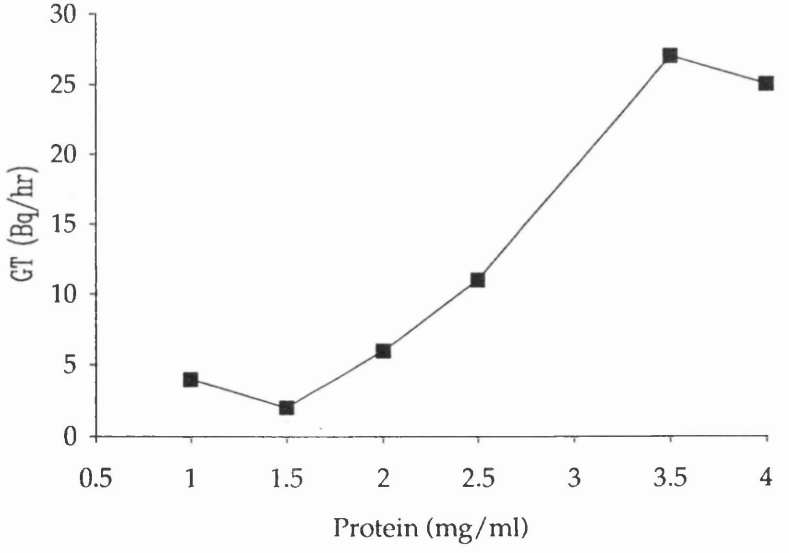


Fig. V.5: Effect of variations of the protein concentration of particulate Golgi membranes on glucuronyltransferase activity at a fixed LDAO concentration.

LDAO concentration = 1.6 mg/ml; GT activity in control = 170 Bq/hr

decreased from 23% to 5% as the concentration of the detergent was increased from 0.5 to 10 $mg \cdot ml^{-1}$ LDAO.

5. 2. 3. 3. 2. Variations of the protein concentration of the particulate Golgi membranes.

Particulate Golgi membranes, brought to a protein concentration of 1, 1.5, 2, 2.5, 3, 3.5 and 4 $mg \cdot ml^{-1}$, were assayed for glucuronyltransferase activity in the presence of 1.6 $mg \cdot ml^{-1}$ LDAO. The results are shown in fig. V.5. Changing the protein concentration in the assay from 1 $mg \cdot ml^{-1}$ to 4 $mg \cdot ml^{-1}$ resulted in the detection of more glucuronyltransferase activity. However, inhibition of the enzyme activity by the detergent was still detected at the highest protein concentration when compared to the control without LDAO.

5. 2. 3. 4. Solubilization of Golgi membranes.

Having assayed a range of different detergents for their effect on the activity of the glucuronyltransferase within the Golgi apparatus, solubilization of Golgi membranes was carried out in order to separate the enzyme activity from its membranous environment. Suitable detergents were chosen in order to achieve a maximum solubilization of membrane-bound proteins. Solubilized material was isolated from insoluble material by ultracentrifugation at 230,000 $g_{av.}$ for 1 hour in a Sorvall OTD65 ultracentrifuge with a Sorvall T865.1 fixed angle rotor.

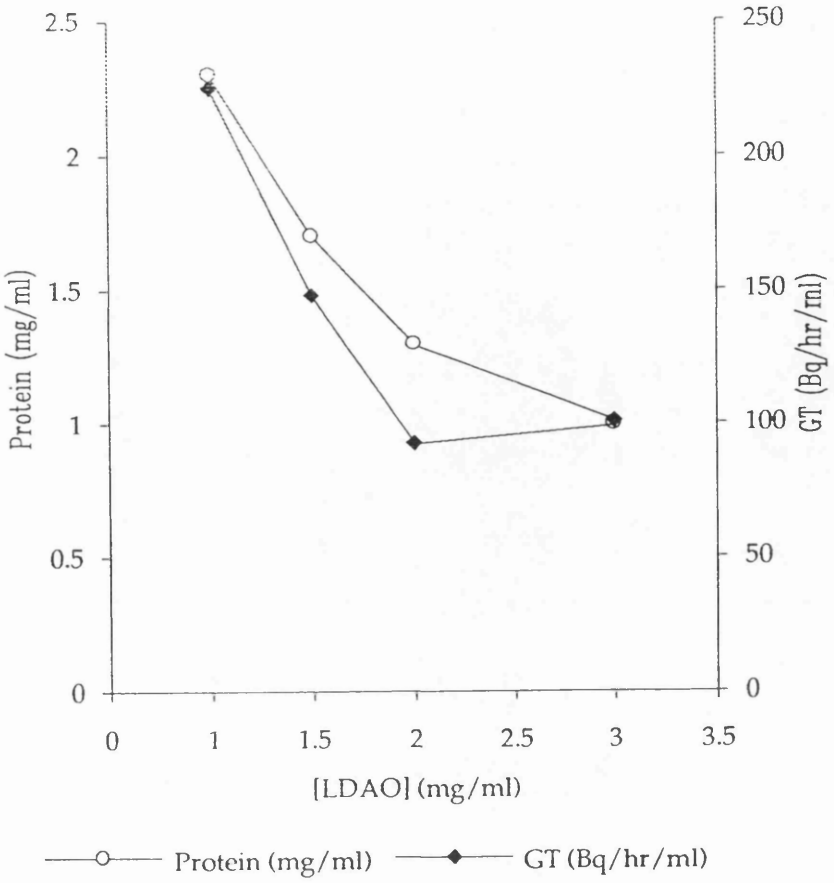


Fig. V.6: Protein concentration and glucuronyltransferase activity in LDAO non-solubilized material.

	Protein			G T	
	Concentration (mg/ml)	Recovery (%)	Solubilization (%)	Bq/hr/ml	Recovery (%)
Control (Detergent + membranes)	2.3	100	-	232	100
Non-solubilized material					
[LDAO] 1mg/ml	2.3	100	0	225	97
[LDAO] 1.5mg/ml	1.7	74	26	148	64
[LDAO] 2mg/ml	1.3	57	43	93	40
[LDAO] 3mg/ml	1.0	44	56	101	44

Table V.6: Protein concentration and recovery, glucuronyltransferase (GT) activity and recovery in the LDAO non-solubilized material.

5. 2. 3. 4. 1. LDAO solubilization.

LDAO solubilization of Golgi membranes was attempted at various concentrations of the detergent or at different ratios of detergent to protein.

First, a range of various concentrations of LDAO (from 1 to 3 $mg \cdot ml^{-1}$) was used to solubilize the glucuronyltransferase activity. No activity was detected in the 230,000 $g_{av.}$ supernatant and estimation of its protein concentration was subject to interference due to the presence of the detergent. Therefore, the percentage of proteins solubilized was estimated by determination of the amount of protein remaining within the pellet produced by the ultracentrifugation. Although the detergent was interfering with the protein assay, causing difficulties in determining the protein concentration of the solubilized material, in figure V.6 and table V.6 it is shown that increasing the concentration of LDAO from 1 $mg \cdot ml^{-1}$ to 3 $mg \cdot ml^{-1}$ for the solubilization resulted in a decrease in proteins remaining in the pellet. This meant that more material was solubilized (up to 56% at 3 $mg \cdot ml^{-1}$ of LDAO) although no glucuronyltransferase activity was detected. The reduced amount of protein found in the insoluble material was associated with a decrease in glucuronyltransferase activity to 44% of the enzyme activity remaining in the pellet when the LDAO concentration for the solubilization was 3 $mg \cdot ml^{-1}$.

Solubilization of Golgi membranes was carried out with LDAO at a concentration of 9 $mg \cdot ml^{-1}$. The protein concentration in the assay was brought to 3 $mg \cdot ml^{-1}$. No glucuronyltransferase activity was detected within the solubilized material. The 230,000 $g_{av.}$ supernatant was then dialysed for 4 hours against 1 litre of 1 mM Mes-NaOH, pH 6 containing LDAO at 0.5 $mg \cdot ml^{-1}$ with 3 changes of buffer.

		GT activity ($Bq \cdot hr^{-1} \cdot ml^{-1}$)
Control (membranes + detergent)		42
Solubilized material		-
Non-solubilized material		105
Dialysed solubilized material		64.5
Microfuged dialysed material: supernatant	(1 mM $MnCl_2$)	10
Microfuged dialysed material: supernatant	(10 mM $MnCl_2$)	16
Microfuged dialysed material: pellet	(1 mM $MnCl_2$)	82
Microfuged dialysed material: pellet	(10 mM $MnCl_2$)	44

Table V.7: Effect of 4 hours dialysis on the recovery of glucuronyltransferase activity from LDAO solubilized Golgi membranes.

Proteins (3 $mg \cdot ml^{-1}$) were solubilized with LDAO (9 $mg \cdot ml^{-1}$).

Microfuged dialysed material was incubated at different $MnCl_2$ concentrations as indicated

	$MnCl_2$ concentration in glucuronyltransferase assay	
	1mM	10mM
Insoluble dialysed material	3124 $Bq \cdot hr^{-1} \cdot ml^{-1}$	1840 $Bq \cdot hr^{-1} \cdot ml^{-1}$
Soluble dialysed material	268 $Bq \cdot hr^{-1} \cdot ml^{-1}$	132 $Bq \cdot hr^{-1} \cdot ml^{-1}$

Table V.8: Glucuronyltransferase activity in LDAO solubilized material after overnight dialysis and ultracentrifugation at 230,000 g_{av} for 1hr.

Dialysis of the solubilized material resulted in precipitation of protein and detergent out of solution. The dialysed sample was assayed for glucuronyltransferase activity. Activity was recovered in the presence of both 1 mM and 10 mM $MnCl_2$. An aliquot (1 ml) of the dialysed solution was then microcentrifuged for 30 min at 10,000 $g_{av.}$ (Table V.7). The resulting pellet and supernatant were assayed for enzyme activity in the presence of 1 mM and 10 mM $MnCl_2$. Activity was found in both pellet and supernatant, but the greatest recovery of enzyme activity was associated with the pelleted insoluble material when 1 mM $MnCl_2$ was used in the glucuronyltransferase assay.

