CELLULOSE BIOSYNTHESIS BY ENZYMES FROM THE HYPOCOTYL OF THE MUNG BEAN (*Phaseolus aureus*), AS JUDGED BY THE EXTRACTION TECHNIQUE OF UPDEGRAFF (1969)

by

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PREFACE

I hereby declare that this thesis was composed by myself, and that the research presented is my own work. Due acknowledgement is made within the text for contributions from other sources.

Andrew F.D. Kennedy
February 1984

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Special acknowledgement must go to my parents for their encouragement during the period of this research and to Dr. D. Richardson for the hard work in the typing of this thesis and deciphering of my handwriting.

ABBREVIATIONS

GDPG	Guanosine diphosphate α-D-glucose
GDPM	Guanosine diphosphate α-D-mannose
UDPG	Uridine diphosphate α-D-glucose
UDPXyl	Uridine diphosphate α-D-xylose
ADPG	Adenosine diphosphate α-D-glucose
CDPG	Cytidine diphosphate α-D-glucose
TDPG	Thymidine diphosphate α-D-glucose
NDPG	Nucleoside diphosphate α-D-glucose
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate
ADP	Adenosine diphosphate
GDP	Guanosine diphosphate
UDP	Uridine diphosphate
UMP	Uridine monophosphate
NTP	Nucleoside triphosphate
NDP	Nucleoside diphosphate
G-1-P	Glucose-1-phosphate
PPi	Inorganic pyrophosphate
Pi	Inorganic phosphate
Gal	Galactose
Glc	Glucose
Man	Mannose
Dol	Dolichol
BSA	Bovine serum albumin
DTT	Dithiothreitol
PVP	Polyvinylpyrrolidone
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl chloride
EDTA	Ethylene diaminotetraacetic acid
DCBN	2,6-dichlorobenzonitrile
L_5	Laminaripentaose
L_4	Laminaritetraose
L_3	Laminaritriose
L_2	Laminaribiose
C_3	Cellotriose
C_2	Cellobiose

CW	Calcofluor white ST
CMC	Carboxymethyl cellulose
V_i	Initial rate of reaction
K_m	Michaelis constant
ER	Endoplasmic reticulum
DPA	Days post anthesis
DP	Degree of polymerisation
MW	Molecular weight
LPS	Lipopolysaccharide
TLC	Thin layer chromatography
TLE	Thin layer electrophoresis
GLC	Gas liquid chromatography
g	Gravitational field
w/w	weight for weight
v/v	volume formvolume
dpm	disintegrations per minute
V_0	column void volume
TS	Transverse section
LS	Longitudinal section
LPS	Lipopolysaccharide

CONTENTS

ABSTRACT

The numbers refer to the chapters concerned.

1. Current knowledge on cellulose biosynthesis was summarised. The section is divided into four main parts concerned with; A. the importance of cellulose; B. the structure of cellulose; C. cellulose biosynthesis, and; D. the cellular location of cellulose biosynthesis. Where relevant, cellulose biosynthesis in the cellulosic bacterium *Acetobacter xylinum* and algal systems was included.

2. This chapter presents the rationale behind this work and the criteria used to assess the cellulosic nature of the synthesised products.

3. The materials and methods used in this work were described.

4. The incorporation of radioactivity from GDP-[U-^{14}C]-G, UDP-[U-^{14}C]-G, [U-^{14}C]-sucrose, [U-^{14}C]-glucose and [U-^{14}C]-glucose-1-phosphate into the water- and chloroform: methanol (3:2 v/v)-insoluble products was investigated. It was found that the sugar-nucleotides were the most efficient substrates, that the incorporation of radioactivity from 1.0 mM UDP-[U-^{14}C]-G into these products appeared to be substrate-activated, and that the products synthesised from UDP-[U-^{14}C]-G at this concentration contained β(1-3) linked glucose. The distribution of the enzymic activity utilising the sugar-nucleotides between wall and particulate fractions and the effect of storage of the enzymes at -20 °C was also investigated.

5. The α-cellulose extraction was explained. Radioactivity from GDP-[U-^{14}C]-G, GDP-[U-^{14}C]-M and UDP-[U-^{14}C]-G was incorporated into α-cellulose. GDP-[U-^{14}C]-M was found to be an efficient substrate and GDPM stimulated the incorporation of radioactivity from GDP-[U-^{14}C]-G into this fraction.

6. The Updegraff extraction was introduced. Both UDP-[U-^{14}C]-G and GDP-[U-^{14}C]-G acted as substrates for Updegraff cellulose (U-cellulose) synthesis. The effect of prolonged periods of extraction of the water-and chloroform:methanol (3:2 v/v)-insoluble products in the Updegraff reagent and the effect of retaining the enzymes at -20 °C was studied.

7. The incorporation of radioactive glucose from GDP-[U-^{14}C]-G (particularly at higher concentrations than that traditionally used by previous researchers) and GDP-[U-^{14}C]-M into U-cellulose was studied. The factors involved in the cessation of U-cellulose synthesis from GDP-[U-^{14}C]-G were investigated and discussed. The products appear to be glucomannan (on the basis of a kinetic study of the effect of GDPM on U-cellulose synthesis from GDP-[U-^{14}C]-G, structural and gel-filtration studies) and possibly mannan (on the basis of a time course study on the synthesis of U-cellulose from 102 μM GDP-[U-^{14}C]-M) and glucan (based on a time-course study of U-cellulose synthesis from 1.0 mM GDP-[U-^{14}C]-G, the effect of E D T A on U-cellulose synthesis from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-M, and a structural study). There was some indication of the presence of a substrate-activated enzyme which synthesised pure glucan from GDP-[U-^{14}C]-G. The non-glucan products could not be solubilised even after prolonged periods of extraction in Updegraff reagent.

8. The incorporation of radioactive glucose from 1-5 μM UDP-[U-^{14}C]-G into U-cellulose was further investigated. The synthesis of U-cellulose from 1 μM UDP-[U-^{14}C]-G showed no distinct pH optimum in the range 4-11 and the factors involved in the cessation of the reaction were discussed. Gel filtration of the water and chloroform:methanol (3:2 v/v)-insoluble products suggested that the majority of the products had a molecular weight of less than 7 x 10^4, although there was some indication of material of a sufficiently large molecular weight to be cellulosic. The U-cellulose synthesised from UDP- U-^{14}C -G does

not appear to be a xyloglucan (based on an investigation of the interaction of UDPG and UDPXyl in U-cellulose synthesis). Structural analysis indicated the presence of β(1-3) and β(1-4) linked glucose. The β(1-3) linked glucose was not removed by prolonged extraction in the Updegraff reagent (6 hours). It was not determined whether the β(1-3) and β(1-4) linked glucose were part of a mixed-link glucan or were derived from separate polymers.

9. The general conclusions on this work and their possible significance for cellulose biosynthesis were evaluated. In particular, a hypothesis is presented by which the purportedly negative results obtained by previous researchers working on GDPG as a precursor of cellulose may be reconciled with cellulose biosynthesis.

CHAPTER 1: INTRODUCTION

A. CELLULOSE

A.1. The abundance of cellulose

Cellulose is the most abundantly produced macromolecule on earth with an annual production of 10^{15} kg, about two orders of magnitude greater than its nearest rival, chitin. By far the greatest part of the mass of cellulose is produced by land rather than marine plants (Colvin, 1980a). Thus, the process of cellulose biosynthesis in higher plants is a subject for which there is ample justification for the attention of researchers.

A.2. The importance of cellulose

A.2.1. The biological importance of cellulose

Cellulose is an essential strengthening and protective component of structures produced by plant, animal and bacterial cells. Cellulose is the major skeletal polysaccharide in higher plant cell walls, where it is found as partially crystalline microfibrils. These microfibrils are surrounded by a matrix of amorphous, non-cellulosic polysaccharides, in a manner analogous to that of a man-made composite material such as glass-reinforced plastic (Northcote, 1972). These matrix polysaccharides may be extracted from the wall by water (pectins) and alkali (hemicelluloses). The partial crystallinity of the cellulose microfibril imparts great tensile strength to the structure. Since the microfibrils are to be found in layers with an ordered orientation, each layer has great strength in the direction parallel to the microfibrils. The microfibrils in one layer have a different orientation to those in the next, and so the wall as a whole has strength to resist tension in any direction.

Cellulose is also a useful reservoir for the stabilization of energy and nutrient in the biosphere, due to its large mass and resistance to degradation (Colvin, 1980a).

A.2.2. The agricultural importance of cellulose

Cellulose is the principal component of such economically important agricultural products as wood and cotton. It is also a major constituent of crop plants.

A.2.3. The industrial importance of cellulose

Before the industrial revolution products containing or derived from cellulose were of paramount technological importance. With the advent of the widespread use of fossil fuels these products have lost some of their interest for our civilization, even though they are being used in greater quantities than ever. However, with the depletion of easily available fossil fuels the relative industrial importance of cellulose and related products as a renewable resource may well increase (Colvin, 1980a). Clearly, if this becomes the case, the ability to accelerate or modify the process of cellulose biosynthesis would be valuable. Before this objective could be achieved, we must have a detailed knowledge of the biosynthesis of cellulose. At the moment this is just what we do not have, despite the effort of a large number of workers over the past twenty-five years. This is largely due to our inability to demonstrate the *in vitro* biosynthesis of cellulose.

B. THE STRUCTURE OF CELLULOSE

B.1. The chemical structure of cellulose

Pure cellulose is a linear polysaccharide consisting of D-glucopyranosyl residues with the chair configuration, linked by $\beta(1\text{-}4)$ glucosidic bonds. The molecule has a

high degree of polymerization (D.P.), which may differ from primary to secondary wall cellulose. In cotton, primary wall cellulose has a non-uniform D P of 2,000-6,000, while secondary wall cellulose is larger with a more uniform D P of 14,000 (Marx-Figini and Schulz, 1966). The chains have a two-fold screw axis, with the result that stereochemically, the repeating unit of the molecule is cellobiose (see Fig. 1).

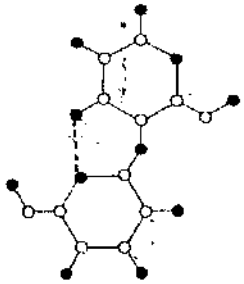


Fig. 1 Cellobiose. ○ carbon; ● oxygen; ---- hydrogen bond.

There are intrachain hydrogen bonds within the cellobiose units between the O-3H group and the O-5 atom of the next residue, suggesting that the cellobiose units have a bent conformation along the chain (Blackwell, 1982). The description of the cellobiose residue above is known as the Herman's cellobiose configuration. Conformational analysis has confirmed that this is the only configuration that is free of steric clashes, while obeying the demand that the chain should have a two-fold screw axis and a projected residue length of 5.15 Å. Further calculation has shown that the total Van der Waal's energy lies close to a minimum, displaced from it slightly to allow for the intrachain hydrogen bond. This is a major factor contributing to the

high stability of cellulose (Preston, 1974).

The description of the cellulose of the crystallographers and organic chemists given above must not be confused with the empirical definition of native cellulose, which is based on the insolubility of cellulose in alkali. The term α-cellulose was introduced in 1947 by Wise and Ratliff to describe the 24% KOH-insoluble fibrous residue obtained upon the extraction of a mixture of polysaccharides such as those found in delignified wood pulps. This fraction has been shown to contain predominantly glucose, but also a small but significant amount of non-glucose residues, mostly xylose and mannose. These non-glucose residues may be integral to the microfibril as part of individual heteroglycan chains containing predominantly glucose, or as separate chains containing little or no glucose mixed with the outer glucan chains. Alternatively, they may arise from non-cellulosic matrix polysaccharides which are so strongly adsorbed to the surface of the microfibril that they are not extracted by alkali (Northcote, 1972; Katō, 1981).

B.2. The physical structure of cellulose

The microfibrillar structure of cellulose is a consequence of the individual glucan chains associating via intermolecular hydrogen bonds between hydroxyl groups. As a result of this, cellulose is very stable and is insoluble in all solvents except those which cause hydrolysis of the glycosidic linkages between the residues, or those which break the hydrogen bonds between the chains (Colvin, 1980a).

In the nineteenth century Carl von Nägeli studied the structure of cellulose by light microscopy. He concluded that cellulose contained separable crystalline entities, which were termed micelles. The "crystals" of cellulose were thin relative to the wavelength of light and were very much longer than broad. The crystallinity of the material was deduced from its appearance under the polarizing microscope and the fact that the crystals were separate long thin entities was deduced from

the swelling behaviour (Preston, 1974). Nägeli's conclusions have to a large extent been confirmed by electron microscopy and X-Ray diffraction studies.

There are at least four different forms of cellulose (cellulose I-IV), recognised by their X-Ray diffraction patterns and infra-red spectra. In each structure, the chains have approximately the same backbone conformation, with two glucose residues repeating in approximately 10.3 Å. The structures differ in terms of the packing of the adjacent chains (Blackwell, 1982) and native cellulose is found as the cellulose I form. The precipitation of native cellulose from solution leads to cellulose II formation and this cellulose I→II transition is irreversible. This implies that cellulose II is the stable form while that of native cellulose I is a metastable structure (Blackwell, 1982). Cellulose I is unstable by 2cal/gm plus an undetermined increase in entropy with respect to cellulose II (Colvin, 1980a). Cellulose III can be prepared from cellulose I or II by treatment with liquid ammonia. Similarly, cellulose IV is prepared from cellulose I or II by treatment in hot glycerol (Blackwell, 1982).

A number of unit cells have been proposed for microfibrillar cellulose from a variety of plants. Meyer and Misch (1937) proposed a two chain unit cell for the cellulose of ramie fibres, in which the individual chains could be parallel or antiparallel. The latter was favoured on the basis of statistical analysis, and by analogy with the chemically and functionally similar polysaccharide, α-chitin (Northcote, 1969). However, it is now thought that the statistical analysis used by Meyer and Misch is invalid (Gardner and Blackwell, 1974). Gardner and Blackwell (1974) found that the chains in the unit cell of *Valonia* cellulose were parallel and that the parallel chain model was favoured over antiparallel chains by 200:1. The unit cell was found to be monoclinic, with dimensions a = 8.17 Å, b = 7.86 Å,

c = 10.38 Å and a γ angle of 97°. The structure contained an O2'-H.....O6 intramolecular hydrogen bond in addition to the previously observed O3-H.....O5' intramolecular hydrogen bond. Thus, there are intramolecular hydrogen bonds on both sides of the glucosidic linkage which should increase the stiffness of the extended conformation. There is also a O6-H.....O3 intermolecular bond along the a axis (see Fig. 2), (Blackwell, 1982).

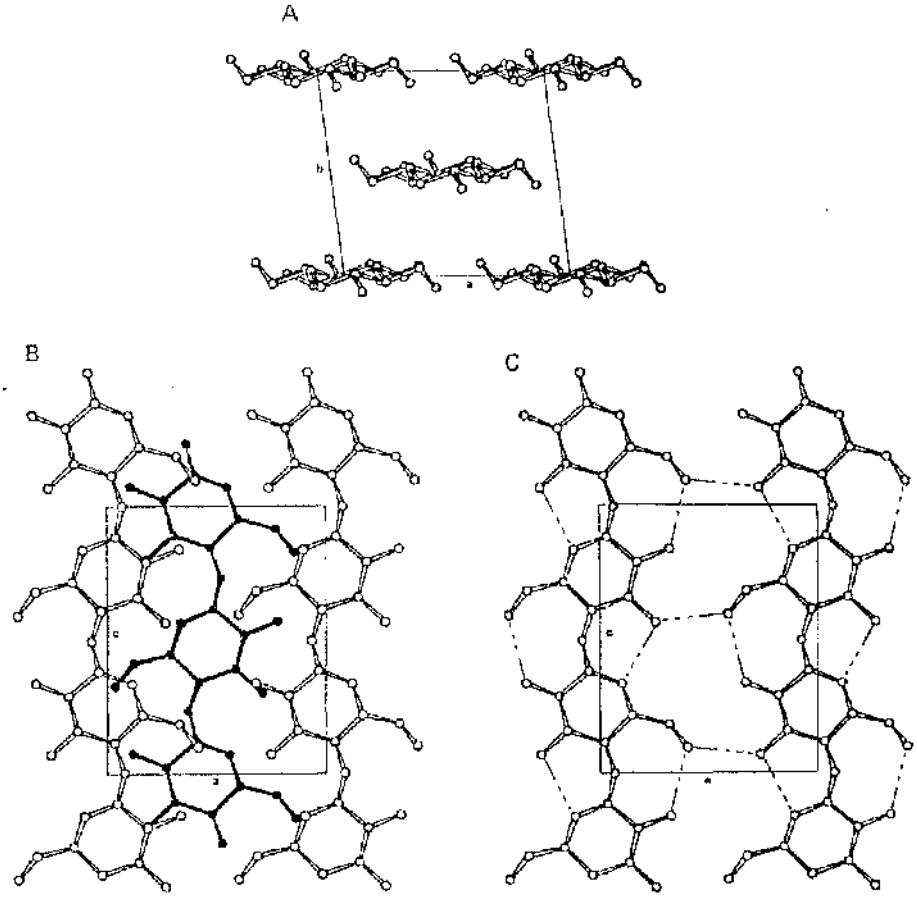


Fig. 2. Structure of cellulose I. (A) ab projection (looking along the chain axes); (B) ac projection; (C) hydrogen bonding network in the sheet parallel to the ac plane. From Blackwell (1982).

A model for the unit cell of cellulose II has also been proposed in which the chains are antiparallel. In this model there are two intermolecular hydrogen bonds per unit cell, which probably explains the greater stability of cellulose II (Blackwell, 1982). In conclusion, it appears that native cellulose has an extended chain conformation, and since this form of cellulose is not the stablest form possible, its structure must be required by the biosynthesis mechanism. As the possibility of folded chains within the microfibril has now been excluded (see B.2.2.), it would be simpler to synthesize the parallel chain structure of cellulose I rather than the antiparallel chains of cellulose II. Cellulose II synthesis would require a sophisticated synthetic apparatus, with two different enzymes in order to produce the "up" and "down" chains. (This assumes tip synthesis of the microfibrils, see C.2.).

Although the paracrystalline, microfibrillar morphology of cellulose is now universally accepted, there is still considerable controversy over the physical disposition of the glucan chains within the crystalline and non-crystalline regions of the microfibril. A number of models for microfibrillar structure have been proposed and they fit into three basic categories.

B.2.1. The elementary fibril concept of microfibrillar structure

Native cellulose microfibrils vary in breadth from around 10 nm in bacterial and wood cellulose, up to 25-30 nm in marine algae (Colvin, 1980a). The elementary fibril concept envisages an extended, uniform, crystalline subdivision of the microfibril which is about 3.5 nm in breadth. Aggregates of these units surrounded by a cortex of less crystalline, although still longtitudinally orientated polymer chains make up the microfibril (see Fig. 3). However, much of the evidence for the existence of an elementary

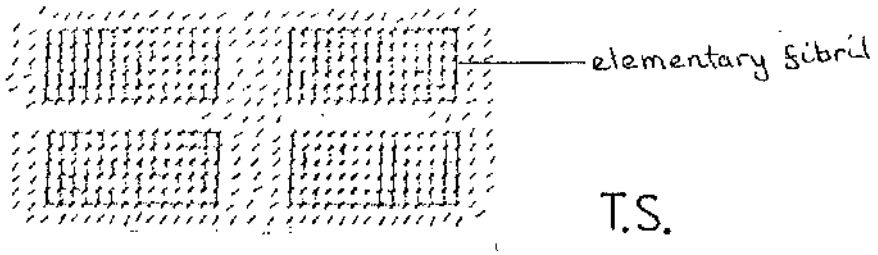


Fig. 3. The elementary fibril concept of microfibrillar structure. From Northcote (1969)

fibril is the result of the misinterpretation of visual images and electron scattering (Colvin, 1980a). Thus, it must be concluded that the case for a uniform 3.5 nm elementary fibril is as yet unproven.

B.2.2. Folded chain models of microfibrillar structure

This concept of microfibrillar structure was derived from the field of synthetic polymers, and was popular in that it easily explained the prevalent viewpoint of the time that the glucan chains were antiparallel. The microfibrils were thought to consist of folded chains, the folds being parallel to the axis of the microfibril (see Fig. 4).

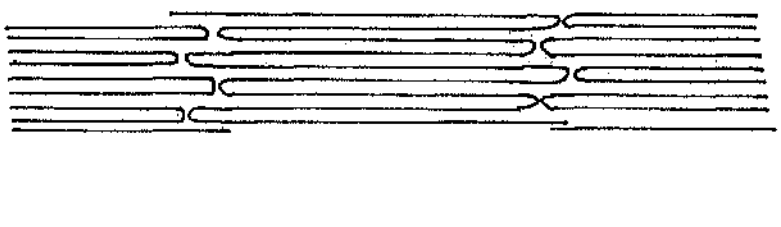


Fig. 4. Folded chain model of Marx-Figini and Schulz (1966). From Northcote (1969).

The folded chain models are now not thought to be correct for the following reasons:-

1) Theoretical and experimental studies on the elasticity of the crystalline regions of the microfibril excluded the possibility of folded chains (Colvin, 1980a).

2) When ramie fibres were cut transversely into thin sections, the D.P. fell from 3,900 to 1,600. This result would only be expected if the constituent glucan chains were extended (Shafizadeh and McGinnis, 1971).

3) X-Ray diffraction studies combined with statistical analysis have indicated that native cellulose has an extended chain conformation (Blackwell, 1982).

B.2.3. The fringed micelle concept of microfibrillar structure

In this model of microfibrillar structure, the individual glucan chains pass repeatedly from regions of disorder within the microfibril, to regions where the chains are part of a periodic lattice or crystallite (see Fig. 5). Stockman (1972) argues on thermodynamic grounds that such an alternation of ordered and disordered regions along the length of the microfibril must occur, and indeed these regions of disorder have been observed by staining with silver (Preston, 1974). Where the chains pass from a crystallite to a region of disorder, a "fringe" of less ordered chains will be created around the crystallite. This model is probably the best representation of native microfibrillar structure to date (Colvin, 1980a).

Regardless of whichever model for microfibrillar structure is favoured, if the non-glucose residues found in such fractions as α-cellulose are indeed integral to the microfibril, then they must be present in the less crystalline regions of the microfibril. This has been confirmed

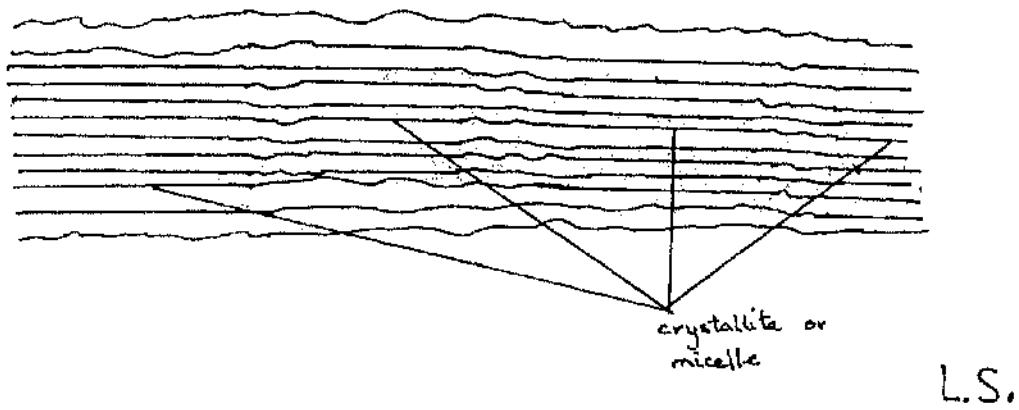


Fig. 5. The fringed micelle concept of microfibrillar structure.

by Dennis and Preston (1961) who demonstrated that the crystalline regions of the microfibrils of elm wood obtained by H_2SO_4 degradation contained only glucose, and that they could only be obtained under conditions that destroyed the integrity of the microfibril.

C. CELLULOSE BIOSYNTHESIS IN HIGHER PLANTS

The biosynthesis of cellulose can be divided conceptually into two stages:-

1) synthesis of the β (1-4) glucan chains
2) assembly of the cellulosic microfibrils.

C.1. The synthesis of β (1-4) glucan

C.1.1. General polysaccharide biosynthesis

Polysaccharide biosynthesis is thought to involve sugar-nucleotides as precursors. Thermodynamically, they are very favourable substrates since their free energy of hydrolysis is considerably more negative than that of other glucose compounds (see Table 1).

Possible precursor	ΔG° cal mole^{-1}
UDPG	-7,600
α-D-glucose phospate	-4,850
α(1-4) link in glycogen	-4,350

Table 1. The free energy of hydrolysis for possible precursors of polysaccharide biosynthesis.

A general scheme for polysaccharide biosynthesis is shown in Fig. 6, in which three distinct stages can be recognised.

First, the glucose residue is activated, resulting in the formation of a sugar-nucleotide. In the second stage, glycosidic bonds are formed by transfer of the sugar residue from the sugar-nucleotide to an acceptor molecule, which may be a polysaccharide, phospholipid, glycophospholipid, protein or glycoprotein. The last stage, if it occurs, is known as transglycosylation and results in the formation of chain branches. A transglycolase attacks a glycosidic linkage at a point in the chain forming a glycosyl enzyme complex, or a stabilised carbonium ion. The severed chain fragment is then transferred to another site on the polysaccharide molecule. The net cost in energy of the incorporation of one sugar residue into a polysaccharide is thus one molecule of ATP plus one molecule of nucleoside triphosphate (NTP).

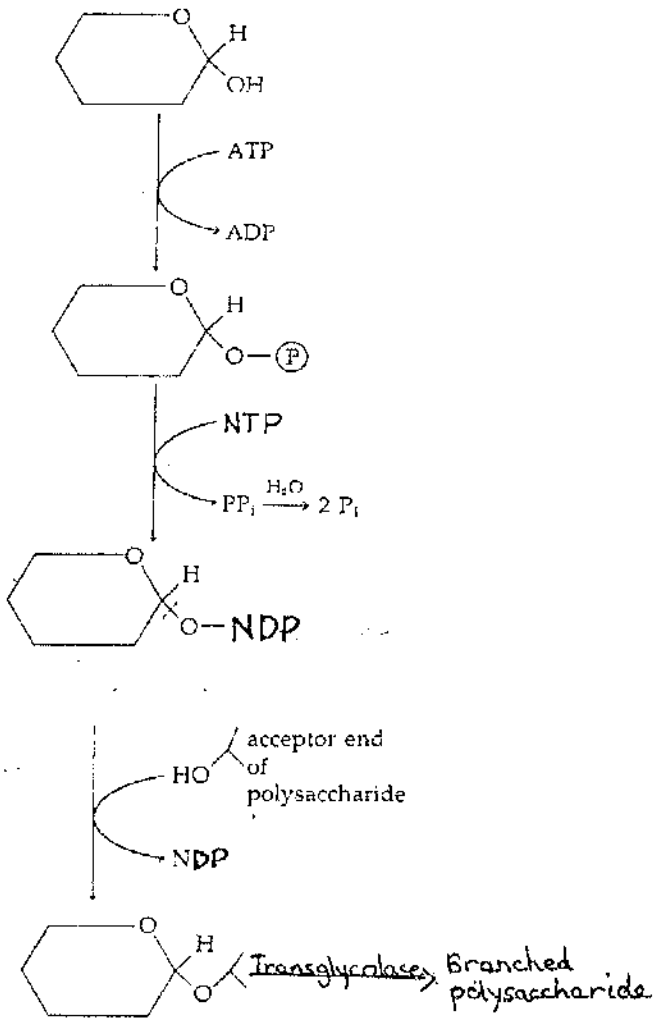


Fig. 6. General scheme for polysaccharide biosynthesis.

C.1.2. The sugar-nucleotide precursors of cellulose biosynthesis.

There have been three main approaches to the study of the sugar-nucleotide precursors of cellulose biosynthesis:-

1) research utilising cell-free enzyme preparations.
2) the use of more intact systems, such as cotton fibres, suspension-cultured cells and tissue slices.
3) *in vivo* studies.

C.1.2.1. Investigations on the sugar-nucleotide precursors of cellulose biosynthesis utilising cell-free enzyme preparations.

C.1.2.1.1. The sugar-nucleotide precursors of β-glucan synthesis.

The enzymes involved in the synthesis of plant cell wall polysaccharides are particle-bound when isolated. This suggests that they are contained within lipoprotein membranes, or they are firmly attached to these membranes. A cell-free homogenate is incubated with a sugar-nucleotide precursor, followed by an analysis of the insoluble products. The use of such crude enzyme preparations has a number of inherent problems which will be discussed in a subsequent section (p 23).

The discovery of uridine diphosphate glucose (UDPG) led to the suggestion that it may be a precursor for β-glucan biosynthesis. Such a role for UDPG was first discovered by Glaser (1958), who demonstrated the synthesis of a β(1-4) glucan from UDPG by a cell-free extract of the cellulosic bacterium *Acetobacter xylinum*. Neither G-1-P nor glucose would act as a substrate for this system.

The nature of the initial glucose donor for higher

plant cellulose biosynthesis is not so clear. In 1958, Feingold *et al* isolated a particulate enzyme preparation from the mung bean *Phaseolus aureus* which catalysed the synthesis of β(1-3) glucan from UDPG. The authors also observed UDPG:β(1-3) glucan glucosyl-transferase activity in particulate preparations from cabbage, spinach, parsley and zucchini squash. This UDPG:β(1-3) glucan glucosyl-transferase activity has subsequently been shown to be of widespread occurrence in particulate preparations from higher plants including *P. aureus* (Villemez *et al*, 1967; Flowers *et al*, 1968; Batra & Hassid, 1969; Chambers & Elbein, 1970; Clark & Villemez, 1972), *Lupinus albus* (Flowers *et al*, 1968; Larsen & Brummond, 1974), *Pisum sativum* (Brett & Northcote, 1975; Chao & Maclachlan, 1978; Raymond *et al*, 1978), *Gossypium hirsutum* (Heineger & Delmer, 1977; Bacic & Delmer, 1981), *Avena sativa* (Tsai & Hassid, 1971, 1973) and *Triticum vulgare* (Peaud-Lenoël & Axelos, 1970).

Initial work implicated guanosine diphosphate glucose (GDPG) rather than UDPG as the sugar-nucleotide involved in higher plant cellulose biosynthesis. Barber *et al* (1964) obtained a particulate enzyme preparation from *P. aureus* which catalysed the incorporation of glucose from GDPG into β(1-4) linked glucan. UDPG, ADPG, TDPG, CDPG, G-1-P and glucose were unable to act as glucose donors for β(1-4) glucan synthesis with this system. The authors also found GDPG: β(1-4) glucan glucosyl-transferase activity in particulate preparations from pea, string bean and corn. The presence of GDPG: β(1-4) glucan glucosyl-transferase activity has been confirmed in particulate preparations from *G. hirsutum* (Barber & Hassid, 1965; Delmer *et al*, 1974), *P. sativum* (Ray *et al*, 1969; Hinman & Villemez, 1975), *L. albus* (Brummond & Gibbons, 1964; Flowers *et al*, 1969) and *A. sativa* (Ordin & Hall, 1967). However, this GDPG: β(1-4) glucan glucosyl-transferase activity is now regarded by most researchers to be involved in the synthesis of glucomannan rather than a β(1-4) linked glucose homopolymer (see pp23-25).

UDPG has also been shown to act as a precursor for β(1-4) glucan synthesis in particulate enzyme preparations from *P. aureus* (Villemez *et al*, 1967; Clark & Villemez, 1972), *L. albus* (Brummond & Gibbons, 1964; Stafford & Brummond, 1970; Larsen & Brummond, 1974), *A. sativa* (Ordin & Hall, 1967; Tsai & Hassid, 1971, 1973), *G. hirsutum* (Bacic & Delmer, 1981), *P. sativum* (Ray *et al*, 1969; Shore *et al*, 1975 ; Chao & Maclachlan, 1978; Raymond *et al*, 1978). However, it is possible that the UDPG:β(1-4) glucan glucosyltransferase activities observed in preparations from dicotyledonous plants are involved in xyloglucan rather than cellulose synthesis as suggested by Ray (1975, 1980) and Villemez & Hinman (1975). UDPG can act as a substrate for the synthesis of both β(1-3) and β(1-4) linked glucan in preparations from several plants (Villemez *et al*, 1967; Ordin & Hall, 1968; Peaud-Lenoël & Axelos, 1970; Tsai & Hassid, 1971, 1973; Clark & Villemez, 1972; Smith & Stone, 1973a; Larsen & Brummond, 1974; Chao & Maclachlan, 1978; Raymond *et al*, 1978; Bacic & Delmer, 1981). In oat, wheat and rye, UDPG can act as a substrate for the synthesis of mixed linked β(1-3) β (1-4) glucans (Ordin & Hall, 1968; Peaud-Lenoël & Axelos, 1970; Smith & Stone, 1973a) and in the case of rye the glucans formed were similar to those found *in vivo* in this plant (Smith & Stone, 1973 a,b).

The above evidence suggests that β-glucan synthesis in plants may be mediated by a number of enzymes utilising UDPG, GDPG or both. Additional evidence that β-glucan synthesis is not mediated by a single enzyme with a broad substrate specificity is as follows:-

1) Brummond & Gibbons (1964) demonstrated that the synthesis of β(1-4) glucan from UDPG and GDPG by particulate preparations from *L. albus* was additive rather than competitive. This suggests the presence of two separate enzymes utilising different substrates.

2) Shore & Maclachlan (1973) found four β(1-4) glucan synthetases in pea epicotyls. Two were GDPG specific and two were UDPG specific. Three of the activities were regulated by auxin, while the fourth (a UDPG specific synthetase) was not.

3) Enzymes involved in β-glucan synthesis have been found to differ in cofactor requirements, pH optima and in K_m values.

4) Chambers & Elbein (1970) separated UDPG: β(1-3) from GDPG: β(1-4) glucan glucosyl-transferase activity in *P. aureus*, on the basis of the enzyme's differential solubilities after extraction of the particles with digitonin. Similarly, Tsai & Hassid (1971) achieved a separation of digitonin extracted UDPG: β(1-3) and UDPG: β(1-4) glucan glucosyl-transferase activities from *A. sativa* coleoptiles.

In conclusion, it appears that the *in vitro* synthesis of β(1-3) glucan utilises UDPG as a substrate, while the sugar-nucleotide precursor for *in vitro* β(1-4) glucan synthesis could be UDPG, GDPG or both. However, the observed rates of *in vitro* β(1-4) glucan synthesis in higher plants are much less than the observed rates of cellulose synthesis (Barber *et al*, 1964; Shore & Maclachlan, 1975). The relative ease by which *in vitro* β(1-3) glucan synthesis is obtained can probably be explained by the fact that plant tissues deposit β(1-3) glucan (wound callose) in response to mechanical injury (Currier, 1957). Thus homogenisation of plant tissue in the preparation of particulate enzymes will inevitably result in rapid synthesis of β(1-3) glucan, which is not normally a major constituent of plant cell walls.

C.1.2.1.2. The conditions required for β-glucan synthesis *in vitro*

In vitro studies on β-glucan synthesis usually involve the homogenisation of plant tissue, isolation of a particulate enzyme by differential centrifugation, and incubation of the enzyme preparation with a sugar-nucleotide substrate. Solubilisation of the enzyme by detergents may be incorporated as an additional step. The conditions employed at any of the steps mentioned above may affect the ability of the enzyme to form β-glucan *in vitro*.

1) Conditions under which the plant is grown. Clark & Villemez (1972) found that the properties of the particulate β-glucan synthetases obtained from *P. aureus* were affected by the temperature at which the seedlings were germinated. When the seedlings were germinated at 21 °C, all of the alkali-insoluble glucan formed from UDPG by the isolated enzyme particles was β(1-4) linked. After germination at 26 °C, the alkali-insoluble glucan was contaminated with polysaccharide containing β(1-3) linked glucose.

2) Nature of the buffering component in the homogenisation and reaction media. The type of buffer used appears not to be of great importance, at least for the UDPG-utilising enzymes. However, it was found that pyrophosphate completely inactivated the *P. aureus* UDPG-utilising enzymes (Clark & Villemez, 1972). Elbein (1969) found that in preparations from the same plant, GDPG:β(1-4) glucan glucosyl-transferase activity was slightly inhibited by phosphate buffers.

3) The method of tissue homogenisation. The method used for tissue homogenisation was not found to influence the properties of the enzyme(s) utilising UDPG in *P. aureus* (Clark & Villemez, 1972), although it has been found to alter the physical properties of the particles produced (Ray *et al.* 1969). However, Maclachlan *et al* (1979) found that preparations

Protective agent	References
D T T	Lui & Hassid, 1970; Heller & Villemez, 1972a,b; Hinman & Villemez, 1975; Delmer *et al*, 1977; Raymond *et al*, 1978; Ray, 1980.
B S A	Tsai & Hassid, 1971; Clark & Villemez, 1972; Heller & Villemez, 1972a,b; Ray, 1980.
sucrose	Villemez *et al*, 1967; Chao & Maclachlan, 1978; Ray, 1980; Barber, 1982.
P V P	Barber & Hassid, 1965.
P E G	Maclachlan *et al*, 1979; Carpita & Delmer, 1980.
E D T A	Lui & Hassid, 1970; Hopp *et al*, 1978; Ray, 1980; Barber, 1982.

Table 2. Some references to workers who have included protective agents in homogenisation and reaction media.

from *P. sativum* were more active in synthesising alkali-insoluble β-glucan from UDPG when the tissue was homogenised by fine chopping with razor blades, rather than grinding in a mortar and pestle or homogenisation in a Polytron homogeniser.

4) The inclusion of protective/stabilising agents. The β-glucan synthetases tend to be unstable and denature over a period of hours even at 0°C. For this reason a number of protective agents have been routinely added to homogenisation and reaction media to stabilise the enzymes (see Table 2).

5) Requirement for divalent cations. Divalent cations have been found to be required for glucan synthesis from UDPG and GDPG in a number of systems. Barber *et al* (1964) and Lui & Hassid (1970) found that after $(NH_4)_2SO_4$ extraction of *P. aureus* seedling particles, there was a requirement for a divalent cation for the incorporation of glucose from GDPG into β(1-4) glucan. Similarly, Chambers & Elbein (1970) demonstrated that digitonin-extracted particles from the same plant resulted in a requirement for $MgCl_2$ before GDPG: β(1-4) glucan glucosyl-transferase activity could be observed. Elbein (1969) demonstrated that particulate mannosyl transferase activity utilising guanosine diphosphate mannose (GDPM) in *P. aureus* was strongly dependent upon the addition of magnesium cations to the reaction media. No such requirement for β(1-4) glucan synthesis from GDPG was observed by these authors. Feingold *et al* (1958) found that divalent cations promoted the synthesis of β(1-3) glucan from UDPG in *P. aureus* systems. However, Larsen & Brummond (1974) found that magnesium chloride inhibited β(1-3) glucan synthesis, but stimulated β(1-4) glucan synthesis from UDPG in a digitonin-solubilised enzyme system from *L. alkus*. Tsai & Hassid (1971) demonstrated a similar stimulation of solubilised UDPG: β(1-4) glucan glucosyl-transferase activity in *A. sativa*. In particulate enzyme preparations from *Zea mays*, Ray (1979) found that UDPG:βglucan synthetase

assayed at micromolar concentrations of UDPG required magnesium cations. UDPG: glucan synthetase assayed at 0.1 mM UDPG had no requirement for divalent cations.

From the above section it is clear that the requirement for divalent cations in glucosyl transferase activity appears to vary according to the source of enzyme, and on the method of enzyme preparation.