Solubilization of the glucuronyltransferase activity was carried out as in [**5. 3. 5. 1. 2.**] except that the LDAO concentration was 10 $mg \cdot ml^{-1}$ and the protein concentration was 2 $mg \cdot ml^{-1}$. The 230,000 $g_{av.}$ supernatant was dialysed overnight against 1 litre of 1 mM Mes-NaOH, pH 6 containing 5 $mg \cdot ml^{-1}$ LDAO. As this resulted in precipitation of material, the sample was centrifuged for 1 hour at 230,000 $g_{av.}$. The supernatant was collected and the pellet resuspended in dialysing buffer. The glucuronyltransferase activity was then assayed in the presence of 1 and 10 mM $MnCl_2$. Activity was found in both pellet and supernatant (Table V.8). More activity was recovered in the pellet than in the supernatant and the recovery of activity was greater when the enzyme was assayed in the presence of 1 mM $MnCl_2$ than when assayed in the presence of 10 mM $MnCl_2$. Overnight dialysis resulted in greater enzyme activity recovery than did dialysis for 4 hours.

5. 2. 3. 4. 2. Lubrol PX solubilization.

Golgi membranes (1 $mg \cdot ml^{-1}$ protein) were solubilized in 5 $mg \cdot ml^{-1}$ Lubrol PX. The glucuronyltransferase activity was detected in the solubilized

	G T	
	Yield $Bq \cdot hr^{-1}$	Recovery %
Control (Golgi membranes, protein 1 $mg \cdot ml^{-1}$)	6016	100
Golgi membranes + Lubrol PX (5 $mg \cdot ml^{-1}$)	400	7
Solubilized material	59	1
Non-solubilized material	1616	27

Table V.9: Solubilization of glucuronyltransferase activity in 5 $mg \cdot ml^{-1}$ Lubrol PX.
GT = glucuronyltransferase activity.

	G T	
	Yield $Bq \cdot hr^{-1}$	Recovery %
Control (Golgi membranes, protein 1 $mg \cdot ml^{-1}$)	4560	100
Golgi membranes + Lubrol PX (7.5 $mg \cdot ml^{-1}$)	480	11
Solubilized material	40	1
Non-solubilized material	150	3
Dialysed solubilized material (1 mM $MnCl_2$)	800	18
Dialysed solubilized material (10 mM $MnCl_2$)	320	7

Table V.10: Solubilization of glucuronyltransferase activity in 7.5 $mg \cdot ml^{-1}$ Lubrol PX followed by dialysis of the solubilized material.
Dialysed material was assayeed for glucuronyltransferase activity at different $MnCl_2$ concentrations as indicated.

material but the recovery was low. Only 1% of the control activity was recovered (Table V.9).

In another solubilization attempt the Golgi membrane-enriched fraction, brought to a protein concentration of 1 $mg \cdot ml^{-1}$, was solubilized in 7.5 $mg \cdot ml^{-1}$ Lubrol PX. The controls used for the assay consisted of aliquots of particulate Golgi membranes (1 $mg \cdot ml^{-1}$ protein) incubated with or without 7.5 $mg \cdot ml^{-1}$ Lubrol PX. Solubilized glucuronyltransferase activity was assayed in the 230,000 $g_{av.}$ supernatant. The 230,000 $g_{av.}$ pellet was resuspended in 0.5 ml of resuspension buffer and assayed for non-solubilized activity.

As shown previously, the effect of this detergent on particulate Golgi membranes was very inhibitory on the activity of the glucuronyltransferase with a loss of almost 100% of activity (Table V.10). Very little activity was detected in the supernatant as its total activity was only 40 $Bq \cdot hr^{-1}$. However the dialysis of the solubilized material against buffer containing a lower concentration (1 $mg \cdot ml^{-1}$) of the detergent resulted in some recovery of the enzyme activity: this shows that the inhibition of the activity caused by the detergent is reversible to some extent.

5. 2. 3. 4. 3. Dodecyl maltoside solubilization.

Golgi membranes brought to a protein concentration of 1 $mg \cdot ml^{-1}$ were solubilized in dodecyl maltoside (10 $mg \cdot ml^{-1}$). The 230,000 $g_{av.}$ supernatant was assayed for solubilized glucuronyltransferase activity and the pellet, resuspended in 0.5 ml of resuspension buffer, was assayed for non-solubilized enzyme activity. Particulate Golgi membranes (1 $mg \cdot ml^{-1}$ protein) were assayed as a control for the presence of glucuronyltransferase activity. An aliquot from

	Glucuronyltransferase activity	Protein concentration
	$Bq \cdot hr^{-1}$	$mg \cdot ml^{-1}$
Golgi membranes	14778	1
Golgi membranes + detergent	1447	1
Solubilized material	63	0.43
Non-solubilized material	784	

Table V.11: Dodecyl maltoside solubilization of the glucuronyltransferase.

Proteins were solubilized in 10 $mg \cdot ml^{-1}$ dodecyl maltoside. Particulate Golgi membranes (1 $mg \cdot ml^{-1}$ protein) was assayed for glucuronyltransferase activity in the absence and presence of the detergent.

	Glucuronyltransferase activity	Protein concentration
	$Bq \cdot hr^{-1}$	$mg \cdot ml^{-1}$
Golgi membranes	18699	1
Golgi membranes + detergent	1926	1
Solubilized material	-	0.4
Non-solubilized material	742	
Dialysed solubilized material	462	
Concentrated dialysed material	100	

Table V.12: Glucuronyltransferase activity after dodecyl maltoside solubilization of Golgi membrane proteins, followed by dialysis and concentration of the solubilized material by ultrafiltration.

Dialysed material (2.5 ml, total glucuronyltransferase activity = 70 $Bq \cdot hr^{-1}$) was concentrated to 1 ml by ultrafiltration with a Centrisart 13229E ultrafiltration unit (5 kDa nominal molecular weight cut off).

the mixture of Golgi membranes and detergent was taken prior to the ultracentrifugation and also assayed for enzyme activity.

The enzyme activity detectable in the solubilized material was very low, as only 0.4% of the activity found in the control (particulate Golgi membranes, no detergent) was recovered, although about 43% of protein was solubilized (table V.11)

The solubilization process was repeated with another Golgi membrane preparation and, in order to attempt the recovery of more enzyme activity, the solubilized fraction, in which no glucuronyltransferase activity was detected, was dialysed for 2 hours against 20 mM Mes-NaOH, pH 6. The dialysed sample was assayed for glucuronyltransferase activity in the presence of 1 mM $MnCl_2$. Due to a high background of radioactivity (non-specific binding of unincorporated radioactive material to the cellulose powder) in the control incubations, no activity was at first detectable. The dialysed sample was assayed again for the transferase activity using, for this incubation, 100 µl of sample incubated with 100 µl of incubation medium (1 mM $MnCl_2$) instead of the usual 50 µl each. Activity was then detected in the dialysed sample (Table V.12).

In order to obtain a more concentrated sample that could be used for anion exchange chromatography, the dialysed material was submitted to ultrafiltration. The filters used for this purpose had a 5 kDa nominal molecular weight cut off. The ultrafiltration was carried out by centrifuging the Centrisart 13229E ultrafiltration unit (Sartorius, BDH, Poole, Dorset), containing 2.5 ml of sample, for 50 min at 2,500 $g_{av.}$ with a Sigma 11133 swing out rotor in a cooled Sigma 3K12 bench centrifuge. The dialysed material was concentrated to 1 ml and assayed for glucuronyltransferase activity. The total glucuronyltransferase

activity found in the concentrated material (100 $Bq \cdot hr^{-1}$) was higher than in the initial 2.5 ml of sample before concentration (70 $Bq \cdot hr^{-1}$) (Table V.12).

5. 3. DISCUSSION.

Waldron *et al.* (1989) showed that the glucuronyltransferase involved in the synthesis of glucuronoxylans in peas could be solubilized in a non-ionic detergent, Triton X-100. In order to solubilize efficiently the enzyme activity, a high concentration of detergent was required. The main evidence for the nature of the enzyme and of the product formed was the maintenance of the stimulatory effect of UDP-xylose on the glucuronyltransferase activity, which had previously been described (Waldron & Brett, 1983). It was also demonstrated that the solubilized form of the enzyme was much more stable than the particulate form, and that it could be stored for long periods at -20°C without a significant loss of activity. The instability of the particulate enzyme at -20°C may be due to the disruption of the membranes upon freezing and de-freezing which does not occur for the solubilized material. A decrease in the optimum Mn^{2+} concentration required for the enzymatic activity was observed upon solubilization. It was suggested that this might result from a slight disorganisation of the Golgi apparatus that permits, under the action of the detergent, an alteration and reduction of the effect of the membrane environment on the access of the bivalent cation to the enzyme, thus lowering the concentration at which it is the most effective.

The two step solubilization of the crude membrane preparation resulted in a differential separation of the membrane-bound proteins. Although some proteins were solubilized at the first step in 1% Triton X-100, very little glucuronyltransferase activity could be detected in this fraction. When the 1%

Triton X-100 insoluble material was submitted to solubilization in 5% NP40, a substantial 14-fold purification of the glucuronyltransferase activity was achieved over the starting homogenate. Using a two step solubilization procedure, Hanley and Chappell (1992) carried out the solubilization and partial purification of a squalene synthase from microsomes isolated from suspension-cultured tobacco cells. The microsomes were initially treated with a mixture of octyl thioglucoside and glycodeoxycholate, at concentrations below their CMC, to remove proteins loosely associated with the membranes. Subsequently, complete solubilization of the squalene synthase activity was achieved after a second treatment at detergent concentrations above their CMC.

The effects of several detergents on Golgi membrane-bound glucuronyltransferase activity were assayed prior to solubilization. This was carried out in order to determine which detergent would be the most effective in solubilizing lipids and proteins, and the least detrimental to enzyme activity. Typically, non-denaturing detergents are used for the solubilization of intrinsic membrane proteins when the preservation of their native structure and biological activity is necessary, and the choice of detergent and its optimal concentration must be determined empirically for each individual membrane protein (Helenius *et al.*, 1979). All the detergents selected were non-ionic except the zwitterionic CHAPS, and provided a range of CMC from 0.05 to 7.5 $mg \cdot ml^{-1}$. They were assayed at concentrations near and above their CMC.