6) The presence of glucose and various glucosides. The presence of glucose and various glucosides in the reaction medium has sometimes been found to stimulate β-glucan synthesis from sugar-nucleotides. An activator (which could be glucose or a variety of glucosides) was found by Feingold *et al* (1958) to be required for β(1-3) glucan synthesis from UDPG by the digitonin solubilised, but not the particulate enzyme, prepared from *P. aureus*. No such activator requirement was found for the *in vitro* synthesis of β(1-4) glucan from GDPG with solubilised or particulate enzyme from this plant (Barber *et al*, 1964; Lui & Hassid, 1970). Tsai & Hassid (1971) found that cellobiose stimulated the synthesis of both β(1-3) and β(1-4) glucan synthesis from UDPG in enzyme preparations from *A. sativa*. Cellobiose was also found to stimulate glucan synthesis from GDPG but not from UDPG in particulate preparations from *P. sativum* epicotyls (Spencer *et al*, 1971). β(1-4) glucan synthesis from UDPG was stimulated by glucose in particulate enzyme preparations and by glucose, methyl-β-D-glucopyranoside and cellobiose in solubilised enzyme from *L. albus* (Stafford & Brummond, 1970; Larsen & Brummond, 1974). In particulate preparations from *G. hirsutum*, it was found that β-linked glucosides stimulated UDPG: β(1-3) glucan glucosyl-transferase activity at, or above 50 μM. Below 50 μM UDPG, UTP had also to be added to the incubation medium to obtain stimulation by β-glucosides. This stimulation by β-linked glycosides and UTP at low UDPG

concentrations was less than that in detached fibres (Delmer *et al*, 1977; Heineger & Delmer, 1977). Thomas *et al* (1969) observed the stimulation of β(1-3) glucan synthesis from UDPG by glycerol in a variety of plants. This stimulatory effect of glycerol was completely inhibited by erythritol. In 1958, Glaser reported a requirement for high molecular weight (M.W.) soluble cellodextrans for the cell-free synthesis of cellulose in preparations from *Acetobacter xylinum*. It was suggested that the cellodextrans were acting as primers. Cooper & Manley (1975a) also noted a stimulation in the yield of alkali-insoluble glucan from UDPG when cellodextrans larger than cellotetraose were added to *A. xylinum* membranes, and they concluded that it was due to both priming and activating effects. However, in both the plant (Feingold *et al*, 1958) and bacterial cell-free systems, the cellodextran concentrations required for stimulation were far in excess of that expected for efficient primer action. Indeed Feingold *et al* (1958) demonstrated that exogenously added D-glucose was not incorporated into the products. This suggests that the stimulatory effects of these glucosides are due to enzyme activation rather than a priming action. Thomas *et al* (1969) demonstrated that the activation of UDPG: glucan glucosyl-transferase by polyols was stereospecific, requiring three closely associated hydroxyl groups. It should be noted however, that this system is unusual in that the allosteric regulators are uncharged.

7) The effect of substrate concentration. When UDPG is used as a substrate its concentration in the incubation may be important in terms of both the quantity, and the nature of the product produced. Tsai & Hassid (1971, 1973) found that at 1 mM UDPG, β(1-3) glucan was the main product formed, while with 10 μM UDPG, β(1-4) glucan predominated in both digitonin-solubilised and particulate enzyme from *A. sativa*. A similar increase in the ratio of β(1-3): β(1-4) linkages in the products with increasing UDPG concentration has been observed in particulate preparations from *A. sativa* (Ordin

& Hall, 1968), *Lolium multiflorum* (Smith & Stone, 1973a), *P. sativum* (Chap & Maclachlan, 1978; Raymond *et al*, 1978), *L. albus* (Stafford & Brummond, 1970), *T. vulgare* (Peaud-Lenoël & Axelos, 1970) and *P. aureus* (Clark & Villemez, 1972). However, Heineger & Delmer (1977) found that UDPG concentration had no effect upon the nature of the product (a linear β(1-3) glucan) in detached cotton fibres.

A number of workers have shown that for a given increase in UDPG concentration, there is a disproportionately large increase in the amount of β(1-3) glucan formed. This suggests that UDPG:β(1-3) glucan glucosyl-transferase is substrate-activated. Substrate activation by UDPG has been noted in particulate enzyme preparations from *A. sativa* (Tsai & Hassid, 1973), *L. multiflorum* (Smith & Stone, 1973a), *P. sativum* (Raymond *et al*, 1978) and *G. hirsutum* (Delmer *et al*, 1977). Tsai & Hassid (1973) postulated that this substrate activation was due to the binding of UDPG to the enzyme causing a conformational change and thereby an activation of the enzyme.

Varying the concentration of GDPG in the incubation medium has not been found to alter the nature of the linkages in the glucan product. However, increasing the GDPG concentration has been found to increase the period of incorporation into polysaccharide products in particulate preparations from *P. sativum* (Hinman & Villemez, 1975). Brett (1981a) found a similar phenomenon in tissue slices from the same plant. To date, there have been no reports of substrate activation of higher plant GDPG:β(1-4) glucan glucosyl-transferases. They do not, however, exhibit normal Michaelis-Menten saturation kinetics in that they have been found to produce non-linear double-reciprocal plots in both particulate systems (Villemez, 1971) and tissue slices (Brett, 1981a).

8) The presence of other sugar-nucleotides in the reaction

media. A major drawback with the particulate enzyme preparations used in the study of plant cell wall polysaccharide biosynthesis is that they contain a large amount of endogenous polysaccharide relative to the amount of radioactive sugar incorporated from added radioactive sugar-nucleotides. As a result, most of the information that can be obtained about the newly synthesised polysaccharide is restricted to the immediate vicinity of the radioactive sugars. Any contribution of sugar residues from endogenous sources may easily go unnoticed, since these contributions would be non-radioactive. This is especially important in view of the fact that a large number of plant cell wall polysaccharides are heteropolysaccharides. To overcome this problem, investigators have studied the effect of exogenously supplied non-radioactive sugar-nucleotide upon the incorporation of radioactivity from a different, radioactively labelled sugar-nucleotide. The effect of exogenously supplied non-radioactive sugar-nucleotides can be studied kinetically, or structurally, to determine whether or not the nature of the products changes.

GDPG was initially thought to be a substrate for higher plant cellulose biosynthesis. However, it was subsequently discovered that the addition of non-radioactive GDPM to an incubation medium containing radioactive GDPG, resulted in a stimulation of the incorporation of radioactivity into polysaccharide. This effect was found in particulate and solubilised enzymes from *P. aureus* (Barber *et al*, 1964; Villemez, 1971; Heller & Villemez, 1972a; Barber, 1982) and in particulate preparations from *P. sativum* (Hinman & Villemez, 1975) and *G. hirsutum* (Barber & Hassid, 1965). Barber *et al* (1964) analysed the products from GDP-^{14}C-G alone and from GDP-^{14}C-G + GDPM by partial acid hydrolysis in fuming HCl. Partial acid hydrolysis of the products from GDP-^{14}C-G resulted in the cellodextran series of oligosaccharides. When GDPM was also present in the incubation medium, partial acid hydrolysis of the products also resulted in the cellodextran series but there was an additional oligosaccharide

series, presumably mannosyl-glucose containing oligosaccharides. This was confirmed by Elbein (1969). From these results, Barber *et al* (1964) concluded that the product from GDPG was indeed a β(1-4) glucan (claimed to be cellulose) and upon the addition of GDPM, an additional glucomannan (see Fig. 7) was synthesised.

-4)-β-D-Manp-(1-4)-β-D-Glcp-(1-4)-β-D-Manp-(1-

Fig. 7 Glucomannan

Villemez and co-workers (Villemez, 1971; Heller & Villemez, 1972a; Hinman & Villemez, 1975) undertook a detailed kinetic analysis of the GDPG and GDPM utilising systems of *P. aureus* and *P. sativum*. It was shown that the reaction utilising GDP-^{14}C-G alone was over within a few minutes. The presence of GDPM extended the period of reaction from GDP-^{14}C-G, resulting in an overall stimulation of the incorporation of radioactivity into the polysaccharide products. However, no stimulation of the initial rate of reaction (V_i) from GDP-^{14}C-G was observed upon the addition of GDPM at any concentration. It was therefore concluded that glucomannan was the sole product from GDPG in these systems since, if the observed stimulation by GDPM was due to the action of an additional glucomannan-synthesising enzyme system, an increase in V_i should be detected. Further evidence was quoted by Villemez and co-workers that would suggest that only a glucomannan is produced from GDPG *in vitro*:-

1) Total ^{14}C polysaccharide and alkali-insoluble ^{14}C-poly-

saccharide exhibit similar kinetic patterns as a function of GDPM concentration.

2) In a study on solubilised mannosyl and glucosyl transferases, Heller & Villemez (1972a) demonstrated the following enzyme properties that would promote the synthesis of glucomannan rather than glucan plus mannan:-

a) The glucosyl and mannosyl transferases apparently use the same type of acceptor molecule
b) The glucosyl transferase required the presence of a mannose-containing acceptor molecule for sustained activity
c) GDPG directly modulates the mannosyl transferase since it is a strong competitive inhibitor
d) GDPM is a weak competitive inhibitor of the glucosyl transferase.

At first sight these results would appear to contradict the results of Barber *et al* (1964). However, Flowers *et al* (1969), using acetolysis rather than partial hydrolysis on the products from GDPG, demonstrated the presence of mannose-containing oligosaccharides. These mannose-containing oligosaccharides were not observed using partial acid hydrolysis, thus explaining the failure of Barber *et al* (1964) to detect glucomannan synthesis from GDPG on its own. It was postulated that these mannose-containing oligosaccharides were only observed upon the addition of GDPM to the incubation medium due to the increased amount of ^{14}C-polysaccharide formed, and the increased proportion of mannose present in the products. On the basis of the evidence presented above, it is now widely thought that glucomannan, not cellulose, is the product from GDPG regardless of the presence or absence of GDPM in the incubation.

More recently the role of at least the golgi-associated UDPG: β (1-4) glucan glucosyl-transferase in cellulose biosynthesis has been questioned. Villemez & Hinmann (1975)

and Ray (1975, 1980) demonstrated that UDPG stimulated the incorporation of radioactivity from UDP-^{14}C-xylose into polysaccharide. Structural analysis of the reaction products indicated that the product was xyloglucan (Ray, 1980), see Fig. 8.

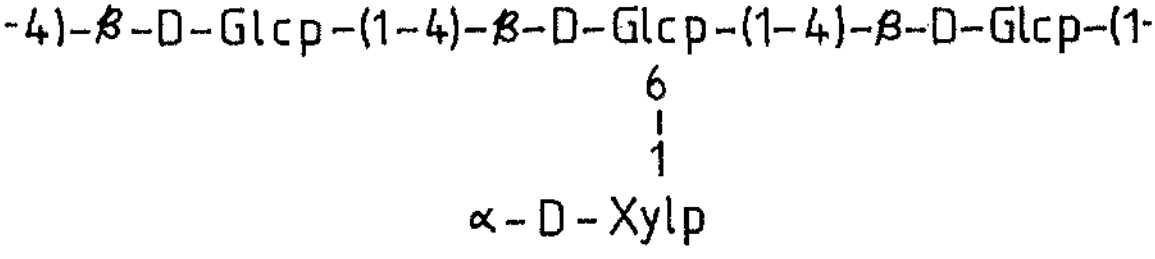


Fig. 8 Xyloglucan

Thus, it is possible that this is the role of the golgi associated UDPG: β(1-4) glucan glucosyl-transferase activities demonstrated *in vitro*.

9) Soluble factors found in tissue homogenates. Soluble factors in tissue homogenates have been found to modulate the activities of β-glucan synthetases. Ordin & Hall (1968) found a heat-stable factor which stimulated the synthesis of β(1-4) relative to β(1-3) glucan in tissue homogenates of *A. sativa*. Chao & Maclachlan (1978) demonstrated the existence of heat-stable, dialysable and a heat-labile, non-dialysable components from extracts of *P. sativum*. Both components primarily affected the amount of β(1-4) rather than β(1-3) glucan in the products. The heat-stable, dialysable component stimulated the production of β(1-4) glucan. The heat-labile, non-dialysable suppressing factor appeared to be a protease. Recently, Aloni *et al* (1982) found a stimulatory protein

factor for β(1-4) glucan synthesis in the supernatant fraction from cell-free prepearations of *A. xylinum*. When GTP, PEG and the protein factor were included in the incubation, *in vitro* rates of β(1-4) glucan synthesis from UDP^{14}C-G were obtained that exceeded 40% of the *in vivo* rates of cellulose synthesis.

10) The effect of membrane potential. Carpita & Delmer, (1980) presented evidence that a membrane potential across the plasmamembrane of detached cotton fibres (see p36) was required for cellulose biosynthesis to occur. Using ionophores, Bacic & Delmer (1981) studied the effect of artificially-induced electrical membrane potentials on β-glucan synthesis in particulate enzyme preparations from *G. hirsutum*. It was found that a membrane potential positive with respect to the inside of membrane vesicles stimulated the synthesis of both β(1-3) and β(1-4) glucan from UDPG. It was shown that this was indeed due to an electrical membrane potential and not a pH gradient. However, the β(1-4) glucan formed was not highly crystalline since it was solubilised by the acetic-nitric acid reagent of Updegraff (1969) (see Section 6.1.)

11) Solubilisation of β-glucan synthetases by detergent. The detergent most commonly used is digitonin, in concentrations ranging from 0.66%-8%. UDPG:β(1-3), UDPG:β(1-4) & GDPG:β(1-4) glucan glucosyl-transferases have all been solubilised, and in some cases separated. Tsai & Hassid (1971) solubilised UDPG: β(1-3) and UDPG: β(1-4) glucosyl-transferase activity by extraction of particles from *A. sativa* in 8% digitonin. Flowers *et al* (1968, 1969) found that the activity of UDPG:β(1-3) glucan glucosyl-transferase was greatly increased by digitonin extraction, while GDPG: β(1-4) glucan glucosyl-transferase was unaffected. Chambers & Elbein (1970) found that UDPG: β(1-3) and GDPG:β(1-4) glucan glucosyl-transferase from *P. aureus* could largely be separated on the basis of their differential solubilities in 0.8% digitonin - 0.5% BSA. In

fact, the UDPG: β(1-3) glucan glucosyl-transferase could be partially solubilised in 0.5% BSA alone, suggesting a loose attachment of the enzyme to the membrane. Solubilisation of the glucan synthetases from *L. albus* by 0.66% digitonin - 0.33% BSA resulted in an increase in the ratio of β(1-4) glucan to total glucan formed from UDPG (Stafford & Brummond, 1970). Lui & Hassid (1970) claimed to have obtained a solubilised GDPG: β(1-4) glucan glucosyl-transferase by extraction of particles from *P. aureus* in 1.6% digitonin - 0.05 M sucrose in water. However, it was subsequently shown that the enzyme was not properly soluble since, after the enzyme was concentrated by $(NH_4)_2SO_4$ precipitation it could be centrifuged down at 200,000 g for 2 hours (Barber, 1982). Heller & Villemez, (1972 a,b) also noted that under the conditions of Lui & Hassid (1970), only trace amounts of mannosyl-transferase activity were solubilised. These authors solubilised both GDPG and GDPM utilising glycosyl-transferase activities from particles of *P. aureus*, by extraction with 0.5% Triton X-100 (Heller & Villemez, 1972 a,b).

In conclusion, it is clear that the conditions required for *in vitro* β-glucan synthesis are determined by a number of factors, mostly concerned with the preservation of activity during isolation, and also activation of the enzymes in the incubation medium.

C.1.2.2. The use of more intact systems to investigate the sugar-nucleotide precursors of cellulose.

In the more recent investigations on cellulose biosynthesis there has been a tendency to use more intact systems, in which the integrity of the cellulose synthetase complex may be better preserved than in cell-free systems. Possible problems with the use of the cell-free systems described previously are outlined below:-

1) Disturbance of the cellulose synthetase complex. Cellulose biosynthesis is thought to occur at the plasma-membrane, mediated by large synthetase complexes which may have been visualised by electron microscopy (see pp. 62-65). It is quite possible that upon homogenisation of the tissue, the structural integrity of the complex may be destroyed, or that the association of the complex with the microfibril which may be required as a primer (or any other priming agent) is disrupted.

2) Turgor changes. There are reports that cellulose synthesis may be very sensitive to alterations in turgor (Ray, 1973; Delmer, 1977). Carpita & Delmer (1980) demonstrated that by circumventing high turgor loss in detached cotton fibres by the application of a non-cell-wall-penetrating osmoticum (PEG 4000), the loss of synthetic activity upon detachment of the fibre from the ovule was prevented. However, not all of the protection afforded by the addition of PEG 4000 was due to the reduction of turgor prior to detachment, since mannitol at equal osmotic concentrations only protected 25% of the activity relative to the intact controls (see p. 36).

3) Membrane asymmetry. Membranes are regarded as aysmmetric structures, particularly the plasma-membrane (Robinson & Quader, 1981). As a result vesicles formed from cellular membranes may have an orientation reflecting the *in vivo* situation (i.e. outside-out), or they may be inverted (inside-out). In terms of substrate accessibility, inside-out vesicles would be preferable since sugar-nucleotides penetrate membranes only slowly (Depierre & Dallinger, 1975). In plants it is not clear which orientation the vesicles formed take. However, in some animal systems the plasma-membrane vesicles have been shown to be outside-out (Robinson & Quader, 1981). If this was the case in cell-free preparations from higher plants, little *in vitro* cellulose synthesis could be expected from exogenous sugar-nucleotide substrates,

given that cellulose is thought to be synthesised at the plasma-membrane (see Section D.).

4) Interaction with cytoskeleton. There is some evidence that the organisation of cellulose microfibril deposition is achieved via the participation of cortical microtubules which underly the plasma-membrane (Hepler & Palevitz, 1974). Upon homogenisation this association will be lost, which could result in the loss of synthetic activity.

5) Release of inhibitors upon homogenisation. Homogenisation destroys cellular compartmentation and as a result, substances whose actions on particular processes were previously negligible may no longer be so. Chao & Maclachlan (1978) found such an inhibitor in preparations from *P. sativum* (see p. 26). It is also possible that plant extracts may contain enzymes which degrade either the substrates or products of a reaction.

6) Loss of membrane potential. Bacic & Delmer (1981) have shown that β(1-3) and β(1-4) glucan synthetase activities from *G. hirsutum* are affected by electrical membrane potentials. Thus, homogenisation may result in inhibitory membrane potentials in the vicinity of the synthetase.

C.1.2.2.1. Investigations into the sugar-nucleotide precursors of cellulose biosynthesis using tissue-slices.

This system may be more favourable for the detection of cellulose synthetase, since the slicing of a cell may be less disruptive than a more thorough homogenisation. It has been claimed that *P. sativum* epicotyl tissue-slices can synthesise β-glucan from extracellular UDPG by highly active cell-surface synthetases (Shore & Maclachlan, 1975; Shore *et al*, 1975). These authors claimed that β(1-4) linked alkali-insoluble glucan was synthesised when tissue slices were

incubated with 600 μM UDPG. The synthetase activities associated with the slices approached that of *in vivo* cellulose deposition rates and were 20-30 times more active than particulate enzyme preparations. However, it is now thought that β(1-3) glucan, not cellulose, is the product synthesised by the UDPG-utilising cell-surface synthetases (see Section C.1.2.2.4. , p. 33). High cell-surface activity was not observed with GDPG as a substrate, although tissues slice synthetase activity was as much as 3 times greater than that of particulate enzymes with this substrate.

C.1.2.2.2. The location of synthetic activity

Anderson & Ray (1978) demonstrated that this UDPG-utilising cell-surface β-glucan synthetase activity is associated with the plasma-membrane. Their evidence for this was based on a comparison of the sub-cellular distribution of synthetic activity with membrane marker enzymes, and an analysis of the lipid content of the membranes.

It was further shown that UDPG was being used directly, and not by breakdown to glucose followed by uptake into the cells, since the isopycnic density distribution of *in vivo* incorporated particle bound radioactivity from UDP-^{14}C-G was entirely different from that obtained by feeding with ^{14}C-glucose in that there was no indication of the incorporation of radioactivity into the golgi system from UDP-^{14}C-G. It was argued from these results that the UDPG: glucan glucosyl-transferase activity was located at the external surface of intact cells and was not due to utilisation of UDP-^{14}C-G by the damaged cells at the cut surfaces of the slices. Raymond *et al* (1978) investigated in more detail the location of the plasma-membrane UDPG: glucan glucosyl-transferase activity within the tissue-slices. It was shown by these workers that the initial rate of glucosyl transfer from 5 mM UDPG was directly proportional to the number of slices

into which a pea epicotyl segment was cut. There was little, if any, lag period for the synthesis of alkali-insoluble material. Thus, it was concluded that the UDP-^{14}C-G was not diffusing more than a few cell-layers into the slices. The tissue-slices were plasmolysed in hypertonic, non-ionic osmotica which have been shown not to inhibit synthetase activities *in vitro*, although under these circumstances, the plasma-membrane retracts from the wall and the deposition of wall material is inhibited (Robinson & Cummings, 1976). No inhibition of the UDPG: glucan synthetase activity was observed, implying that the plasma-membrane adhering to the walls of cut cells is the probable location of synthetic activity. Further indirect evidence for this conclusion comes from the work of Brett (1978) on soybean suspension-cultured cells, and by Carpita & Delmer (1980) on detached cotton fibres, where it has been shown that the plasma-membrane must be ruptured before extracellular UDPG could act as a substrate for β-glucan synthesis. Recently, Maclachlan (1982) has confirmed that it is indeed the cut cells at the surface of the slices that are responsible for β-glucan synthesis from extracellular UDPG. When tissue-slices were floated in solutions containing UDP-^{3}H-G and subsequently subjected to autoradiography, radioactivity was associated only with the cut cells at the slice surface. Radioautographs at the electron microscopic level further demonstrated that synthesis was confined to regions where the plasma-membrane remains in contact with the cell wall (Mueller & Maclachlan, 1980).

C.1.2.2.3. Properties of the UDPG-utilising enzyme system.

The enzyme(s) utilising UDPG in tissue-slices appears to be substrate-activated, requiring very high substrate levels (mM range) to saturate the system. The kinetic parameters of the slice system (pH, Mg^{2+} optimum, apparent K_m) were found to be similar to those of the less active particulate preparations from the same plant (Raymond *et al*, 1978).

C.1.2.2.4. Nature of the reaction products

It was initially thought that β(1-4) glucan was the primary product from UDPG in the tissue slice system (Shore & Maclachlan, 1975; Shore *et al*, 1975). However, subsequent workers have shown that the majority of the product is β(1-3) glucan (Anderson & Ray, 1978; Raymond *et al*, 1978; Heineger & Delmer, 1977). Brett (1981a) demonstrated that GDPG could act as a substrate for the synthesis of alkali-insoluble material containing β(1-4) linked glucose. As in particulate enzyme systems, with low concentrations of GDPG (1 μM) the reaction proceeded for only a few minutes and could be extended by the addition of GDPM, implicating the synthesis of glucomannan (see p. 23). With 50 μM GDPG, GDPM could still stimulate synthesis of product but the reaction from GDPG alone could continue for up to 2 hours. This would suggest that pure β(1-4) glucan is being produced since endogenous sources of mannose required for glucomannan synthesis should be used up, at least in the latter period of the incubation. Indeed, a greater proportion of the products from the higher concentration of GDPG resembled cellulose in terms of its insolubility in 4.4 N NaOH and increased M.W.

C.1.2.3. Investigations into the sugar-nucleotide precursors of cellulose biosynthesis using the detached cotton fibre system.

In the form of a single-celled epidermal hair (fibre), the cotton plant produces the purest form of cellulose known. The cotton fibre system offers the major advantage that the primary and secondary phases of wall deposition can be studied independently, since they are separated in time. This cannot be done with whole plant organs or even single tissue types (Delmer *et al*, 1974). Marx-Figini (1966) and Marx-Figini & Schulz (1966) studied the synthesis of primary

and secondary wall cellulose from a kinetic point of view. It was found that primary and secondary wall cellulose were distinct in terms of their rates of synthesis. The primary cellulose had a non-uniform DP and its synthesis was slow, while secondary cellulose had a homogeneous DP (14,000) and was synthesised more rapidly. It was proposed that primary and secondary wall cellulose were synthesised by two distinct mechanisms and that the synthesis of β(1-4) glucan from GDPG demonstrated in particulate preparations from cotton by Barber & Hassid (1965) may represent primary wall cellulose synthesis, while secondary wall cellulose may proceed via another enzyme system which may require a template to produce the homogenous DP. That the enzyme for secondary wall cellulose synthesis might utilize UDPG as a substrate was indicated by Franz & Meier (1969), who demonstrated that radioactivity from UDP-^{14}C-G could be incorporated into ethanol-4N NaOH insoluble material in this system. The product resembled cellulose in that it yielded cellobiose upon partial acid hydrolysis. The capacity for the synthesis of product from UDPG increased with increasing age of the fibre, and was very high in older fibres where secondary wall cellulose deposition was actively occurring. Delmer and co-workers investigated this model for cellulose biosynthesis in detached cotton fibres in detail. GDPG was found to be active as a substrate only during the period of primary wall synthesis. The activity of particulate preparations from detached fibres of increasing age followed a similar pattern (Delmer *et al*, 1974). The incorporation into hot-alkali-insoluble product from UDPG followed the reverse pattern, lowest during primary wall deposition and steadily increasing during secondary wall synthesis. Analysis of the products revealed that GDPG acted as a substrate for alkali-insoluble material containing β(1-4) linked glucose which was insoluble after extraction in the acetic-nitric acid reagent of Updegraff (1969). However, it was shown that 90% of the radioactivity incorporated from UDP-^{14}C-G into hot-alkali-insoluble material was acetylated and non-acetylated steryl-glucosides (Delmer *et al*, 1974;

Delmer, 1977). Since these molecules do not exhibit turnover and are not good donors of glucose, they are not considered to be involved in cellulose biosynthesis. However, traces of glucosyl-phosphoryl-polyprenol of the type reported by Forsee & Elbein (1972, 1973) (see p 48) were occasionally found (Delmer, 1977). Thus, it is still possible that UDPG could have been acting as a substrate for secondary wall cellulose synthesis in this system if the glucosyl-phosphoryl-polyprenols had very low steady-state levels with a rapid turnover.

There is now considerable evidence presented below, against a model utilising GDPG and UDPG for primary and secondary cellulose synthesis respectively.

1) Incorporation from GDPG into the cellulosic product is stimulated by GDPM, both in detached fibres (Delmer, 1977), and particulate enzyme preparations (Delmer *et al*, 1974). This suggests the synthesis of glucomannan (see p.23) from GDPG rather than cellulose.

2) The low levels or absence of GDPG in plant tissues actively synthesising cellulose (Villemez & Heller, 1970).

3) Coumarin and 2,6 dichlorobenzonitrile (DCBN) are purportedly selective inhibitors of cellulose biosynthesis *in vivo* (Hara *et al*, 1973; Hogetsu *et al*, 1974a). Both compounds were found to inhibit specifically the incorporation of radioactivity from ^{14}C-glucose into the cellulose fraction of cotton fibres cultured *in vitro*. Coumarin (1 mM) was equally effective in inhibiting cellulose synthesis in cultured ovules synthesising primary wall cellulose and those synthesising secondary wall cellulose. This suggests that primary and secondary wall cellulose biosynthesis share a common coumarin-sensitive step in the pathway, implying one rather than two pathways (Montezinos & Delmer, 1980). However, since the mode of action of these inhibitors is unknown, it is possible that they may be acting before a divergence in the pathways.

4) Delmer and co-workers have shown that the polysaccharide products from UDPG are β(1-3) not β(1-4) linked (i.e. definitely not cellulose) in the detached fibre system. With 0.5 μM UDPG, the vast majority of the products were sucrose or steryl glucosides. However, by increasing UDPG concentration to 1 mM the synthesis of an insoluble glucan could be demonstrated. It was shown that this UDPG utilising system was substrate-activated. It was subsequently shown that the product from UDPG was a linear β(1-3) glucan regardless of UDPG concentration (Heineger & Delmer, 1977) and that it was a natural constituent of the fibre cell wall which was deposited at the onset of secondary wall deposition (Maltby *et al*, 1979).

Thus, from the evidence given in 4) above it would seem that UDPG acts as a precursor for the synthesis of a β(1-3) glucan rather than cellulose in the detached fibre system. GDPG could be the glucosyl donor for primary wall cellulose synthesis although points 1) and 2) do shed considerable doubt on this hypothesis.

Carpita & Delmer (1980) studied the synthesis of cellulose from ^{14}C-glucose in detached cotton fibres. It was found that detachment of the fibres from the ovule resulted in the loss of 90% of the *in vivo* capacity for cellulose synthesis. However, over 50% of the capacity for cellulose synthesis in the detached fibre population was protected when PEG 4000 (0.06molal) was included in the detachment and incubation media. It was argued that this effect of PEG 4000 was due to resealing of the plasma-membrane and that in the presence of PEG 4000 there were two populations of cells, 50% of which could synthesis cellulose from ^{14}C-glucose at rates comparable to intact controls, and 50% of which could not synthesise cellulose at all as judged by radioautography. Since all fibres detached in the presence of PEG 4000 were capable of synthesising polysaccharide from ^{14}C-glucose, and therefore both populations retained adequate energy

supplies for polysaccharide synthesis from glucose, it was proposed that there was an additional requirement for those fibres that retained the capacity for cellulose synthesis. It was hypothesised that a threshold membrane potential was required for cellulose synthesis to occur and subsequently, Bacic & Delmer (1981) demonstrated that particulate $\beta(1\text{-}4)$ and $\beta(1\text{-}3)$ glucan synthetases utilising UDPG were stimulated by a positive potential with respect to the inside of vesicles (see p. 27).

Thus a paradox may exist in the detached cotton fibre system (and perhaps others) in that, while membrane integrity must be disrupted to allow access of an impermeant sugar-nucleotide substrate to the site of synthesis, such disruption destroys the catalytic activity of the cellulose synthetase complex.

C.1.2.4 Investigations into the sugar-nucleotide precursors of cellulose biosynthesis using suspension-cultured cells.

Brett (1978) studied the synthesis of β-glucan in suspension-cultured soybean cells (*Glycine max*) from extracellularly supplied UDPG. The product was shown to be a $\beta(1\text{-}3)$ glucan and was synthesised at a low rate in shaken incubations. No evidence for the synthesis of $\beta(1\text{-}4)$ glucan from UDPG was obtained. $\beta(1\text{-}3)$ glucan synthesis was stimulated thirty-fold when the incubation was stirred with a glass rod, a procedure which was shown to damage some of the cells, indicating that $\beta(1\text{-}3)$ glucan production was a wound response.

C.1.2.5 *In vivo* studies on the sugar-nucleotide precursors of cellulose biosynthesis.

In vivo studies on cellulose biosynthesis have centred

upon the incubation of the system in use with a radioactive source of carbon ($^{14}CO_2$, ^{14}C-glucose, ^{14}C-sucrose) and subsequent observation of the kinetics of the incorporation of radioactivity into possible precursors and cell wall components. In some of these studies, use has been made of the purportedly specific cellulose biosynthesis inhibitors, DCBN and coumarin. These inhibitors have been applied *in vivo* to seedlings (Hara *et al*, 1973), Azuki bean epicotyl sections (Hogetsu *et al*, 1974a,.) and cotton fibres (Montezinos & Delmer, 1980). They were shown to inhibit cellulose biosynthesis without significantly affecting non-cellulosic polysaccharide biosynthesis. Thus these inhibitors may prove useful in further *in vivo* studies since the steady-state levels of precursors to cellulose would be expected to change upon the addition of such an inhibitor.

Carpita & Delmer (1981) investigated the concentration and metabolic turnover of UDPG in developing cotton fibres *in vivo*. UDPG was the major sugar nucleotide found, with steady-state levels of 0.2 µmol/boll at 13 days post anthesis (DPA) which increased to over 2.1 µmol/boll by 24 DPA, just prior to the maximum rate of secondary wall cellulose and β(1-3) glucan synthesis. The UDPG levels dropped precipitously at the time when cellulose synthesis ceased. Subsequently, fibres cultured *in vitro* with their ovules (undetached), were pulse-labelled with ^{14}C-glucose. UDPG was the predominant sugar-nucleotide to become labelled. Measurements were made of pool sizes and the rates of labelling of glucose, G-1-P and UDPG. These data were analysed using a computer simulation model and it was shown that the rate of UDPG synthesis and turnover was more than sufficient to account for the combined rates of synthesis of sucrose, steryl-glucosides, β(1-3) glucan and cellulose. The above work of Carpita & Delmer is strong *in vivo* evidence that UDPG is a precursor for cellulose biosynthesis in the cotton fibre.

C.1.2.6. Conclusions on the sugar-nucleotide precursors of cellulose biosynthesis.

From the previous sections, it is clear that both UDPG and GDPG can be utilised for the synthesis of polysaccharide containing $\beta(1-4)$ linked glucose, and there is strong *in vivo* evidence for the involvement of UDPG in cellulose biosynthesis. The fact that they have been shown to be involved in the synthesis of non-cellulosic polysaccharides in no way rules them out as precursors of cellulose. UDPG and the enzymes by which it is synthesised are ubiquitous in higher plants. However, this is of no great significance since UDPG is an important intermediate in a number of metabolic pathways other than cellulose biosynthesis. GDPG has been formed from GTP and glucose-1-phosphate by enzyme extracts from peas, mung bean, spinach , buckwheat and parsley (Barber & Hassid, 1964; Peaud-Lenoël & Axelos, 1968; Delmer, 1977). The fact that the capacity for GDPG synthesis in plant extracts is often only found with difficulty or not at all (Delmer, 1972 ; Delmer, 1977; Heineger & Franz, 1980) does not exclude GDPG as a precursor, since the enzymes by which it is synthesised may be extremely labile. It is also possible that GDPG may be synthesised by an as yet undiscovered pathway. However, GDPG itself does not appear to be present in appreciable quantities in the plants investigated (Elnaghy & Nordin, 1966; Boothby, 1972; Carpita & Delmer, 1981). It has been postulated that this may be due to rapid utilisation and effecient feedback control of its synthesis resulting in very low steady-state levels (Nikaido & Hassid, 1971).

The possibility of a molecule other than a sugar-nucleotide acting as a precursor for cellulose biosynthesis must not entirely be ruled out. Sucrose and the raffinose oligosaccharide series are efficient donors of glucose and are therefore possible precursors. Low energy glucose donors may also be possible precursors since, although the equilibrium

of glycosidase-catalysed reactions favours hydrolysis, *in vivo* factors such as localised high substrate concentrations or rapid utilisation of products, may induce polysaccharide formation by this route.

Thus the question of the initial glucosyl donor of cellulose biosynthesis is an open question. However, GDPG and UDPG must be regarded as the most promising candidates.

C.1.3. The initial acceptor of glucose

The overall reaction for glucan synthesis is now established to be of the form

$$n.NDPG \longrightarrow (Glc)_n + n.NDP$$

Each individual addition of glucose is therefore a reaction of the form

$$NDPG + (Glc)_x \longrightarrow NDP + (Glc)_{x+1}$$

However, these simple reaction schemes may be an oversimplification and conceal rather more complicated biosynthetic pathways, as has been shown to be the case in the synthesis of glycoproteins and bacterial exopolysaccharides. There is no information in the above reaction sheme as to how the glucan chains are initiated or whether there are any intermediate steps in the addition of the glucose units to the growing chain.

Very little is known of chain initiation in the β-glucan synthesis of plants. This is in contrast to the synthesis of the α-glucans, starch and glycogen, where it has been shown that synthesis occurs by transfer of a single monomer unit from a sugar-nucleotide to the non-reducing end of accessible chains of preformed α-glucan. The introduction of new non-reducing chain ends into the primer by partial

hydrolysis etc, often results in an enhanced priming capacity of the system (Maclachlan, 1982). Primers for polysaccharide synthesis may also include proteins and glycoproteins as the initial acceptors for glycosyl transfer. A protein primer has been found in the synthesis of starch $\alpha(1\text{-}4)$ glucan in potato tubers. The synthesis appears to be initiated by glucose transfer from UDPG to a protein and the glucoprotein then acts as an acceptor for the synthesis of the rest of the α-glucan chain (Lavintman & Cardini, 1973). Elongation of the glycoprotein by repeated transfer to the non-reducing end of the glycone could result in the protein components remaining at the core of the completed polysaccharide. Such a mechanism has been proposed for both starch and glycogen biosynthesis (Tandecarz & Cardini, 1978).

It is usually taken that β-glucan synthesis may require a primer in the same sense as starch or glycogen biosynthesis. However, added glucose and soluble glucosides have not been observed to be incorporated into β-glucan chains *in vitro*. The fact that detergent-solubilised enzymes are capable of synthesising β-glucan in the absence of any added glucan primer suggests that such external primers are not required for synthesis. Thus the acceptor for the first glucose residue of the β-glucan must be part of the enzyme-complex, or so tightly adsorbed by the enzyme that it is solubilised with the enzyme by the action of the detergent. This suggestion has a precedent in that it is often difficult to be certain that an apparently purified enzyme does not contain small but crucial amounts of tightly adsorbed primer polysaccharide (Hawker *et al*, 1974). However, if the primer of β-glucan synthesis is an integral part of the enzyme complex, then it is likely to be a protein, glycoprotein or a lipid.

Franz (1976) demonstrated that particulate preparations from *P. aureus* could use UDPG to synthesise a glycoprotein containing $\beta(1\text{-}4)$ linked glucose. Turnover experiments showed

that the glycoprotein possessed acceptor functions for the initiation and subsequent elongation of glucan chains. The addition of isolated non-radioactive glycoprotein to the incubation medium resulted in the enhanced synthesis of alkali-insoluble glucan. Brett & Northcote (1975) demonstrated the synthesis from UDPG of a substance containing β(1-3) and β(1-4) linked glucose oligosaccharides attached to or very strongly adsorbed to protein in membrane preparations from *P. sativum*. It was argued that the protein-linked oligosaccharides were involved in β-glucan synthesis. Pont-Lezica *et al* (1975) also summarised evidence for a glucoprotein containing β(1-4) linked oligoglucan in membranes from the same plant. Nowak-Ossaric (1976) found cellulose tightly bound to a protein fraction in cotton fibres. The cellulose contained a constant, though very small fraction of protein throughout fibre maturation. It was postulated that the protein was a residual part of the synthetic apparatus. In the Chlorophycean alga, *Prototheca zopfii*, Hopp *et al* (1978) observed the possible priming action of protein in the cellulose biosynthesis of this organism (see Section C.1.4.2.).

The observations above need further study and confirmation, particularly in the demonstration that the glucoproteins formed are actually involved in cellulose biosynthesis. However, they do open up the possibility of a priming role for a protein acceptor in β-glucan synthesis.

C.1.4. Intermediate steps in the formation of β-glucan.

Glucolipids, glucoproteins and short chain glucans have all been suggested as intermediates in cellulose biosynthesis. Studies with *A. xylinum* and green algae have provided more information on this question than have those on higher plants.