Two of the detergents selected, octyl glucoside and dodecyl maltoside, belong to the alkyl glycoside group. When tested, they led to opposite effects on the activity of the membrane-bound glucuronyltransferase. Octyl glucoside was a strong inhibitor whereas, in the presence of dodecyl maltoside, almost 100% of the enzyme activity was recovered. The dissimilar effects of these two detergents could result from the difference in the sugar residue carried at their

hydrophilic pole, namely glucose and maltose. The inhibitory effect of octyl glucoside might be caused by competition, with the substrate, of the glucose moiety of the detergent that probably interferes with or occupies the catalytic site of the glucuronyltransferase, and which then prevents the glucuronic acid from UDP-glucuronic acid from obtaining access to the enzymatic site. The hydrophilic pole of the other alkyl glycoside detergent is a maltose residue, a disaccharide formed of two glucose residues linked by a α-(1→4) bond. This detergent had almost no inhibitory effect on the glucuronyltransferase activity, and this may be due to the presence of a disaccharide residue that is too big to fit in the active site of the enzyme and compete with its substrate.

Lubrol PX was the only detergent to show stimulation of the enzyme activity. This stimulation may have resulted from a disruption of the membranes. The detergent would act in the same way as Triton X-100 stimulates the activity of the latent IDP'ase in the Golgi apparatus membranes (Green, 1983) and/or when Mn^{2+} optimum concentration for glucuronyltransferase activity is lowered upon solubilization (Waldron & Brett, 1983). A similar effect was observed during the solubilization of a UDP-glucose-ceramide glucosyltransferase from the Golgi membranes of porcine submaxillary ganglia (Durieux *et al.*, 1990). Isolated Golgi vesicles were subjected to the effect of various detergents at concentrations near and above their CMC. Only the zwitterionic detergent CHAPS showed some activation of the enzyme and was later used alone and in combination with another detergent, Zwittergent 3-14, to solubilize the enzyme.

It was interesting to note that the inhibitory effect of the detergents tested on particulate Golgi membrane-bound glucuronyltransferase increased as the value of their CMC increased. With all the detergents tested, a relative decrease in glucuronyltransferase activity was observed when assayed at a detergent

concentration greater than the CMC. Additionally, the recovery of particulate enzyme activity decreased with increasing levels of Lubrol PX and LDAO in solution. Depending on the properties of each individual detergent, the effects observed on enzyme activity could result from an inhibition of the activity, or could reflect a variation in the rate of catalysis as well as possible inactivation of the enzyme, as the membrane structure is altered by increasing amounts of membrane-bound detergent.

Solubilization of the Golgi membrane-enriched fraction led to various results, depending on which detergent was used. Generally a high concentration of detergent was necessary in order to obtain efficient solubilization of the proteins from their membranous environment. LDAO seemed to be very efficient in solubilizing the intrinsic membrane proteins but was later abandoned because of its high inhibitory effect on the enzyme activity. This was probably caused by its effect on the pH during the glucuronyltransferase incubation and by its insolubility in aqueous media at relatively weak acidic pH values (Hjelmeland, 1990). This resulted in its precipitation out of solution upon dialysis against pH 6 buffer. Moreover, the interferences of LDAO with the micro-Lowry protein assay made the estimation of the protein content of LDAO solubilized material difficult. Solubilization of proteins with Lubrol PX was efficient at high concentrations of the detergent but resulted in inhibition of glucuronyltransferase activty. This inhibition of enzyme activity could be reversed to a small extent by dialysis against buffer containing a lower concentration of the detergent. A similar result was obtained during the solubilization of the Golgi membranes with dodecyl maltoside. It is possible that the substitution of the lipids by the detergents affected the structure and conformation of the solubilized proteins, in an reversible way, by a slight unfolding of the polypeptides (Neugebauer, 1988).

In this chapter it was shown that the activity of particulate Golgi membrane-bound glucuronyltransferase is affected by the presence of detergents. The enzyme can be solubilized from its membranous environment with various detergents with relative success in the recovery of its activity.

CHAPTER 6. CHROMATOGRAPHY.

6. 1. INTRODUCTION.

After solubilization, the next step in the purification of a membrane-bound enzyme involves the use of chromatography. Affinity chromatography of the solubilized material was tried because of its high potential resolution. Agarose-linked UDP-glucuronic acid was used since the specificity of this matrix for the enzyme was previously exploited for the purification of glucuronyltransferases in animal systems (Singh *et al.*, 1981). Anion exchange chromatography of the solubilized material was also used to attempt the purification of the glucuronyltransferase.

6. 2. METHODS.

6. 2. 1. Affinity chromatography.

A gel of uridine 5'-diphospho glucuronic acid crossed-linked to agarose (Sigma) was used to prepare an affinity chromatography column (1 ml) in a Pasteur pipette. This column was equilibrated with several ml of buffer containing NP40 (5%), Tris - Mes (10 mM, pH 6). Proteins were solubilized from a whole membrane preparation [section **2. 3. 1** and **4. 3. 1**] in 5% NP40. Solubilized proteins (1 ml) were loaded onto the column and eluted with equilibration buffer (2 ml). Elution was continued with the same buffer (3 ml) containing UDP-xylose (1.25 mM) and UDP-glucuronic acid (2.5 mM). A third elution was carried out with the equilibration buffer (3 ml) containing NaCl (1 M). Eluted fractions (1 ml) were collected and assayed for glucuronyltransferase activity. Aliquots of the different fractions were

separated by SDS-PAGE on a gradient of acrylamide concentration as described in **2. 6. 1** . The gels were subsequently silver-stained according to Morrissey (1981) [see section **2. 7. 1** .].

6. 2. 2. Anion exchange chromatography.

6. 2. 2. 1. DEAE-Sephadex G-25 anion exchange chromatography.

Golgi membranes solubilized in LDAO (5 $mg \cdot ml^{-1}$) at a protein concentration of 2 $mg \cdot ml^{-1}$ were subjected to anion exchange using a DEAE Sephadex G25 anion exchanger. The column was equilibrated with the elution solution (1 mM Tris-HCl, pH 6; 5 $mg \cdot ml^{-1}$ LDAO). The sample (2 ml) was eluted first with the elution solution (20 ml) then with the same solution containing NaCl at respective concentrations of 0.1 M (20 ml), 0.4 M (10 ml), 0.8 M (10 ml) and 1 M (10 ml) (Fig. VI.2). The protein elution profile was obtained by measuring the O.D. of each fraction (1 ml) by UV spectrophotometry at 280 nm.

6. 2. 2. 2. Dowex 1x2 (200) anion exchange chromatography.

Anion exchange chromatography of LDAO solubilized Golgi membranes was carried out in the same conditions as for the DEAE Sephadex chromatography except that the elution buffer was at pH 7.6. The sample (2 ml) was first eluted with elution buffer (20 ml) (Fig. VI.3), then with the same buffer containing NaCl (0.4 M, 25 ml). The elution was continued with buffer conaining NaCl (1 M, 20 ml). Fractions (1 ml) were collected and the presence of protein in the fractions was checked by UV spectrophotometry at 280 nm.

6. 2. 2. 3. Anion exchange chromatography with the FPLC.

Anion exchange chromatography was performed with a Mono Q anion exchange gel on the Pharmacia FPLC (Fast Protein Liquid Chromatography) system, and Golgi membrane proteins solubilized with LDAO, Lubrol PX and dodecyl maltoside.

The anion exchange resin was equilibrated with the elution buffer before loading Golgi membrane proteins solubilized with the appropriate detergent. After loading of the sample onto the column, the elution of material was carried out with buffer until no proteins could be detected eluting off the column. The elution was continued with a gradient of sodium chloride from 0 to 1 M. Protein elution was monitored at 280 nm with a UV monitor and eluting peaks of protein were recorded, as well as the NaCl gradient shape, with a chart recorder.

6. 2. 2. 3. 1. Anion exchange of LDAO solubilized material.

A Mono Q anion exchange column was first washed and equilibrated with the elution buffer (10 mM Mes-NaOH (pH 6), 1 $mg \cdot ml^{-1}$ LDAO). Pea Golgi membranes solubilized in 10 $mg \cdot ml^{-1}$ LDAO were diluted with buffer to bring the detergent concentration to 2 $mg \cdot ml^{-1}$ and the sample (3 ml) was submitted to anion exchange. The elution of material off the column was carried out at a constant flow rate of 2.5 $ml \cdot min^{-1}$ and the protein elution profile was monitored at 280 nm and recorded on a chart. Protein peaks were collected manually and assayed for glucuronyltransferase activity.

6. 2. 2. 3. 2. Anion exchange of Lubrol PX solubilized material.

A Mono Q anion exchange column was first washed and equilibrated with the elution buffer (10 mM Mes-NaOH (pH 6), 1 $mg \cdot ml^{-1}$ Lubrol PX). Plant material solubilized in Lubrol PX (5 $mg \cdot ml^{-1}$) was diluted with 10 mM Mes-NaOH (pH 6) buffer to bring the Lubrol PX concentration to 1 $mg \cdot ml^{-1}$. The sample (3 ml) was loaded onto the column. The elution flow rate was set at 5 $ml \cdot min^{-1}$. The first elution was carried out with elution buffer for 10 min, followed by a linear gradient of buffer containing sodium chloride from 0 to 1 M NaCl. Peaks of protein eluting from the column, detected by monitoring at 280 nm, were collected and assayed for glucuronyltransferase activity.

6. 2. 2. 3. 3. Anion exchange of dodecyl maltoside solubilized material.

A Mono Q anion exchange column was equilibrated with elution buffer containing 20 mM Mes-NaOH, pH 6 in 1 $mg \cdot ml^{-1}$ dodecyl maltoside. Golgi membranes were prepared as described previously and were solubilized in 10 $mg \cdot ml^{-1}$ dodecyl maltoside (in 20 mM Mes-NaOH, pH 6) at a protein concentration of 1 $mg \cdot ml^{-1}$. The solubilized material was dialysed for 2 hours against 2 litres of 20 mM Mes-NaOH, pH 6. The dialysed material was then concentrated to 50% of its initial volume by ultrafiltration [see section **5. 3. 5. 3.**] and its glucuronyltransferase activity assayed prior to the chromatography.

The concentrated solubilized material (6 ml) was loaded onto the anion exchange column and eluted with the equilibration buffer before a NaCl linear gradient was applied. The elution of material off the column was carried out at a constant flow rate of 3 $ml \cdot min^{-1}$. Peaks of protein eluting off the column were detected by UV monitoring, and fractions of variable volume, depending on

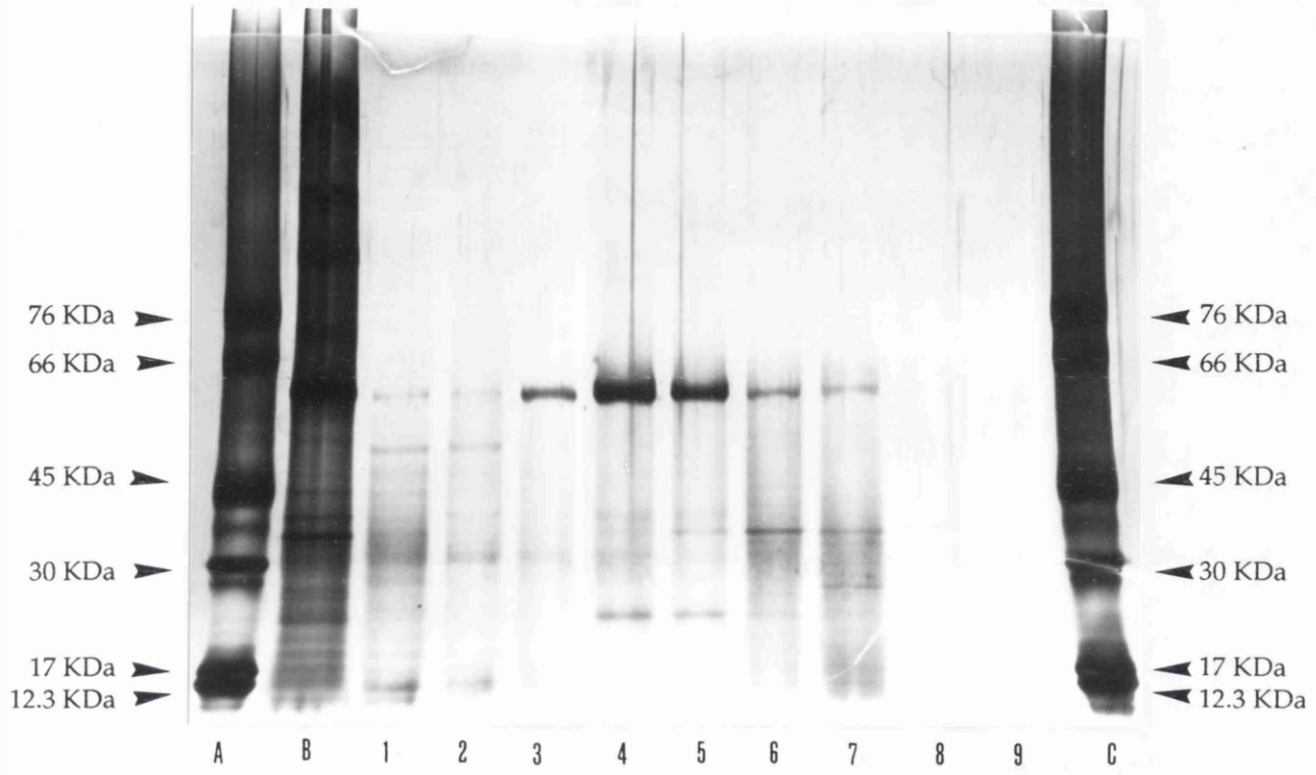


Fig. VI.1a: SDS polyacrylamide gradient gel electrophoresis of fractionscollected during the affinity chromatography.
Lanes 1 to 3: elution with 5% NP 40 buffer,
Lanes 4 to 6: elution with 5% NP 40, UDP-xyl (1.25 mM) and UDP-glcA (2.5 mM) buffer.
Lanes 7 to 9: elution with NaCl (1 M) buffer.
Lanes a and c: molecular weight markers,
Lane b: 5% NP 40 solubilized membranes.

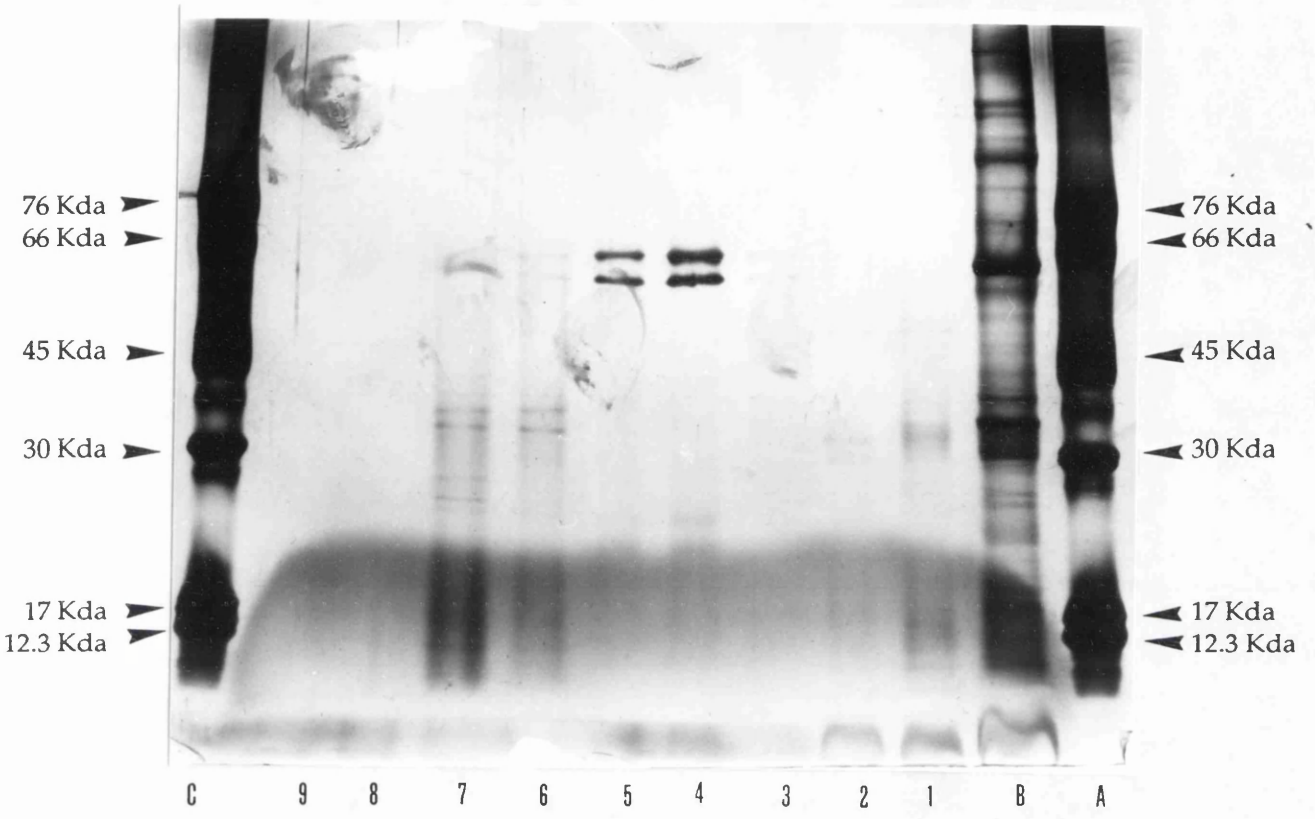


Fig. VI.1b: SDS polyacrylamide gradient gel electrophoresis of fractionscollected during the affinity chromatography.
Lanes 1 to 3: elution with 5% NP 40 buffer,
Lanes 4 to 6: elution with 5% NP 40, UDP-xyl (1.25 mM) and UDP-glcA (2.5 mM) buffer.
Lanes 7 to 9: elution with NaCl (1 M) buffer.
Lanes a and c: molecular weight markers,
Lane b: 5% NP 40 solubilized membranes.

the peak shape, were collected manually. Those composing peaks of proteins were pooled and concentrated before assaying for the glucuronyltransferase activity.

Aliquots of the fractions (1 ml) were subjected to acetone precipitation and the resulting pelleted material was resuspended in suitable buffer to carry out SDS polyacrylamide (12% acrylamide) gel electrophoresis (as described in section **2. 6. 2.**] The gels were subsequently silver-stained according to Oakley *et al.* (1980) [section **4. 3. 1**].

6. 3. RESULTS.

6. 3. 1. Affinity chromatography of NP40 solubilized material.

Affinity chromatography of NP40 solubilized membranes on a column packed with a gel of agarose-linked UDP-glucuronic acid did not permit the recovery of the glucuronyltransferase activity. The enzyme activity was estimated by measuring the incorporation of radioactive glucuronic acid into newly synthesized glucuronoxylan. The total incorporation of glucuronic acid was estimated by using the ratio of cold to radioactive UDP-glucuronic acid present in the assay. The SDS (7.5 to 15% polyacrylamide) gel electrophoresis of aliquots of the fractions showed the appearance of a large protein band when the column was eluted with elution buffer containing UDP-xylose and UDP-glucuronic acid (Fig. VI.1.a). Proteins were also eluted with the NaCl buffer but no glucuronyltransferase activity was recovered from the different fractions. Electrophoresis of the same samples in a shallower gradient of polyacrylamide (9~15%) resulted in the dissociation of a major protein band into two subunits (Fig. VI.1.b).

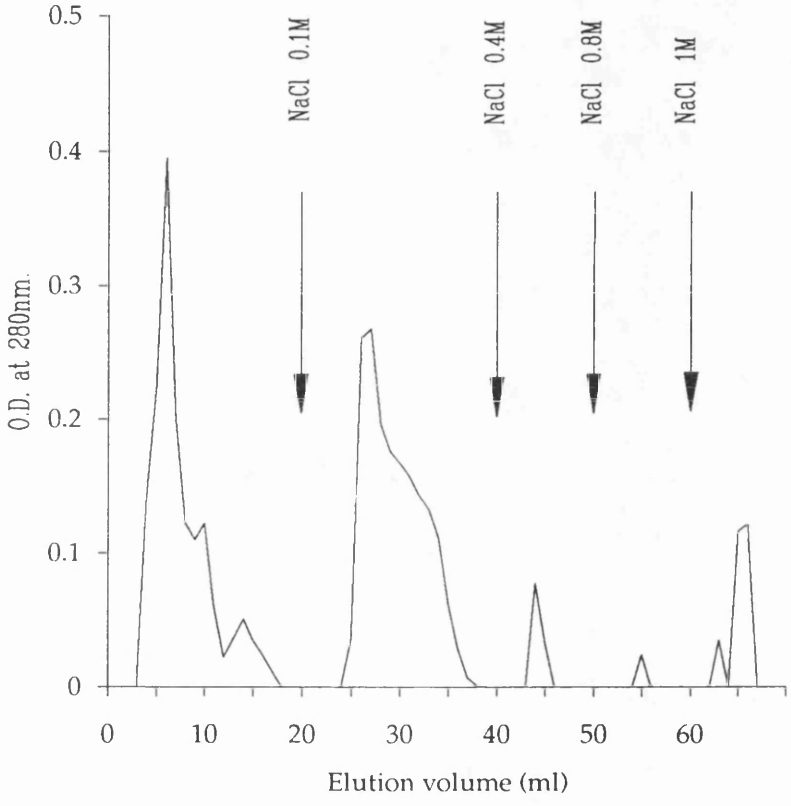


Fig. VI.2: DEAE Sephadex G25 anion exchange of LDAO solubilized Golgi membranes. Protein elution profile and elution buffer changes.