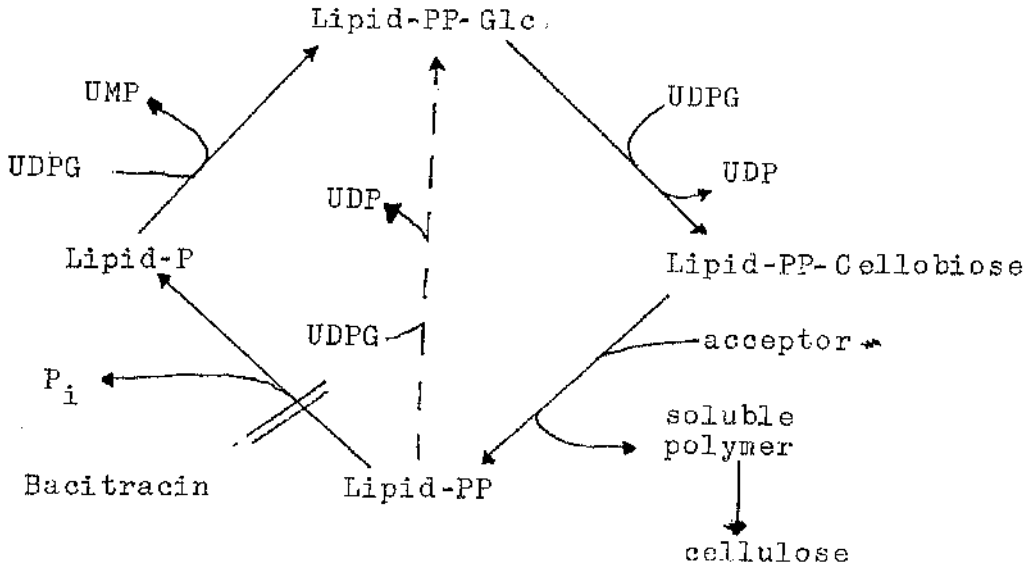


Fig. 9. Colvin's proposed scheme for cellulose biosynthesis in *A. xylinum* (After Colvin, 1980a)

– – – → proposed route of Aloni & Benziman (1982)

C.1.4.1. The case for glucolipid intermediates in *A. xylinum*

Colvin (1959) found that 80% ethanol extracts of *A. xylinum* cells contained a substance which was rapidly converted to cellulose microfibrils when placed in water. A glycolipid was isolated from the ethanol extract and was rapidly converted to cellulose when placed in an aqueous medium containing the extracellular *Acetobacter* enzyme (Khan & Colvin, 1961). A similar extract from *A. sativa* and *P. sativum* seedlings formed microfibrillar material when incubated in a medium containing the extracellular bacterial enzyme (Colvin, 1961). The authors suggested that the above result indicated that either the substrates in higher plants and the bacteria were identical and were thus both glycolipids, or that specificity was restricted to the glucose part of the molecule. It was not possible to replace the *Acetobacter* enzyme with an aqueous extract of peas. This is not unexpected however, since β-glucan synthetases in plants are not soluble in water. Garcia *et al* (1974), using E D T A - treated cells which were subsequently frozen and thawed, further investigated the possibility of glucolipid intermediates. The authors assayed the incorporation of radioactivity from UDP-^{14}C-G and UDP-^{14}C-galactose into the butanol-soluble products which were shown to contain radioactively labelled lipid diphosphate-α-glucose, lipid diphosphate-α-cellobiose and lipid monophosphate-β-galactose. The time-courses of formation of the lipid diphosphate -α-glucose and lipid-diphosphate-α-cellobiose indicated that they rapidly reached plateaus which could be interpreted as steady-state levels, while the incorporation of radioactivity into the total products continuously increased, suggesting but not proving that they may be intermediates. The nature of the lipid moieties was not clearly established although they were probably prenols. Kjosbakken & Colvin (1973) obtained similar results with cell-free extracts from the bacteria. This system formed lipid pyrophosphate compounds, which were attached to glucose, cellobiose and possibly cellotriose

and cellotetraose. The kinetics of formation of these compounds suggested that they were synthesised prior to the formation of the final cellulosic product and UDP and UMP were found to be potent inhibitors of both lipid carrier and cellulose formation. However bacitracin, which in other systems inhibits the dephosphorylation of lipid diphosphate to lipid phosphate, did not appear to inhibit cellulose synthesis. From the results of Kjosbakken & Colvin (1973) and Garcia *et al* (1974), Colvin (1980a) proposed the scheme shown in Fig. 9. It is interesting to note that in this scheme, cellobiose is the functional unit in cellulose biosynthesis, since for steric reasons it is an attractive unit to act as a cellulose precursor (Delmer, 1977).

There remain a number of drawbacks to this scheme:-

1) Neither Garcia *et al* (1974) nor Kjosbakken & Colvin (1973) demonstrated that the purported glucolipid intermediates had a precursor-product relationship with cellulose. Garcia *et al* (1974) did note however, that in E D T A treated cells from *A. xylinum* mutants lacking cellulose, the glucolipids were not synthesised. Also, Aloni & Benziman (1982) demonstrated that coumarin and DCBN inhibited the formation of glucolipids *in vivo* and *in vitro*. These two latter points add further indirect evidence for a role of glucolipid intermediates in cellulose biosynthesis in *A. xylinum*.

2) Couso *et al* (1980) found that the lipid-diphosphate-cellobiose formed by the system used by Garcia *et al* (1974) is in fact a precursor for a lipid trisaccharide identified as β-mannosyl (1-3) β-cellobiosyl-1-diphosphate prenol, with UDPG and GDPM acting as glycosyl donors. Further doubt is shed upon the results of Garcia *et al* (1974) and Kjosbakken & Colvin (1973) in that Sanderman (1977), using the same methods as these groups, identified the disaccharide attached to lipid as maltose, not cellobiose.

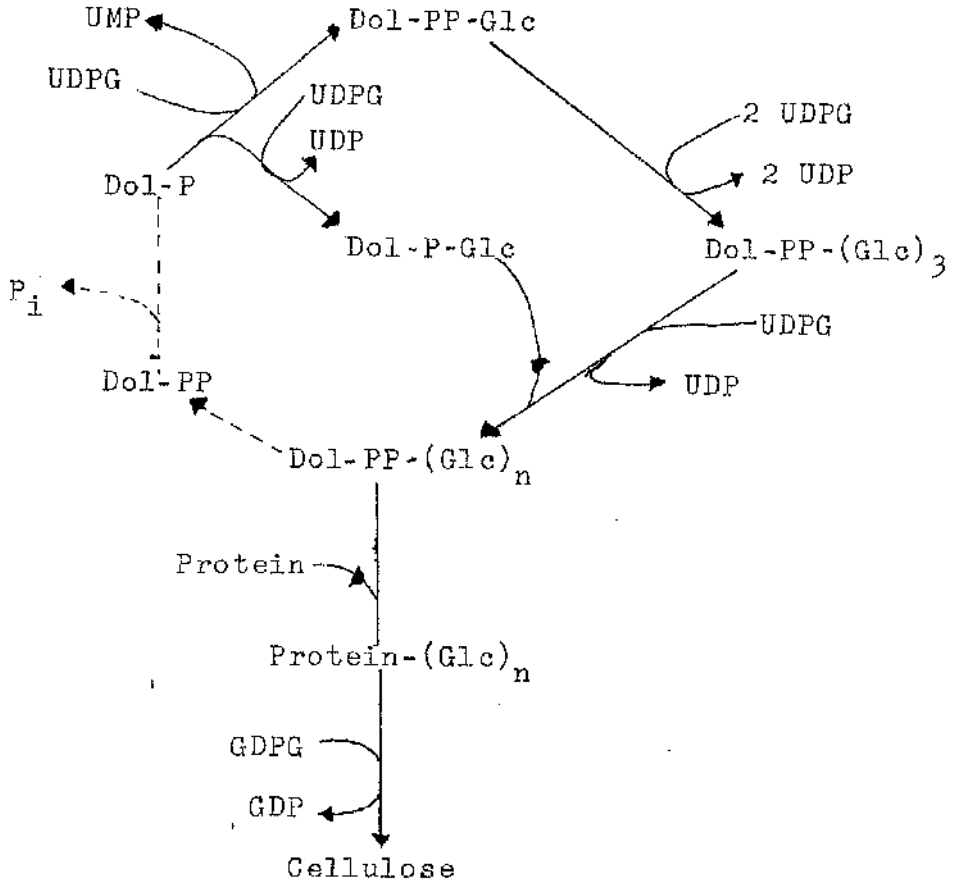


Fig. 10. Proposed scheme for the synthesis of glucoprotein and cellulose in *P. zopfii*. Dotted lines indicate reactions not detected but which probably occur. From Hopp *et al*, 1978.

3) Aloni & Benziman (1982) reported that UDP was a far more potent inhibitor than UMP of the glucosylation of lipid-soluble materials from UDPG *in vitro*. Indeed, the inhibitory capacity of UDP + UMP did not exceed that of UMP alone. This is in contrast to the results of Kjosbakken & Colvin (1973) and would suggest that UDP is the product of the transfer reaction. Thus, a non-phosphorylated glucose moiety must be transferred from UDPG to the acceptor in Aloni & Benziman's system. This would explain the lack of inhibition of cellulose synthesis by bacitracin observed by Kjosbakken & Colvin (1973) (see Fig. 9).

C.1.4.2. Evidence for glucolipid intermediates in algae and higher plants.

In particulate preparations from the Chlorophycean alga *Prototheca zopfii*, Hopp *et al* (1978) demonstrated the synthesis of lipid-phosphate-glucose, lipid-diphosphate-glucose and lipid diphosphate $\beta(1\text{-}4)$ oligoglucan (Glc_n, n = 2-10). UDP and UMP were found to inhibit glucolipid synthesis and the lipid moiety was thought to have the properties of dolichol. The glucolipids were shown to be precursors for a water-soluble glucoprotein, containing $\beta(1\text{-}4)$ linked glucose. Upon the addition of GDPG, the water-soluble polymer became insoluble in hot-alkali and had cellulosic properties. A reaction scheme for cellulose biosynthesis in this organism was constructed (see Fig. 10).

The above work by Hopp *et al* (1978) is extremely important and probably the best evidence for these intermediates, since precursor-product relationships were established between the glucolipids, glucoprotein and alkali-insoluble residue. However, a more rigorous demonstration that the alkali-insoluble product is indeed cellulose would be desirable.

The role of glucolipids in higher plant cellulose biosynthesis is unclear. Forsee & Elbein (1972, 1973) obtained a particulate enzyme preparation from cotton fibres which catalysed the incorporation of glucose from UDPG into an acidic glycolipid, the lipid portion of which was shown to be a phosphoryl polyprenol. The glycolipid was acid-labile and its synthesis was reversible. Thus the "high sugar transfer potential" of the sugar-nucleotide is retained in the glycolipid, which would therefore be able to donate the sugar residue to another acceptor and act as an intermediate. Delmer *et al* (1974) also demonstrated the synthesis of glucolipid material from UDPG in detached cotton fibres. The synthesis of the glucolipid material closely paralleled that of secondary wall cellulose deposition. However, it was shown that 95% of this material was steryl glucoside, which is not thought to be involved in cellulose biosynthesis (Delmer, 1977). Trace amounts of an acidic glycolipid that resembled that found by Forsee & Elbein (1973) were occasionally found. Brett & Northcote (1975) observed the synthesis from UDPG of polyprenol-like-lipid linked via phosphodiester or pyrophosphate bonds to glucose oligosaccharides containing β (1-3) and β (1-4) links in membrane preparations from *P. sativum* roots. It was proposed that they may be intermediates in β-glucan synthesis on the basis of the distribution of enzymic activities and the chemical properties of the glucolipids. The authors failed however, to demonstrate a precursor-product relationship between the glucolipids and β-glucan (Brett, personal communication). Other studies have also failed to obtain evidence for the participation of glycolipids during polysaccharide biosynthesis (Barber *et al*, 1964; Storm & Hassid, 1972; Helsper, 1979).

C.1.4.3. The evidence for glycoprotein intermediates

Glycoproteins that may be involved as intermediates in β-glucan synthesis have been reported in higher plants (see

pp41-42 for refs), algae (Hopp *et al*, 1978) and bacteria (Aloni & Benziman, 1982). In the case of the bacterial glycoprotein, *in vivo* pulse-chase studies have demonstrated a precursor-product relationship between a glycoprotein which contained β(1-4) linked glucose, and cellulose. No such relationship was demonstrated for the glycosylated lipid in this system.

C.1.4.4. Evidence for short-chain glucan intermediates

Colvin and co-workers have presented evidence for the involvement of a polymeric intermediate in the cellulose biosynthesis of *A. xylinum*. This will be discussed in Section C.2. pp 52-55). Mortimer (1967) obtained results suggesting the existence of a polymeric intermediate in the cellulose biosynthesis of beet and barley leaves. It was demonstrated that a trichloro-acetic acid-soluble glucosan that was readily hydrolysed by cellulase, became labelled from $^{14}CO_2$ during short term photosynthesis. Upon the withdrawal of $^{14}CO_2$, a slow transfer of radioactivity from the acid-soluble glucan to acid-insoluble residual cellulose was observed. Satoh *et al* (1976) also raised the possibility of a β(1-4) linked polymeric intermediate in the *in vivo* biosynthesis of cellulose in *P. aureus*. A cytoplasmic β(1-4) glucan was found to have a similar distribution to UDPG: glucan glucosyl-transferase activity after subcellular fractionation by differential centrifugation. The β(1-4) glucan turned over and coumarin inhibited the incorporation of radioactivity into the cytoplasmic β(1-4) glucan to the same extent as that into the cell wall cellulose of the seedling hypocotyl.

Using pulse-chase techniques in cotton fibres, β(1-3) glucan was found to have a high turnover. While radioactivity in β(1-3) glucan decreased after a pulse of ^{14}C-sucrose, radioactivity in cellulosic β(1-4) glucan increased (Meier

et al, 1981). From these results it was argued that β(1-3) glucan may be an intermediate in the synthesis of β(1-4) glucan possibly mediated by β (1-3) glucanases acting as transglucosylases. However, it must be noted that there was no evidence in the results presented by Meier *et al* for a lag-phase of incorporation into cellulose with respect to β(1-3) glucan synthesis, which thus sheds some doubt upon the conclusions drawn. Further evidence against the conclusions of Meier *et al* comes from the work of Maltby *et al* (1979), who failed to demonstrate turnover of β(1-3) glucan in a similar cotton fibre system. It is interesting to note however, that the β(1-3) glucan synthesis does coincide with the onset of secondary wall cellulose synthesis. Also Swissa *et al* (1980) found that radioactivity which is incorporated into *A. xylinum* cellulose, passes through water and alkali-insoluble fractions which contained primarily β (1-2) glucan.

C.1.4.5. General conclusions on intermediates in cellulose biosynthesis

The previous section has surveyed the evidence for intermediates beyond the sugar-nucleotides on the pathway to cellulose. The case for glucolipid intermediates must be regarded as unproven as the evidence stands. Although the kinetics of formation of these glucolipids are consistent with the idea that they are intermediates of a biosynthetic pathway, there is very little evidence to suggest that they are specifically involved in cellulose biosynthesis. Initially it was thought that the attachment of a saccharide moiety from an impermeant sugar-nucleotide to a lipid would facilitate passage of the molecule through membranes although this has not been proven to be the case. However, it must be borne in mind that glucolipid intermediates have been widely found in the synthesis of glycoproteins and bacterial cell

wall components.

The case for polymeric and glycoprotein intermediates is as yet also unresolved, and requires further investigation. There are a number of strong arguments against polymeric intermediates in cellulose biosynthesis (see Section C.2. pp 54-55). In view of the fact that Aloni & Benziman (1982) have recently clearly demonstrated a precursor-product relationship between a glucoprotein containing $\beta(1-4)$ linked glucose, and cellulose in *in vivo* pulse-chase studies on *A. xylinum*, the case for a glucoprotein intermediate is reinforced, particularly since it is possible that they may be functionally analogous to the proposed glucoprotein precursor of α-glucan synthesis.

C.2. The assembly of the cellulose microfibril

The biosynthesis of cellulose poses an additional problem over and above the synthesis of the $\beta(1-4)$ glucan chains of which it consists. This is the formation of the morphological entity, the paracrystalline microfibril with the cellulose I structure. No *in vitro* system to date has been able to produce this.

Various hypotheses have been suggested to explain the formation of cellulose I microfibrils. Synthetic fibres spun from solution, or extruded from melt and subsequently drawn by several times their initial length, reveal a fibrillar texture with longitudinally running chains and cellulose microfibrils might be biosynthesised by a similar mechanism. However, fibrillar material produced by the above means consist of folded molecules with a predominantly uniform period. Since present data strongly suggest that native cellulose has an extended chain structure (see pp5-9), spinning or extruding as a potential fibrillar growth

mechanism is unlikely (Stockman, 1972). The following crystallisation conditions are known to produce extended chain structures.

a) Elevated hydrostatic pressure:- application of 7000 bars during melt crystallization of polyethylene produces extended crystals 100,000 Å long. However, since cell turgor pressure is more than two orders of magnitude lower than that applied above, high pressure cannot be involved in cellulose biosynthesis (Stockman, 1972).

b) Shear stress:- mechanical agitation, such as rapid stirring during solution crystallization, generates fibrous crystals with extended macromolecules. However, although it is known that the cytoplasmic solution inside the protoplast is in a state of continuous motion and streaming, there is no evidence of microfibril synthesis in the cytoplasmic solution (see pp66-69). Thus shear stress as a mechanism must be dismissed.

c) Crystallization with polymerization:- there are three possible courses leading from activated units to a crystalline fibril. These differ in the timing of crystallization and polymerization.

c.i) Successive polymerization and crystallization. Colvin and co-workers have postulated a two stage synthesis of cellulose microfibrils in *A. xylinum*.

Stage 1 - the synthesis of a soluble polyglucosan intermediate

Stage 2 - subsequent extracellular association of these polyglucans to form a nascent microfibril which forms a consilidated microfibril on elimination of water.

Kjosbakken & Colvin (1975) using sucrose-density gradient centrifugation of cell-free particulate enzymes which had been incubated with UDPG, demonstrated the presence of a transient insoluble polymer(s) of glucose, covalently attached to the particles. It was removed from the particles by treatment with alkali and could form short segments of microfibril when precipitated from alkaline solution by ethanol. The authors claimed that this polyglucosan was an intermediate in cellulose synthesis. A portion of the polymer(s) was isolated and shown by electron microscopy to have a length corresponding to a D.P. of 180. It was also shown that if a borate-soluble $\beta(1\text{-}4)$ glucan synthesised from UDP-^{14}C-G was isolated and added to a standard enzyme preparation containing non-radioactive UDPG, it became alkali-insoluble after two hours (King & Colvin, 1976). It was further reported that the proposed intermediate polymers emerged in a roughly linear fashion from a polar, funnel-shaped opening in the wall of the cell (Colvin *et al*, 1977a; Colvin & Leppard, 1977). In aqueous suspension, the emergent filament of glucan associated spontaneously to form what was called a "nascent" microfibril. The "nascent" microfibrils were highly hydrated and were up to 100 nm in breadth, with a dense core surrounded by an amorphous sheath. With the progressive removal of water, the core became denser and upon drying they formed a normal consolidated microfibril (Leppard *et al*, 1975; Colvin & Leppard, 1977). Occasionally, occluded granules from the medium and associated swellings in "nascent" microfibrils were observed. It was argued from this that tip synthesis of the microfibril could not occur, since this mechanism would push the granules aside rather than enclose them. Thus it was postulated that the granules were trapped by the association of preformed macromolecules in the "nascent" microfibril as they associated to form the consolidated microfibril (Colvin, 1976). Colvin also stresses that formation of the microfibril is a totally extracellular process that does not require an intact membrane, distinct portion of the cell or any other organelle

(Colvin, 1980b). It was shown that in a particulate preparation from *A. xylinum* the synthetic enzyme was adsorbed onto whole, dense ovoid particles, 50-250 nm long with a typical unit membrane around each. When these particles were incubated with UDPG, linear wisps of fibrils were produced which subsequently associated to form microfibrils. No visible attachment of the wisps to the particles could be observed (Colvin, 1980b).

There are, however, a number of serious problems with Colvin's extracellular, non-cell-directed two-stage mechanism for cellulose microfibril formation.

1) β(1-4) glucan polymers of D.P. greater than 10 tend to be insoluble in aqueous solution. Thus, a polymer such as that found by King & Colvin (1976) to have a D P of 180 must be substituted with polar groups or branched if it were to remain soluble during its synthesis previous to extra-cellular microfibril formation. Indeed, a soluble, non-dialysable β(1-4) linked polymer of glucose was isolated and purified from the supernatant of a culture of *A. xylinum* actively producing cellulose. The β(1-4) glucan was linear with single glucose residues as branches on carbon 2 of every third glucose residue in the chain (Colvin *et al*, 1977b). It was proposed that these branches would be cleaved off by an enzyme before association of the polymers to form a microfibril. However, there is no evidence that this particular molecule has any relationship with cellulose synthesis (Burgess, 1979; Colvin, 1980a).

2) As pointed out in section B.2., native cellulose has the metastable cellulose I structure. It is thought that tip synthesis is a pre-requisite to generate cellulose I (see p. 57) since it is known that if free polymer chains associate in an aqueous environment a microfibril with the cellulose II structure is produced. Thus, it is difficult to envisage how Colvin's model for microfibril construction

could result in native cellulose I. Indeed microfibrils formed by Colvin (1980b) were shown to be of the cellulose II type after preparation, thus casting doubt as to whether this mechanism reflects the *in vivo* process.

3) Brown *et al* (1976) were unable to observe a sheath around forming microfibrils as visualised by Leppard *et al* (1975). Indeed, Willison *et al* (1980) argue that the amorphous sheath observed by Leppard *et al* was an artefact, due to the replicating carbon. Furthermore, a key point in the arguments of Colvin and co-workers was that these "hydrated" nascent microfibrils were not found in air-dried cellulosic pellicles of *A. xylinum*. If this was the case, fibrils with this appearance should never be observed in replicas of air dried specimens. This is clearly not the case since sheathed microfibrils are observed in the air-dried walls of *Glaucocystis* and *Oocystis* (Willison, *et al*, 1980). Thus Willison *et al* (1980) conclude that there is no evidence for a hydrated nascent microfibril in bacteria or higher plants.

4) Stockman (1972) argues that above a critical molecule length (1000 Å) flexible linear molecules will not crystallize with an extended chain conformation as found in native cellulose. Thus if polymerization was to occur before crystallization, then it could only be to a limited extent. Furthermore, polymerization followed by crystallization should lead to microfibrils with a splaying tip as in Fig. 11 (assuming tip synthesis). It is known that at least in algae and in bacteria the microfibrils have a blunt tip.

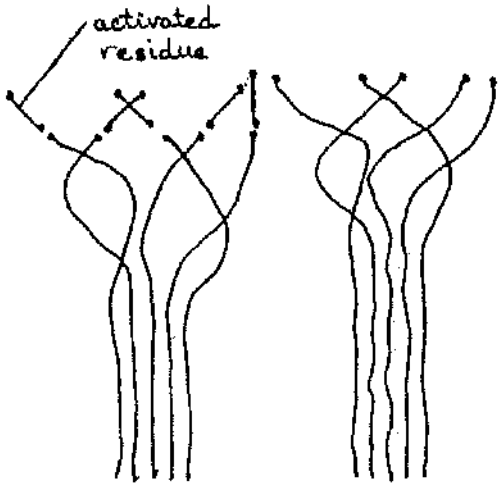


Fig. 11 Successive polymerization and crystallization
From Stockman (1972)

c.ii) Crystallization followed by polymerization (see Fig. 12). Polymerization from the solid state is unlikely because of the complicated machinery it would require (Stockman, 1972):-

1) Each already crystallized activated residue would require a nucleating agent to obtain the metastable cellulose I state.

2) It is difficult to envisage the means by which the crystallized residues would obtain the same width as the microfibrils.

Furthermore there is no electron microscopic evidence for the existence of residual crystals.

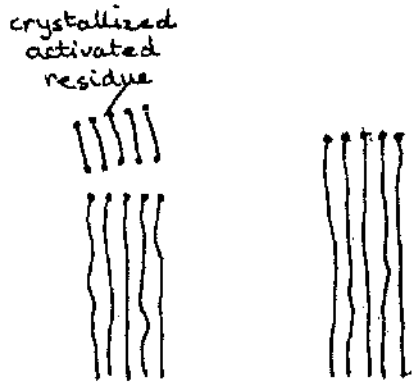


Fig. 12 Polymerization of crystallized residues. From Stockman (1972).

c.iii) Simultaneous crystallization with polymerization
It has been proposed that crystallization with polymerization must occur as a one step process at the tip of an already crystalline microfibril as a prerequisite to the formation of the unfavourable cellulose I lattice type (Stockman, 1972; Preston, 1974).

Thus, if the activated residues are transferred immediately to the crystalline plane with both covalent and crystalline bonds being set simultaneously, the result would be the "freezing in" of the metastable cellulose I structure. This mechanism also satisfies the requirement for a blunt microfibrillar tip (see Fig. 13).

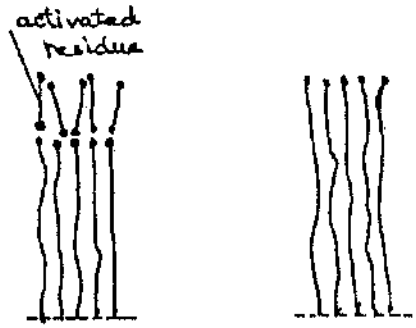


Fig. 13 Simultaneous crystallization and polymerization. From Stockman (1972).

Thus, theoretically speaking at least, tip synthesis involving simultaneous polymerization and crystallization is by far the most likely mechanism for microfibrillar construction. Recently, experimental evidence from the *A. xylinum* system has been presented for a microfibrillar growth mechanism with some similarities to the theoretical hypothesis above.

Cooper & Manley (1975 a,b,c) indicated a close relationship between the bacterial cell envelope, the cellulose polymer and its biosynthesis. Brown *et al* (1976), using dark field light microscopy, visualised tip synthesis of microfibrils in the form of a ribbon projecting from the pole of the bacterial rod. The ribbon consisted of a number of microfibrils which were assembled in association with rows of particles (presumed to be groups of multienzyme complexes) along the longitudinal axis of the bacterial rod. The particles were situated below extrusion pores in the L.P.S. layer of the bacterium (see Fig. 13) (Brown *et al*, 1976; Zaar, 1979).

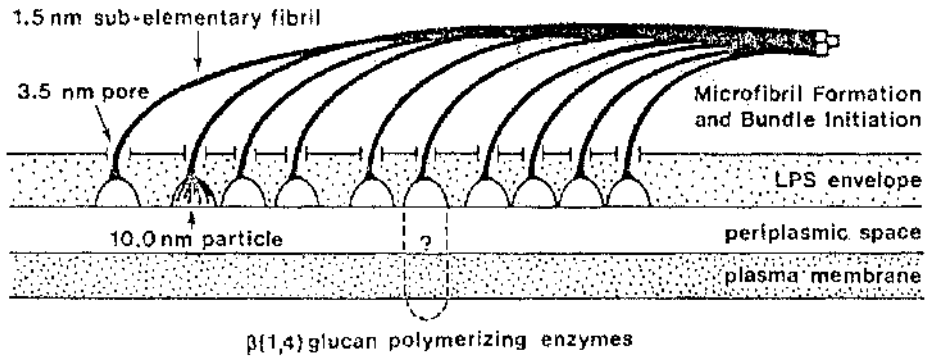


Fig. 14 Model of cellulose microfibril assembly in *A. xylinum*. From Haigler & Benziman (1982).

The ribbon was observed to elongate at a rate of 2 μm min^{-1}. The bacterial rod turned on its axis as it was propelled forward by the elongating ribbon, suggesting an inherent kinetic force in the crystallization process. Using the fluorescent brightener calcofluor white S.T (C W) and carboxy-methyl-cellulose (CMC), evidence has been presented that indicates that cellulose ribbon synthesis is a hierarchical, cell-directed, self-assembly process.

When C W at a concentration above a threshold value of 0.1 mM was added to cultures of *A. xylinum* actively synthesizing cellulose, it interrupted the synthesis of the cellulosic ribbon. This is achieved by the planar C.W. molecule binding to β-glucan chains via hydrogen bonding and dipolar interactions, thus prohibiting microfibril crystallization (Haigler & Benziman, 1982). As a result, the bacterium synthesizes broad, non-crystalline, cellulosic bands perpendicularly to the longitudinal axis of the cell, rather than the normal twisting ribbons. This effect of C.W. was shown to be reversible (Haigler & Benziman, 1982).

Negatively stained preparations revealed that the bands were composed of bent fibrils originating along the longitudinal cell surface. The smallest fibres averaged 1.5 nm in diameter, smaller than any fibril normally observed in *A. xylinum* cultures. The 1.5 nm fibrils curved and undulated, implying low crystallinity. Larger fibrils measuring 3.0 nm, 4.5nm and 6.0 nm were formed by fasciation of the 1.5 nm fibrils. These larger fibrils showed cracking sites suggesting that they were rigid and crystalline. The low crystallinity of the 1.5 nm fibrils was probably due to the fact that 60-80% of their glucan chains are located at the surface of the fibril, thus preventing the possibility of a high degree of lateral order (Haigler & Benziman, 1982). Upon drying of the band cellulose, metastable cellulose I was regenerated (Benziman *et al*, 1980), implying that although the altered cellulose does not contain cellulose I crystallites, it must contain extensive parallel chain ordering if it is to generate metastable cellulose I upon drying. That the altered cellulose does contain ordered chains was confirmed by birefringence studies (Haigler & Benziman, 1982). The ordered assembly of glucan chains is probably achieved by the extrusion pores (Haigler & Benziman, 1982).

In addition to changing the morphological appearance and crystallinity of cellulose, C W also induces a large increase in the rate of glucose polymerization, which is not directly linked to the energy metabolism of glucose. This stimulation was not the result of the direct interaction of C W and the cellulose polymerizing enzymes (Benziman *et al*, 1980). Thus, it was concluded that C W 's stimulatory effect upon glucose polymerization was due to interference with the crystallization phase of the biosynthetic process, which must be occurring after polymerization. It was further concluded that the two stages of cellulose I biogenesis (polymerization + crystallization) are tightly coupled and that the rate of crystallization must limit the

rate of polymerization. This limitation has two possible explanations:-

1) If microfibril crystallization and ribbon assembly require that parallel glucan-chain aggregates from adjacent synthetic sites interact, then the time required for the proper interaction to occur could be rate-limiting (Haigler & Benziman, 1982).

2) If a perfect ribbon is assembled from a large number of synthesising complexes, the individual complexes must be synthesising cellulose at the same rate. Therefore each enzyme will be restrained to the rate of the slowest ones. Thus upon the addition of C.W , the synthesising complexes will be autonomous, and each can proceed at its maximum efficiency (Haigler & Benziman, 1982). Haigler *et al* (1982) demonstrated that CMC also increased the rate of glucose polymerization although to a much lesser extent than C.W. This stimulation was associated with an interference of ribbon assembly, but at a higher level of organisation than that caused by C.W. Instead of a band composed of bent 1.5-6.0 nm fibrils, long 10-20 nm wide bundles originated along the cell-surface at approximately regularly spaced intervals in the presence of CMC. It was proposed that CMC prevented fasciation of microfibril bundles to form the twisting ribbon.

In summary, multi-enzyme complexes below the extrusion pores synthesise the β-glucan chains. The chains are subsequently extruded through the pores which probably facilitate later microfibril crystallization by aggregating the glucan chains into non-dissociable aggregates of parallel chains (this implies a cell-directed process). The formation of such ordered aggregates minimises entropy and facilitates the formation of the cellulose I lattice. The nascent fibril extruded from each pore is too small to crystallize into true cellulose I, thus fibrils from more than one

extrusion site fasciate to form the microfibril (C.W. interferes here). The microfibrils then aggregate into bundles which then fasciate to form the twisting ribbon (CMC interferes here). Thus the synthesis of the cellulosic ribbon is a cell-directed, hierarchical self-assembly process (see Fig. 14).

It must be noted that in the above hypothesis, polymerization and crystallization are consecutive, not simultaneous. However, the two processes are tightly coupled and co-ordinated through the synthesis and extrusion of ordered glucan aggregates that co-crystallize while attached to their forming enzyme complexes. When Stockman (1972) proposed that covalent and hydrogen bonds had to be set simultaneously if extended chain cellulose I microfibrils are to be formed, he was assuming that the activated residues were added to the growing ends of microfibrils far from the cell surface (Haigler & Benziman, 1982) without cell mediation in the biogenetic process.

Experimental evidence on the mechanism of microfibril formation in algae and higher plants lags considerably behind studies on the bacterial system. Freeze etch/freeze fracture techniques have produced some evidence for tip synthesis, in that putative intramembrane enzyme complexes located in the plasma-membrane which have a close association with impressions of microfibril tips have been observed with the algae, *Oocystis* (Brown & Montezinos, 1976 ; Montezinos, 1982), *Glaucocystis*(Willison & Brown, 1978) and *Micrasterias* (Giddings *et al*, 1980). The intramembranous complexes pack together in lines which are associated with the impressions of microfibrils on the external face of the plasma-membrane. Fig. 15 is a diagrammatic portrayal of the hypothetical model of cell wall biogenesis in *Oocystis* (after Montezinos, 1982). Mueller *et al* (1976) presented electron micrographs of the external face of fractured plasma-membrane from corn stelar tissue, showing globular complexes attached to the

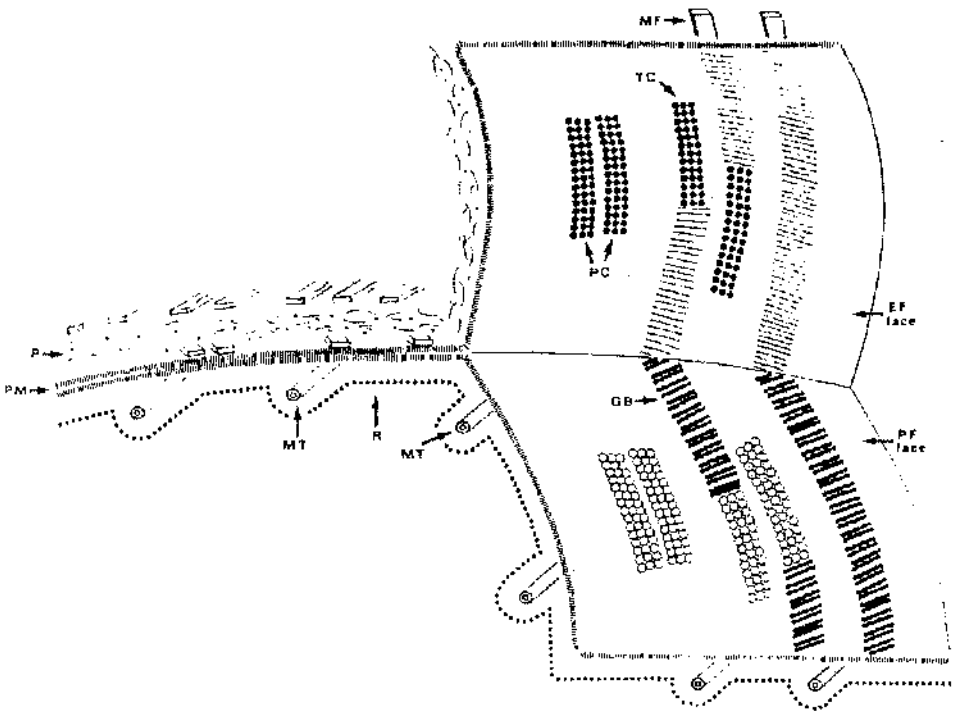


Fig. 15 Diagrammatic protrayal of the hypothetical model of cell-wall biogenesis in *Oocystis*. The components of the model include: (1) Periplasm (P). (2) Plasma membrane (PM). Terminal complexes (TC) associate with the ends of ridges that are the impressions of microfibrils (MF) or with each other in pairs (PC) on the EF face of the plasma membrane. The ridges are crosshatched by the impressions of granule bands (GB) that associate with the PF face of the plasma membrane. The granule bands form rows underlying the ridges and end at the impression in the PF face produced by terminal complexes. (3) Microtubules (MT). Also note a region (R) of possible transmembrane control that is yet to be elucidated, underlying the plasma membrane and surrounding the microtubules. From Montezinos (1982).

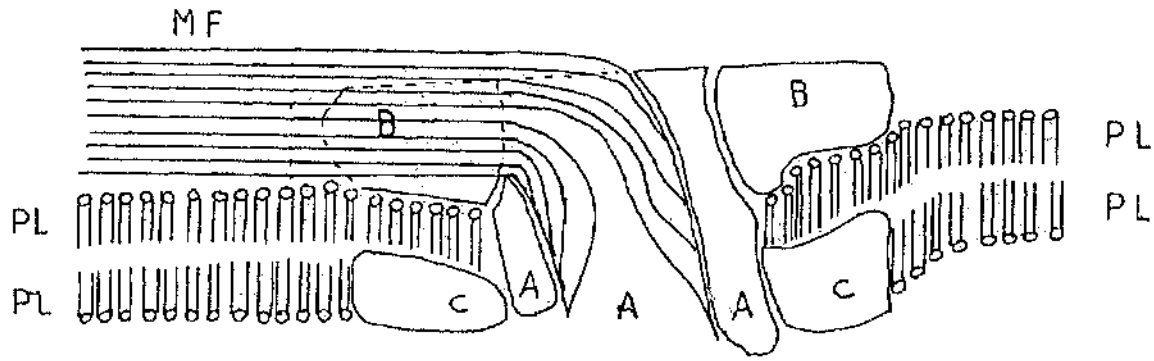


Fig. 16 A model demonstrating the proposed relationship of terminal complexes (A and B) and rosettes (C) in association with the plasma-membrane. The membrane (PL), microfibrils (MF) and microfibril associated structures are drawn to approximately equivalent scale. From Mueller & Brown (1980).

end of nascent microfibrils. Willison & Grout (1978) also observed similar globules attached to the end of microfibrils in radish roots. However, Grout (1975) failed to show any association of microfibril deposition with terminal complexes or differentiated regions of the plasma-membrane on the naked surfaces of tobacco mesophyll protoplasts. The microfibrils had tapering ends, or were sunk into the membrane surface and did not make any impressions on the plasma-membrane. Lloyd (1980) however, argued that no association between microfibrils and particles would be observed in this system. Without their walls, protoplasts are fragile and are prevented from swelling and bursting by regulating the osmotic concentration of the suspension medium. Thus, a wall in the process of being regenerated by a protoplast may be physically incapable of printing its image through to the membrane's fracture plane where the particles are observed. Mueller & Brown (1980) have subsequently confirmed the presence of specialised particle rosettes (see Fig. 16) in the plasma-membranes of corn, pine and mung bean seedlings. The rosettes were associated with impressions of microfibrils on the internal leaflet of the membrane. These authors further proposed, on the basis of the pattern of tears in the plasma-membrane caused by microfibrils, that the complexes moved in the plane of the membrane during synthesis and that the direction of membrane flow plays an important role in the cellular control over the direction of microfibril deposition (Mueller & Brown, 1982a, b). It is interesting to note that while the terminal complexes found in algae and higher plants are mobile, those found in *A. xylinum* are stationary.

Thus there is circumstantial, cytological evidence for cell-directed tip synthesis of microfibrils in algae and higher plants. It must be borne in mind however, that there is as yet, no direct evidence of a biosynthetic role for these putative terminal enzyme complexes. It is encouraging to note that Willison (1982) calculated that, if the particles

observed in cotton fibres were involved in microfibril deposition, then their rate of movement would be 1.3 - 2.6 $\mu m\ min^{-1}$, a value very close to the microfibril growth rate observed in *Acetobacter* (Brown *et al*, 1976).

D. CELLULAR LOCATION OF CELLULOSE BIOSYNTHESIS.

The enzymes concerned with the synthesis of plant cell wall polysaccharides are particulate when isolated. Thus they are either contained within vesicles, or firmly attached to membranes. There is considerable evidence implicating the internal membrane system (endoplasmic reticulum (E.R.), golgi body and vesicles) in the synthesis of the matrix polysaccharides of the plant cell wall (Northcote, 1969). This was accomplished using autoradiographic techniques associated with chemical analysis (Northcote, 1969 and references therein) and subcellular fractionation with associated enzymic assays (Bowles & Northcote, 1972; Ray *et al*, 1976). It is thought that the direction of flow for the precursors to the wall is as follows:

cytosol → E.R. → Golgi → Golgi vesicles →

fusion of vesicles to the plasma-membrane by reverse pinocytosis → wall

Pickett-Heaps (1968) further demonstrated that polymerization of the precursors contained in the golgi vesicles occurred as they moved across the cytoplasm. The above is in general agreement with the endomembrane flow concept already well established in animal cells.

Cellulose synthesis, however, is not thought to be associated with these endomembranes (at least in the final

stages), but rather with the plasma-membrane, for the following reasons:-

1) Cellulose is deposited in the wall as a highly organised group of chain molecules within the microfibril. As the wall grows and develops the microfibrils are laid down with an ordered orientation and are to be found in layers (Northcote, 1969). Thus the synthesis of cellulose is more likely to involve the incorporation of soluble precursors at a site at, or just outside the plasma-membrane since any alternative hypothesis would pose considerable organizational problems. Indeed the formation of microfibrils within the endomembrane system has never been observed in electron micrographs of higher plant cells. This would appear to contrast with the numerous reports of $\beta(1\text{-}4)$ glucan synthetase that are associated with the golgi apparatus vesicles (Shore & Maclachlan, 1975; Shore *et al*, 1975; Ray *et al*, 1976). However, it is now thought that these synthetases are not the loci for cellulose synthesis *in vivo* but may be either or both of the following:

1) Inactive cellulose synthetase in transit to the site of action at the wall: protoplast interface where they may be activated in some manner. This situation corresponds to that of the chitosomes in the biosynthesis of the functionally and chemically similar polysaccharide, chitin.

2) Enzymes involved in the synthesis of matrix polysaccharides containing $\beta(1\text{-}4)$ linked glucose, eg xyloglucan, glucomannan.

The assembly of microfibrils within the golgi apparatus has been observed in Chrysophycean alga *Pleurochrysis* (Brown *et al*, 1970). However, in this organism, the microfibrils are arranged within scales rather than in continuous layers round the cell, and thus the production of the scale unit and its intususception into the wall does not pose the same

organisational problems found in other algae and higher plants.

2) It has been demonstrated that in stems of sycamore fed with radioactive glucose for 30 min and chased with non-radioactive glucose, the α-cellulose fraction of the cell wall becomes labelled, while the hemicellulose and pectic fractions do not. Autoradiographic study of these cells showed that the labelled material was present just outside the plasma-membrane and no accumulation of label occurred over any other organelle (Wooding, 1968).

3) Bowles & Northcote (1972), working on maize roots supplied with radioactive glucose, studied the incorporation of radioactivity into cell wall, golgi, smooth and rough microsomal fractions. The incorporation of radioactivity into polymeric glucose in the golgi and E.R. was low, while incorporation into the sugars characteristic of matrix polysaccharides was high. The cell wall contained a much greater proportion of radioactive glucose than the cytoplasmic organelles. This implies that cellulose synthesis does not occur in these cytoplasmic organelles.

4) Robinson & Ray (1977) demonstrated that KCN inhibited the incorporation of matrix substances into the cell walls of pea stem cells. It did not inhibit incorporation into cell wall cellulose. In the presence of KCN matrix polysaccharides are synthesised, but not transported to the cell wall and the removal of KCN allowed transport of the matrix polysaccharide to the wall. Thus cellulose does not appear to be involved in the cytoplasmic transport system by which the matrix polysaccharide are transported to the wall.

5) Native cellulose is of the cellulose I form and not cellulose II, which is produced when free β(1-4) glucans in solution are allowed to crystallize (Robinson & Quader, 1981). From this observation it has been argued that the

molecular chains must be synthesised collectively as a microfibril by tip synthesis (Preston, 1974; Stockman, 1972). The necessity for tip synthesis would require an enzyme complex close to at least one end of a microfibril. As discussed in pages 62-65 these enzyme complexes at the termini of microfibrils may have been visualised.

These 5 points together make up a powerful argument for the plasma-membrane being the *in vivo* locus for cellulose biosynthesis. This is now widely accepted to be the case.

CHAPTER 2: INTRODUCTION TO EXPERIMENTAL WORK

The purpose of this project was to obtain information concerning the pathway of cellulose biosynthesis in higher plants, and to establish whether the $\beta(1\text{-}4)$ glucan synthetase activities found in particulate enzyme preparations are involved in cellulose synthesis.

In past *in vitro* studies of cellulose biosynthesis, it has proved very difficult to demonstrate that the products are genuine cellulose or even whether or not they are early stages of cellulose biosynthesis. The incorporation of glucose from a radioactive substrate into an alkali-insoluble polymer containing $\beta(1\text{-}4)$ linkages by an *in vitro* enzyme system is not proof of the formation of cellulose for the following reasons:-

1) Alkali-insolubility is no longer regarded as being specifically a property of $\beta(1\text{-}4)$ glucan (see Sections 5.1 & 6.1).

2) Other non-cellulosic heteropolysaccharides of the plant cell wall contain $\beta(1\text{-}4)$ linked glucose, eg xyloglucan, glucomannan and mixed-linked $\beta(1\text{-}3)$, $\beta(1\text{-}4)$ glucan. The particulate enzyme systems used in these studies contain endogenous non-radioactive substrates. Thus the demonstration that a product contains $\beta(1\text{-}4)$ linked radioactive glucose does not rule out the possibility that the molecule contains non-glucose residues derived from endogenous sources. These non-radioactive components may be only detected with difficulty.

3) Cellulose is thought to be a linear polymer with a high D P., up to 10,000 in some systems, and is probably at least an order of magnitude greater than other cell wall polysaccharides (Brett, 1981b). Robinson & Preston (1972) concluded from an X-ray diffraction investigation that the glucans synthesised from UDPG and GDPG by *P. aureus* particulate enzyme preparations were short chain oligosaccharides,

probably $\beta(1\text{-}4)$ linked. This therefore casts doubt over the cellulosic nature of these products and because of this result, Robinson & Preston emphasised the need for physical as well as chemical evidence when examining the cellulosic nature of the polysaccharide products from these systems.

As it is difficult to see how any solid progress in unravelling the mechanism of cellulose biosynthesis can be made before the *in vitro* biosynthesis of cellulose is unequivocably demonstrated, it was decided to re-evaluate the products of these particulate enzyme systems using the criteria outlined below:-

1) Sugar composition. This was determined by the total acid hydrolysis technique described in Chapter 3. A cellulosic product would be expected to contain glucose as the major constituent.

2) Sugar-sugar linkage. This was examined by partial acid hydrolysis (see Chapter 3) and partial acetolysis (Clark & Villemez, 1972). Cellulose contains only $\beta(1\text{-}4)$ linkages.

3) Molecular weight. This is the simplest physical assay for cellulose. The molecular weight distribution of the products synthesised from UDP-$[U\text{-}^{14}C]$-G and GDP-$[U\text{-}^{14}C]$-G was investigated by gel filtration on Sepharose CL-6B in the cellulose solvent, cadoxen. Any large product detected (D.P. $>$ 1000) is likely to be cellulose, since other water-insoluble cell wall polysaccharides are thought to have a much lower D.P., generally less than 200 (Brett, 1981b). As extraction of the products with acid or alkali may cause a reduction in molecular weight, the analysis was conducted on the products insoluble in water and chloroform-methanol (3:2 v/v).

4) The effect of other sugar-nucleotides. Even if structural analysis indicates that $\beta(1\text{-}4)$ linked glucose is the only radioactive sugar residue in the product, other non-radioactive residues may be present. If so, the addition of

other sugar-nucleotides (eg GDPM or UDP-Xylose) to the assay system is likely to affect glucose incorporation and a kinetic investigation such as that done by Villemez (1971) may indicate whether a heteropolysaccharide is being synthesised.

5) The use of purportedly cellulose-specific extractions. The solubilities of the products were tested in strong alkali (24% KOH) and the acidic hydrolytic extraction of Updegraff (1969). Products that are insoluble after these extractions are liable to be present as part of consolidated microfibrils (see Sections 5.1 & 6.1), which would be good evidence for their cellulosic nature. Soluble products however, might represent cellulose not fully incorporated into microfibrils.

Some of these criteria are very stringent and in every case a negative result does not rule out the possibility that the product is cellulose. Positive results should however, provide evidence that genuine cellulose is being synthesised.

The tissue chosen as a source of enzyme was dark-grown *P. aureus* hypocotyls for the following reasons:-

1) *P. aureus* is easy to grow and work with.

2) Enzyme preparations from this plant have been shown to synthesise products containing $\beta(1\text{-}4)$ linked glucose from both UDPG and GDPG.

3) Many of the previous investigations on *in vitro* β-glucan have used this plant and thus direct comparisons can be drawn.

CHAPTER 3: MATERIALS AND METHODS

This chapter describes the materials and general methods used in this work. Methods relevant only to specific chapters are described in those chapters.

3.1. Chemicals

All chemicals were of analar or laboratory grades and glass distilled water was used for all solutions.

3.2. Radioactive chemicals

The radioactive substrates were UDP-[U-^{14}C]-G, GDP-[U-^{14}C]-G, GDP-[U-^{14}C]-M, [U-^{14}C]-sucrose, [U-^{14}C]-glucose and ^{14}C-glucose-1-phosphate, all of which were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. UDP-[U-^{14}C]-Xyl was obtained from New England Nuclear, 2 New Rd., Southampton, U.K.

3.3 Plant material

Seeds of the mung bean (*Phaseolus aureus*) were used throughout this work. The seeds were pre-soaked in water overnight at 22 °C. The seeds were subsequently planted in trays (58 x 30 cm) which contained wetted vermiculite and were left in the dark at 22 °C for approximately 5 days, until the hypocotyls reached 4-6 cm in length.

3.4. Particulate enzyme preparation

The hypocotyls (70 gm) were harvested and kept on ice until homogenisation in 200 ml 0.1M tris-HCl buffer, pH 7.5 at 0-4 °C using a polytron kinematica homogeniser at a minimum setting for 5 seconds. The homogenate was filtered through four crossed layers of muslin (to remove large cellular debris and intact cells) and centrifuged at 97,000 g (average) in 6 centrifuge tubes for 35 min in a Sorvall OTD-65B ultracentrifuge (temperature set at 1 °C) using a

Sorvall AH-627 swinging bucket rotor. The supernatant was discarded and the pellets, which were used as an enzyme source, were stored at -20°C until further use. The pellets were each resuspended in 0.5 ml 0.1 M tris-HCl, pH 7.5 (unless otherwise stated) using a glass rod and vorticer, ensuring the temperature did not rise above 4°C.

3.5 Incubation conditions

The incubations were carried out at 25°C. The incubation media contained 10 mM $MgCl_2$, [U-^{14}C]- sugar-nucleotide made up to the required concentration with non-radioactive sugar-nucleotide and enzyme suspension (containing 0.5 mg protein as determined by the Bio-Rad protein assay with BSA standard), all in a total volume of 100 µl. The incubations were terminated by adding 4 ml boiling water (unless otherwise stated) and boiling for 15 min. This was the first water extraction.

3.6 Routine extractions

The insoluble residue was extracted as above a further twice. The water-insoluble material was extracted a further two times with 1 ml chloroform:methanol (3:2 v/v), once with 1 ml methanol and once with 1 ml water.

3.7 Alkaline extraction

Nitrogen gas was bubbled through a 24% KOH solution containing 0.1 M $NaBH_4$. The KOH/$NaBH_4$ solution (1 ml) was added to the insoluble residue from the routine extractions. Nitrogen was blown gently over the sample for 3 min. The tubes were sealed with a double layer of nescofilm, vorticed and left on an orbital shaker at 25°C for 24 hours. The insoluble residue after this extraction was washed in 1 ml 24% KOH and subsequently several times in 1 ml water. The resulting 24% KOH-insoluble residue is termed the "α-cellulose" fraction (Wise & Ratliff, 1947).

3.8 Updegraff extraction

This was carried out as by Updegraff (1969). After the routine extractions had been conducted on the sample, 3 ml of acetic-nitric acid reagent was added (composition - 100 ml 80% acetic acid and 10 ml conc. nitric acid). The sample was then extracted at 100°C for 30 min unless otherwise stated. The insoluble residue (henceforth termed Updegraff or "U-cellulose") was washed several times in 1 ml water.

3.9 Liquid scintillation counting of fractions obtained by extraction methods

Liquid scintillation was carried out by the methods of Waldron and Brett (1983).

3.10 Analytical methods

a) Total acid hydrolysis. This was carried out by the methods of Harris & Northcote (1970). The residue to be analysed was pre-treated with 72% (w/w) H_2SO_4 overnight. The sample was diluted to 3% (w/w) and subjected to hydrolysis at 15 lb/in^2 (103.4 kN/m^2) and 120°C for 1 hour, and subsequently neutralised overnight with the bicarbonate form of Amberlite IR-45 ion exchange resin. The resin was filtered off, the filtrate and washings rotary evaporated and the residue resuspended in a few drops of water.

b) Identification of sugars. The hydrolysate produced by the methods of 3.10a) above was run on paper chromatography with appropriate markers in ethyl acetate-pyridine-water (8:2:1 v/v) for a stated period. The chromatogram regions containing the radioactive sugars were cut into 1 cm strips and counted as described by Harris and Northcote (1970). The markers were detected by the method of Trevelyan *et al*, (1950).

c) Partial acid hydrolysis. The sample was pre-treated in 72% (w/w) H_2SO_4 overnight, diluted to 3% (w/w) and subjected to hydrolysis at 15 lb/in^2 and 120°C for a stated period. The hydrolysate was neutralised and recovered as in Section 2.10a) and run on a paper chromatogram along with β(1-3) and β(1-4) linked gluco-oligosaccharide markers in n-propanol:ethyl acetate: water (7:1:2 v/v) for a stated period. Radioactive oligosaccharides and markers were detected as in 3.10b) above.

d) Acetolysis. The method of Clark and Villemez (1972) was used. Samples were incubated with acetic acid-acetic anhydride-sulphuric acid (1:1:0.1 v/v) for a stated period at room temperature. They were then diluted with an ice/water mixture and neutralised with 5 N NaOH, keeping the temperature below 4°C. The acetylated material was extracted with chloroform, washed with 1 M $NaHCO_3$ and 1 M KCl solutions, and deacetylated with 0.03 M BaOMe in methanol. The barium was precipitated with CO_2. Paper chromatography, scintillation counting and the detection of markers was as in 3.10c) above.

e) Thin layer electrophoresis (T L E) After the incubations were terminated and diluted to 500 µl, the particulate material was centrifuged down on an Eppendorf bench centrifuge. A sample volume (25 µl) of the supernatant was subjected to thin layer electrophoresis in acetic acid-formic acid-water on silica plates, pH 2 at 3 kV. Plates were cut into 1 cm strips and counted as in 3.10b). Markers were detected by the method outlined in Waldron & Brett (1983).

f) Thin layer chromatography (T.L.C.) The chloroform: methanol (3:2 v/v)-soluble products were extracted by the methods of Folch *et al* (1957). The sample was applied to silica gel plates and run in ascending chromatography with chloroform:methanol:water (65:25:4). The plates were dried, cut into 1 cm strips and counted as in 3.10b).

g) Gas liquid chromatography (G.L.C.) G.L.C. analysis was conducted on the sugars produced by total acid hydrolysis as in 3.10a) using the methods of Albersheim *et al* (1967).

3.11 Gel filtration on sepharose CL-6B or CL-2B

Gel filtration was carried out on the water- and chloroform:methanol (3:2 v/v)-insoluble products rather than U or α-cellulose since the extraction procedures used in the production of these residues may cause a reduction in molecular weight.

Sample preparation: After the routine extractions had been carried out, the residual pellet was desiccated in vacuo over NaOH for 3 hours. Cadoxen (0.125 ml), a cellulose solvent, (Cadoxen composition - diaminoethane:water:CdO, 126 ml: 228 ml: 40 gm; Brett, 1981b). Undissolved material was centrifuged down and the supernatant retained. To the supernatant, 0.125 ml water was added resulting in a final sample volume of 0.25 ml in 50% cadoxen. This was applied to the column.

The column. The gel filtration was carried out on Sepharose CL-6B or CL-2B. Cross-linked Sepharose was used to ensure stability of the gel in the elution solvent, 50% cadoxen. The fractions were collected on a Gilson microcol TDC 80 fraction collector. A sample volume (100 µl unless otherwise stated) of each fraction was counted as in 3.9.

3.12 Presentation of results

Where applicable, the results are expressed as the mean ± the standard error of the mean for triplicate assay samples.

CHAPTER 4: SUBSTRATES FOR THE SYNTHESIS OF WATER-AND CHLOROFORM:METHANOL (3:2 v/v)-INSOLUBLE PRODUCTS BY THE PARTICULATE ENZYME PREPARATION

4.1 Introduction

In this chapter experiments are described which were carried out to determine whether the particulate enzyme preparation prepared as in chapter 3 contained enzymes which catalysed the formation of water-and chloroform:methanol (3:2 v/v)-insoluble products from GDP-[U-^{14}C]-G, UDP-[U-^{14}C]-G, [U-^{14}C]-sucrose, [U-^{14}C]-glucose and [U-^{14}C]-glucose-1-phosphate. The experiments also deal with the distribution of the enzymic activity between the wall and particulate fractions, and the effect of storage of the enzyme preparation at -20°C.

4.2 Results and discussion

The results in Tables 3 and 4 were obtained using the methods outlined in chapter 3. The modification of the general procedures used to obtain the wall (W) and particulate (P) fractions in Table 5 are outlined below that table. All the incubations in Tables 3-5 were for 15 minutes, except those with 1 μM GDP-[U-^{14}C]-G which were for 5 minutes. Individual enzyme preparations were found to vary in activity. Thus the results in different tables and figures in this and subsequent chapters are not quantitatively comparable unless otherwise stated. Boiled enzyme controls did not incorporate radioactivity from the [U-^{14}C]-substrate at any concentration into the insoluble products.

The results in Table 3 show that radioactivity from both UDP-[U-^{14}C]-G and GDP-[U-^{14}C]-G is incorporated into water-and chloroform:methanol (3:2 v/v)-insoluble material.

In terms of residues incorporated from the sugar-nucleotides, most incorporation occurs at a concentration of 1.0 mM, UDP-[U-^{14}C]-G being a twenty-six-fold better substrate than GDP-[U-^{14}C]-G at this concentration. The GDP-[U-^{14}C]-G utilising system is almost saturated at 101 µM, whereas incorporation from UDP-[U-^{14}C]-G is not saturated at 1.0 mM. There is some indication that the synthesis of water- and chloroform:methanol (3:2 v/v)-insoluble products synthesised from UDP-[U-^{14}C]-G is substrate-activated since for a thousand-fold increase in UDPG concentration the synthesis of insoluble products has been found to be stimulated by 2,900 - 10,000 fold in numerous experiments. Figure 17 is a logarithmic plot of UDPG concentration against pmol. residue incorporated into the products. If the UDPG concentration was plotted on a linear scale, the resulting relationship between UDPG concentration and residues incorporated would be sigmoidal rather than the hyperbolic relationship expected for an enzyme which exhibited normal Michaelis-Menten saturation kinetics. Substrate activation of enzyme preparations by UDPG has been reported many times in the synthesis of β(1-3) glucan by *in vitro* preparations from different plants (Smith & Stone, 1973a; Tsai & Hassid, 1973; Delmer *et al*, 1977; Raymond *et al*, 1978).

The solubilities of the products from 1 µM UDP-[U-^{14}C]-G were investigated further. It was found that about 80% of the water-insoluble products were soluble in chloroform: methanol (3:2 v/v). During TLC (see 3.10.8) all the radioactivity ran at or near the solvent front, strongly suggesting that the product was a neutral-lipid-glucoside, probably steryl-glucoside, rather than polyprenol-P-monosaccharide or polyprenol-P_2-oligosaccharide (Brett, 1981b) which have been reported as intermediates in β-glucan synthesis (see section C.1.4.1.) This result is similar to those obtained by Delmer and co-workers (Delmer, 1977) with the detached cotton fibre system. It is therefore unlikely that radioactivity incorporated into chloroform:methanol (3:2 v/v)-soluble products from 1 µM UDP-[U-^{14}C]-G is involved in cellulose biosynthesis

unless there are very small, undetectable amounts of the acidic glycolipids of the type found by Forsee and Elbein (1972, 1973) present.

Radioactivity from [U-^{14}C]-sucrose, [U-^{14}C]-glucose and [U-^{14}C]-glucose-1-phosphate is also incorporated into the products, although they are not as efficient substrates as the sugar-nucleotides (Table 3). This implies that the sugar-nucleotides are used directly by the enzyme preparation, rather than being metabolized to either breakdown products or sucrose before the synthesis of insoluble product. Sucrose and glucose-1-phosphate may be used directly as substrates, or they may be converted to a sugar-nucleotide derivative via sucrose synthetase (reaction scheme 1) or pyrophosphorylase (reaction scheme 2) activity prior to insoluble-product synthesis.

1. sucrose + NDP $\rightleftharpoons$ NDPG + fructose

2. glucose-1-phosphate + NTP $\longrightarrow$ NDPG + PPi

Both of these enzymes have been shown to be present in enzyme preparations from *P. aureus* (Delmer 1972, 1977). It is unlikely that glucose could be incorporated into the products via reactions 1 or 2 to any great extent, since the energy-generating capacity of the hypocotyl cells would be destroyed upon homogenisation.

The enzymes utilising GDP-[U-^{14}C]-G and 1.0 mM UDP-[U-^{14}C]-G remain stable for at least 24 hours at -20°C (Table 4). However, the synthesis of insoluble products from 1 µM UDP-[U-^{14}C]-G is reduced by 50% after storage for 24 hours at this temperature, although the activity did not decrease further with longer periods of storage. Thus, in the subsequent experiments described in this chapter, unfrozen pellets were used as an enzyme source to assay the synthesis of insoluble products from 1 µM UDP-[U-^{14}C]-G.

Table 3: The incorporation of radioactive residues from [U-^{14}C]-sucrose, [U-^{14}C]-α-D-glucose, [U-^{14}C]-α-D-glucose-1-phosphate, GDP-[U-^{14}C]-G and UDP-[U-^{14}C]-G into the water- and chloroform:methanol (3:2 v/v)-insoluble products by particulate enzyme preparation from unfrozen pellets.

substrate	radioactivity in incubation (nCi)	concentration (mM)	pmol incorporated
GDP-[U-^{14}C]-G	25	0.001	20 ± 1
GDP-[U-^{14}C]-G	25	0.101	262 ± 27
GDP-[U-^{14}C]-G	25	1.000	292 ± 32
UDP-[U-^{14}C]-G	25	0.001	2.6 ± 0.6
UDP-[U-^{14}C]-G	25	1.000	7680 ± 337
[U-^{14}C]-sucrose	200	0.012	0.34± 0.0
[U-^{14}C]-sucrose	200	1.000	32 ± 0.4
[U-^{14}C]-α-D-glucose	199	0.006	0.23± 0.0
[U-^{14}C]-α-D-glucose	199	1.000	40 ± 2
[U-^{14}C]-α-D-glucose-1-phosphate	100	0.003	0.9 ± 0.0
[U-^{14}C]-α-D-glucose-1-phosphate	100	1.000	121 ± 4

Table 4: The incorporation of radioactive residues from UDP-[U-^{14}C]-G and GDP-[U-^{14}C]-G into the water- and chloroform:methanol (3:2 v/v)-insoluble products by particulate enzyme preparation derived from pellets retained at -20°C for 24 hours.

substrate	concentration (mM)	pmol incorporated	% relative to unfrozen enzymes
GDP-[U-^{14}C]-G	0.001	19 ± 0.6	95
GDP-[U-^{14}C]-G	0.101	304 ± 8	116
GDP-[U-^{14}C]-G	1.000	306 ± 22	105
UDP-[U-^{14}C]-G	0.001	1.3 ± 0.1	50
UDP-[U-^{14}C]-G	1.001	8540 ±268	111

The pellets used in this experiment were prepared at the same time from the same batch of plants as those in Table 3. The results are thus directly comparable to those in Table 3. Each incubation contained 25 nCi of radioactivity.

Fig. 17 The effect of varying UDPG concentration upon the incorporation of radioactive residues into water- and chloroform:methanol (3:2 v/v)-insoluble products from UDP-[U-^{14}C]-G (25 nCi) by the particulate enzyme preparation. Incubations were for 1 minute and terminated by 5% trichloroacetic acid.

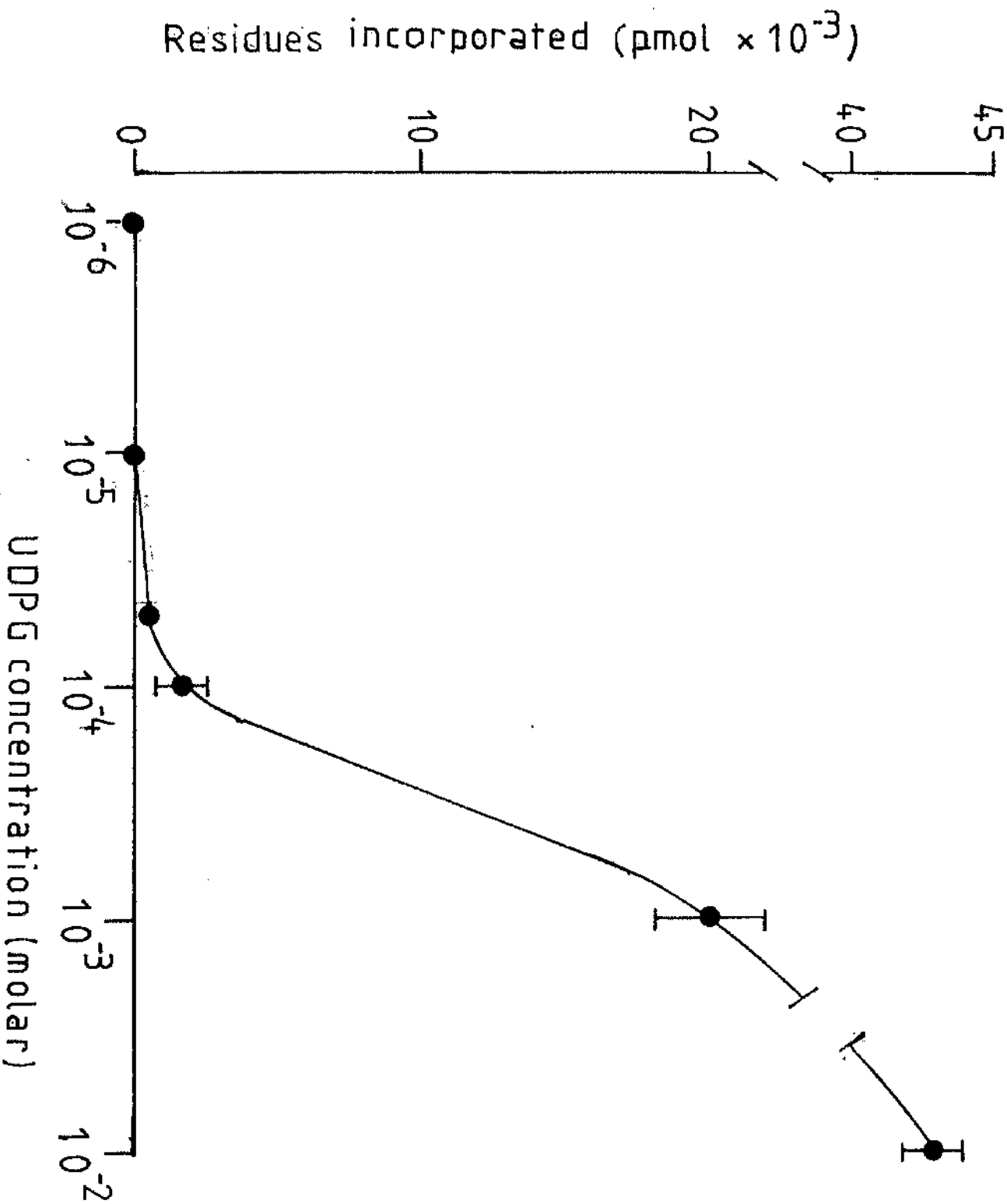
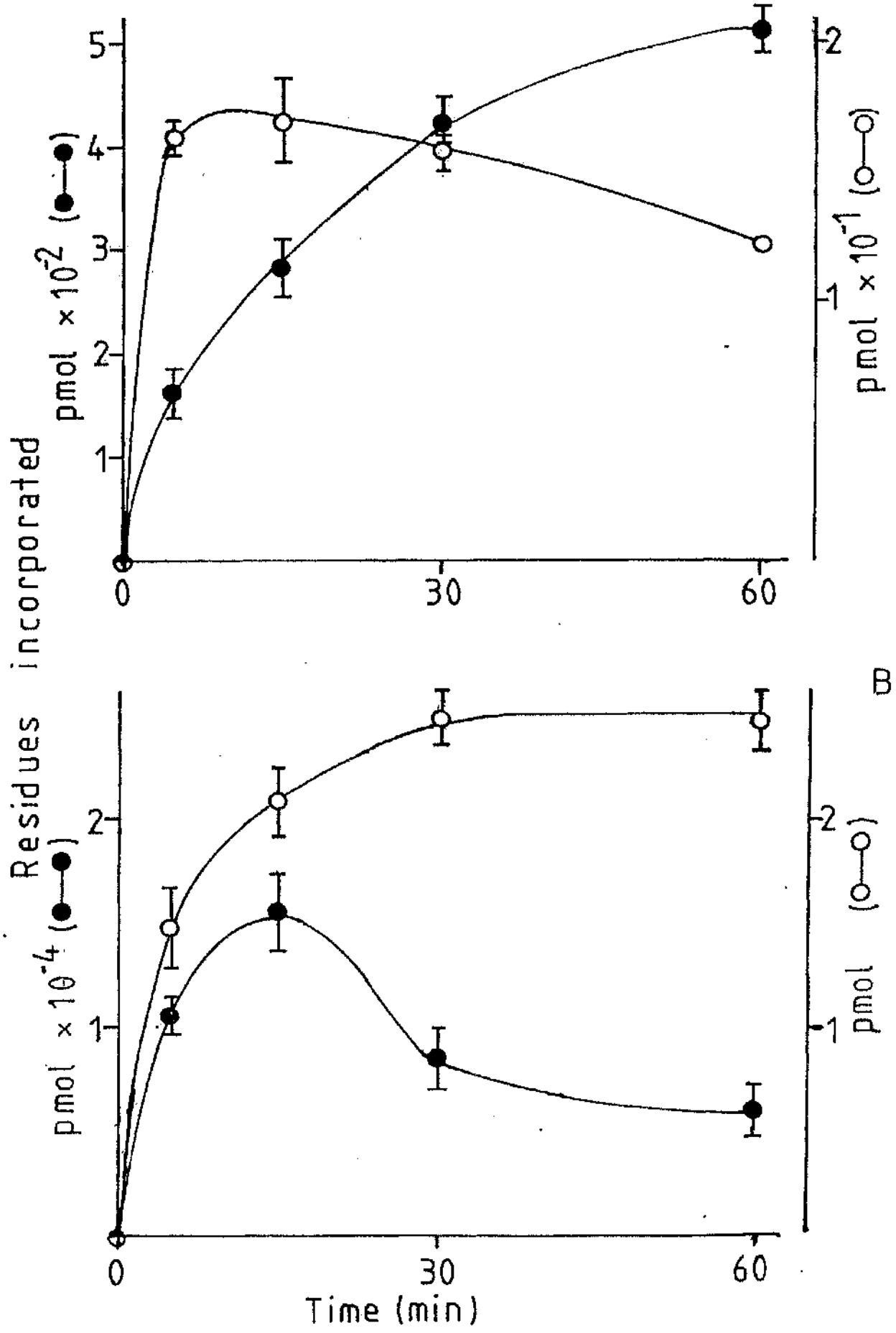

Residues incorporated (pmol x 10-3)
0
10
20
40
45
UDPG concentration (molar)
10-6
10-5
10-4
10-3
10-2

Fig. 18A Time-course of incorporation of radioactive residues into water- and chloroform:methanol (3:2 v/v)-insoluble products from 1 μM GDP-[U-^{14}C]-G (○——○, 25 nCi) and 1.0 mM GDP-[U-^{14}C]-G (●——●, 50 nCi) by the particulate enzyme preparation.

Fig. 18B Time-course of incorporation of radioactive residues into water- and chloroform:methanol (3:2 v/v)-insoluble products from 1 μM UDP-[U-^{14}C]-G (○——○, 25 nCi) and 1.0 mM UDP-[U-^{14}C]-G (●——●, 25 nCi) by the particulate enzyme preparation.


A
B
Residues incorporated
pmol ×10⁻² (●—●)
pmol ×10⁻¹ (○—○)
pmol ×10⁻⁴ (●—●)
pmol (○—○)
0
30
60
Time (min)

The results in Fig 18A and B demonstrate that net synthesis of the insoluble products reaches a maximum in less than 5 minutes with 1 μM GDP-[U-^{14}C]-G, after 60 minutes with 1.0 mM GDP-[U-^{14}C]-G, 30 minutes with 1 μM UDP-[U-^{14}C]-G and 15 minutes with 1.0 mM UDP-[U-^{14}C]-G. The products from 1 μM GDP-[U-^{14}C]-G and 1.0 mM UDP-[U-^{14}C]-G exhibit turnover. This is not surprising as crude enzyme preparations such as the one used in this investigation may well contain degradative enzymes. The presence of these endogenous degradative enzymes offers a possible explanation for the non-linearity of the synthesis of insoluble products with respect to time.

The enzyme preparation used in this study is unusual in that the cell wall material is retained. In the large majority of previous investigations on *in vitro* β-glucan synthesis, the cell wall material has been removed by a short precentrifugation of 1000 - 2000g.

In view of the fact that *in vitro* cellulose biosynthesis with these non-cell wall containing enzyme preparations has not yet been demonstrated, despite much effort, it was decided to retain the cell wall material in this investigation for the following reasons:-

1) The wall material may contain priming molecules for cellulose synthesis, such as the cellulose microfibrils.

2) There may be a cofactor or activator required for cellulose synthesis present in the wall.

3) It was hoped that the wall fraction might contain pieces of plasma-membrane still attached to the cell wall. In such fragments the structural integrity of any plasma-membrane enzyme complex may be better preserved than in membraneous vesicles obtained in the particulate (P) fraction.

Most of the enzymic activity utilising UDP-[U-^{14}C]-G

Table 5: The effect of removal of cell wall material from the enzyme preparation on the incorporation of radioactive residues from GDP-[U-^{14}C]-G and UDP-[U-^{14}C]-G into the water-and chloroform; methanol (3:2 v/v)-insoluble products

substrate	radioactivity in incubation (nCi)	conc. (mM)	enzyme preparation	pmol incorporated
GDP-[U-^{14}C]-G	50	0.002	N	36 ± 3
GDP-[U-^{14}C]-G	50	0.002	W	10 ± 2
GDP-[U-^{14}C]-G	50	0.002	P	30 ± 8
GDP-[U-^{14}C]-G	25	0.101	N	304 ± 8
GDP-[U-^{14}C]-G	25	0.101	W	84 ± 3
GDP-[U-^{14}C]-G	25	0.101	P	327 ± 16
UDP-[U-^{14}C]-G	25	0.001	N	1.3± 0.1
UDP-[U-^{14}C]-G	25	0.001	W	0.2± 0.0
UDP-[U-^{14}C]-G	25	0.001	P	1.3± 0.0
UDP-[U-^{14}C]-G	25	1.000	N	6390 ± 238
UDP-[U-^{14}C]-G	25	1.000	W	1910 ± 36
UDP-[U-^{14}C]-G	25	1.000	P	5570 ± 868

The hypocotyl tissue homogenate was produced and filtered as in chapter 3. Normal (N) enzyme pellets were produced as in chapter 3. Otherwise, the tissue homogenate was centrifuged for 5 minutes at 2000 g to produce the wall (W) pellet. The supernatant was centrifuged for 35 minutes at 97000 g to produce the particulate (P) pellet.

and GDP-[U-^{14}C]-G was shown to reside in the particulate rather than the wall fraction (Table 5). However, some enzymic activity was still to be found in the wall fraction. This could be due to:

1) large membraneous material which is centrifuged down at 2000 g for 5 min.
2) enzymic activity associated with the cell wall itself
3) membrane-bound enzymes occluded in, or adsorbed to the wall.

The results in Table 3 are similar to those of Spencer *et al* (1971) using GDPG as a substrate and Machlachlan *et al* (1979) using UDPG, although Machlachlan *et al* found that a greater proportion of the enzymic activity was associated with the wall fraction in particulate enzyme preparations from *P. sativum* epicotyls. Machlachlan *et al* (1979) also found that while some of the wall-associated enzymic activity utilising UDPG could be removed from the wall to the particulate fraction by rehomogenisation, most of the activity was destroyed. This tends to add weight to the arguments in point 3 above, since rehomogenisation of the wall fraction may well result in the disruption of the structural integrity of enzyme complexes in the putative plasma-membrane wall fragments.

The fact that the products assayed in the experiments in this chapter are insoluble after the routine extractions outlined in chapter 3 indicates that they are one of the following classes of molecules:

1) Glycoprotein. The carbohydrate synthesised may be covalently linked to a membrane-bound or water-insoluble protein. Alternatively, the carbohydrate might be tightly adsorbed on to such a protein, in which case it would constitute a protein-carbohydrate complex rather than a glycoprotein. However, treatment of the products with 1 mg ml^{-1} Proteinase K did not result in the solubilisation of any radioactivity. Thus, either the molecule is not a glycoprotein or protein-carbohydrate complex, or if it is,

Fig. 19 Paper chromatogram of the water- and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 1.0 mM UDP-[U-^{14}C]-G (50 nCi) after partial acid hydrolysis for 15 minutes at 15 lb in^{-2}, and 3 days chromatography. The gluco-oligosaccharide markers are termed C_2 (cellobiose), C_3 (cellotriose), L_2 (laminarobiose), L_3 (laminarotriose), etc, and Glc (glucose).

d. p. m. × 10⁻¹
0
5
10
0
20
40
Distance from origin (cm)
L5
L4
L3
L2
Glc
G3
G2

then the carbohydrate portion of the molecule must itself be water-insoluble after digestion of the protein moiety.

2) Complex-oligosaccharide-pyrophosphate-lipid. The products are unlikely to be a member of this class of molecules since extraction of the products with chloroform:methanol: water (1:1:0.3 v/v) reported by Behrens *et al* (1971) to extract such molecules did not result in the loss of significant amounts of radioactivity from the products unless, again, the oligosaccharides are water-insoluble.

3) Water-insoluble polysaccharide. There are abundant reports in the literature of enzyme preparations which synthesize polysaccharide from UDPG and GDPG (see Chapter 1). The water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from UDP-[U-^{14}C]-G (at least at higher concentrations) appear to contain β (1-3) glucan since:

1) The synthesis of the insoluble products from UDP-[U-^{14}C]-G by the particulate enzyme preparation is substrate-activated which, as outlined previously, is a well-documented property of the UDPG:β(1-3) glucan glucosyltransferase of higher plants.

2) Partial acid hydrolysis of the insoluble products synthesised from 1.0 mM UDP-[U-^{14}C]-G results in the production of the β(1-3) glucan series of oligosaccharides (Fig. 19).

The fact that no radioactivity co-chromatographs with cellobiose does not exclude the possibility that β(1-4) glucan is present, since the hydrolysis conditions used in this experiment may not have been rigorous enough to hydrolyse the more acid stable β(1-4) glucosidic linkage. Thus β(1-4) glucan may be present in the undegraded material remaining at the origin in Fig 19.

The chemical nature of the polysaccharide products synthesised from GDP-[U-^{14}C]-G and 1 µM UDP-[U-^{14}C]-G will be discussed in later sections.

CHAPTER 5: THE ∝-CELLULOSE EXTRACTION

5.1. Introduction

Most of the previous studies on cellulose biosynthesis *in vitro* have examined the products insoluble in alkali at 2 N or lower concentrations. However, the standard alkali extraction used to produce "∝-cellulose" from plant material involves a hydrogen-bond breaking extraction using about 4 N alkali, usually 24% KOH. This treatment was originally used to obtain "∝-cellulose" from a mixture of polysaccharides such as those found in de-lignified wood pulps (Wise & Ratliff, 1947). The term ∝-cellulose is used to describe the insoluble residue obtained after this extraction. Alkali of this strength was used in this investigation because of the numerous reports of non-cellulosic cell wall polysaccharides that are insoluble in milder concentrations of alkali (see page 96 for refs.).

$NaBH_4$ was included in the extraction medium to minimise oxidation from the reducing end of the polysaccharides.