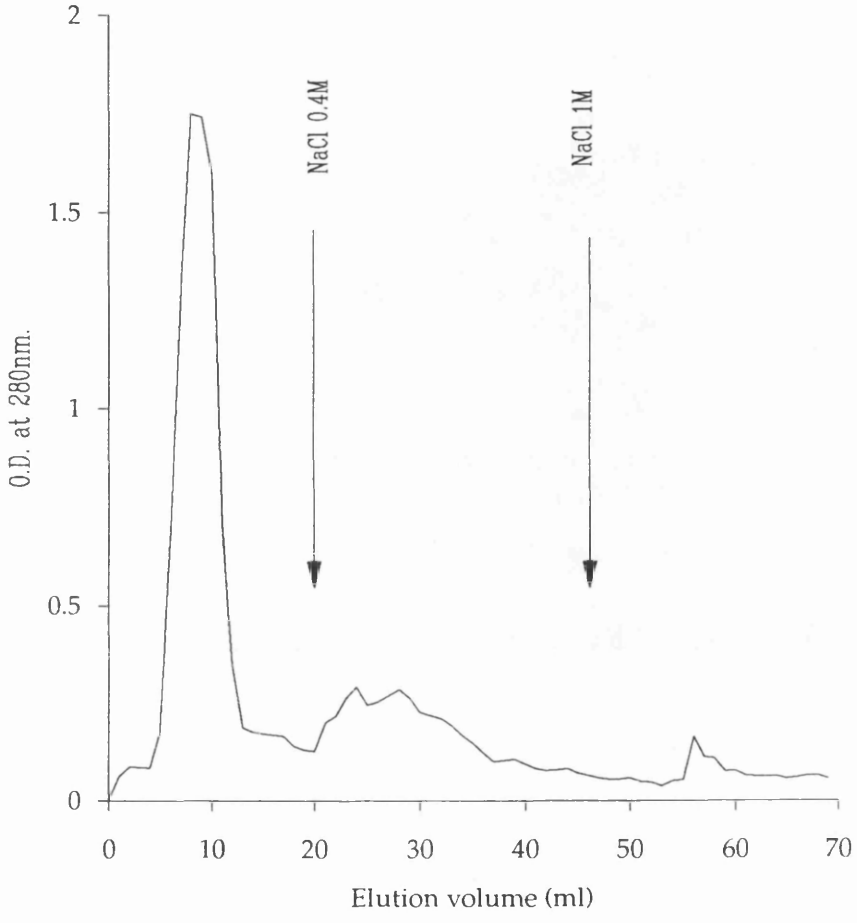


Fig. VI.3: Dowex 1x2 (200) anion exchange of LDAO solubilized Golgi membranes. Protein elution profile and elution buffer changes.

6. 3. 2. Anion exchange chromatography.

Anion exchange chromatography was performed in order to attempt the purification of glucuronyltransferase from the Golgi membrane solubilized proteins since affinity chromatography did not lead to any positive results.

6. 3. 2. 1. DEAE Sephadex G25 anion exchange.

As shown in Fig. VI.2, a first peak of protein, not binding to the anion exchanger, was eluted off the column with buffer containing no NaCl. Each subsequent increase in the ionic strength, obtained by changing the NaCl concentration in the elution buffer, was followed by the elution of proteins. No enzyme activity was recovered from the different protein peaks assayed.

6. 3. 2. 2. Dowex 1x2 (200) anion exchange.

The protein elution profile of this chromatography is shown on Fig. VI.3. A major peak of proteins, not binding to the anion exchange resin, was detected within the first 12 ml of elution with buffer containing no NaCl. After 20 ml of elution with buffer containing no NaCl, the elution was followed with buffer containing 0.4 M NaCl. This change of buffer resulted in the elution of two smaller peaks of proteins. When the elution volume was 45 ml, the buffer was changed to one which contained 1 M NaCl. The increase the ionic strength of the buffer was followed by the elution of three smaller peaks of material off the column. However, no enzyme activity was recovered from any of the fractions collected.

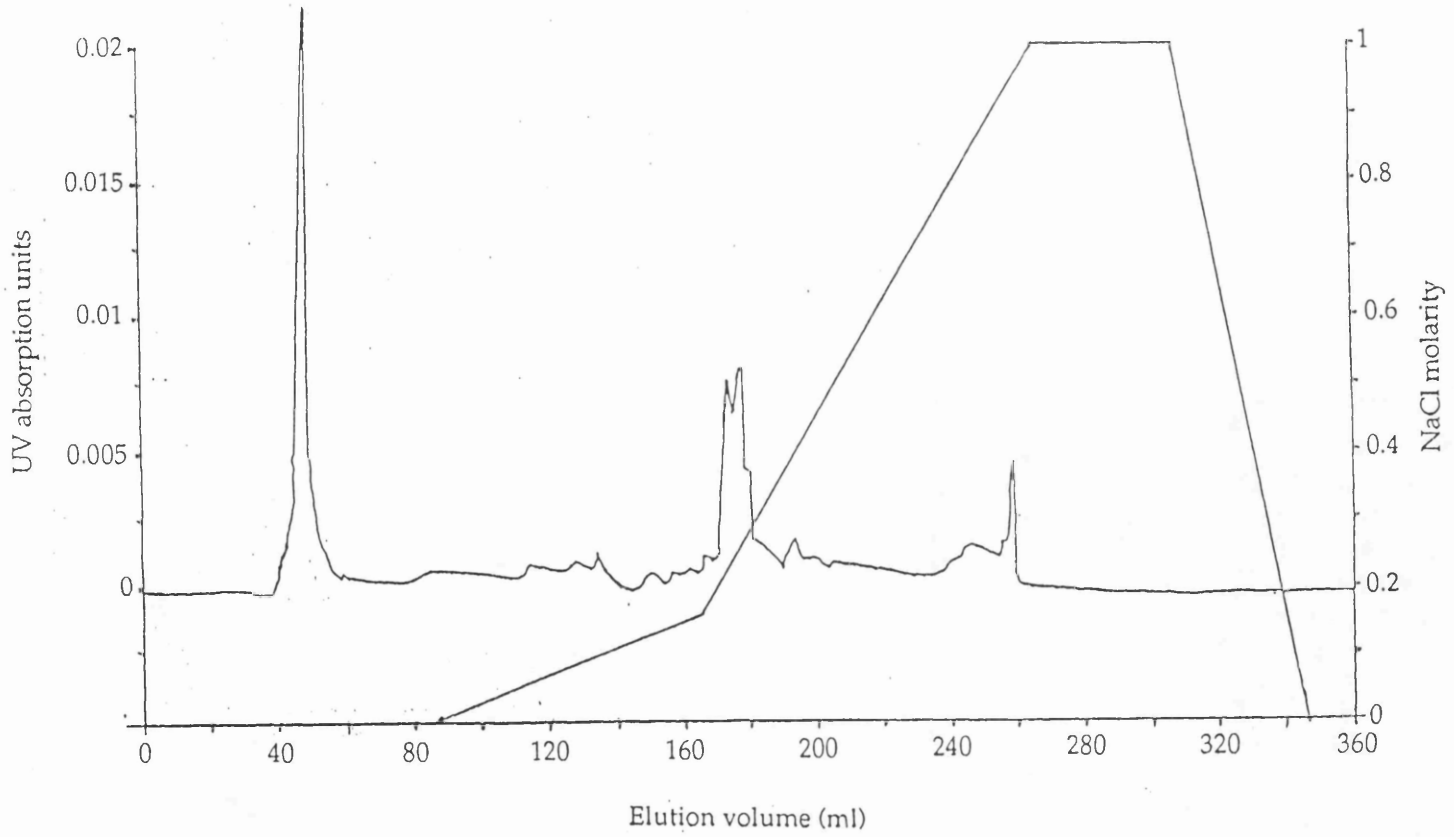


Fig. VI.4: Mono Q anion exchange on the FPLC of LDAO solubilized Golgi membranes. Protein elution and NaCl gradient profiles.