5.2 Results and discussion

Radioactivity from GDP-[U-^{14}C]-G and UDP-[U-^{14}C]-G is incorporated into ∝-cellulose (Table 6). However, the incorporation of sugar residues from GDP-[U-^{14}C]-M (106 μM) was greater than that from either of the glucose-containing substrates. This result is surprising since the ∝-cellulose fraction of higher plants contains mostly glucose residues, although some non-glucose residues (mostly mannose and xylose) are found (Preston, 1974). Heller & Villemez (1972a) demonstrated that after a 20 hour extraction in 20% NaOH a greater percentage (>95%) of the mannan synthesised from GDPM by Triton X-100 solubilised mannosyl transferase was solubilised than glucomannan or glucan synthesised by similarly solubilised enzymes. The higher incorporation of

Table 6. The incorporation of radioactive residues from GDP-[U-^{14}C]-G, GDP-[U-^{14}C]-M and UDP-[U-^{14}C]-G into ∝-cellulose.

substrate	radioactivity in incubation (nCi)	concentration (mM)	pmol incorporated
GDP-[U-^{14}C]-G	125	0.005	8 ± 1
GDP-[U-^{14}C]-G	50	0.102	56 ± 4
GDP-[U-^{14}C]-G + GDPM (100 µM)	50	0.102	341 ± 18
GDP-[U-^{14}C]-M	125	0.106	385 ± 22
UDP-[U-^{14}C]-G	50	1.000	136 ± 0.9
UDP-[U-^{14}C]-G	25	0.001	0.3 ± 0.0

All incubations were for 15 minutes except those with 5 µM GDP-[U-^{14}C]-G which were for 5 minutes.

sugar residues from 106 µM GDP-[U-^{14}C]-M into ∝-cellulose may simply be due to the synthesis of greater amounts of water-and chloroform:methanol (3:2 v/v)-insoluble products from this substrate. An alternative hypothesis is that the insoluble products synthesised from GDP-[U-^{14}C]-M may be rendered insoluble in 24% KOH by an association with endogenous cellulose microfibrils in the particulate enzyme preparation. This may be by non-covalent binding, or it might be that the radioactive mannose residues were attached to the ends of polysaccharides already present in the microfibrils. If these primer polysaccharides were glucans, this would result in glucomannan formation. Table 6 shows that 100 µM GDPM stimulated the incorporation of radioactive residues from 102 µM GDP-[U-^{14}C]-G into ∝-cellulose, indicating the synthesis of a glucomannan (see pp. 23-25). Indeed, glucomannans have been found in the ∝-cellulose fractions of white birch (Timmell, 1964). However, preliminary experiments on the sugar composition of the ∝-cellulose fraction produced from the mung bean particulate enzyme preparation by a G.L.C. analysis of the alditol acetates produced by the methods outlined in chapter 3 failed to reveal the presence of mannose in the ∝-cellulose fraction although mannose was found in the water-and chloroform:methanol (3:2 v/v)-insoluble fraction.

Thus, the incorporation of radioactivity from a radioactive substrate into ∝-cellulose cannot be taken to indicate genuine *in vitro* cellulose biosynthesis if by the term "cellulose" one is referring to pure β(1-4) glucan. It was therefore decided to investigate another, purportedly more specific, cellulose isolation technique, that of Updegraff (1969).

CHAPTER 6: THE UPDEGRAFF EXTRACTION

6.1. Introduction

The major setback to research on cellulose biosynthesis in the past has been the lack of an adequate assay for cellulose. Much of the previous work used the criterion of alkali-insolubility to indicate the presence of cellulosic $\beta(1\text{-}4)$ glucan (see chapter 5). However, there are now numerous reports of non-cellulosic polysaccharides, notably $\beta(1\text{-}3)$ glucan, which are insoluble in alkali (Peaud-Lenoel & Axelos, 1970; Herth *et al*, 1974; Raymond *et al*, 1978; Heineger & Delmer, 1977). In cotton fibres $\beta(1\text{-}3)$ glucan has even been found in the α-cellulose fraction (Huwyler *et al*, 1978; Maltby *et al*, 1979). Thus the assumption that alkali-insolubility denotes $\beta(1\text{-}4)$ glucan polymers is clearly invalid, casting doubt on much of the previous literature on *in vitro* cellulose biosynthesis.

Recently, an apparently more specific analytical method for the detection of cellulose has been used. This is the Updegraff extraction (Updegraff, 1969) which involves a hydrolytic extraction of the water- and chloroform:methanol (3:2 v/v)-insoluble products in acetic acid: nitric acid: water (8:1:2 v/v) at 100 °C for 30 minutes. This extraction is purported to solubilise essentially all protein, lignin, lipid and non-cellulosic glucan leaving the cellulose fibres intact (Sloneker, 1971; Delmer *et al*, 1974), presumably due to the crystalline nature of cellulose (Bacic & Delmer, 1981). The Updegraff extraction has been widely used as an assay for cellulose (eg Hogetsu *et al*,1974b; Freeze & Loomis, 1978, 1979; Ginnivan *et al*, 1977; Heineger & Delmer, 1978; Carpita & Delmer, 1980, 1981; Dugger & Palmer, 1980; Montezinos & Delmer, 1980). To date there are no reports of a cell-free enzyme system which is capable of synthesising non-extractable material from any substrate, although detached cotton fibres have been shown to incorporate radioactivity into "Updegraff" cellulose from GDP-^{14}C-G

(Delmer *et al*, 1974). This chapter describes the preliminary experiments carried out to determine whether GDP-[U-^{14}C]-G or UDP-[U-^{14}C]-G could act as substrates for the synthesis of Updegraff cellulose by the particulate enzyme preparation.

6.2 Results and Discussion

The results in this chapter were obtained by the methods described in chapter 3. All incubations were for 15 minutes.

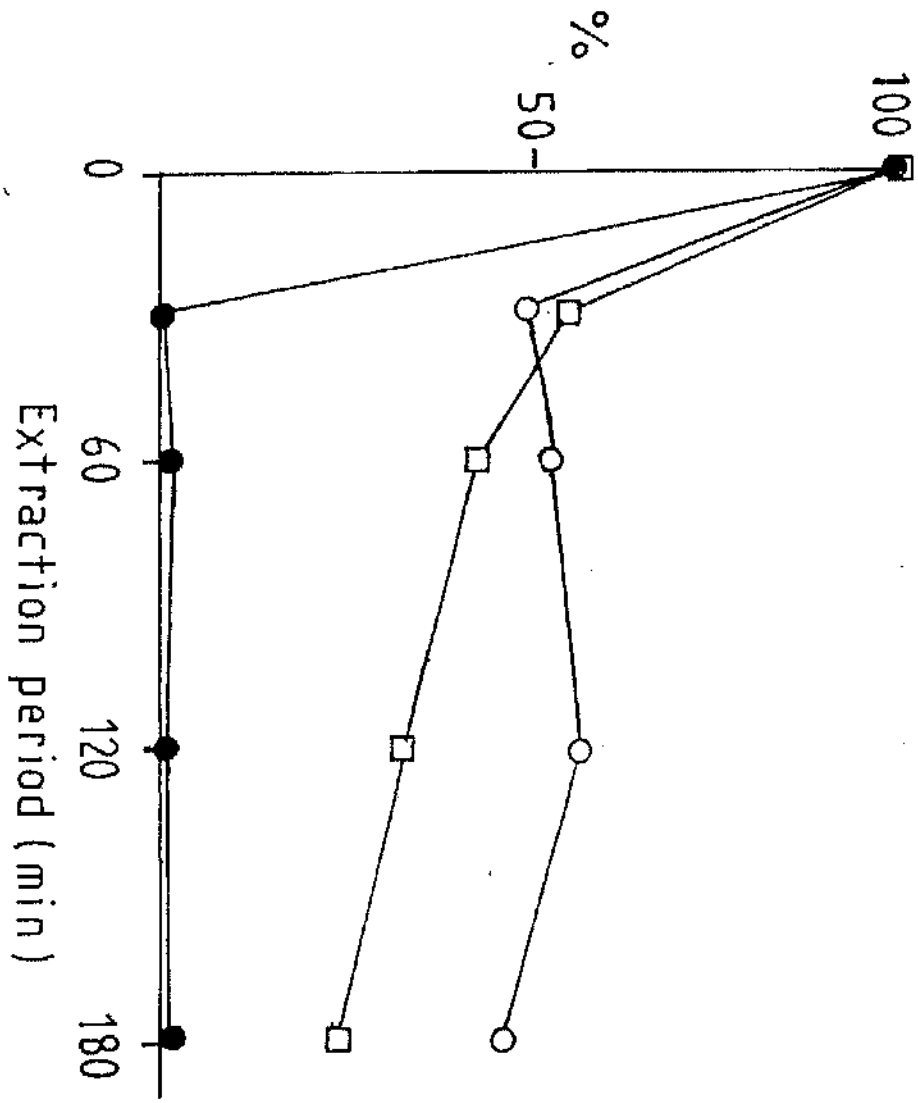
Both UDP-[U-^{14}C]-G and GDP-[U-^{14}C]-G can act as substrates for the synthesis of U-cellulose by the enzyme preparation. No incorporation of radioactivity into U-cellulose was observed from [U-^{14}C]-sucrose, [U-^{14}C]-glucose-1-phosphate or [U-^{14}C]-glucose. Neither D T T , BSA, PMSF, PEG 4000, nor sucrose stimulated the incorporation of radioactive glucose from any of these substrates into U-cellulose. The capacity for the synthesis of U-cellulose from the sugar-nucleotides remained stable for at least up to 24 hours at -20°C (Table 7). Thus, in all subsequent experiments, either unfrozen pellets, or pellets retained at -20°C for less than 24 hours were used as an enzyme source. On the basis of the previous studies outlined in the introduction to this chapter, the U-cellulosic products from GDP-[U-^{14}C]-G and UDP-[U-^{14}C]-G should be crystalline and microfibrillar in nature. Thus, the radioactive residues from the sugar-nucleotides may be added on to the ends of endogenous microfibrils which are acting as primers, or crystalline microfibrillar material is synthesised *de novo*. The alternative possibility that the products are non-microfibrillar and are not extracted by the Updegraff extraction due to a strong non-covalent association with the endogenous microfibrils is unlikely since, at least with GDP-[U-^{14}C]-G as a substrate, Triton X-100 solubilised enzyme was capable of synthesising U-cellulosic material. UDP-[U-^{14}C]-G (1.0 mM) is an efficient donor of glucose for cellulose synthesis (Table 7), although only 1-2% of the water-and

Table 7: The effect of storage of the pellets at -20°C for 24 hours upon the incorporation of radioactive glucose from UDP-[U-^{14}C]-G and GDP-[U-^{14}C]-G into U-cellulose.

substrate	concentration (mM)	enzyme source	incorporation pmol glucose
GDP-[U-^{14}C]-G	0.101	unfrozen pellet	145 ± 10
GDP-[U-^{14}C]-G	0.101	frozen pellet	143 ± 16
UDP-[U-^{14}C]-G	0.001	unfrozen pellet	0.7± 0.0
UDP-[U-^{14}C]-G	0.001	frozen pellet	0.7± 0.0
UDP-[U-^{14}C]-G	1.000	unfrozen pellet	310 ± 7
UDP-[U-^{14}C]-G	1.000	frozen pellet	299 ± 14

The pellets were either used immediately (unfrozen pellet) or were stored at -20°C for 24 hours (frozen pellet) before use. After the routine extractions were conducted, the water-and chloroform:methanol (3:2 v/v)-insoluble products were extracted in acetic acid: nitric acid: water (8:1:2 v/v) at 100°C for 30 minutes as outlined by Updegraff (1969).

Fig. 20. Figure shows the percentage of the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised form 1 μM UDP-[U-^{14}C]-G (□——□, 25 nCi), 1.0 mM UDP-[U-^{14}C]-G (●——●, 25 nCi), and 101 μM GDP-[U-^{14}C]-G (○——○, 25 nCi) remaining insoluble after 30, 60, 120 and 180 minutes extraction with acetic acid: nitric acid: water (8:1:2 v/v) at 100°C.


100
50
%
0
60
120
180
Extraction period (min)

chloroform:methanol (3:2 v/v)-insoluble products remain insoluble after the extraction (Fig 20). This is doubtless due to the removal of the large amounts of β(1-3) glucan shown to be synthesised from 1.0 mM UDP-[U-^{14}C]-G in chapter 4. As a result, the synthesis of U-cellulose from UDP-[U-^{14}C]-G does not appear to be substrate activated. Carpita and Delmer (1980) demonstrated that β(1-3) glucan synthesised from UDPG was entirely extracted from detached cotton fibres by the Updegraff extraction.

Radioactive glucose from GDP-[U-^{14}C]-G (101 µM) was also incorporated into U-cellulose and less than 54% of the water- and chloroform:methanol (3:2 v/v)-insoluble products were removed by the extraction.

With the water- and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 101 µM GDP-[U-^{14}C]-G and 1.0 mM UDP-[U-^{14}C]-G, no further significant extraction occurs after 30 minutes. However, with the products synthesised from 1 µM UDP-[U-^{14}C]-G the extraction is biphasic and does not plateau. The continued slow rate of extraction could be due to two possibilities:

1) The extraction of residual β(1-3) glucan which is less susceptible to the Updegraff reagent due to an association with the cellulose microfibrils, or alternatively, because a fibrillar β(1-3) glucan is synthesised. There is a precedent for the latter hypothesis in the Herth *et al* (1974) found fibrillar β(1-3) glucan, which was insoluble in 20% NaOH, in the cell walls of *Lilium longiflorum* pollen tubes.

2) The gradual hydrolysis of the radioactive residues at the ends of newly synthesised polysaccharide chains. This would be more apparent with the products from 1 µM UDP-[U-^{14}C]-G as there was no isotopic dilution of the radioactive substrate in these incubations.

These two possibilities will be discussed further in chapter 8.

CHAPTER 7: GDP-[U-^{14}C]-G AS A SUBSTRATE FOR THE SYNTHESIS OF U-CELLULOSE

7.1. Introduction

There have been many reports of GDPG acting as a substrate for the synthesis of alkali-insoluble polysaccharide containing β(1-4) linked glucose by particulate and detergent-solubilised enzyme preparations from higher plants. However, there is now some considerable evidence that the product synthesised from GDPG by these preparations is a glucomannan rather than cellulose (chapter 1, pp 23-25) particularly at the low concentrations of GDPG traditionally used. In view of the inadequacy of alkali-insolubility as a criterion to assay for cellulose and the fact that Brett (1981a) demonstrated that the polysaccharide synthesised from GDPG was more cellulosic in nature at higher concentrations of substrate (50 µM), it was decided to re-evaluate the polysaccharide(s) synthesised from high concentrations of GDP-[U-^{14}C]-G by the *P. aureus* particulate enzyme preparation, using the criteria outlined in chapter 2.

7.2. The incorporation of radioactive glucose from GDP-[U-^{14}C]-G into U-cellulose by the particulate enzyme preparation.

With 1 µM GDP-[U-^{14}C]-G as a substrate, the synthesis of U-cellulose comes to an abrupt halt after 1 min (see Fig 21). This very short duration of the synthesis of alkali-insoluble ^{14}C-polysaccharide from low concentrations of GDP-[U-^{14}C]-G is well documented in particulate (Flowers *et al*, 1969; Villemez, 1971; Hinman & Villemez, 1975) and soluble enzyme preparations (Heller & Villemez, 1972a) from a number of plants. However, the reason for the cessation of polysaccharide synthesis in the preparation used in this investigation differs from that found by previous workers. It was found by Flowers *et al* (1969),

Table 8: The effect of preincubating the particulate enzyme preparation at 25°C upon the incorporation of radioactive glucose from GDP-[U-^{14}C]-G into U-cellulose

substrate	conc. (mM)	preincubation (minutes)	incubation (minutes)	incorporation pmol glucose
GDP-[U-^{14}C]-G	0.002	5	-	29 ± 5.0
GDP-[U-^{14}C]-G	0.002	5	5	33 ± 2.4
GDP-[U-^{14}C]-G	0.102	15	-	87 ± 6.4
GDP-[U-^{14}C]-G	0.102	15	15	76 ± 5.7

The enzyme preparation was prepared as usual. GDP-[U-^{14}C]-G (50 nCi) was added immediately, or after a preincubation at 25°C.

Table 9: The effect of the addition of either active or boiled particulate enzyme preparation after an initial incubation with GDP-[U-^{14}C]-G upon the incorporation of radioactive glucose into U-cellulose.

1st incubation				2nd incubation		
substrate	concentration (mM)	incubation (minutes)	incorporation pmol glucose	added enzyme	incubation (minutes)	incorporation pmol glucose
GDP-[U-^{14}C]-G	0.001	1	12 ± 0.8	-	-	-
GDP-[U-^{14}C]-G	0.001	1	-	buffer	1	11 ± 0.5
GDP-[U-^{14}C]-G	0.001	1	-	active	1	12 ± 0.5
GDP-[U-^{14}C]-G	0.001	1	-	boiled	1	12 ± 0.6
GDP-[U-^{14}C]-G	0.101	5	87 ± 0.5	-	-	-
GDP-[U-^{14}C]-G	0.101	5	-	buffer	5	75 ± 5.5
GDP-[U-^{14}C]-G	0.101	5	-	active	5	117 ± 4.5
GDP-[U-^{14}C]-G	0.101	5	-	boiled	5	100 ± 3.4

The enzyme preparation was prepared as normal and incubated with 1 µM GDP-[U-^{14}C]-G (25 nCi) or 101 µM GDP-[U-^{14}C]-G (25 nCi) for 1 and 5 minutes respectively. Subsequently, the reaction was terminated, or a further 100 µl of either buffer (0.1 M tris-HCl pH 7.5), boiled enzyme preparation, or active enzyme preparation was added and incubated for a further 1 or 5 minutes before termination.

Table 10: The effect of the addition of further GDP-[U-^{14}C]-G after an initial incubation with CDP-[U-^{14}C]-G upon the incorporation of radioactive glucose into U-cellulose.

	1st incubation				2nd incubation		
substrate	radioactivity initially added to incubation (nCi)	conc. (mM)	incubation (minutes)	incorporation (dpm)	added radio-activity (nCi)	incubation (minutes)	incorporation (dpm)
GDP-[U-^{14}C]-G	25	0.001	1	6500 ± 280	-	-	-
GDP-[U-^{14}C]-G	25	0.001	1	-	25	1	12530 ± 40
GDP-[U-^{14}C]-G	25	0.001	1	-	-	1	5470 ± 280
GDP-[U-^{14}C]-G	50	0.102	5	1560 ± 40	-	-	-
GDP-[U-^{14}C]-G	50	0.102	5	-	50	5	1760 ± 70
GDP-[U-^{14}C]-G	50	0.102	5	-	-	5	1450 ± 50

The enzyme preparation was prepared as normal and incubated with 1 µM GDP-[U-^{14}C]-G (25 nCi) or 102 µM GDP-[U-^{14}C]-G (50 nCi) for 1 and 5 minutes respectively. The reaction was then terminated or 100 ul of either buffer (0.1 M tris HCl pH 7.5), the same buffer containing either 1 µM GDP-[U-^{14}C]-G (25 nCi) or 102 µM GDP-[U-^{14}C]-G (50 nCi) was added and incubated for a further 1 or 5 minutes before termination. The results are expressed as dpm due to the uncertainty in the amounts of GDP-[U-^{14}C]-G present in the 2nd incubations.

Fig. 21 Time-course of the incorporation of radioactive glucose into U-cellulose from 1 μM GDP-[U-^{14}C]-G (●——●, 25 nCi), 10 μM GDP-[U-^{14}C]-G (○——○, 25 nCi), and 1.0 mM GDP-[U-^{14}C]-G (□——□, 50 nCi) by the particulate enzyme preparation.

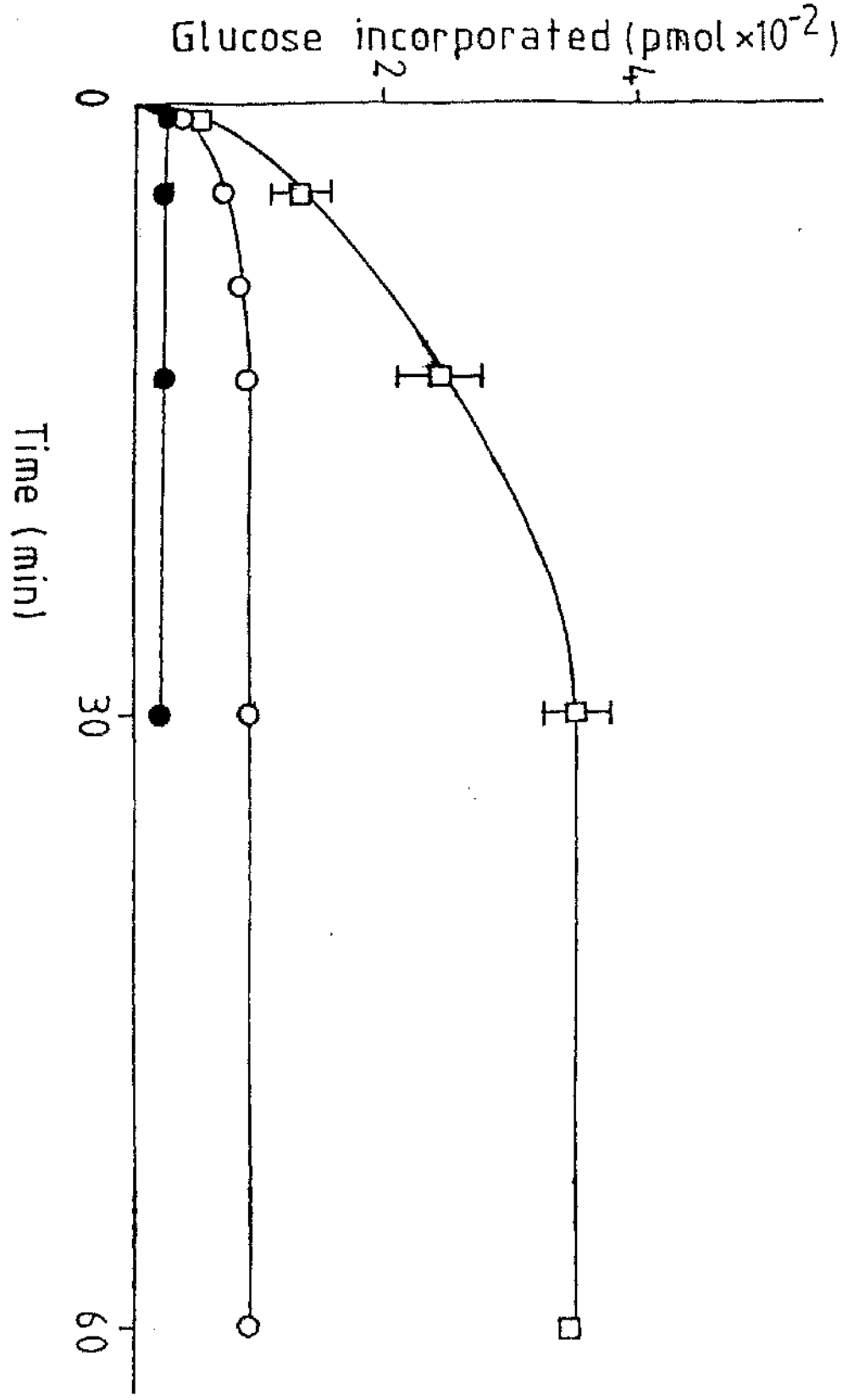

Glucose incorporated (pmol×10⁻²)
2
4
0
30
60
Time (min)

Fig. 22A Thin layer electrophoretogram of the water-soluble radioactive components after a 30 minute incubation of the particulate enzyme preparation with 101 μM GDP-[U-^{14}C]-M (25 nCi).

Fig. 22B Thin layer electrophoretogram of the water-soluble radioactive components after a 1 minute incubation of the particulate enzyme preparation with 1 μM GDP-[U-^{14}C]-G (25 nCi).

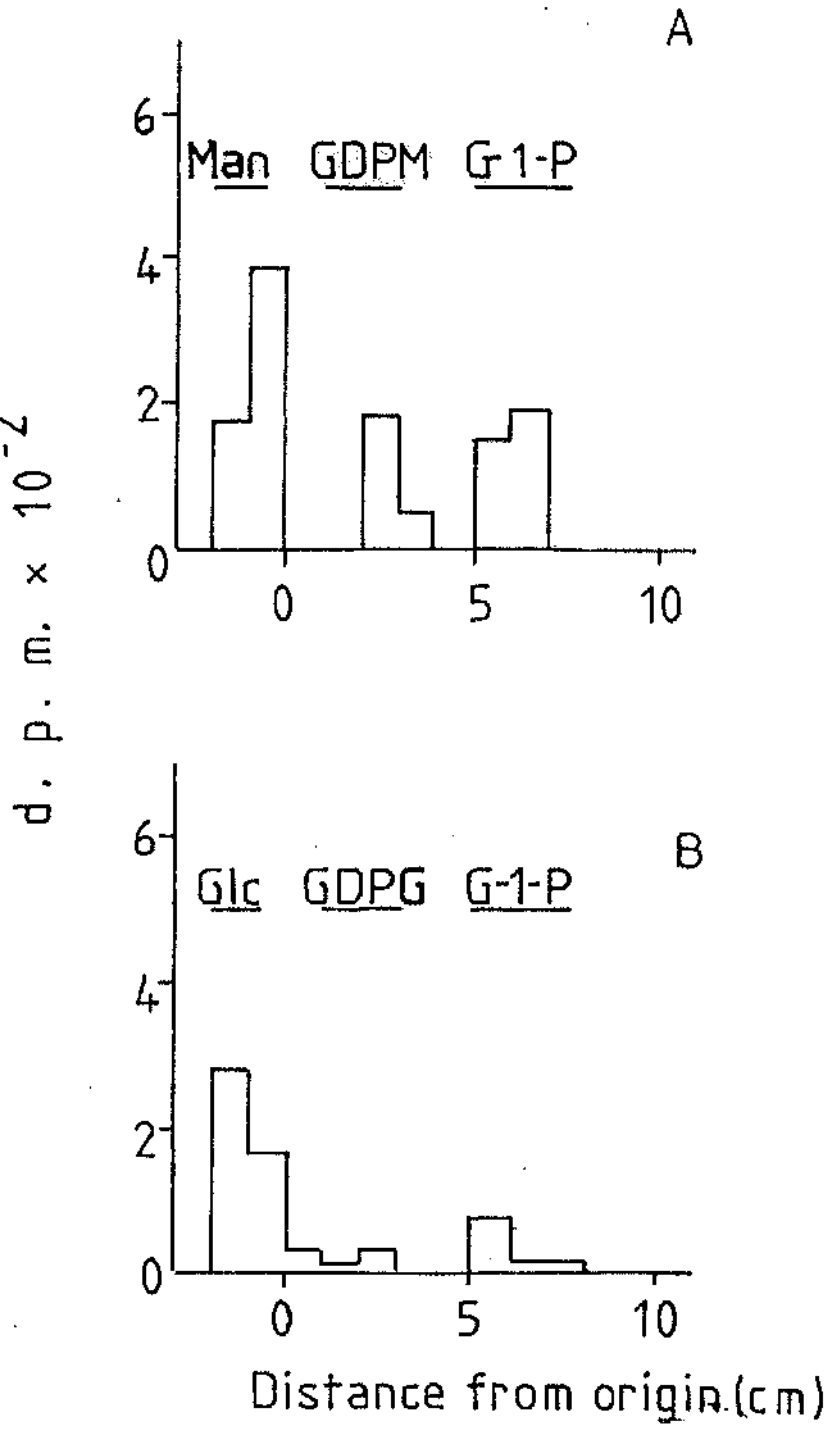

A
Man GDPM Gr1-P
B
Glc GDPG G-1-P
d. p. m. x 10^{-2}
0
2
4
6
0
5
10
Distance from origin (cm)

Fig. 23 Thin layer electrophoretogram of the water-soluble radioactive components after a 1 minute (A), 5 minute (B) and 15 minute (C) incubation of tho particulate enzyme preparation with 101 μM GDP-[U-^{14}C]-G (25 nCi).

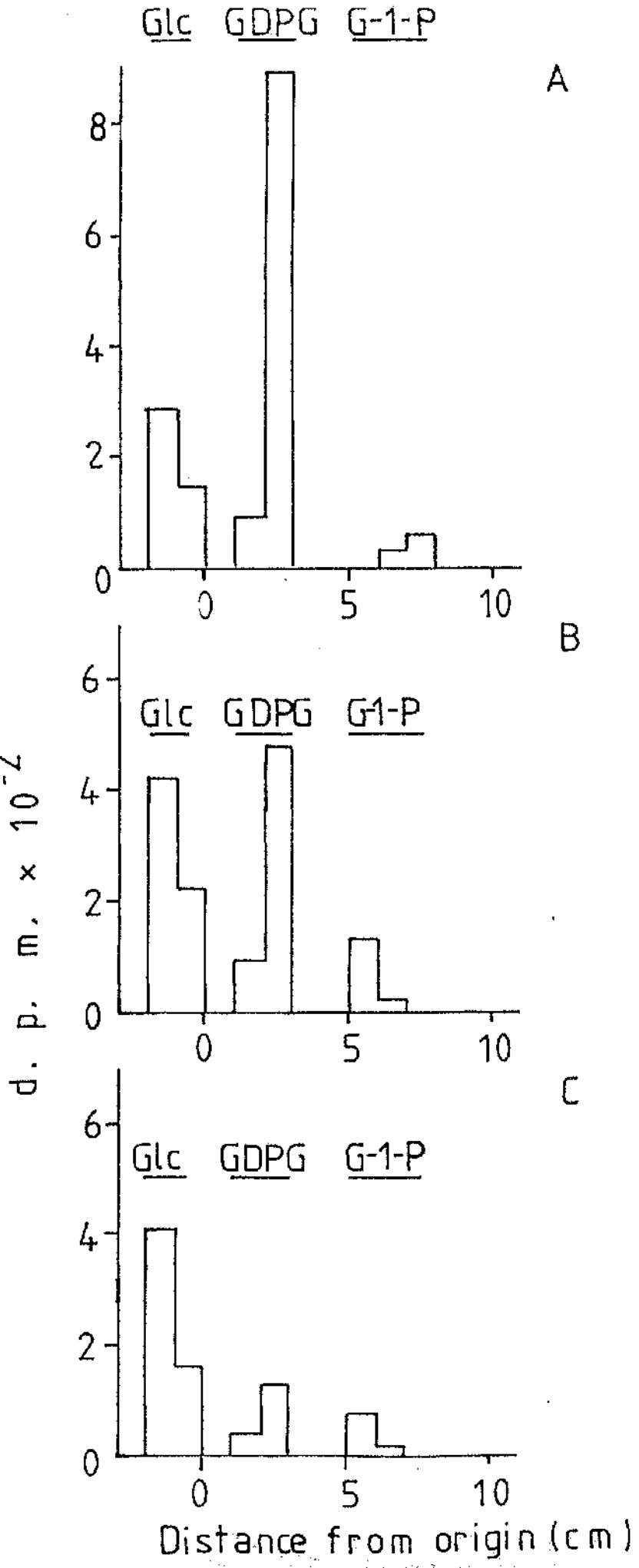
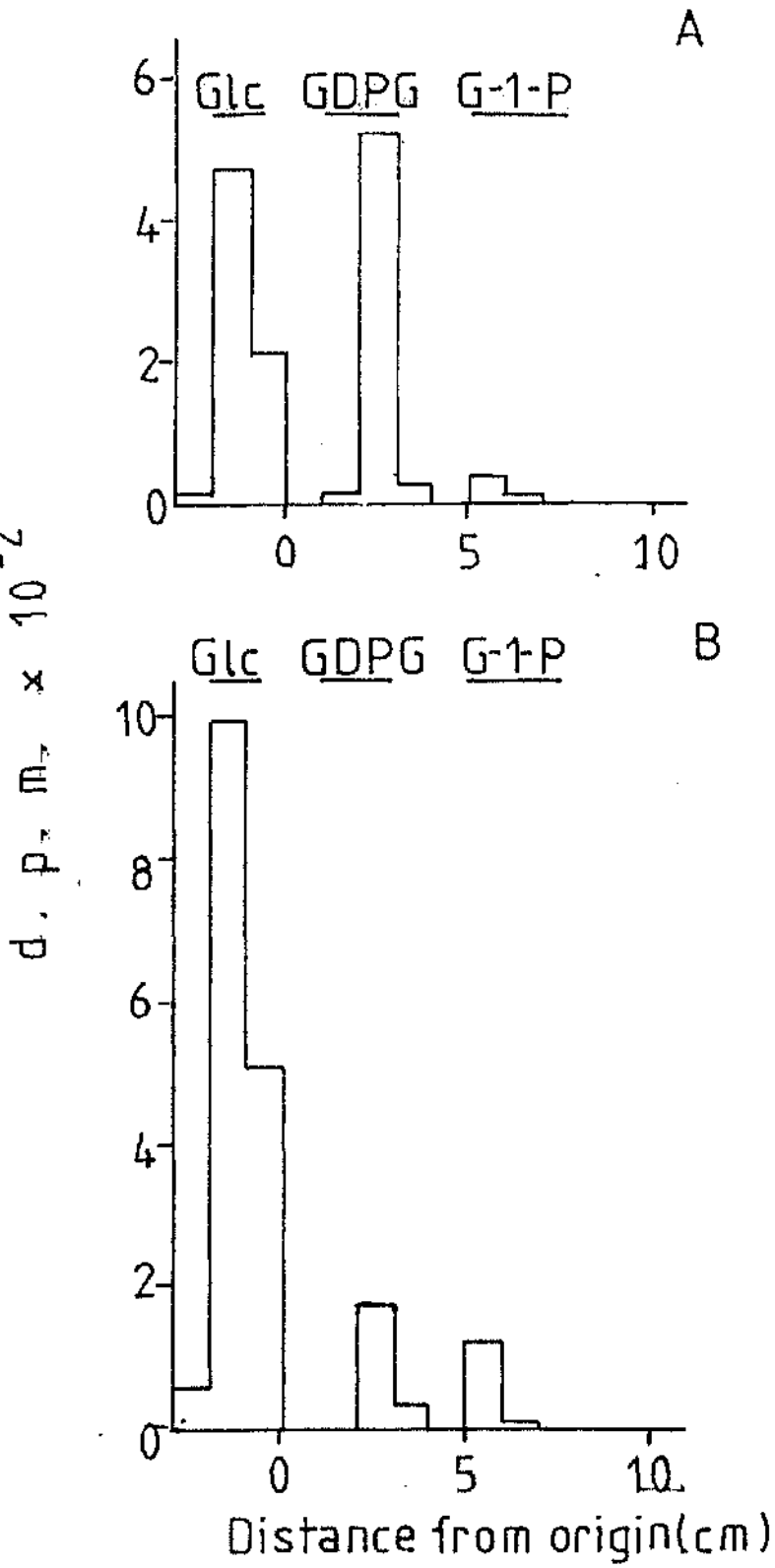

Glc GDPG G-1-P
A
8
6
4
2
0
0
5
10
B
Glc GDPG G1-P
6
4
2
0
0
5
10
C
Glc GDPG G-1-P
6
4
2
0
0
5
10
d. p. m. x 10^{-2}
Distance from origin (cm)

Fig. 24 Thin layer electrophoretogram of the water-soluble radioactive components after a 5 minute (A) and 15 minute (B) incubation of the particulate enzyme preparation with 101 µM GDP-[U-^{14}C]-G (25 nCi) plus 100 µM GDPM.


A
Glc GDPG G-1-P
6
4
2
0
0
5
10
B
Glc GDPG G-1-P
10
8
6
4
2
0
0
5
10
d. p. m. x 10⁻²
Distance from origin(cm)

Lui & Hassid (1970) and Villemez (1971) that the addition of fresh enzyme to incubations after synthesis had ceased, resulted in additional synthesis of ^{14}C-polysaccharide, suggesting that the reaction from low concentrations of GDP-^{14}C-G was not stopping due to a lack of radioactive substrate. I have found however, that in the enzyme preparation used in this investigation the reaction from 1 µM GDP-[U-^{14}C]-G ceased due to a depletion of radioactive substrate. The evidence for this is outlined below:

a) The enzyme(s) are not denaturing during the incubation as the results in Table 8 demonstrate that the capacity for synthesis of U-cellulose remains stable for up to 10 min. Villemez (1971) obtained a similar result in particulate enzyme preparations from the same plant. However, with particulate enzyme preparations from *P. sativum* Hinman and Villemez (1975) observed a bifunctional inactivation of the enzyme although it was clearly shown that this inactivation was not sufficient to account for the termination of the reaction.

b) T.L.E. analysis of the incubation supernatant after a 1 min incubation revealed very little radioactivity co-chromatographing with GDPG (see Fig 22B).

c) The addition of fresh enzyme did not result in a significant increase in the incorporation of radioactive glucose into U-cellulose (Table 9).

d) The addition of fresh GDP-[U-^{14}C]-G after the synthesis of U-cellulose had ceased resulted in a 2-fold increase in the incorporation of radioactivity relative to the control (Table 10). This result also indicates that the reaction does not stop due to the production of an inhibitor during the reaction.

If the concentration of GDPG in the incubation

is increased, the duration of U-cellulose synthesis is also increased (Fig 21). This phenomenon has been observed in particulate enzyme preparations (Hinman & Villemez, 1975) and tissue slices (Brett,1981a) from *P. sativum*.

U-cellulose synthesis from 101 μM GDP-[U-^{14}C]-G is virtually over after 5 minutes. The cessation of synthesis is not due to enzyme inactivation (Table 8), or lack of radioactive substrate since:

a) T L E. analysis reveals that significant amounts of GDP-[U-^{14}C]-G were present in the reaction medium even after a 15 minute incubation (Fig 23).

b) The addition of fresh enzyme preparation to the reaction medium after a 5 minute incubation resulted in a 56% increase in the incorporation of glucose into U-cellulose (Table 9).

c) The addition of further GDP-[U-^{14}C]-G resulted only in a 2% increase in the incorporation of radioactivity into U-cellulose (Table 10).

The above results suggest that there is some limiting endogenous factor in the enzyme preparation which is required for continued U-cellulose synthesis from 101 μM GDP-[U-^{14}C]-G This factor is temperature stable, since the addition of boiled enzyme preparation results in a 33% increase in U-cellulose synthesis, only 23% less than when active enzyme preparation is added. This result lends credence to the argument of Delmer (1977) who suggested that one of the factors involved in the reaction may be the number of endogenous glucan chains able to act as primer molecules for the synthesis of glucan from GDP-[U-^{14}C]-G in detached cotton fibres. Another factor definitely involved in the cessation of the reaction, that of endogenous mannose sources, will be discussed in section 7.3.

The incorporation of glucose into U-cellulose from 1.0

mM GDP-[U-^{14}C]-G continues at an ever decreasing rate for up to 30 minutes (Fig 21). This would suggest the presence of an enzyme which is activated by increased GDPG concentrations and which apparently does not require the presence of mannose donors or mannose-containing acceptor molecules for continued U-cellulose synthesis, since in the latter periods of the incubation at least, endogenous sources of mannose would be expected to be depleted. Thus the product of this reaction may be long stretches of microfibrillar glucan which, if so, has obvious implications for cellulose biosynthesis and will be discussed in chapter 9.

7.3. The effect of other non-radioactive sugar-nucleotides upon the incorporation of radioactive glucose from GDP-[U-^{14}C]-G into U-cellulose by the particulate enzyme preparation.