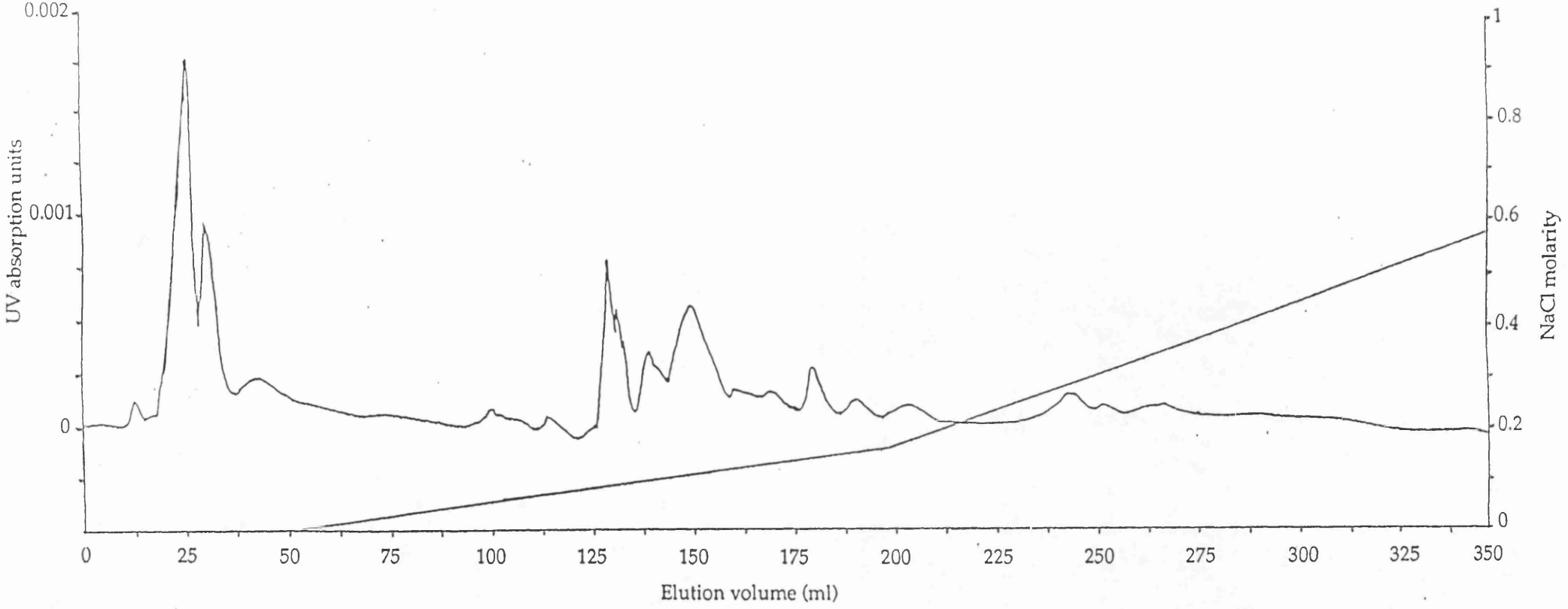


Fig. VI.5: Mono Q anion exchange on the FPLC of Lubrol PX solubilized Golgi membranes. Protein elution and NaCl gradient profiles.

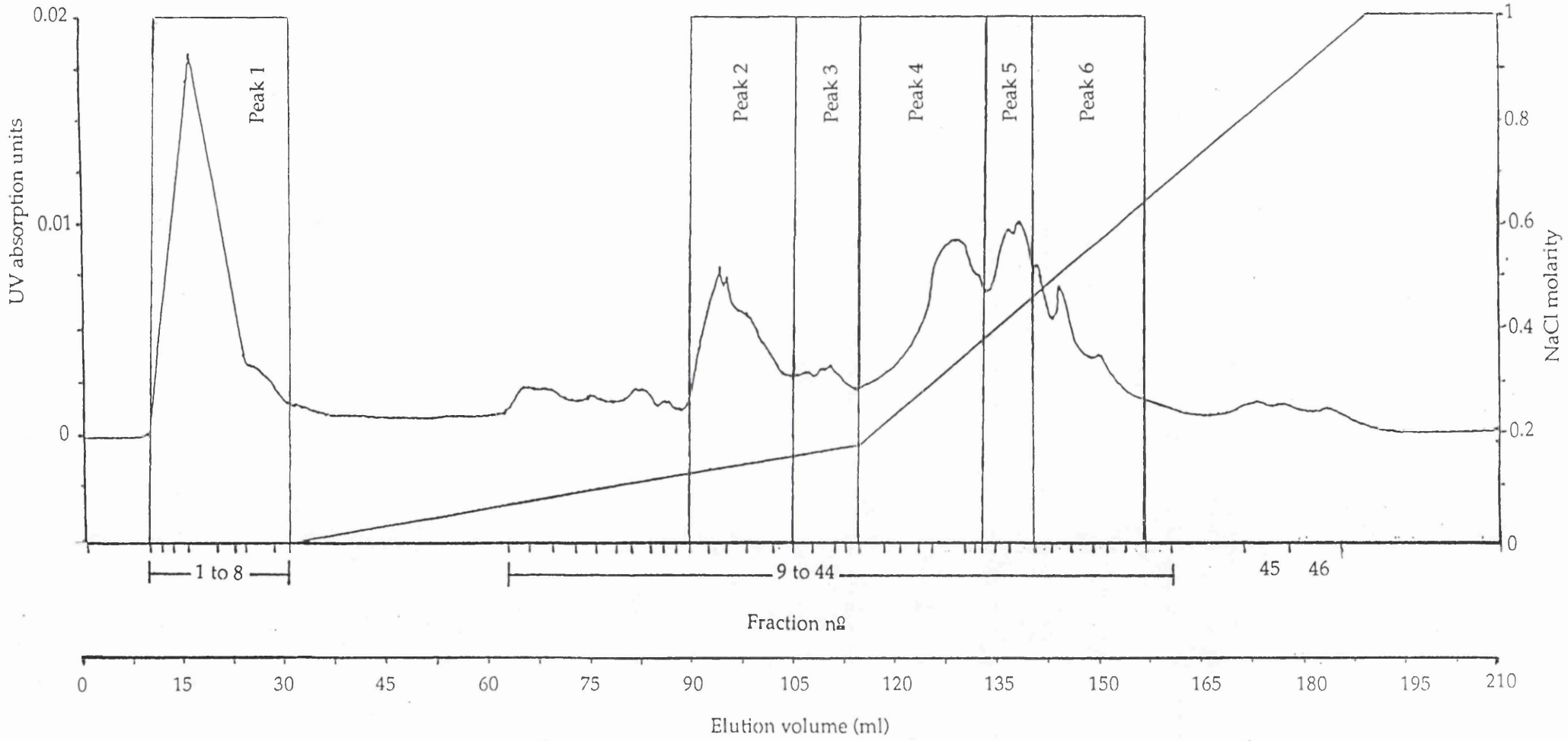


Fig. VI.6: Mono Q anion exchange on the FPLC of dodecyl maltoside solubilized Golgi membranes. Protein elution and NaCl gradient profiles.

Fractions of variable volume, depending on peak geometry, were collected for analysis as shown.

6. 3. 2. 3. Anion exchange with the FPLC.

6. 3. 2. 3. 1. Anion exchange of LDAO solubilized material.

Material solubilized in LDAO was fractionated by anion exchange chromatography on the FPLC Mono Q exchanger as shown by the protein elution profile recorded in Fig. VI.4. Elution of material was first carried out with buffer containing no NaCl and resulted in the elution of one major peak of protein. Further elution with a gradient of NaCl concentration resulted in the elution of several protein peaks as the concentration of NaCl in the elution buffer increased. No glucuronyltransferase activity was recovered from the fractions and peaks of protein collected.

6. 3. 2. 3. 2. Anion exchange of Lubrol PX solubilized material.

Elution profile of plant material is shown in Fig. VI.5. A fraction of the solubilized material did not bind to the column and proteins were eluted with the first elution buffer, which did not contain NaCl. These proteins eluted in three peaks, the first two following each other closely and the third one eluting a little later. Three protein peaks eluted when the gradient was between 0.07 to 0.11 M NaCl. No enzyme activity was recovered in the different fractions corresponding to the peaks of proteins.

6. 3. 2. 3. 3. Anion exchange of dodecyl maltoside solubilized material.

The pattern of peaks of proteins eluting off the column was very similar to the results obtained when proteins present in Golgi membranes were

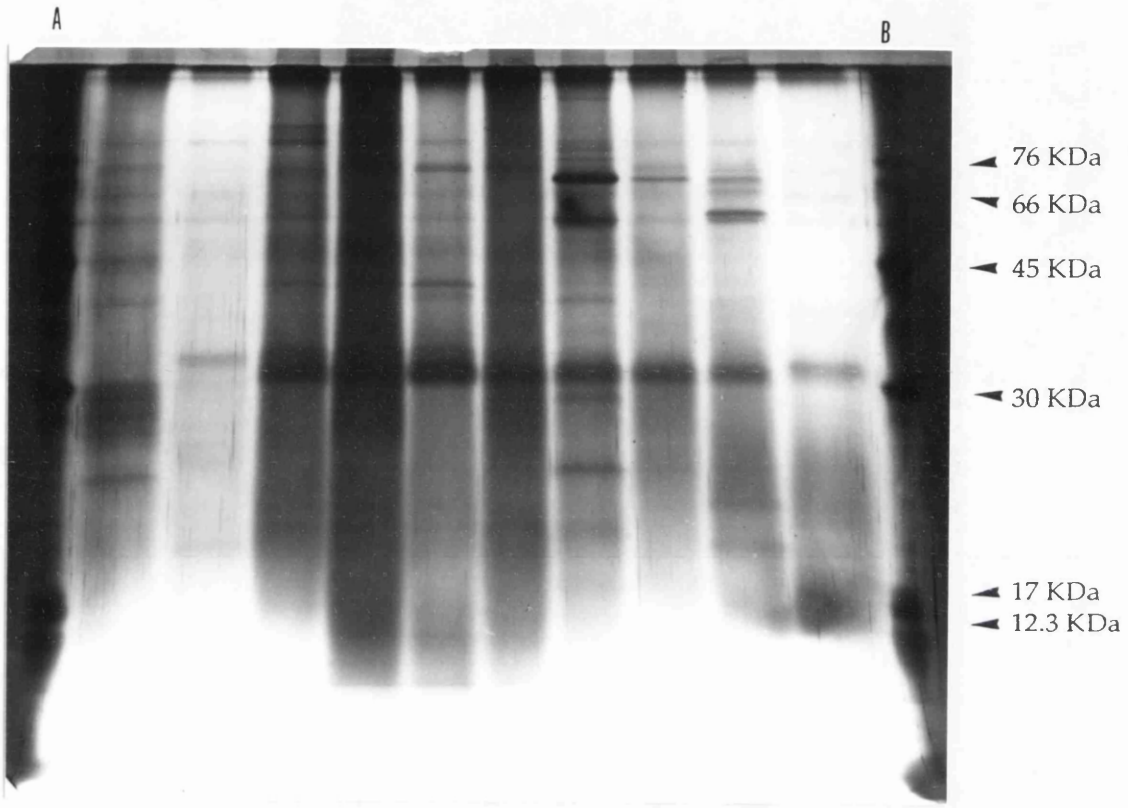


Fig. VI. 7a: SDS polyacrylamide electrophoresis gel of protein fractions collected during the FPLC anion exchange of dodecyl maltoside solubilized material [see fig. VI.6].

Proteins were acetone precipitated and resuspended in electrophoresis sample buffer as described in section 6. 2. 2. 3. 3. prior to the electrophoresis.