In view of the considerable weight of evidence for the synthesis of glucomannan from GDPG by enzyme preparations from higher plants (see chapter 1, pp 23-25) it was decided to investigate the effect of GDPM (the mannose donor for glucomannan synthesis) upon the synthesis of U-cellulose from 101 µM GDP-[U-^{14}C]-G. It was found that 100 µM GDPM stimulated the synthesis of U-cellulose from 101 µM GDP-[U-^{14}C]-G and that the stimulation was due to an extension of the reaction period (Fig 25). No increase in the initial rate of reaction (determined after 6 seconds) from 101 µM GDP-[U-^{14}C]-G could be observed in the presence of 10 µM, 100 µM or 1 mM GDPM in the reaction media. These results are similar to those obtained by Villemez (1971) with *P. aureus* enzyme preparations and Hinman and Villemez (1975) with preparations from *P. sativum*, both using low concentrations of GDPG in the synthesis of polysaccharides. Neither UDP-Xyl nor UDPG were observed to stimulate U-cellulose synthesis from 101 µM GDP-[U-^{14}C]-G (Table 11). The prolonged period of incorporation was not due to a preservation of GDP-[U-^{14}C]-G by GDPM since T L E analysis of the supernatants after a 5 minute incubation demonstrated

Table 11: The effect of GDPM (100 µM), UDPG (100 µM) and UDPXyl (100 µM) upon the incorporation of radioactive glucose from 101 µM GDP-[U-^{14}C]-G into U-cellulose.

substrate	added sugar-nucleotide	incorporation pmol glucose
GDP-[U-^{14}C]-G	-	118 ± 11
GDP-[U-^{14}C]-G	UDPG	127 ± 2
GDP-[U-^{14}C]-G	UDPXyl	133 ± 5
GDP-[U-^{14}C]-G	GDPM	358 ± 22

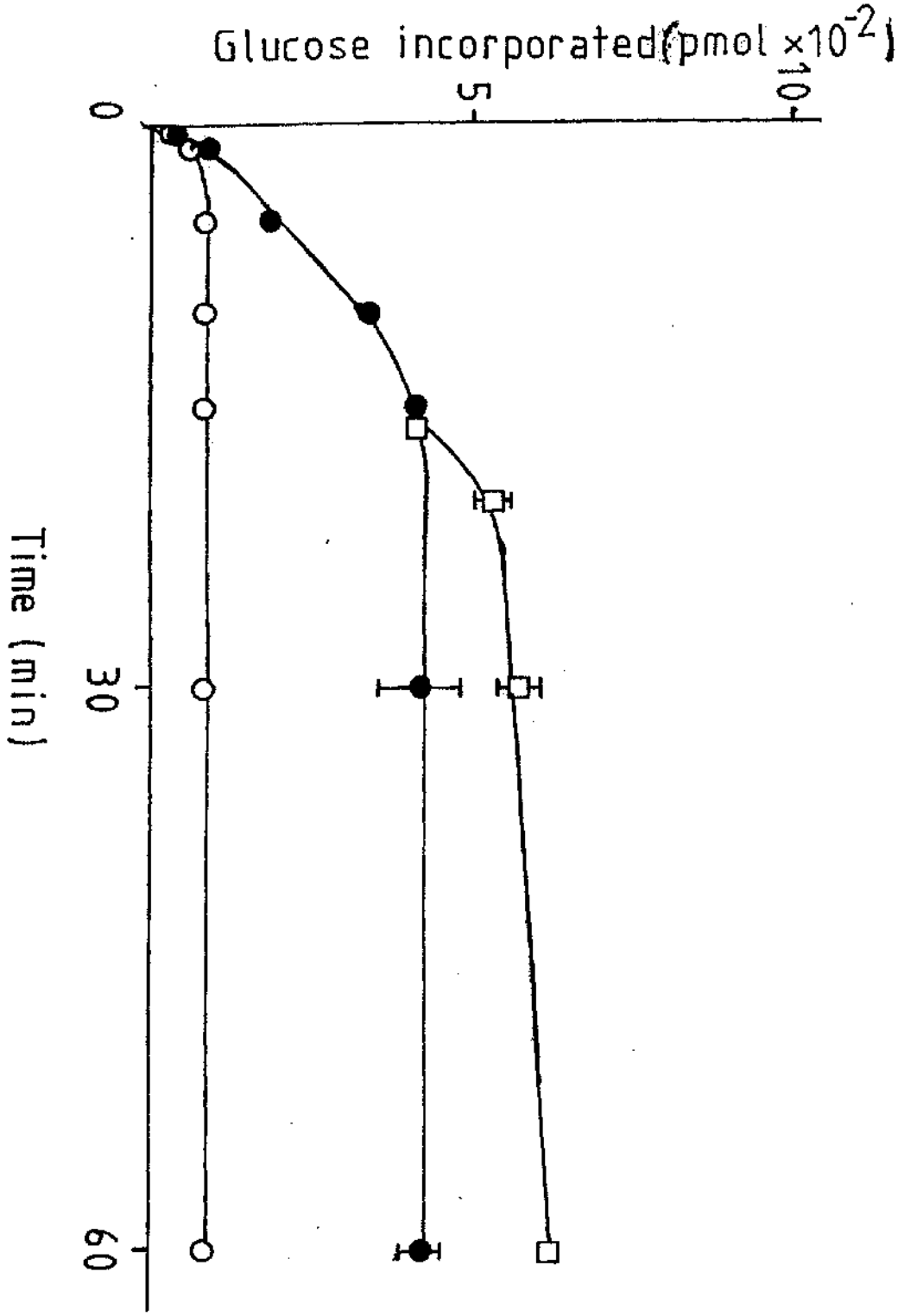
Incubations were as outlined in chapter 3 except that water, UDPG, UDPXyl or GDPM was added to the reaction media. Each incubation contained 25 nCi of radioactivity and was incubated for 15 minutes.

Table 12: The effect of pre-incubation with 100 µM GDP-mannose upon the incorporation of radioactivity from 101 µM GDP-[U-^{14}C]-G into U-cellulose.

preincubation	incubation	incorporation dpm	pmol incorporated
GDPM (100 µM)	GDP-[U-^{14}C]-G	323 ± 12	58 ± 2
H_2O	GDP-[U-^{14}C]-G	296 ± 36	54 ± 7
H_2O	GDP-[U-^{14}C]-G + 100 µM GDPM	1360 ± 41	247 ± 45

The enzyme preparation was incubated as normal ± GDPM (100 µM) for 15 min and the membranes recovered by centrifugation at 97000 g for 35 min after a 250-fold dilution with 0.1 M tris HCl buffer, pH 7.5 at 1° C. The pellets were resuspended and the process repeated to remove residual GDPM. The membranes were then resuspended in buffer and incubated with 101 µM GDP-[U-^{14}C]-G with or without 100 µM GDPM for 15 min. Each incubation contained 25 nCi.

Fig. 25 Time-course of the incorporation of radioactive glucose from 101 µM GDP-[U-^{14}C]-G (25 nCi) plus (●——●) and minus (O——O) 100 µM GDPM. Boiled enzyme preparation (100 µl) was added to incubations containing GDPM after incorporation from GDP-[U-^{14}C]-G had ceased (□——□).


Glucose incorporated (pmol ×10⁻²)
0
5
10
Time (min)
30
60

that there were similar amounts of GDP-[U-^{14}C]-G regardless of the presence or absence of GDPM in the incubations (Figs 23B and 24A). Heller and Villemez (1972a), using Triton X-100 solubilised enzyme preparations, found that GDPM did not stimulate the synthesis of ^{14}C-polysaccharides by isolated GDPG:β(1-4) glucan glucosyl-transferase, but rather it was a weak competitive inhibitor. Thus it was argued that it was the product(s) of the mannosyl transferase that was responsible for the prolonged synthesis of polysaccharide from GDPG i.e. the GDPG:β(1-4) glucan glucosyl-transferase activity was dependent upon a mannose-containing acceptor molecule for continued synthesis. Barber (1982) also found that with a solubilised enzyme preparation from *P. aureus*, preincubation with non-radioactive GDPM resulted in the usual stimulation of polysaccharide synthesis from GDPG observed when both sugar nucleotides are present simultaneously in the reaction media. It was suggested that mannosyl units may act as glucosyl acceptors, perhaps anchoring or initiating the synthesis of the cellulose polymer. The results in Table 12 indicate that, in my system, the product(s) from GDPM do not act as priming molecules for the subsequent, continued U-cellulose synthesis from 101 µM GDP-[U-^{14}C]-G but rather, GDPM must be present simultaneously with GDP-[U-^{14}C]-G to cause stimulation of U-cellulose synthesis.

The reaction utilising 101 µM GDP-[U-^{14}C]-G plus 100 µM GDPM does not stop after 15 minutes due to a lack of either substrate (Figs 22A and 24B). Neither does it stop due to enzyme denaturation of either the glucosyl transferase (Table 8) or the mannosyl transferase (Fig 26). (This assumes that the mannosyl transferase which synthesises U-cellulose from GDPM alone is the same enzyme as that involved in glucomannan synthesis. This assumption is probably justified on the basis of the observations by Villemez (1971) and Heller and Villemez (1972a) working with *P. aureus* enzymes. (See chapter 1. pp 23-25). It is further supported by the fact that under conditions when

the β-mannosyl transferase utilising GDPM is inhibited, no stimulation of U-cellulose synthesis from 101 μM GDP-[U-^{14}C]-G by 100 μM GDPM is observed (see Table 13). The possibility that the reaction terminates due to the production of an inhibitor during the reaction also does not seem likely in the light of the results in this section and 7.2. Thus, as was the case with incubations containing 101 μM GDP-[U-^{14}C]-G alone, there appears to be a requirement for a consumable, heat-stable, endogenous factor in the enzyme preparation as indicated by the further synthesis of U-cellulose from GDP-[U-^{14}C]-G plus GDPM upon the addition of boiled enzyme preparation to the incubation (Fig. 25). As argued in 7.2., this factor may be the ends of glucan chains, perhaps within the endogenous cellulose microfibrils to which, even in the presence of GDPM, only a limited number of sugar residues may be added. This restriction could easily be envisaged as it is thought that microfibril deposition is a complicated process involving membrane flow and cortical microtubules (Mueller & Brown, 1982a, b). It is very likely that homogenisation of the hypocotyl tissue will result in the disruption of the organisation of the microfibril deposition apparatus required for continued microfibril synthesis.

In this investigation GDPM was also found to stimulate U-cellulose synthesis from 1 μM GDP-[U-^{14}C]-G. This result would appear to be contradictory to the observation that U-cellulose synthesis from 1 μM GDP-[U-^{14}C]-G stops due to depletion of radioactive substrate (Fig. 22B). However, it can be explained if the insertion of a mannose residue into a glucan chain allowed further elongation of the glycan chain resulting in a product with a larger molecular weight. Indeed, it has been shown that the presence of GDPM in the incubation increases the molecular weight of the water-and chloroform:methanol (3:2 v/v)-insoluble products from GDP-[U-^{14}C]-G (see section 7.5). Thus the inclusion of GDPM in the incubation could result in the increased synthesis of U-cellulose at the expense of water-soluble oligosacc-

Fig. 26 Time-course of the incorporation of radioactive mannose into U-cellulose from 102 µM GDP-[U-^{14}C]-M (50 nCi) by the particulate enzyme preparation.

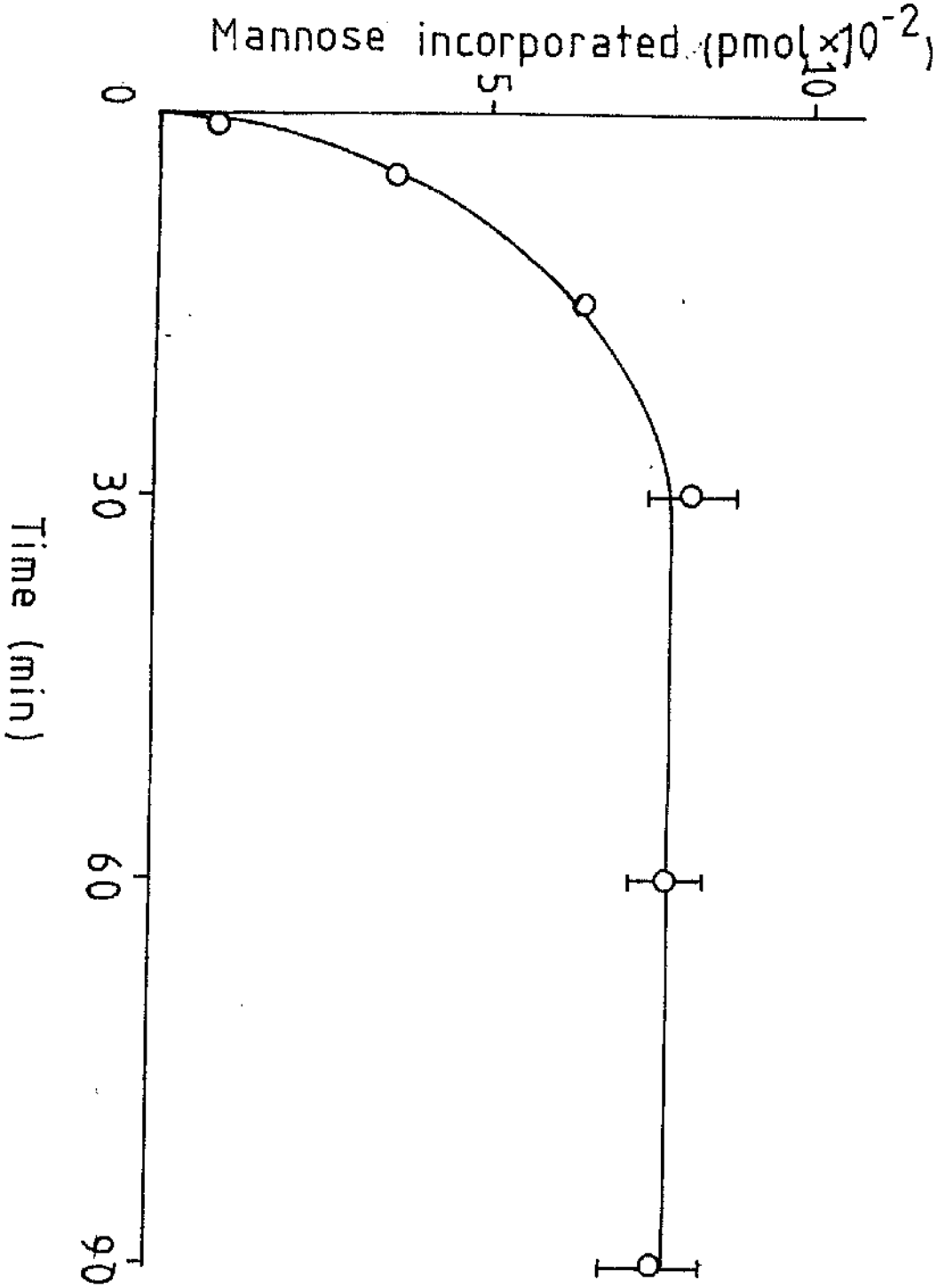
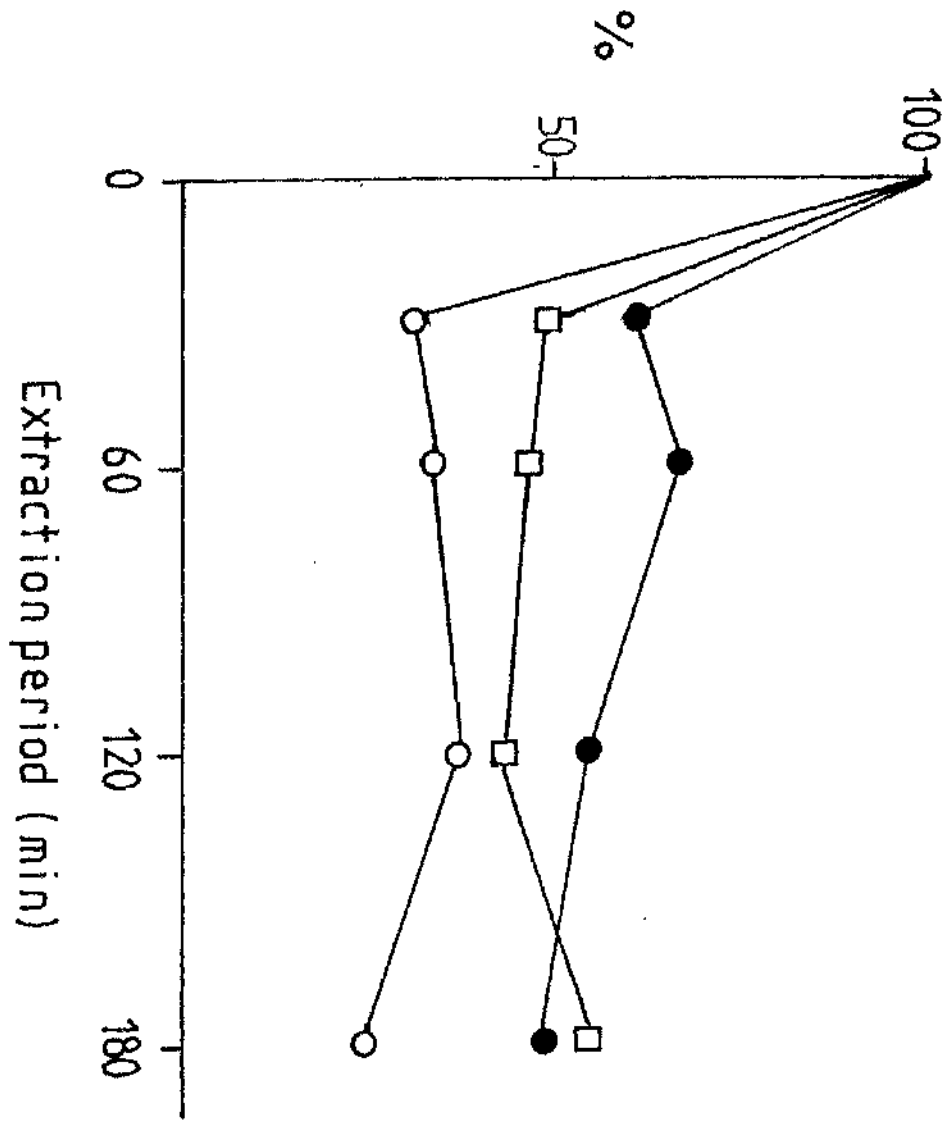

Mannose incorporated (pmol(×10⁻²))
0
5
10
Time (min)
0
30
60
90

Fig. 27 This figure shows the percentage of the water and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 102 µM GDP-[U-^{14}C]-G (●——●), 102 µM GDP-[U-^{14}C]-G + 100 µM GDPM (□——□) and 102 µM GDP-[U-^{14}C]-M (○——○) remaining insoluble after 30, 60, 120 and 180 minutes extraction with acetic acid: nitric acid: water (8:1:2 v/v) at 100°C. Each incubation was for 15 minutes and contained 50 nCi of radioactivity.


%
100
50
0
60
120
180
Extraction period (min)

harides which may in part be responsible for the peak at the origin in Fig. 22B. If this hypothesis is correct, then it would suggest the the water-soluble oligosaccharides are in fact intermediates in U-cellulose synthesis. Indeed there is some previous evidence for the participation of short chain glucan intermediates in cellulose biosynthesis (see section C.1.4.4.)

The previous results and these obtained by gel filtration (section 7.5) and linkage analysis (see section 7.6) indicate that a glucomannan which is insoluble after the Updegraff extraction is synthesised from exogenously supplied GDP-[U-^{14}C]-G by the particulate enzyme preparation. GDP-[U-^{14}C]-M is also utilised for the synthesis of U-cellulose (Fig. 26). Indeed, it appears to be a better substrate than GDP-[U-^{14}C]-G and synthesis from 10Ω μM GDP-[U-^{14}C]-M proceeds at an ever decreasing rate for 30 minutes.

Thus GDPG and GDPM both act as substrates for the formation of Updegraff-insoluble material containing a glucomannan and possibly mannan and glucan. Since it was possible that the standard extraction time, 30 minutes, was not long enough to remove all non-cellulosic materials, longer periods of extraction were conducted. As shown in Fig. 27, none of the products exhibited any significant change with extraction periods of up to 3 hours. Thus it must be concluded that all the products are indeed U-cellulose as defined by the extraction procedure. However, as was the case with the α-cellulose fraction of the mung bean particulate enzyme preparation, no mannose was detectable after a G.L.C analysis of the alditol acetates produced from the U-cellulosic fraction of the enzyme preparation. It must be stressed however, that the results obtained were far from satisfactory in terms of reproducibility and the quality of chromatography. It is surprising indeed that non-glucose residues were not found in view of the considerable evidence for microfibrillar cellulose containing

amounts of non-glucose residues including mannose (Preston, 1974) which are detectable by paper chromatographic techniques. As pointed out in ch. 1;B.2.3., these residues are only removed under conditions which destroy the integrity of the microfibril. Work is being continued by Dr C.T. Brett to develop an improved method for G.L.C. analysis of the sugar residues found in the cellulosic fractions of plant tissues.

7.4. The effect of calcofluor white ST on U-cellulose synthesis from GDP-[U-^{14}C]-G by the particulate enzyme preparation

In *Acetobacter xylinum*, calcofluor white ST prevents the formation of crystalline microfibrils resulting in the increased synthesis of non-crystalline β(1-4) glucan (see chapter 1. section C.2 p. 60). Since the Updegraff extraction is purported to leave only crystalline cellulose in the insoluble residue, it was hoped that the inclusion of calcofluor white ST would result in an inhibition of the synthesis of U-cellulose synthesis and a corresponding increase in the synthesis of water- and chloroform: methanol (3:2 v/v)-insoluble product from GDP-[U-^{14}C]-G. This would provide further evidence for the microfibrillar nature of the products.

It was found that U-cellulose synthesis from 101 uM GDP-[U-^{14}C]-G by the particulate enzyme system was inhibited by 43% with 1 mg ml^{-1} calcofluor white ST in a 15 minute incubation. However, the synthesis of the water-and chloroform:methanol (3:2 v/v)-insoluble products was also inhibited by 40%. This would suggest that the effect was due to a direct inhibition of the polymerase enzymes involved, rather than an interference with the crystallization process. This is in contrast with the results of Benziman *et al* (1980).

7.5. Gel filtration of the water- and chloroform:methanol (3:2 v/v)-insoluble products synthesised from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-M by the particulate enzyme preparation.

As pointed out in chapter 2 it may be necessary to use a physical criterion to aid in the identification of cellulose. The simplest physical assay for cellulose is that of molecular weight since cellulose is the only polysaccharide of dicotyledonous plant cell walls that may have a D P of several thousand. Thus, any high molecular weight material found in the radioactive products may well be cellulosic. As can be seen in Figs. 28-31 all the products were eluted with, or just before Dextran T70 and therefore have a probable molecular weight of less than or equal to 7×10^4. Different enzyme preparations were used for the results in Figs 28 and 30 explaining the slight variation in the elution volume of the products from 101 µM GDP-[U-^{14}C]-G.

There is some justification for doubting the appropriateness of these dextran markers in assessing the molecular weight of the products, as dextrans are highly branched molecules, while β-glucans, glucomannans and mannans are mostly linear. It is clear however, that none of the products are of the size expected of cellulose. However this negative result alone does not exclude the possibility that the products are cellulosic for the following reasons:

1) Spencer & Machlachlan (1972) have shown that in *P. sativum* epicotyls, low molecular weight (D.P $<$ 500) cellulose does exist, particularly in young tissues.

2) The products may be degraded by endogenous cellulases. Indeed there is some evidence that these degradative enzymes are present in the particulate enzyme preparation used in this investigation (see chapter 4, p 87).

Fig 28 Gel filtration of the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 101 μM GDP-[U-^{14}C]-G (25 nCi) plus (O——O) and minus (●——●) 100 μM GDPM on Sepharose CL-6B (column volume 9 cm^3; fraction volume, 0.375 cm^3). Incubations were for 15 minutes and whole fractions were counted. The Dextran markers, Dextran T500 and T70 (abbreviated in figure to T500 and T70) were obtained from Pharmacia Fine Chemicals and were detected in the eluant by the phenol-sulphuric assay of Dubois *et al* (1956).

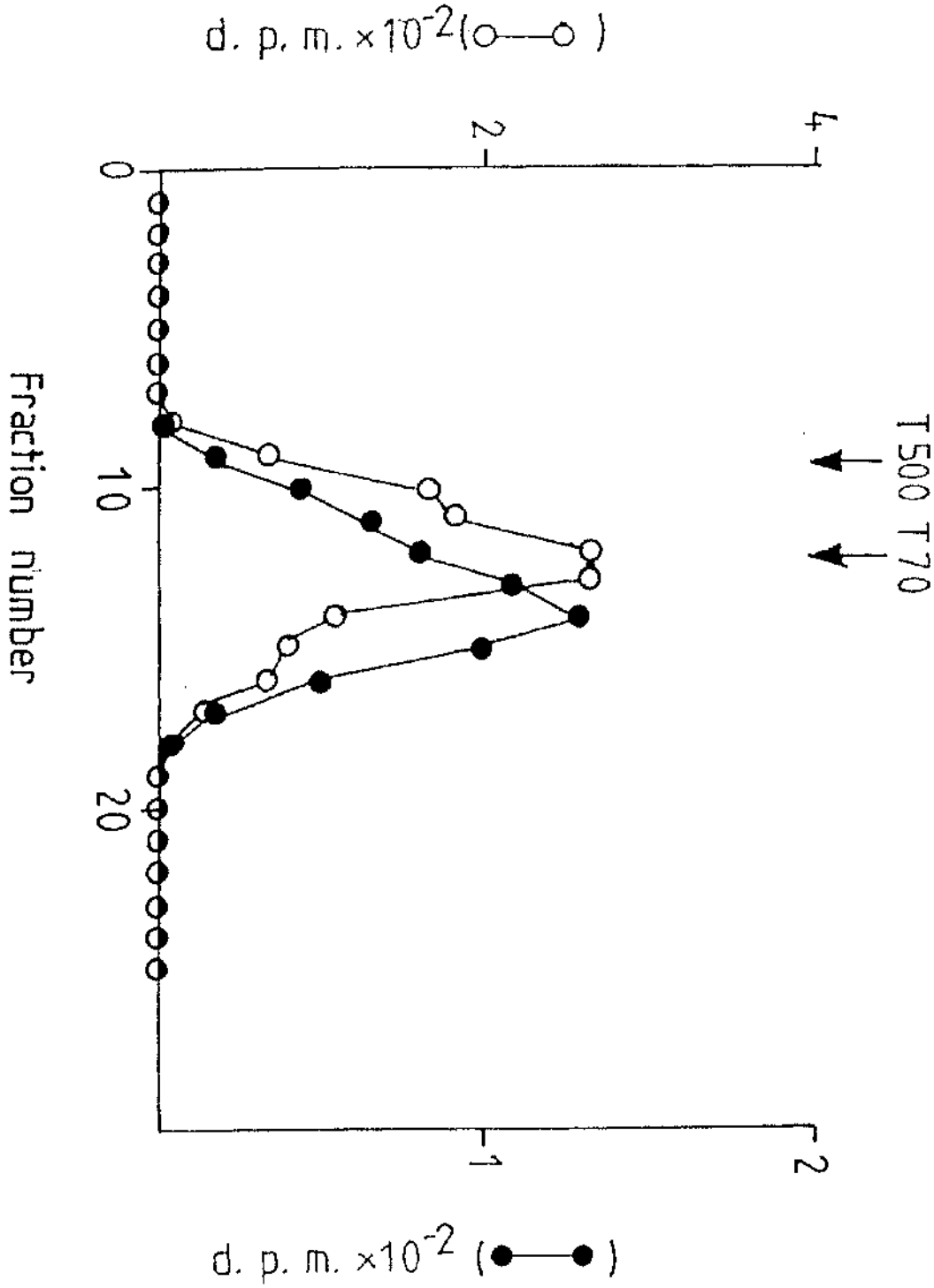

d. p. m. ×10⁻²(o—o)
4
2
0
10
20
Fraction number
T50 T70
2
1
d. p. m. ×10⁻² (●—●)

Fig. 29 Gel filtration of the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 101 μM GDP-[U-^{14}C]-M (25 nCi) on Sepharose CL-6B (column volume 9 cm^3; fraction volume, 0.375 cm^3). The products used in this figure and in Fig. 28 were synthesised by the same enzyme preparation and thus the results are directly comparable. The incubation period was 15 minutes and whole fractions were counted. Dextran markers and their detection as in Fig. 28.

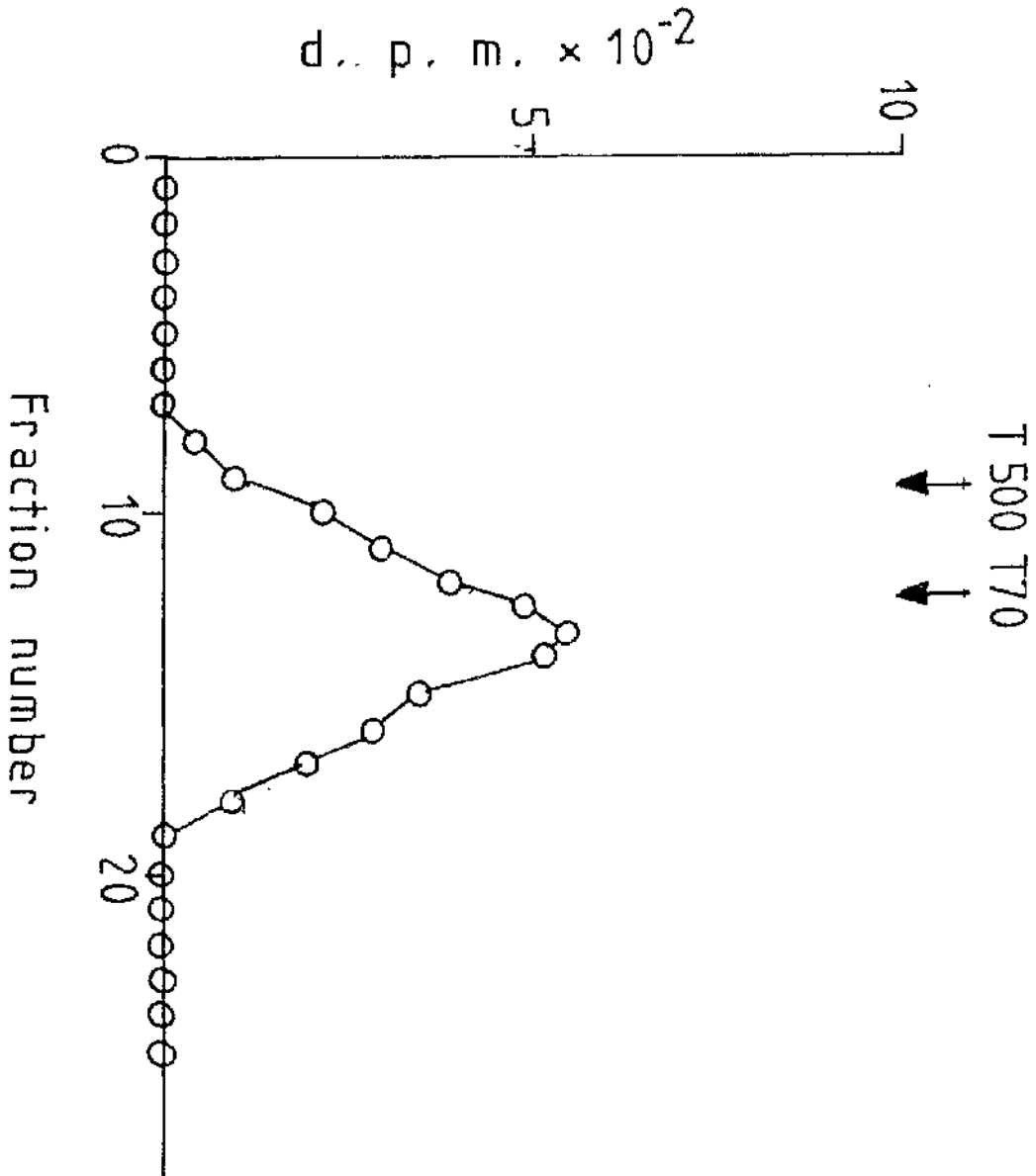

d. p. m. × 10-2
5
10
0
10
20
Fraction number
T 500 T70

Fig. 30 Gel filtration of the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 1 μM (O—O) and 101 μM (●—●) GDP-[U-^{14}C]-G (25 nCi) on Sepharose CL-6B (column volume 9 cm^3; fraction volume 0.375 cm^3). The incubations were for 5 minutes and whole fractions were counted. The Dextran markers and their detection are as in Fig. 28.

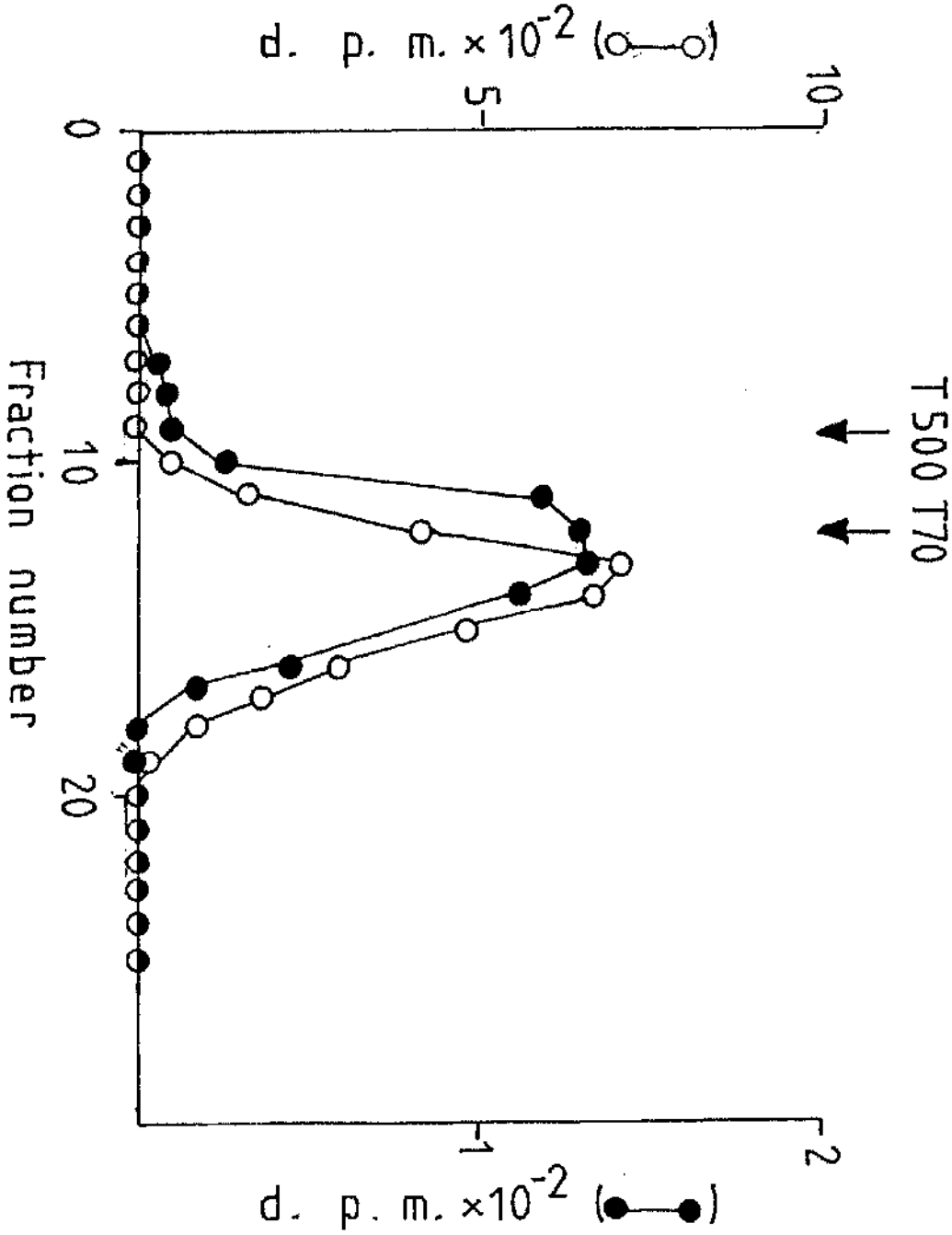
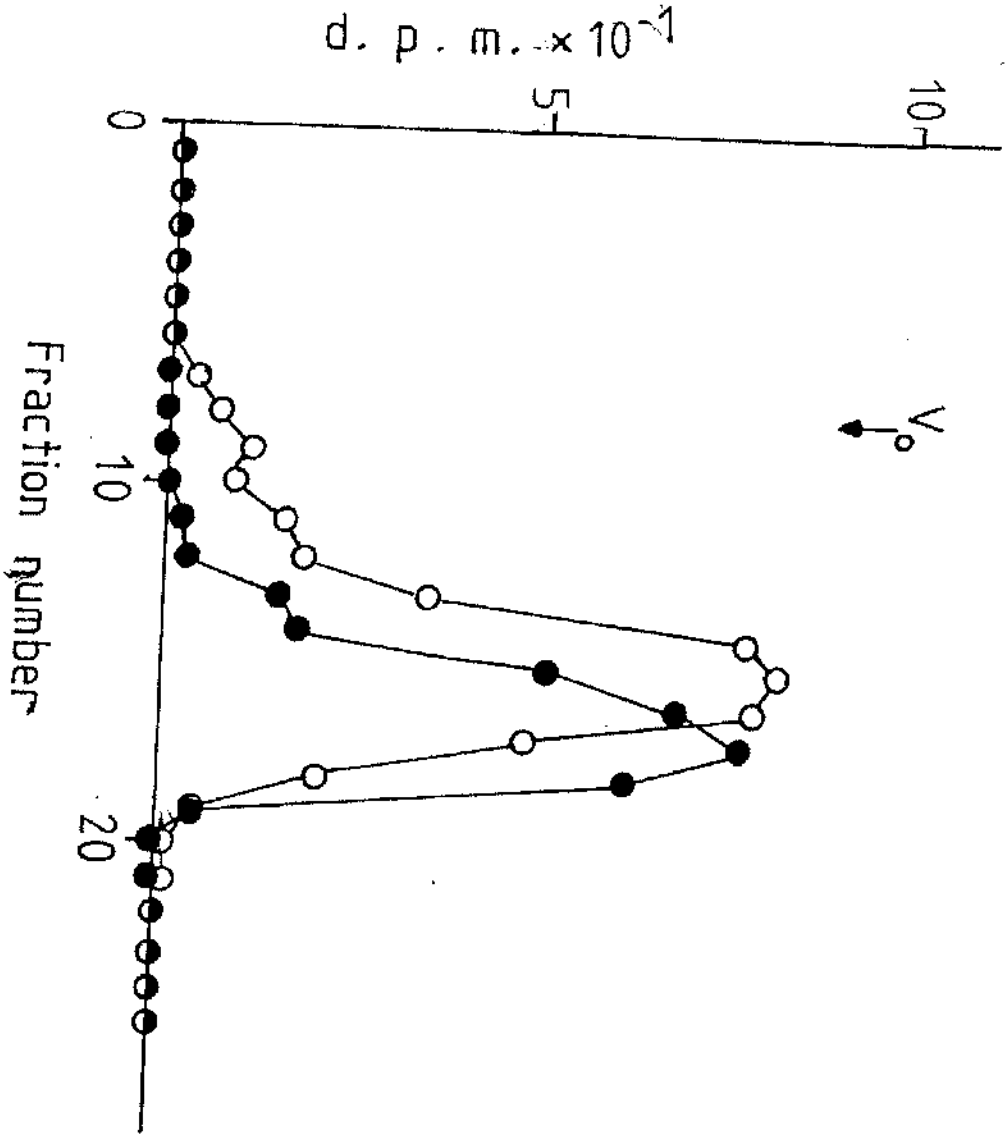

d. p. m. × 10⁻² (o—o)
5
10
0
10
20
Fraction number
T500 T70
1
2
d. p. m. ×10⁻² (●—●)

Fig. 31 Gel filtration of the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 1 μM (●——●) and 101 μM (O——O) GDP-[U-^{14}C]-G (25 nCi) on Sepharose CL-6B (column volume 25 cm^3; fraction volume, 1 cm^3). Incubations were for 5 minutes and in the case of the products synthesised from 101 μM GDP-[U-^{14}C]-G, 300 μl of the fraction was counted. Column void volume (V_0) was determined using Blue Dextran 2000 (Pharmacia Fine Chemicals).


d. p. m. × 10⁻¹
0
5
10
Fraction number
10
20
V_o

3) The products may be short-chain intermediates whose further incorporation into high molecular weight cellulose is blocked in some manner.

It is interesting to note that the products from GDP-[U-^{14}C]-G, GDP-[U-^{14}C]-M and GDP-[U-^{14}C]-G plus GDPM are not of equivalent size. Hinman and Villemez (1975) reported that the major products synthesised from low concentrations of GDPG, GDPM, and GDPG plus GDPM by particulate enzyme preparations from *P. sativum* had similar molecular weights. It was argued from this result that the same product (a glucomannan) was synthesised from both these substrates and the suggestion of Flowers *et al* (1969), that the GDPG:β(1-4) glucan glucosyl-transferase thought to be involved in cellulose biosynthesis required the insertion of mannosyl residues to continue chain elongation, was incorrect. This hypothesis of Flowers *et al* is attractive as it offers an explanation for the mannose residues commonly found in the cellulosic fractions of plant material. The results of the experiments in the present investigation are consistent with this hypothesis, in that the inclusion of GDPM in the incubation increases the molecular weight of the products from GDP-[U-^{14}C]-G. Upon closer inspection, the results of Hinman and Villemez are also consistent with the hypothesis of Flowers *et al*. When either GDPG or GDPM was incubated alone, a smaller molecular weight product was detected. When both these sugar-nucleotides were included in the incubation, the size of the low molecular weight peak was considerably reduced, implying that they were intermediates in the chain forming process which required the insertion of either a glucose or mannose residue (depending on radioactive substrate) for further chain elongation. The fact that the products were not of the size expected for cellulose even when both sugar-nucleotides were present in no way excludes the possibility that they are cellulosic for the reasons outlined earlier.

Increasing GDP-[U-^{14}C]-G concentration from 1 μM to

101 µM also resulted in an increase in the molecular weight of the products (Fig. 30) This effect was confirmed on a larger column (Fig. 31). A similar result has been reported for the products synthesised from GDP-[U-^{14}C]-G by pea epicotyl slices (Brett, 1981a).

7.6. Further analysis of U-cellulose synthesised from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-M by the particulate enzyme preparation.

In this section experiments are described which were conducted to determine the sugar composition and the sugar-sugar linkage of the U-cellulose synthesised from the substrates.

Total acid hydrolysis of the products from GDP-[U-^{14}C]-G plus GDPM and GDP-[U-^{14}C]-M gave rise to radioactive glucose and mannose respectively as the only radioactive monosaccharides (Figs 33 and 34) indicating that the radioactive residues from the sugar-nucleotides are incorporated into U-cellulose without prior modification of the sugar-residues. Total acid hydrolysis of the U-cellulose synthesised from GDP-[U-^{14}C]-G alone did not result in a complete hydrolysis of the products (Fig. 32). Neither did increased periods of hydrolysis significantly reduce the amount of undegraded material. This may be some evidence of the cellulosic nature of these products, as it is known that cellulose is especially resistant to acid hydrolysis under these conditions (Brett, 1981b). Fig 32 demonstrates that glucose is the only radioactive residue found in the hydrolysed portion of U-cellulose synthesised from 103 µM GDP-[U-^{14}C]-G.

Dennis and Preston (1961) showed that the pure glucan crystallites of the cellulose microfibril, termed rodlets, were resistant to extraction with 2.5 N H_2SO_4 at 100°C for 24 hours. The rodlets had a characteristic cellulose

Fig. 32 Paper chromatogram of the U-cellulosic products synthesised from 103 μM GDP-[U-^{14}C]-G (75 nCi) after total acid hydrolysis at 15 lb in^{-2} for 1 hour and 2 days chromatography. The incubation was for 5 minutes. (Glc : glucose, Gal : galactose, Man : mannose).

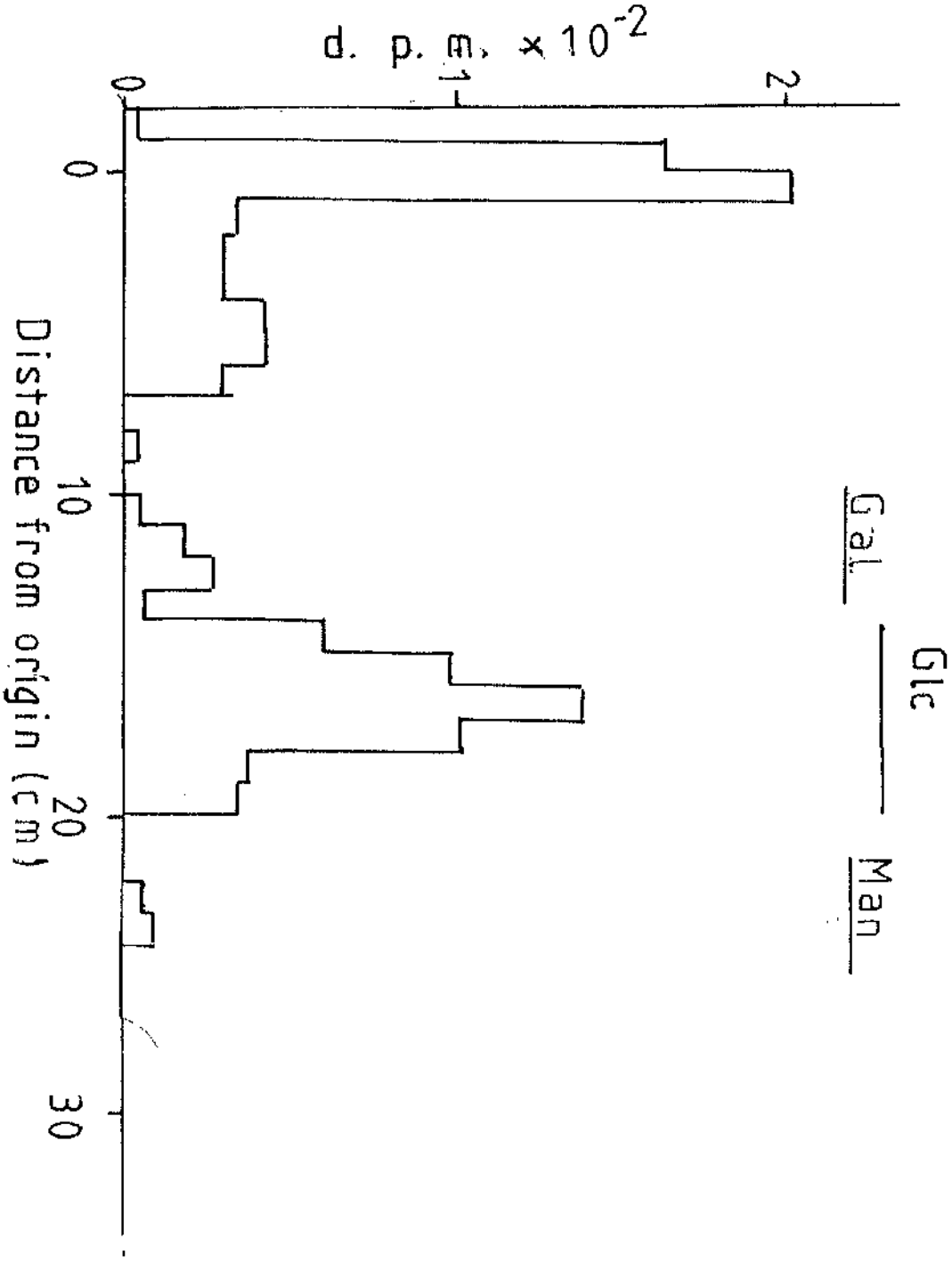

d. p. m. x 10^-2
0
1
2
0
10
20
30
Distance from origin (cm)
Gal
Glc
Man

Fig. 33 Paper chromatogram of the U-cellulosic products synthesised from 102 µM GDP-[U-^{14}C]-G (50 nCi) plus 100 µM GDPM after total acid hydrolysis at 15 lb in^{-2} and 2 days chromatography. The incubation was for 15 minutes. (Glc : glucose, Gal : galactose, Man : mannose)

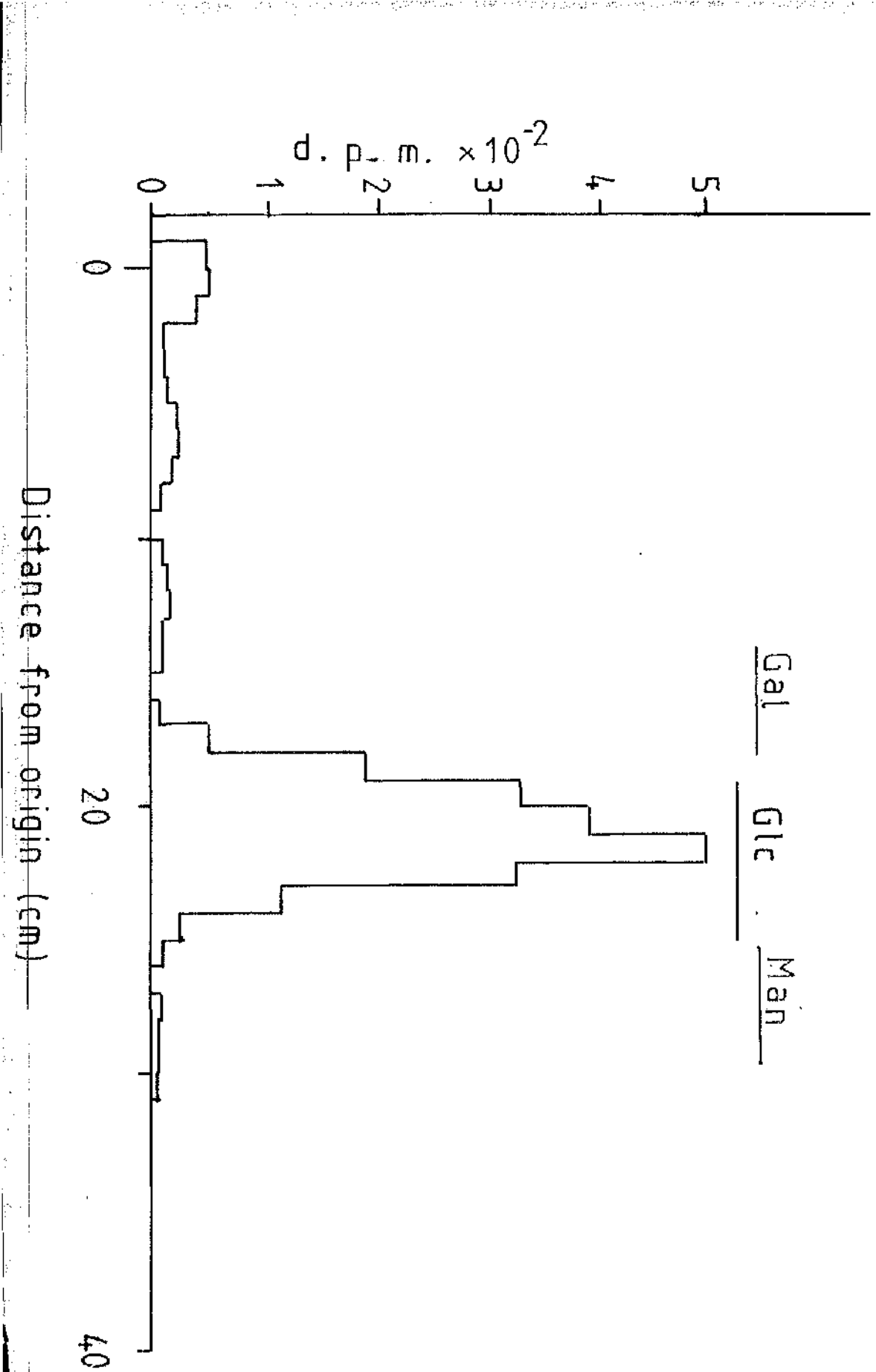
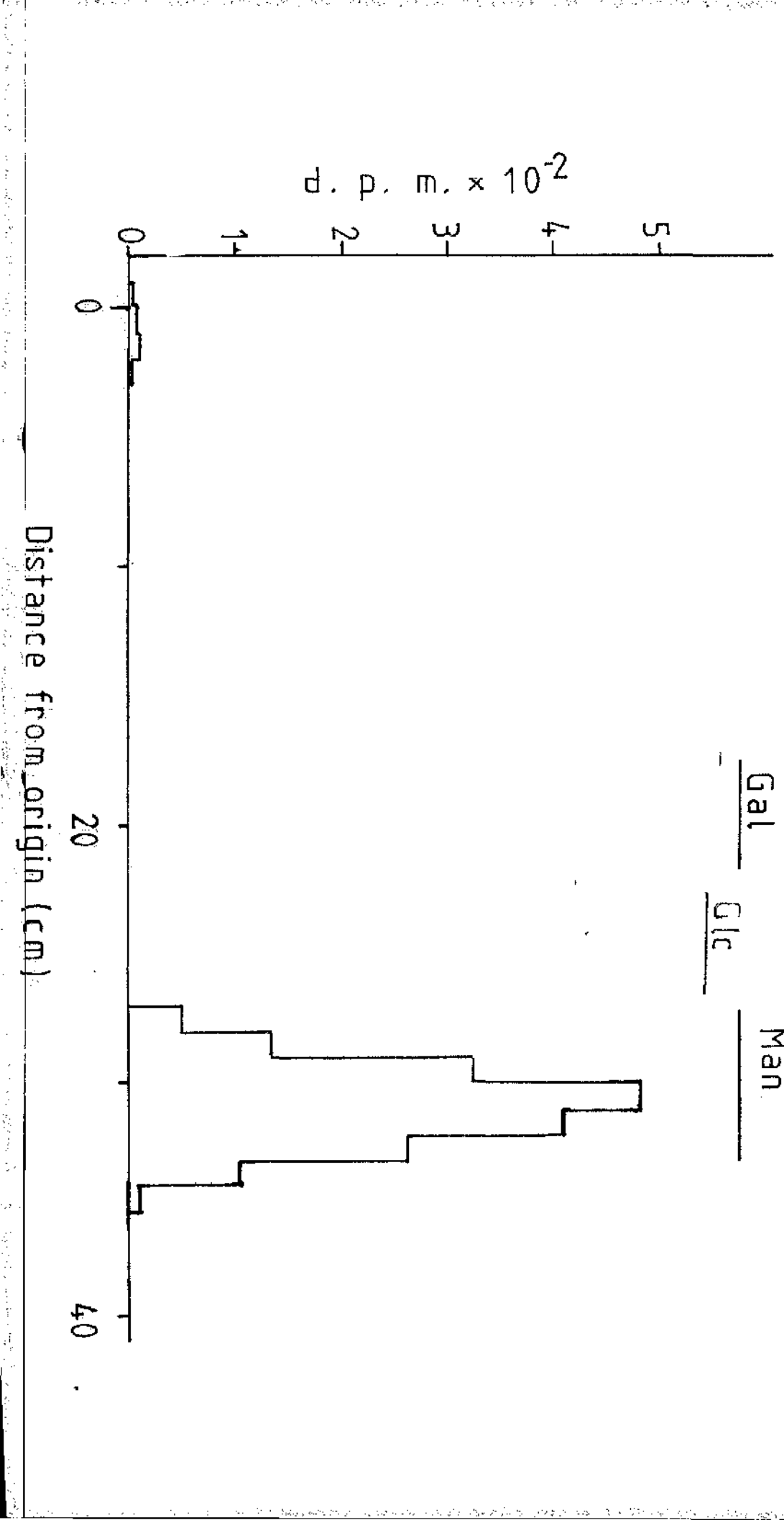

d. p. m. ×10^-2
0
1
2
3
4
5
0
20
40
Distance from origin (cm)
Gal
Glc
Man

Fig. 34 Paper chromatogram of the U-cellulosic products synthesised from 102 µM GDP-[U-^{14}C]-M (50 nCi) after total acid hydrolysis at 15 lb in^{-2} and 2 days chromatography. The incubation was for 15 minutes (Man : mannose, Gal : galactose, Glu : glucose)


d. p. m. × 10-2
0
1
2
3
4
5
0
20
40
Distance from origin (cm)
Gal
Glc
Man

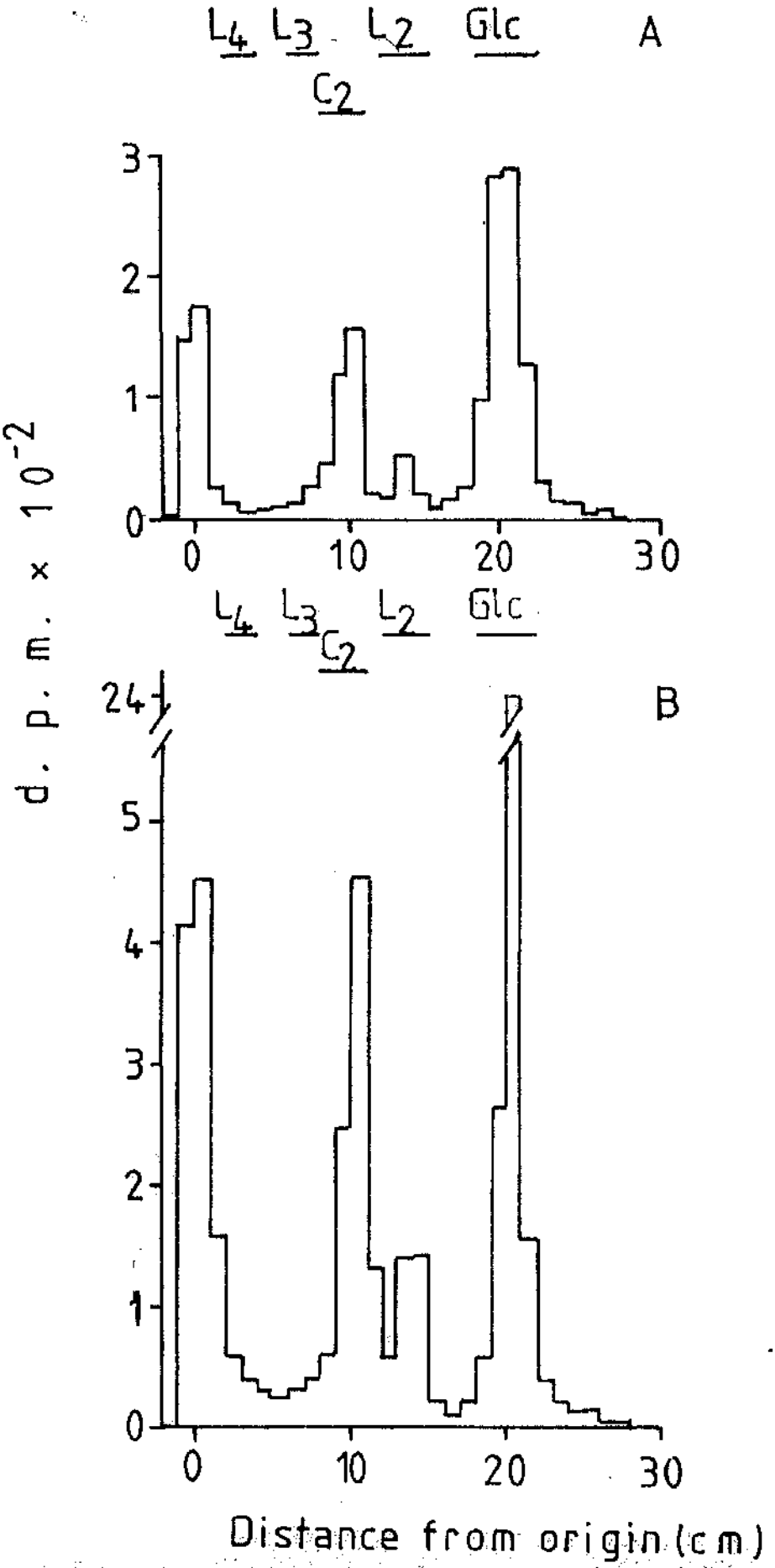
X-ray diffractogram and formed a colloidal solution upon removal of the acid by washing with water.

In this investigation an attempt was made to obtain these rodlets since the incorporation of radioactivity from a radioactive substrate into these rodlets would be strong evidence for the synthesis of the pure $\beta(1\text{-}4)$ glucan crystallites of the microfibril. Unfortunately, although radioactivity from 101 μM GDP-[U-^{14}C]-G was incorporated into material insoluble in 2.5 N H_2SO_4 under the conditions of Dennis and Preston (1961), no colloidal solution of rodlets was obtained upon washing. However, the fact that the products were to some extent insoluble under the conditions of Dennis and Preston (1961) and in the total hydrolysis conditions in Fig. 32 is some evidence for the crystalline nature of the products.

The nature of the sugar-sugar linkage in the products from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-G + GDPM was investigated. The partial acid hydrolysis techniques outlined in chapter 3 were found to be inadequate in that difficulty was encountered in obtaining adequate amounts of oligosaccharide material. Flowers *et al* (1969) also demonstrated that this technique is inappropriate for the analysis of products synthesised from GDPG as the presence of mannose containing oligosaccharides may not be detected (see chapter 1, p 25) It was therefore decided to subject the U-cellulosic products to the partial acetolysis methods of Clark and Villemez (1972). Partial acetolysis of the products from 104 μM GDP-[U-^{14}C]-G and 104 μM GDP-[U-^{14}C]-G + 100 μM GDPM gave rise to glucose, cellobiose and a third radioactive peak co-chromatographing with laminaribiose in n-propanol: ethyl acetate: water (7:1:2 v/v) (Fig. 35). The size of the third peak relative to cellobiose increased upon the addition of 100 μM GDPM to the incubation. Material from this peak was chromatographed on paper in butanol:pyridine: water (10:10:3 v/v) and it ran with an $R_{cellobiose}$ of 1.5.

Fig.35A Paper chromatogram of the U-cellulosic products synthesised from 104 μM GDP-[U-^{14}C]-G (100 nCi) after a 9 day acetolysis and 3 days chromatography. the incubation was for 5 minutes and markers are as described in Fig.19.

Fig.35B Paper chromatogram of the U-cellulosic products synthesised from 104 μM GDP-[U-^{14}C]-G (100 nCi) plus 100 μM GDPM after a 9 day acetolysis and 3 days chromatography. The incubation was for 15 minutes and the markers are as described in Fig.19.


L4 L3 L2 Glc
C2
A
3
2
1
0
0
10
20
30
d. p. m. x 10-2
L4 L3 L2 Glc
C2
B
24
5
4
3
2
1
0
0
10
20
30
Distance from origin (cm)

This is the value reported by Heller and Villemez (1972a) for glucosyl-β(1-4)-mannose in this solvent. Since laminaribiose runs close to glucosyl-β(1-4)-mannose in this chromatography system, the hydrolysate was subjected to T L E in sodium tetraborate in water (0.05 M, pH 9.4) at 3 kV for 30 minutes. The unknown peak was clearly separated from laminaribiose and thus may be tentatively identified as glucosyl-β(1-4)-mannose, an oligosaccharide to be expected on partial acetolysis of a glucomannan. The fact that the glucosyl-β(1-4)-mannose oligosaccharide is found in the products of incubations without added GDPM confirms the presence of endogenous mannose donors or mannose containing acceptors in the enzyme preparation, since total acid hydrolysis of the U-cellulosic products from GDP-[U-^{14}C]-G glucose alone did not indicate the presence of epimerase activity.

7.7. The effect of E D T A on the synthesis of U-cellulose from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-M by the particulate enzyme preparation.

Elbein (1969) reported that in particulate enzyme preparations from *P. aureus*, the mannosyl transferase utilising GDPM as a substrate was strongly dependent upon the addition of Mg^{2+} to the incubation. The glucosyl transferase using GDPG had no such requirement. It was therefore decided to test the effect of 10 mM E D T A upon the synthesis of U-cellulose from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-M.

The results of Table 13 indicate that the mannosyl transferase activity is totally inhibited by the presence of E D T A, presumably due to the chelation of endogenous divalent cations in the incubation. E D T A also eliminated the stimulation by GDPM of the synthesis of U-cellulose from 101 µM GDP-[U-^{14}C]-G. The synthesis of U-cellulose from 101 µM GDP-[U-^{14}C]-G is also inhibited by 76% relative

Table 13: The effect of 10 mM EDTA upon the incorporation of radioactive glucose from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-M into U-cellulose

substrate	radioactivity in incubation	conc. (mM)	EDTA	incorporation pmol glucose
GDP-[U-^{14}C]-M	12.5	0.101	-	809 ± 46
GDP-[U-^{14}C]-M	12.5	0.101	+	2 ± 2
GDP-[U-^{14}C]-G	25	0.101	-	159 ± 20
GDP-[U-^{14}C]-G	25	0.101	+	38 ± 10
GDP-[U-^{14}C]-G + 100 µM GDPM	25	0.101	-	549 ± 18
GDP-[U-^{14}C]-G + 100 µM GDPM	25	0.101	+	32 ± 3
GDP-[U-^{14}C]-G	25	0.001	-	18 ± 0.0
GDP-[U-^{14}C]-G	25	0.001	+	0 ± 0.0

The incubations were as normal except that $MgCl_2$ was ommitted and either 0.1 M tris HCl pH 7.5 buffer or 0.1 M tris HCl buffer containing EDTA was added to result in a final EDTA concentration of 10 mM.

to the controls by 10 mM E D.T.A., while synthesis from 1 μM GDP-[U-^{14}C]-G is totally inhibited. These results suggest the presence of two glucosyl transferases utilising GDP-[U-^{14}C]-G in the enzyme preparation; one which is totally inhibited by E.D.T.A and is probably involved in glucomannan synthesis, and another which is at least partially active in the presence of E.D.T.A. This enzyme may be substrate activated (Table 13). As a result of the mannosyl transferase activity being totally inhibited under these conditions and the fact that no stimulation of U-cellulose synthesis from GDP-[U-^{14}C]-G by GDPM is observed, it is likely that the product is a pure glucan. Initial experiments were conducted to determine the pH optimum of the reaction and the nature of the sugar-sugar linkages. The pH optimum was found to be 5.5 in tris-MES-acetate buffer (all at 50mM) although the activity at pH 7.5 was only slightly lower than at pH 5.5. A partial acetolysis of the U-cellulosic products synthesised from 110 μM GDP-[U-^{14}C]-G in the presence of 10 mM E D.T.A in 0.1 M tris-HCl, pH 7.5 indicated the presence of cellobiose after paper chromatography of the hydrolysate in n-propanol: ethyl acetate: water (7:1:2 v/v). This demonstrates the presence of β(1-4) linked glucose in the products. No radioactivity was observed to co-chromatograph with the tentative glucosyl-β(1-4)-mannose oligosaccharide observed in the U-cellulosic products synthesised from GDP-[U-^{14}C]-G in the absence of E.D.T.A (Fig. 35A), raising the possibility that the product may indeed be a β(1-4) glucan. Unfortunately, insufficient radioactive oligosaccharide material was present on the paper chromatogram to make these results conclusive. Work is being continued on this matter by Dr. C.T. Brett.

CHAPTER 8: UDP-[U-^{14}C]-G AS A SUBSTRATE FOR THE SYNTHESIS OF U-CELLULOSE

8.1. Introduction

In chapters 6 and 7 it was shown that glucose from GDP-[U-^{14}C]-G was incorporated into U-cellulose. However, UDPG is now widely considered to be the most likely initial glucosyl donor for cellulose biosynthesis in higher plants for the following reasons:

1) UDPG is the major sugar-nucleotide found in the plants so far investigated, whereas GDPG is found only at low levels or not at all.

2) Recently compelling *in vivo* evidence for the involvement of UDPG in the cellulose biosynthesis of cotton fibres has been presented by Carpita and Delmer (1981) (see p 38).

In vitro studies have shown that enzyme preparations from numerous plants can synthesise products containing β(1-4) linked glucose from UDPG (see section C.1.2.1.1. and references therein), particularly with low concentrations of substrate (1-10 µM). A major problem with the studies on the products synthesised from UDPG by *in vitro* systems has been the massive production of alkali-insoluble β(1-3) glucan by the substrate-activated UDPG:β(1-3) glucan glucosyl transferase. As Carpita and Delmer (1980) found that the extraction of Updegraff (1969) removed all the β(1-3) glucan synthesised from UDP-[U-^{14}C]-G by detached cotton fibres, it was hoped that this extraction would act similarly in this investigation, allowing a direct study of the UDPG: β(1-4) glucan glucosyl-transferase to be undertaken.

8.2. The incorporation of radioactive glucose from UDP-[U-^{14}C]-G into U-cellulose by the particulate enzyme preparation

In chapter 6 it was shown that UDP-[U-^{14}C]-G could act as a substrate for U-cellulose synthesis and that various protective agents added to homogenisation and reaction media did not result in any stimulation of U-cellulose synthesis. The results in Fig. 36 show that U-cellulose synthesis ceases after 2 minutes from 1 µM UDP-[U-^{14}C]-G and after 4 minutes from 1.0 mM UDP-[U-^{14}C]-G. With both UDP-[U-^{14}C]-G concentrations, turnover of the U-cellulosic products is observed. This is more marked with the products from 1 µM UDP-[U-^{14}C]-G, probably due to the dilution of the radioactive glucose residues by non-radioactive glucose in the products synthesised from 1.0 mM UDP-[U-^{14}C]-G.

It was decided to investigate further the U-cellulosic products from low (<10 µM) rather than higher concentrations of UDP-[U-^{14}C]-G for two reasons. First, the UDPG:β(1-4) glucan glucosyl-transferases found in particulate enzyme preparations are active at low UDPG concentrations. Secondly the highly active β(1-3) glucan synthetase is activated at higher UDPG concentrations and it has been shown that the β(1-3) glucan produced may be remarkably resistant to extraction procedures (see chapter 6 p. 96).

Attempts were made to determine the pH optimum for U-cellulose synthesis from 1 µM UDPG-[U-^{14}C]-G. However, no pH optimum between pH 4-11 was demonstratable. Although this result is unusual, it is not unprecedented since the protease papain also has a very broad pH optimum (Stockell & Smith, 1957). Another possible explanation is that the active site of the synthetase is totally enclosed within the hydrophobic environment of the membrane and would therefore not necessarily respond to variations in external pH. The alternative possibility that the enzyme has a pH

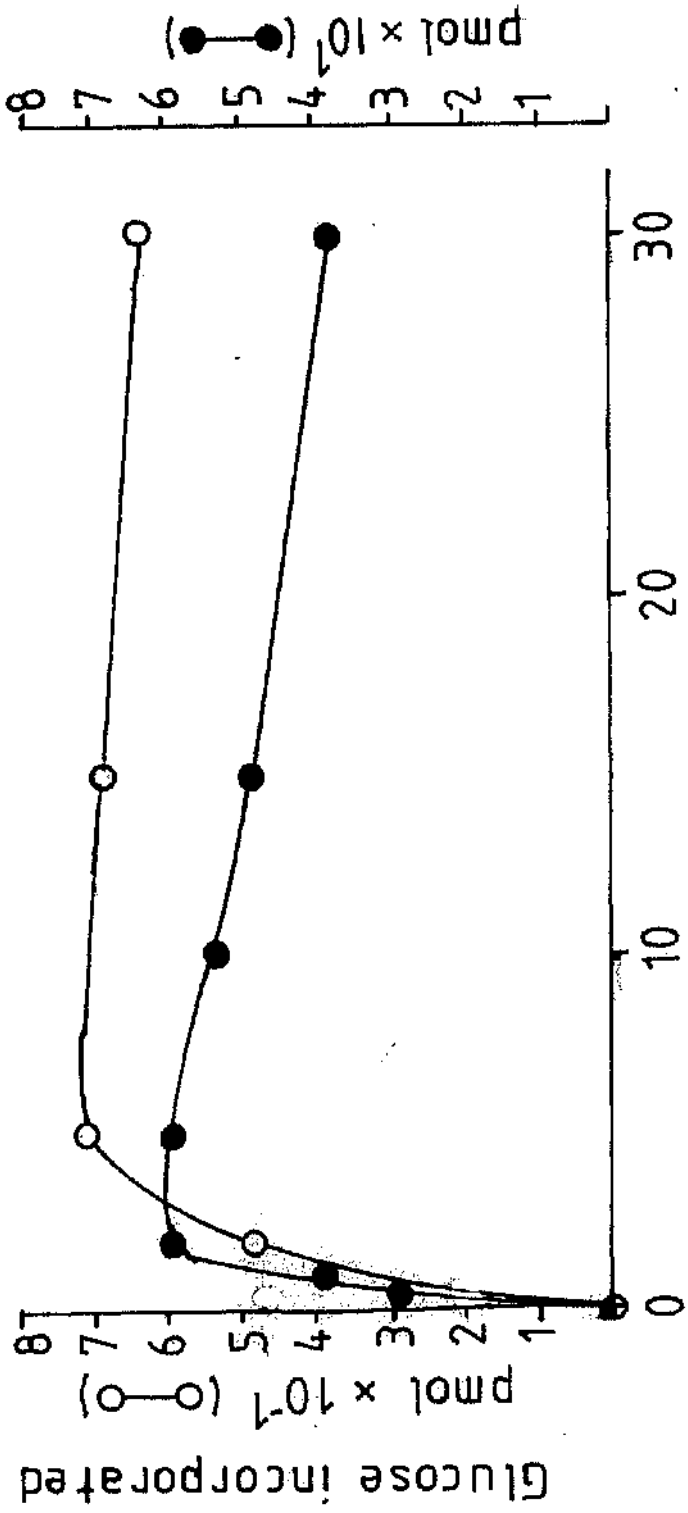


Fig. 36 Time-course of the incorporation of radioactive glucose into U-cellulose from 1μM (●—●, 25 nCi) and 1.0 mM (O—O, 75 nCi) UDP-[U-^{14}C]-G by the particulate enzyme preparation.

Table 14 The effect of preincubating the particulate enzyme preparation at 25°C upon the incorporation of radioactivity from 1 μM UDP-[U-^{14}C]-G into U-cellulose.

preincubation (minutes)	incubation (minutes)	incorporation pmol glucose
-	2	0.50 ± 0.00
2	2	0.53 ± 0.02
15	2	0.25 ± 0.01

The enzyme preparation was prepared and UDP-[U-^{14}C]-G was either added immediately, or after a preincubation at 25°C. Each incubation contained 25 nCi of radioactivity.

Table 15: The effect of the addition of either active enzyme preparation, boiled enzyme preparation or more UDP-[U-^{14}C]-G after an initial incubation with 1 µM UDP-[U-^{14}C]-G upon the incorporation of radioactive glucose into U-cellulose.

1st incubation		2nd incubation			
radioactivity initially added to incubation (nCi)	incubation (minutes)	added enzyme	added radio-activity (nCi)	incubation (minutes)	incorporation dpm
25	2	-	-	-	245 ± 4
25	2	buffer	-	2	253 ± 6
25	2	buffer	25	2	249 ± 7
25	2	boiled	-	2	238 ± 12
25	2	active	-	2	310 ± 10

The enzyme preparation was prepared as normal and incubated with 1 µM UDP-[U-^{14}C]-G (25 nCi) for 2 minutes. Subsequently the reaction was terminated, or a further 100 µl of either buffer (0.1 M tris HCl pH 7.5), the same buffer containing 25 nCi of UDP-[U-^{14}C]-G, boiled enzyme preparation or active enzyme preparation was added and incubated for a further 2 minutes before termination. The results are expressed as dpm due to the uncertainty in the amounts of UDP-[U-^{14}C]-G present in the 2nd incubation after further UDP-[U-^{14}C]-G was added.

optimum outside the range 4-11 is unlikely considering the loci of the glucan synthetases involved in β-glucan synthesis.

The reaction from 1 μM UDP-[U-^{14}C]-G does not stop as a result of enzyme inactivation at 25°C since the enzyme is stable for at least 4 minutes at this temperature (Table 14), although after 17 minutes the enzyme preparation is only 50% as active. Nor does the reaction stop due to a lack of radioactive substrate since:

1) the incorporation of radioactivity into the water-and chloroform:methanol (3:2 v/v)-insoluble products continues for 30 minutes.

2) the addition of further active enzyme preparation results in a small but significant increase in the synthesis of U-cellulose while the addition of more radioactive substrate has no effect (Table 15).

These results would suggest that an inhibitor may be produced during the course of the reaction. The increased incorporation of radioactivity into U-cellulose upon the addition of active enzyme to the incubation media could not be solely due to a dilution of the proposed inhibitor as it is clear from the results in Table 15 that extra active enzyme in addition to inhibitor dilution is required to observe a significant effect. The nature of the inhibitor was not determined due to a lack of time. However, likely candidates are UDP or UMP since these molecules may be produced as the result of glucosyltransferase activity.

8.3. The interaction of UDPG and UDPXyl in the synthesis of water-and chloroform:methanol (3:2 v/v)-insoluble products and U-cellulose by the particulate enzyme preparation.

Ray (1980) presented evidence that the golgi-associated

UDPG:β(1-4) glucan glucosyl-transferase activity assayed at low substrate concentrations in enzyme preparations from higher plants may be involved in xyloglucan rather than cellulose biosynthesis. It was found that UDPG stimulated the incorporation of radioactivity from UDP-[U-^{14}C]-Xyl into polysaccharide and that the observed stimulation was due to the production of glucan acceptor molecules from UDPG to which xylose residues were added by the xylosyl-transferase. Structural analysis of the product synthesised in the presence of UDPXyl and UDPG confirmed that is was a xyloglucan with a glucan backbone and xylose side chains.

In the light of the above work, it was decided to investigate the possibility that the UDPG:β(1-4) glucan glucosyl-transferase assayed at low substrate concentrations in the *P. aureus* particulate enzyme system is involved in xyloglucan biosynthesis. This series of experiments has important implications for the specificity of the Updegraff extraction since xyloglucan is thought to have a very close association with cellulose microfibrils via hydrogen bonding in the primary walls of dicotyledonous plants. Ray (1980) reported that the xylosyl transferase activity found in *P. sativum* particulate enzyme preparations was obligately dependant on the presence of free sugar, sucrose being most effective. Thus sucrose (0.25 M) was included in the homogenisation and reaction media of the experiments described in this chapter.

The results in Table 16 are similar to those obtained by Ray (1980) and suggest the synthesis of a water- and chloroform:methanol (3:2 v/v)-insoluble xyloglucan from 0.4 μM UDP-[U-^{14}C]-Xyl in the presence of 10-100 μM UDPG. The possibility that UDPG has a direct modulating effect upon the xylosyl-transferase cannot be excluded on the basis of the results in Table 16. However, in view of the findings of Ray (1980) this possibility is unlikely. Also in agreement with Ray (1980), high concentrations of UDPXyl

Table 16: The effect of UDPG upon the incorporation of radioactive residues from UDP-[U-^{14}C]-Xyl into the water-and chloroform:methanol (3:2 v/v)-insoluble products and U-cellulose.

substrate	concentration µM	added sugar-nucleotide	pmol incorporated water-and chloroform:methanol (3:2 v/v)-insoluble products	U-cellulose
UDP-[U-^{14}C]-Xyl	0.4	-	0.7 ± 0	0
UDP-[U-^{14}C]-Xyl	0.4	UDPG (10 µM)	1.0 ± 0	0
UDP-[U-^{14}C]-Xyl	0.4	UDPG (100 µM)	1.2 ± 0	0

Homogenisation and reaction media were as normal except that they contained 0.25 M sucrose. The incubations contained 0.01 µCi of radioactivity and were incubated for 5 minutes with or without UDPG.

Table 17: The effect of UDP-Xyl upon the incorporation of radioactive glucose from UDP-[U-^{14}C]-G into the water-and chloroform:methanol (3:2 v/v)-insoluble products and U-cellulose.

substrate	concentration µM	added sugar-nucleotide	pmol incorporated water-and chloroform:methanol (3:2 v/v)-insoluble products	U-cellulose
UDP-[U-^{14}C]-G	1	-	1.6 ± 0.1	1.1 ± 0.2
UDP-[U-^{14}C]-G	1	UDPXyl (10 uM)	1.6 ± 0	1.0 ± 0.1
UDP-[U-^{14}C]-G	1	UDPXyl (100 uM)	1.1 ± 0.1	1.1 ± 0.1

Homogenisation and reaction media contained 0.25 M sucrose. The incubations contained 25 nCi of radioactivity and were incubated for 5 minutes.

(100 μM) appears to inhibit the incorporation of radioactive residues into the water-and chloroform:methanol (3:2 v/v)-insoluble products from UDP-[U-^{14}C]-G (Table 17). No concentration of UDPXyl tested significantly affected the synthesis of U-cellulose from 1 μM UDP-[U-^{14}C]-G. Neither GDPG nor GDPM affected the synthesis of U-cellulose from UDP-[U-^{14}C]-Xyl or UDP-[U-^{14}C]-G.