From left to right, fractions nº 1 to 10. Lanes a and b: molecular weight markers.

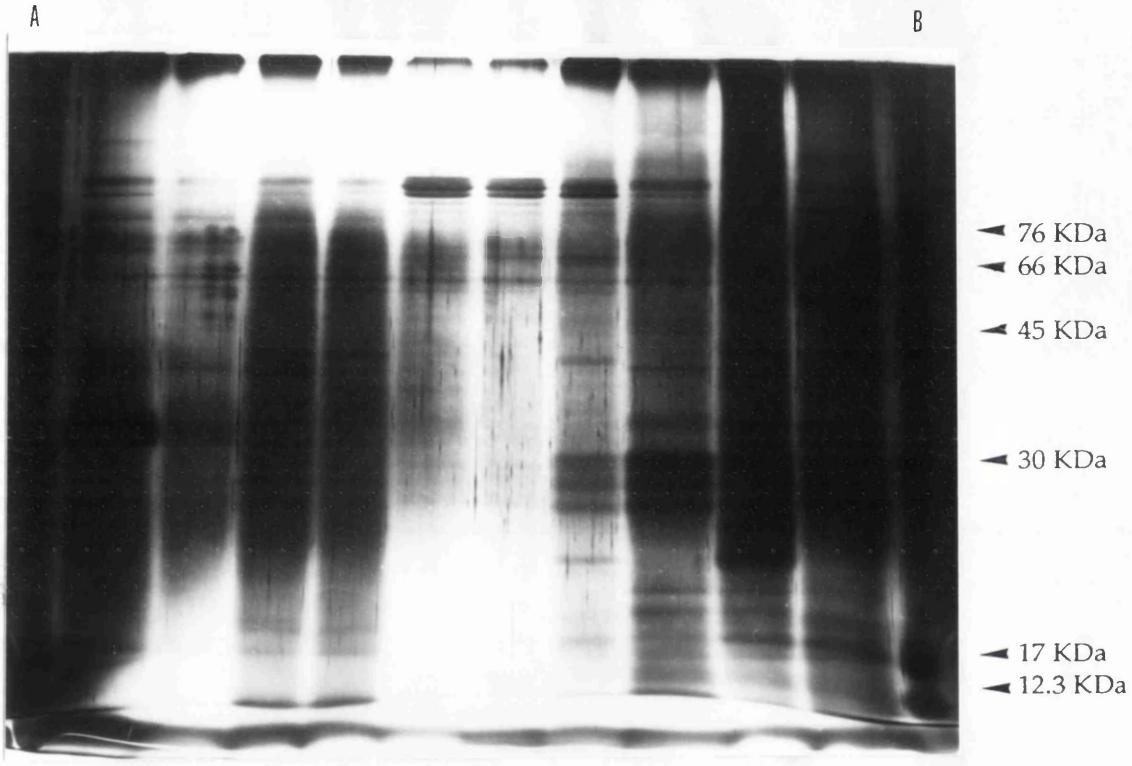


Fig. VI. 7b: SDS polyacrylamide electrophoresis gel of protein fractions collected during the FPLC anion exchange of dodecyl maltoside solubilized material [see fig. VI.6].

Proteins were acetone precipitated and resuspended in electrophoresis sample buffer as described in section **6. 2.** 2. 3. 3. prior to the electrophoresis.

From left to right, fractions nº 11 to 20. Lanes a and b: molecular weight markers.

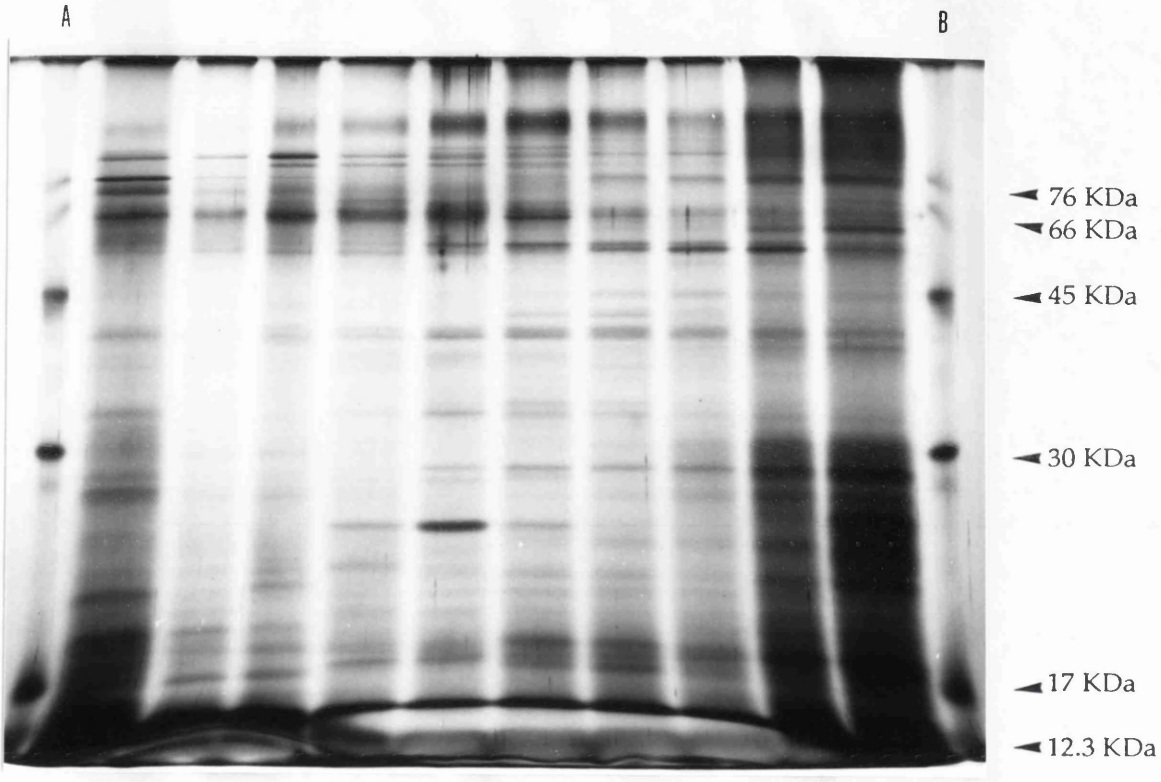


Fig. VI. 7c: SDS polyacrylamide electrophoresis gel of protein fractions collected during the FPLC anion exchange of dodecyl maltoside solubilized material [see fig. VI.6].

Proteins were acetone precipitated and resuspended in electrophoresis sample buffer as described in section **6. 2.** 2. 3. 3. prior to the electrophoresis.

From left to right, fractions nº 21 to 30. Lanes a and b: molecular weight markers.

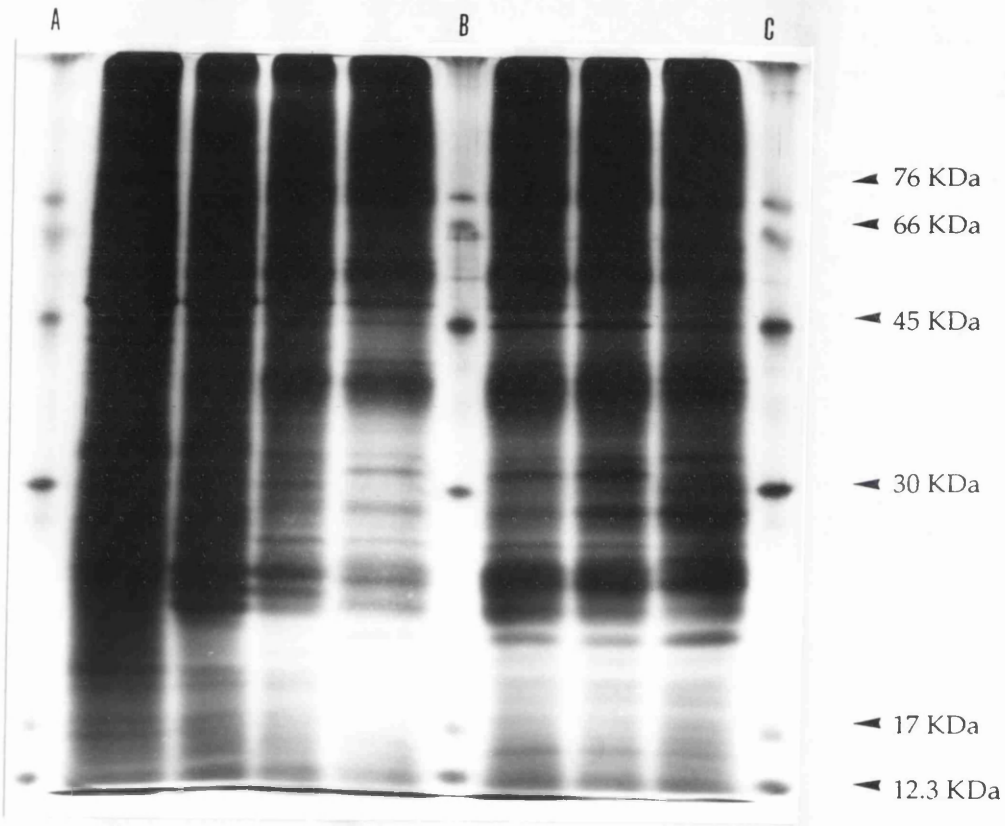


Fig. VI. 7d: SDS polyacrylamide electrophoresis gel of protein fractions collected during the FPLC anion exchange of dodecyl maltoside solubilized material [see fig. VI.6].

Proteins were acetone precipitated and resuspended in electrophoresis sample buffer as described in section **6. 2.** 2. 3. 3. prior to the electrophoresis.

From left to right, fractions nº 31 to 37. Lanes a, b and c: molecular weight markers.

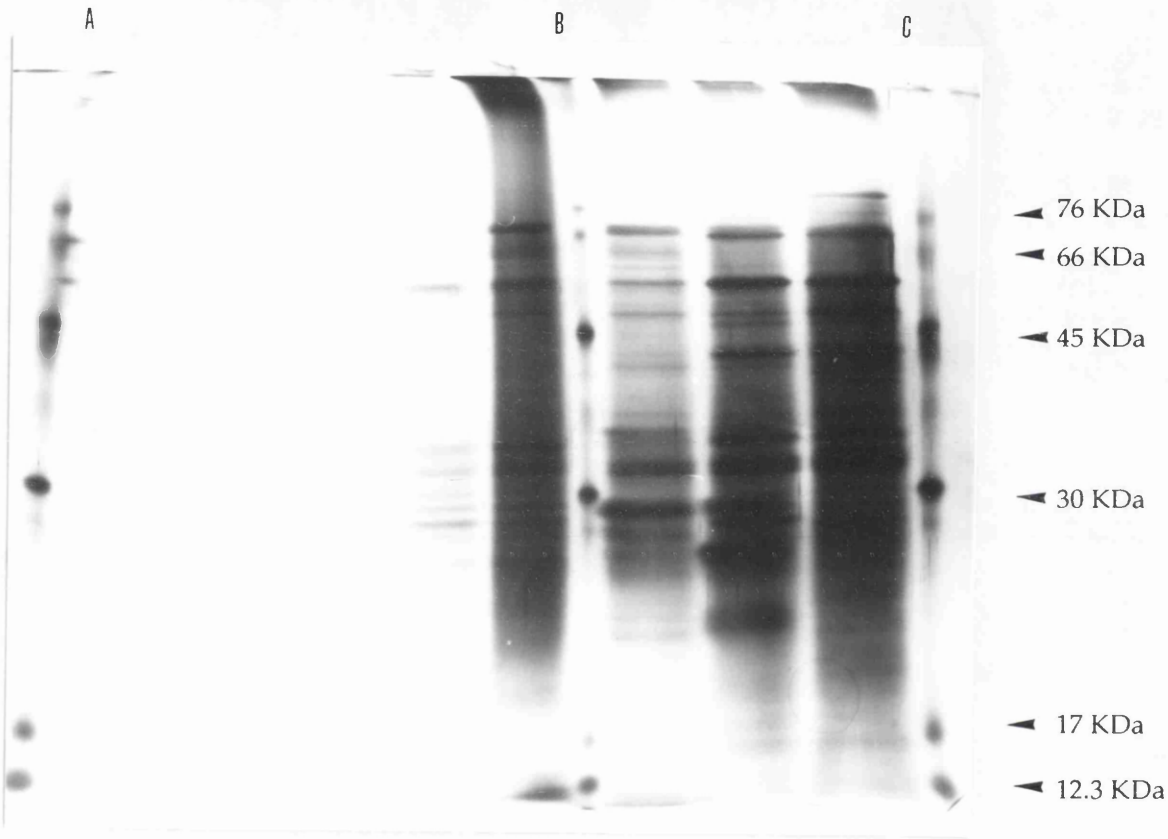


Fig. VI. 7e: SDS polyacrylamide electrophoresis gel of protein fractions collected during the FPLC anion exchange of dodecyl maltoside solubilized material [see fig. VI.6].

Proteins were acetone precipitated and resuspended in electrophoresis sample buffer as described in section **6. 2.** 2. 3. 3. prior to the electrophoresis.

From right to left, fractions nº 38 to 46. Lanes a, b and c: molecular weight markers.

solubilized with Lubrol PX (Fig. VI.6). No glucuronyltransferase activity was recovered from the different peaks of proteins collected during the chromatography. Aliquots of the fractions collected during the chromatography were precipitated in acetone (1 volume of sample/10 volumes of acetone, kept overnight at -20°C, resuspended in electrophoresis sample buffer [section **2. 7. 2.**]). Electrophoresis of protein was carried out through a 12% polyacrylamide gel (Fig. VI.7.a to VI.7.e). Many protein bands were detected on the different electrophoresis gels and it was possible to distinguish changes in the protein pattern of the fractions eluting from the column. However, due to the lack of glucuronyltransferase activity recovery, it was difficult to relate any protein band to the enzyme.

6. 4. DISCUSSION.

The different techniques of chromatography used in order to attempt further purification of the solubilized glucuronyltransferase (affinity and anion exchange chromatography with or without the use of the FPLC system) did not lead to the recovery of the enzyme activity in any of the fractions collected from the columns. It is possible that this lack of success in recovering the enzyme activity was caused by the differential elution, in separate fractions, of the two transferases (xylosyl- and glucuronyl-) required for the synthesis of the backbone of the glucuronoxylan. Since the glucuronyltransferase activity is maintained in the presence of UDP-xylose (Waldron *&* Brett, 1983), and therefore in the presence of the xylosyltransferase activity, a separation of the two transferases may have resulted in the loss of the glucuronyltransferase activity. Triton X-100 solubilized transferases were submitted to gel filtration chromatography through Sepharose CL-6B and CL-2B (Waldron *et al.*, 1989). It was suggested that the enzymes do not necessarily form an enzymatic complex

since gel filtration on Sepharose CL-6B appeared to partially separate the stimulatory effect of UDP-xylose from the glucuronyltransferase activity. Thus, the two transferases might not be contained in the same detergent-protein micelle, and, during the process of chromatography, react differently with the column matrix. It is also possible that the lack of enzyme recovery during anion exchange and affinity chromatography is due to the loss of a small protein subunit, a membrane-bound associated cofactor, or a priming factor essential for the activity of the enzyme(s) to be expressed. It is thought that a protein primer may be required for the initiation of the polysaccharide synthesis. This primer or any endogenous acceptor for the xylose and glucuronyl residues may have been lost or separated from the enzymes during the chromatography, preventing the enzymatic elongation of the glucuronoxylan polymer. In addition, separation of enzyme proteins by charge or hydrophobicity is a problem when trying to resolve detergent-solubilized proteins. Detergent moieties can interfere with the specificity of protein binding, or elution from the column material may destabilize the protein-detergent complex and result in the loss of catalytic activity (Hjelmeland & Chrambach, 1984)

Although the glycosyltransferase involved in the synthesis of homopolysaccharide, β-(1→3) glucan, has been solubilized with many detergents and characterized from different materials (Thelen & Delmer, 1988; Henry & Stone, 1982; Sloan *et al.*, 1987; Morrow & Lucas, 1986; Quigley *et al.*, 1988), previous attempts to purify this and other enzymes involved in the biosynthesis of cell wall heteropolysaccharides have been unsuccessful. Heller and Villemez (1972a,b) solubilized the glucosyl- and mannosyltransferases associated with glucomannan biosynthesis using Triton X-100, but gel filtration of the solubilized enzymes resulted in the almost complete inactivation of the mannosyltransferase.

With regard to the results obtained with anion exchange chromatography and the lack of success in recovering the glucuronyltransferase activity from any of the fractions collected during the elution of solubilized Golgi membranes, an alternative approach to the problem should be considered. The purification of the xylosyltransferase, that synthesizes the xylan backbone, may be useful. If the xylosyltransferase could be purified it could be added to fractions collected from anion exchange chromatography of solubilized membranes. Glucuronyltransferase activity could then be assayed in the presence of purified xylosyltransferase. Since the glucuronyltransferase activity is sustained in the presence of UDP-xylose (Waldron *&* Brett, 1983), the addition of xylosyltransferase to the glucuronyltransferase assay may help the detection of glucuronyltransferase activity, especially if the two enzymes are separated during chromatography. Identification of the suggested protein primer may also be useful. This primer may be lost or separated from the glucuronyl- and/or xylosyltransferase during chromatography. If this protein primer is necessary for the expression of the enzyme activities, once purified, its addition to the glucuronyltransferase assay may help to detect the enzyme activity.

CHAPTER 7: CONCLUSION.

The subcellular location of the transferases involved in the biosynthesis of the basic polymer of the hemicellulosic glucuronoxylan was investigated in pea epicotyls. Pea Golgi apparatus was fractionated by continuous sucrose density gradient centrifugation. Three distinct subcompartments (*cis-*, *medial-* and *trans-*Golgi cisternae) were obtained and characterised by their latent IDP'ase activity. The activity of the glucuronyltransferase was found to be associated chiefly with the *cis*-Golgi cisternae. It is highly probable that the xylosyltransferase, synthesizing the xylan backbone onto which glucuronic acid is transfered, is also located in the same subcompartment of the Golgi apparatus. A procedure for rapid isolation of a Golgi membrane-enriched fraction was derived from the protocol designed for the fractionation of the Golgi apparatus. The effects of selected detergents on particulate Golgi membrane-bound glucuronyltransferase activity were tested. The glucuronyltransferase activity was solubilized with different detergents from Golgi membrane-enriched fractions. Efficient protein solubilization was only achieved at high concentration of detergent which, in turn, was highly inhibitory of the enzyme activity. The inhibition of enzyme activity upon solubilization could be reversed by dialysis of the solubilized material against buffer containing a lower concentration of the detergent. Unfortunately, further attempts to purify the enzyme from Golgi membrane-solubilized proteins, using chromatography techniques, did not lead to the recovery of the enzyme activity.

In the future, it is hoped that the isolation of the xylosyltransferase, responsible for the synthesis of the xylan backbone, and of the other transferases involved in the "decoration" of the basic polymer, will permit a better understanding of the interactions of all the enzymes required to process

the polysaccharide. Furthermore, the subcellular localization of the enzymes responsible for the methylation, acetylation and feruloylation of the glucuronoxylan may shed some light on the sequence of events occurring, during the biosynthesis of cell wall heteropolysaccharides, within the different subcompartments of the Golgi apparatus. The identification of the presumed protein primer involved in the initiation of the biosynthesis of the polysaccharide is also important for the understanding of the earliest stages of the elaboration of the polymer, and for assessing the role of the endoplasmic reticulum in this process.

The ability of hemicellulose to bind to cellulose microfibrils might be exploited for the purification of the enzyme by entrapment. The technique of entrapment of a polysaccharide synthase with its product has previously been used for the isolation of a chitin synthase (Kang *et al.*, 1984), a β-(1,3) β-(1,4) glucan synthase (Bulone & Fèvre, 1989) and a callose synthase from red beet storage tissue (Wu *et al.*, 1991). It is possible that the addition of a small amount of cellulose powder to the glucuronyltransferase assay would result in the co-precipitation of the enzyme with the newly-formed glucuronoxylans bound to the cellulose. The development of photoaffinity-labelling of UDP-sugar binding proteins, as used in animal (Drake *et al.*, 1991) and plant systems (Lawson *et al.*, 1989), will provide photoprobes useful for the identification of the different glycosyltransferases.

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