No incorporation of radioactive residues into U-cellulose from any concentration of UDP-[U-^{14}C]-Xyl (0.4 μM - 1 mM) was observed. Neither did the presence of UDPG (10 - 100 μM) result in any incorporation of radioactive residues into U-cellulose from 0.4 μM UDP-[U-^{14}C]-Xyl (Table 16). The above observation demonstrates that the proposed xyloglucan synthesised from UDP-[U-^{14}C]-Xyl is solubilised by the Updegraff extraction, suggesting that the Updegraff-insoluble product synthesised from UDP-[U-^{14}C]-G alone is not xyloglucan. The possibility that the extraction removes the xylose side-chains while the glucan backbone remains insoluble is unlikely as U-cellulose synthesis from UDP-[U-^{14}C]-G does not show the inhibition by UDPXyl observed in the synthesis of water-and chloroform:methanol (3:2 v/v)-insoluble products (Table 17). This implies that xyloglucan and U-cellulose synthesis from UDPG involve separate enzyme systems. Thus it may be concluded that U-cellulose synthesised from UDP-[U-^{14}C]-G by the *P. aureus* particulate enzyme preparation is not xyloglucan.

8.4. Gel filtration of the water- and chloroform:methanol (3:2 v/v)-insoluble products synthesised from UDP-[U-^{14}C]-G by the particulate enzyme preparation.

In this section, the molecular weights of the products synthesised from UDP-[U-^{14}C]-G was investigated in a similar manner to that in section 7.5.

The major product from 5 μM UDP-[U-^{14}C]-G elutes just

before Dextran T70 and therefore has a probable molecular weight of less than 7×10^4. There is also a minor peak eluting just after Dextran T500 which corresponds to a molecular weight of approximately 5×10^5 (Fig. 37). This is of a large enough molecular weight to indicate that the product may be cellulosic. Unfortunately, insufficient radioactivity could be incorporated into this peak to allow a structural analysis to be undertaken. Neither could the peak be observed after extraction of the products in Updegraff reagent or 24% KOH. This result is not unexpected as these powerful extractions may well degrade the products. The possibility that the smaller molecular weight peak is cellulosic cannot be ruled out on this result alone for the reasons outlined on pp 129 and 138.

A large amount of the products synthesised from 1.0 mM UDP-[U-^{14}C]-G would appear to have a molecular weight in excess of 1×10^6 since they are excluded from Sepharose CL-6B as judged by Blue Dextran 2000. The fractionation range for polysaccharides by Sepharose CL-6B is 1×10^4 - 1×10^6. There is also a smaller-sized peak which co-elutes with the major peak obtained from the products synthesised from 5 μM UDP-[U-^{14}C]-G in Fig. 37.

The products from 1.0 mM UDP-[U-^{14}C]-G were subjected to gel filtration on a larger column of Sepharose CL-2B (fractionation range for polysaccharides 1×10^5 - 2×10^7). The results in Fig. 39 demonstrate that in terms of molecular weight there are indeed two components in the products, the larger of which appears to be excluded suggesting a molecular weight of greater than 2×10^7. Unfortunately as was the case for the high molecular weight product synthesised from 5 μM UDP-[U-^{14}C]-G, insufficient radioactivity could be incorporated to allow for a structural analysis of the peak. However, using the more active *P. sativum* tissue slice system, the high molecular weight product synthesised from 1.0 mM UDP-[U-^{14}C]-G that was excluded from Sepharose CL-2B was shown to be a β(1-3)

Fig. 37 Gel filtration of the water- and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 5 μM UDP-[U-^{14}C]-G (125 nCi) on Sepharose CL-6B (column volume 9 cm^3: fraction volume 0.375 cm^3). Dextran markers are as in Fig. 28.

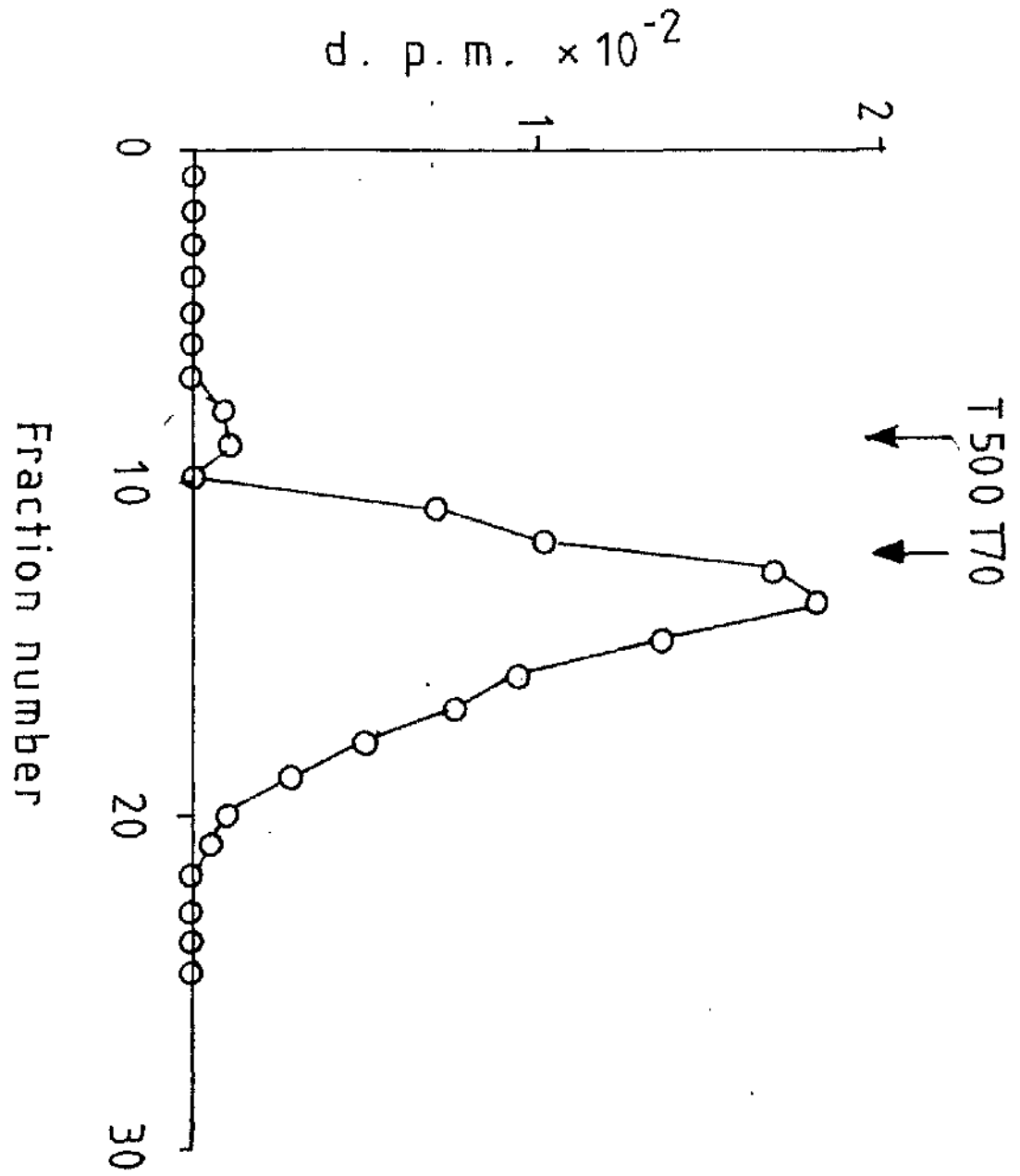

d. p. m. × 10⁻²
0
1
2
Fraction number
0
10
20
30
T50
T70

Fig. 38 Gel filtration of the water- and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 1.0 mM UDP-[U-^{14}C]-G (50 nCi) on Sepharose CL-6B (column volume 9 cm^3; fraction volume 0.375 cm^3). Dextran markers and void volume (V_o) determination are as in Fig. 28. The products used in this figure and in Fig. 37 were synthesised by the same enzyme preparation and thus the results are directly comparable.

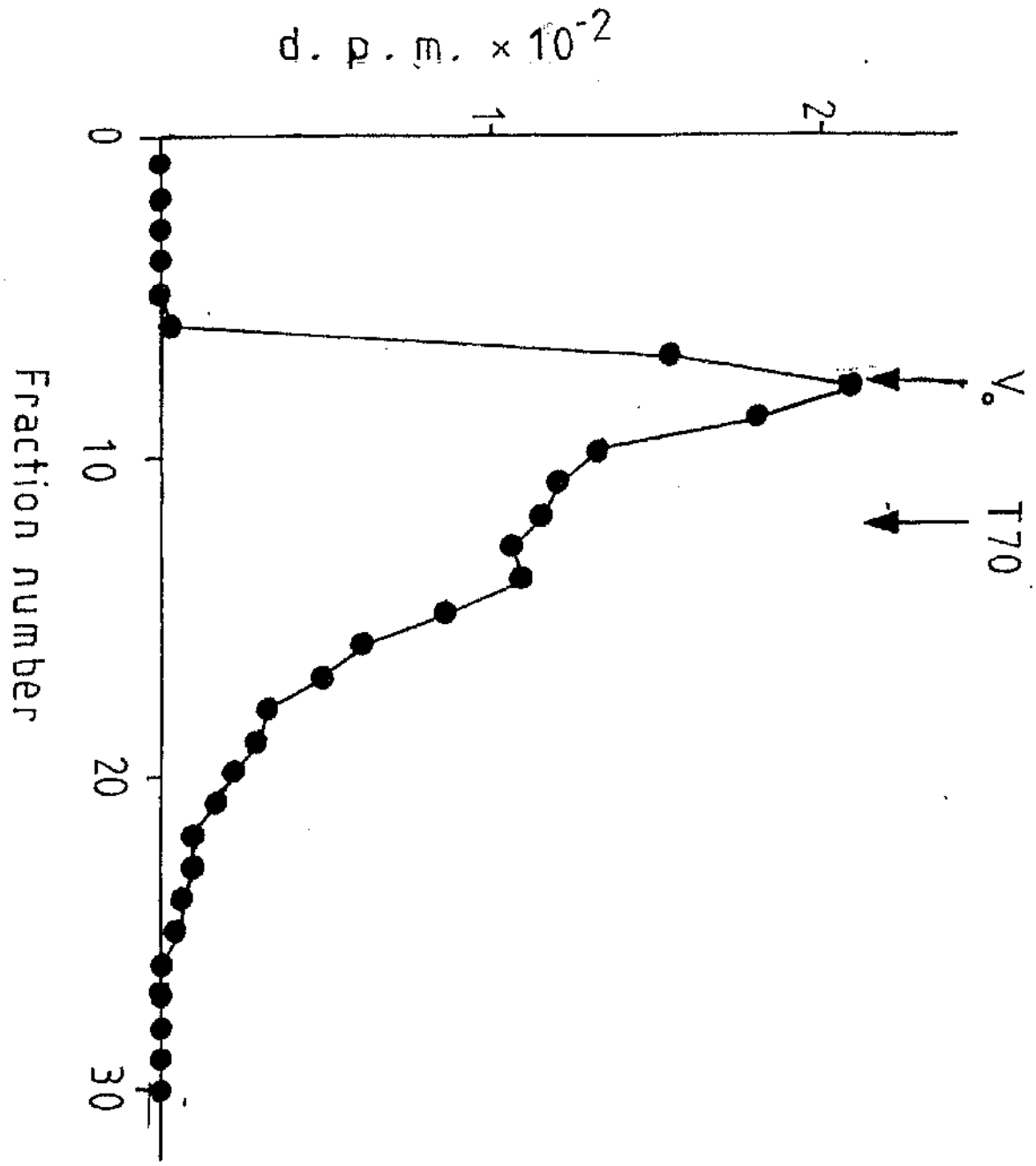
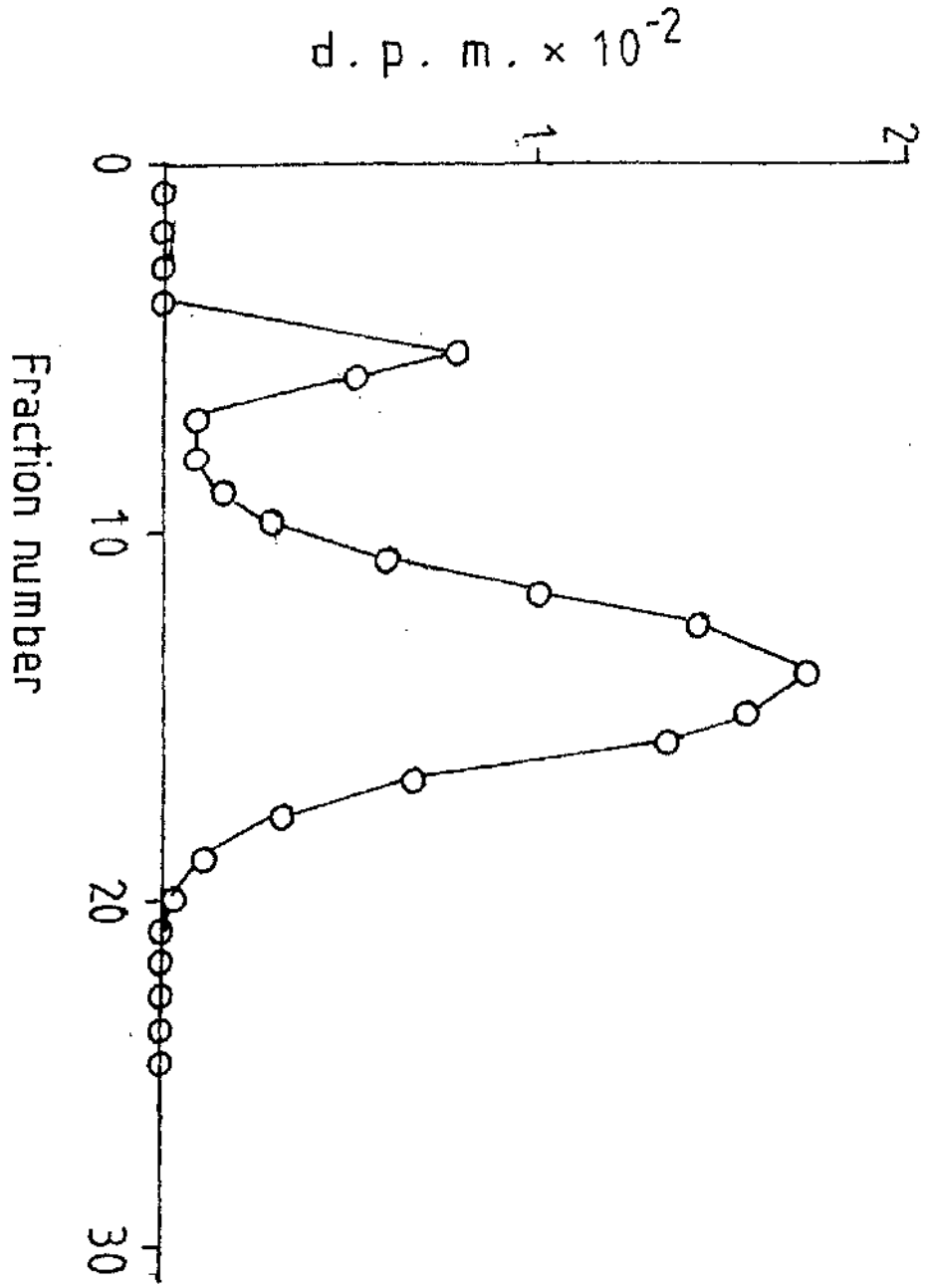

d. p. m. × 10^-2
1
2
0
10
20
30
Fraction number
V°
T70

Fig. 39 Gel filtration of the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 1.0 mM UDP-[U-^{14}C]-G (50 nCi) on Sepharose CL-2B (column volume 21 cm^3; fraction volume 1.1.cm^3).


d. p. m. × 10-2
1
2
0
10
20
30
Fraction number

glucan (Brett, personal communication). The lower molecular weight peak synthesised from 1.0 mM UDP-[U-^{14}C]-G by the *P. aureus* particulate enzyme preparation has also been shown to contain β(1-3) glucan by partial acid hydrolysis and subsequent paper chromatography.

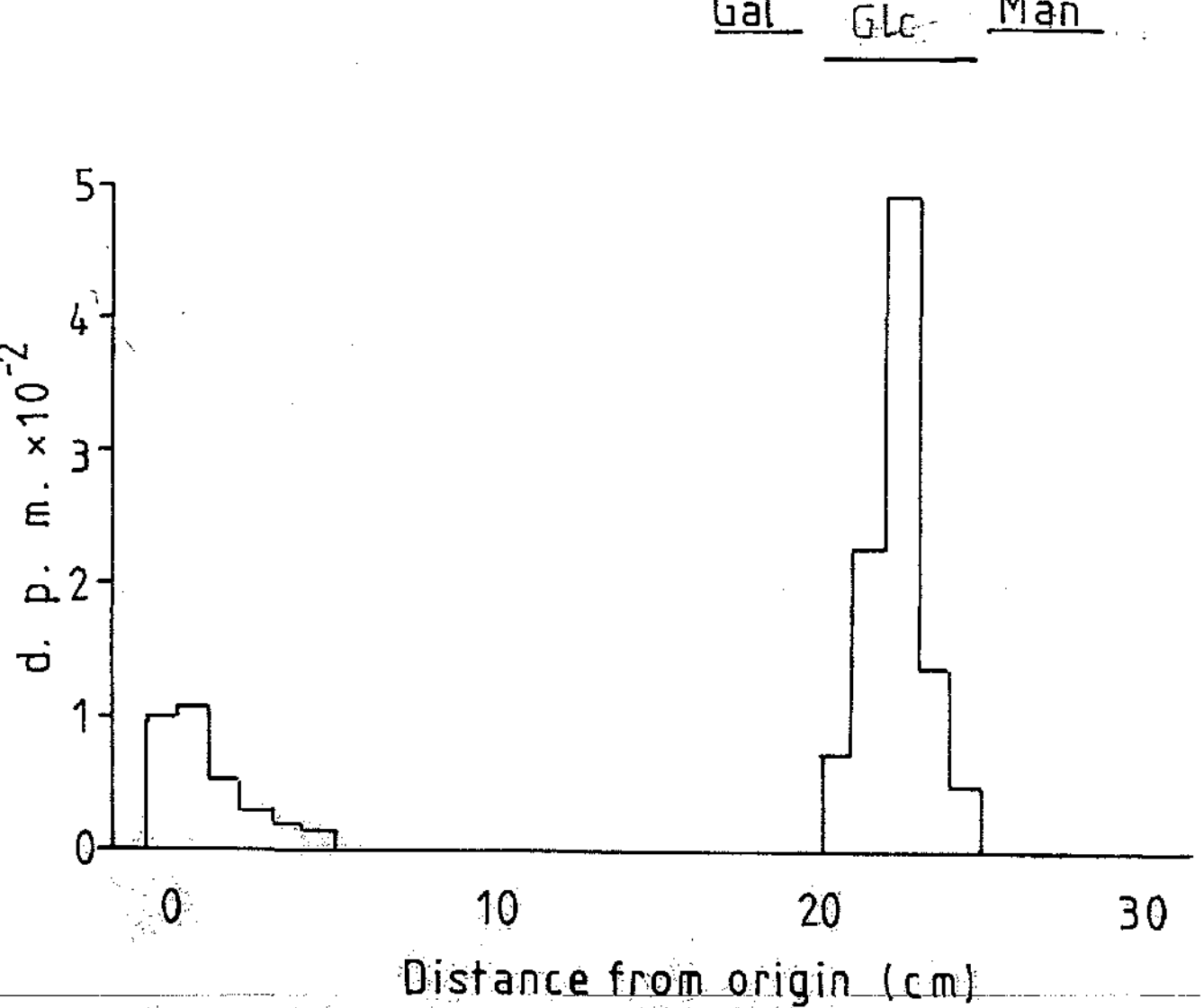
8.5. Further analysis of U-cellulose synthesised from 1 μM UDP-[U-^{14}C]-G by the particulate enzyme preparation.

In this section experiments are described which were conducted to determine the sugar composition and the sugar-sugar linkage of the U-cellulose synthesised from 1 μM UDP-[U-^{14}C]-G. Because of the small amounts of radioactivity incorporated into U-cellulose from UDP-[U-^{14}C]-G, the incubations were scaled up by a factor indicated below the figures.

Radioactive glucose was the only radioactive residue detected after total acid hydrolysis of U-cellulose synthesised from 1 μM UDP-[U-^{14}C]-G (Fig. 40). Partial acid hydrolysis was found to be unsatisfactory as a procedure to investigate the sugar-sugar linkage since only small amounts of radioactive oligosaccharides were detectable relative to undegraded material and glucose.

Partial acetolysis of the products gave rise to glucose laminaribiose, laminaritriose and cellobiose (Fig. 41), indicating that the U-cellulosic product contains β(1-3) and β(1-4) linked glucose. The β(1-3) and β(1-4) linked glucose could be part of a mixed-linked β-glucan or they could be two separate polysaccharides. A water-soluble mixed-link β-glucan has been identified in *P. aureus* cell walls (Buchala & Franz, 1974). The presence of β(1-3) glucan in the U-cellulosic fraction after 30 minutes offers an explanation for the continued slow phase of extraction of the water- and chloroform:methanol (3:2 v/v)-insoluble

Fig. 40 Paper chromatogram of the U-cellulosic products synthesised from 1 μM UDP-[U-^{14}C]-G (125 nCi) after a total acid hydrolysis at 15 lb in^{-2} and 2 days chromatography. The incubation was scaled up in size by a factor of 5 (contains 2.5 mg protein) relative to that outlined in chapter 3. The incubation period was for 5 minutes. (Glc : glucose)


Gal
Glc
Man
d. p. m. ×10⁻²
0
1
2
3
4
5
0
10
20
30
Distance from origin (cm)

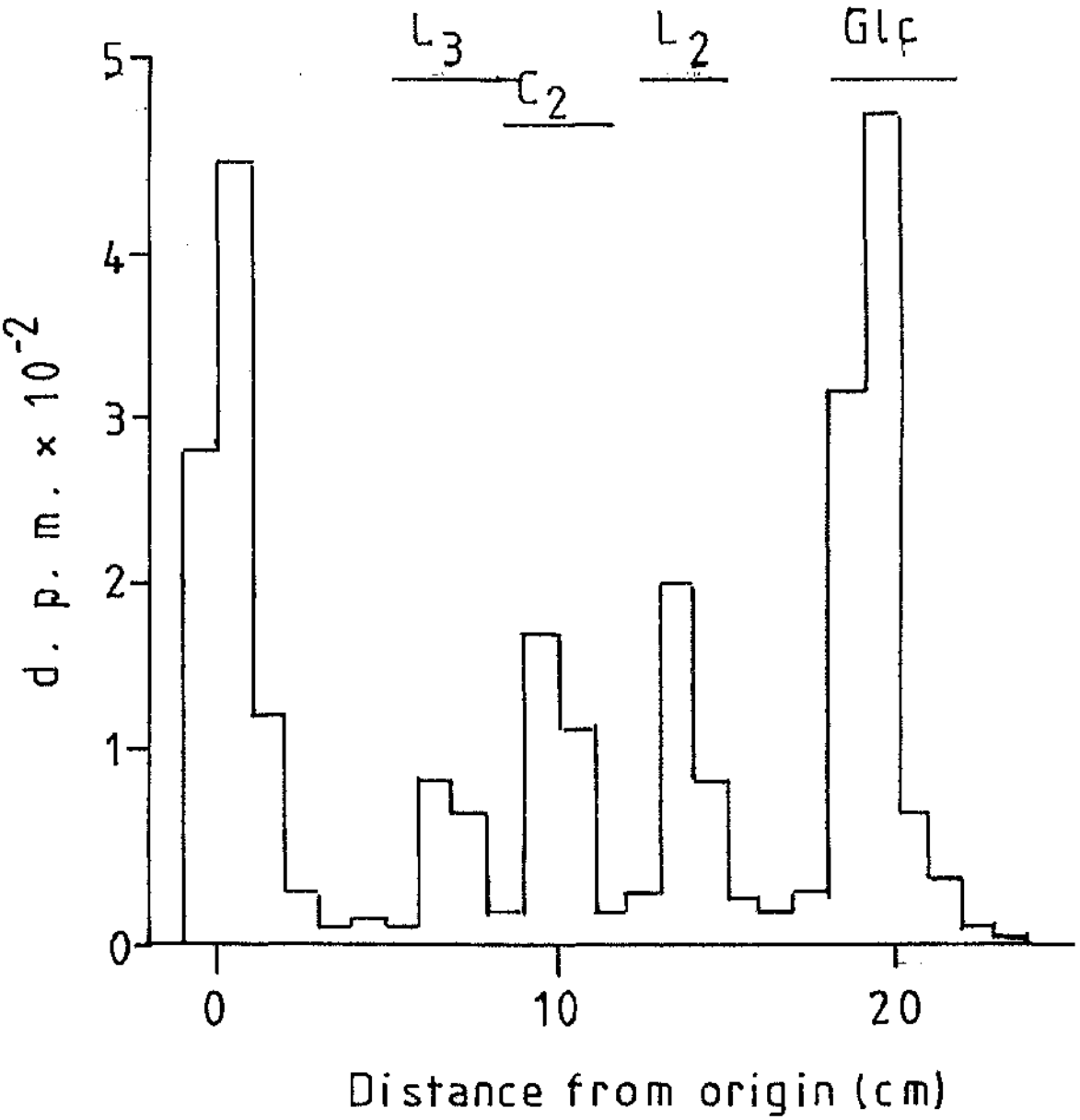
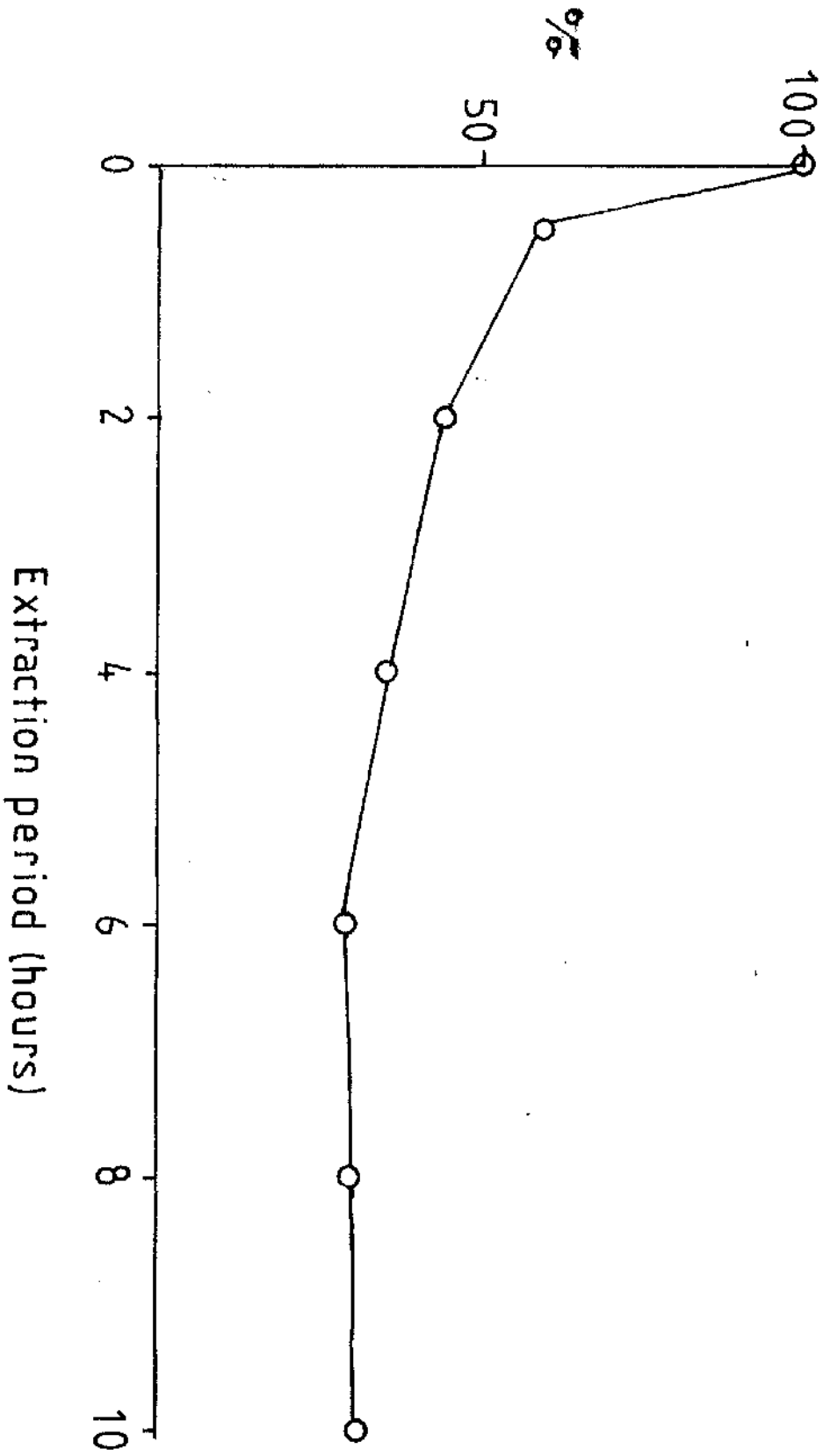


Fig. 41 Paper chromatogram of the U-cellulose synthesised from 1 μM UDP-[U-^{14}C]-G (250 nCi) after a 9 day acetolysis and 3 days chromatography. The incubation was scaled up by a factor of 10 (contains 5 mg of protein) relative to that outlined in chapter 3. The incubation was for 5 minutes and the markers were as described in Fig. 19.

Fig. 42 This figure shows the percentage of the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from 2 μM UDP-[U-^{14}C]-G (50 nCi) after a 5 minute incubation remaining insoluble after 30, 120, 240, 360, 480 and 600 minutes after extraction with acetic acid: nitric acid: water (8:1:2 v/v).


%
100
50
0
2
4
6
8
10
Extraction period (hours)

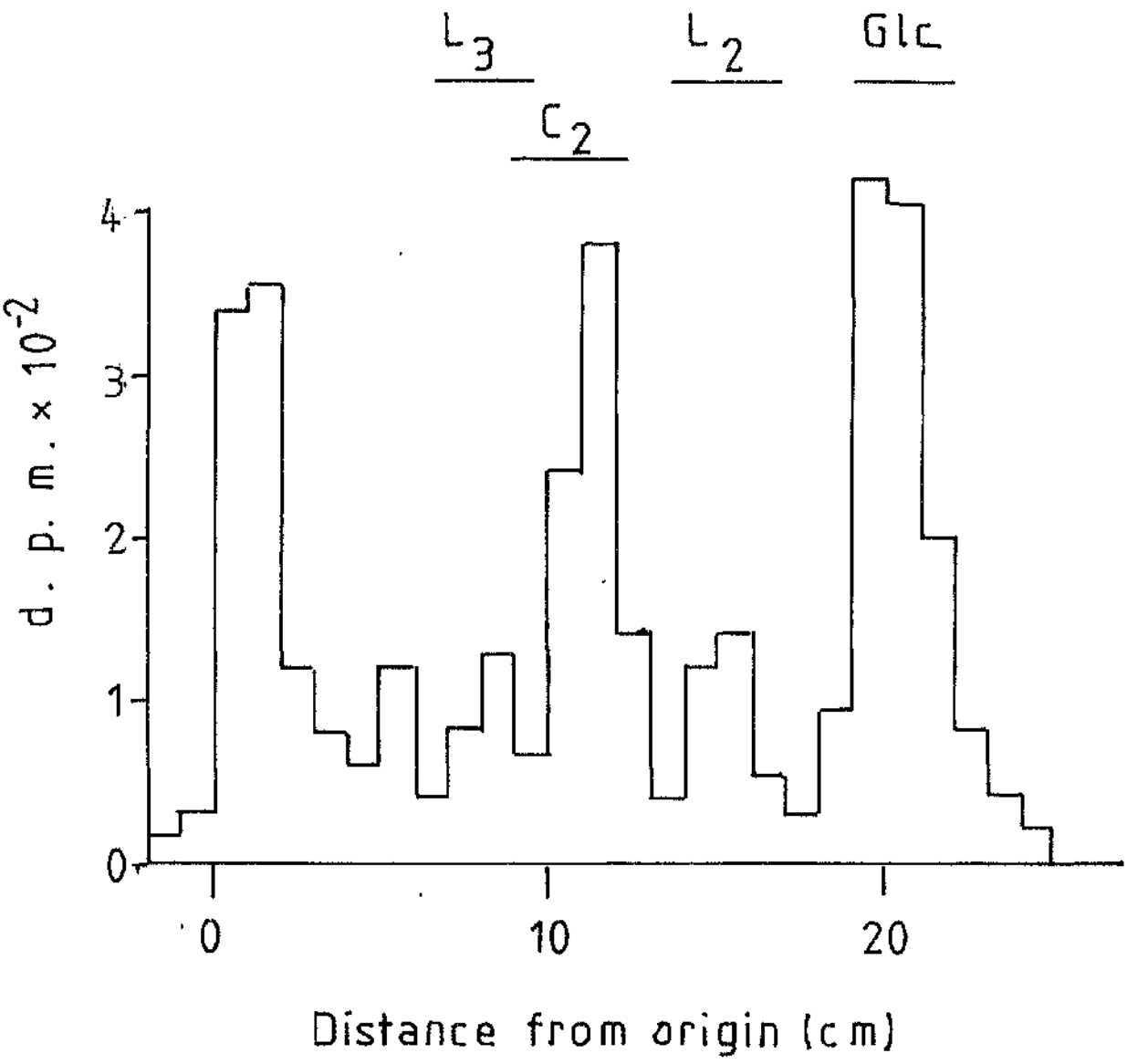


Fig. 43 Paper chromatogram of the U-cellulose (6 hour extraction) synthesised from 1 μM UDP-[U-^{14}C]-G (250 nCi) after a 9 day acetolysis and 3 days chromatography. Incubation conditions were as in Fig. 41. Markers were as described in Fig. 19.

products observed in Fig. 20 (see chapter 6 , p 100). Thus it would appear that the standard 30 minute extraction in acetic acid: nitric acid: water (8:1:2 v/v) at 100°C used by Updegraff (1969) is not sufficient to remove all the non-cellulosic product produced from UDP-[U-^{14}C]-G. It was therefore decided to extract the water-and chloroform: methanol (3:2 v/v)-insoluble products for longer periods in the Updegraff reagent to determine whether or not the slow phase of extraction would eventually plateau. The results in Fig. 42 indicate that the extraction of the products from 1 μM UDP-[U-^{14}C]-G continues for up to 6 hours after which no further extraction occurs up to 10 hours. The products of a partial acetolysis of the Updegraff-insoluble products after a 6 hr extraction is shown in Fig. 43. It is clear that although proportion of laminaribiose relative to cellobiose is decreased, it is still present, indicating that if a separate β(1-3) glucan molecule is present in the products, then it is not extractable in the Updegraff reagent.

CHAPTER 9: CONCLUSIONS AND IMPLICATIONS FOR CELLULOSE BIOSYNTHESIS

In this chapter the results obtained during this investigation are discussed in terms of their possible significance for cellulose biosynthesis.

It was shown that GDP-[U-^{14}C]-G, GDP-[U-^{14}C]-M and UDP-[U-^{14}C]-G acted as substrates for the synthesis of U-cellulose by the *P. aureus* particulate enzyme preparation. The polymers synthesised from GDP-[U-^{14}C]-G and GDP-[U-^{14}C]-M would appear to be glucomannan (on the basis of kinetic, structural and gel filtration studies) and possibly mannan (on the basis of a time-course study of U-cellulose synthesis from GDP-[U-^{14}C]-M) and glucan (on the basis of a time-course of U-cellulose synthesis from 1.0 mM GDP-[U-^{14}C]-G, studies on the effect of EDTA on U-cellulose synthesis from GDP-[U-^{14}C]-G and a structural investigation). The U-cellulose synthesised from UDP-[U-^{14}C]-G, at least at low concentrations, contained both β(1-3) and β(1-4) linked glucose.

The above results are clearly in contrast to the widely held belief that the Updegraff extraction solubilises all non-crystalline, non-cellulosic glycans and would suggest that one or more of the following possibilities must pertain:

1. The extraction is not specific to crystalline, microfibrillar cellulose, and other non-crystalline, non-fibrillar glycans that have no association with cellulose microfibrils are not solubilised.

2. The extraction solubilises all non-cellulosic glycans except those which have an intimate association with the endogenous cellulose microfibrils via non-covalent bonding.

3. The extraction does not solubilise any fibrillar, crystalline material, whatever its sugar residue and glycosidic linkage composition. Two factors which may contribute to the acid-stability of such material are:-

a) the anhydrous (at least relatively so) nature of the crystallites.

b) the sugar residues in the crystalline state are held in a rigid conformation. This would hinder the adoption of the half-chair conformation formed to stabilise the carbonium ion intermediate formed in the acid hydrolysis of a glycosidic linkage.

4. It is conceivable that the mannose-containing polysaccharides are covalently linked to the β(1-4) glucan chains of cellulose and may therefore be integral to the microfibrillar structure. If this is the case, then they are part of the molecule to be synthesised.

These four possibilities will be evaluated in the following discussion.

It was shown in this investigation that the water-and chloroform:methanol (3:2 v/v)-insoluble products synthesised from UDP-[U-^{14}C]-Xyl and UDP-[U-^{14}C]-Xyl plus UDPG (probably xyloglucan) were totally solubilised by the extraction, while 98% of the same products synthesised from 1.0 mM UDP-[U-^{14}C]-G (containing β(1-3) glucan) were also solubilised. Bacic and Delmer (1981) demonstrated that non-crystalline β(1-4) glucan synthesised from UDPG by a particulate enzyme preparation from cotton fibres was also extracted. These results, combined with the work of other researchers and the drastic nature of the extraction, indicate that 1. above is unlikely. However, it is conceded that very small amounts of non-crystalline, non-fibrillar material may not be extracted, particularly if it contains a high proportion of acid-stable glycosidic linkages (eg. those involving uronic acids). Indeed, even after a 10 hour extraction of the water- and chloroform:

methanol (3:2 v/v)-insoluble material synthesised from 1 μM UDP-[U-^{14}C]-G, some β(1-3) glucan was still present. However, this result could equally well be explained by 2 or 3 above, or if the β(1-3) glucan was covalently linked to cellulose microfibrils. Delmer (1977) has even suggested that the highly active UDPG:β(1-3) glucan glucosyl-transferase activity found in *in vitro* enzyme preparations may be cellulose synthetase which has been modified during preparation. This must be considered unlikely, however, in view of the great stereochemical difference between β(1-3) and β(1-4) glycosidic linkages.

One possibility for the mannose-containing polymers is that they are rendered insoluble in the Updegraff extraction due to an intimate, non-covalent association with endogenous cellulose microfibrils. It is known that certain cell-wall matrix polysaccharides do have a very close relationship with the surface of microfibrils and under certain conditions glucomannans can crystallise onto the surface of cellulose microfibrils, resulting in a shish (cellulose) kebab (glucomannan) structure (Chanzy *et al*, 1982). However, three points would indicate that this hypothesis is incorrect:

1) In preliminary experiments with Triton X-100 solubilised enzyme preparation, GDPM caused the usual stimulation of U-cellulose synthesis from GDP-[U-^{14}C]-G.

2) The proposed xyloglucan synthesised from UDP-[U-^{14}C]-Xyl plus UDPG appears to be totally solubilised by the Updegraff extraction. Xyloglucan is bound to the surface of cellulose microfibrils via extensive hydrogen bonding (Valent & Albersheim, 1974). Thus if the xyloglucan synthesised in this investigation has such a relationship with the endogenous cellulose microfibrils then this result indicates that if the mannose containing polymers were rendered insoluble by such an association with microfibrils, the association must be very intimate indeed.

3) Kjosbakken and Colvin (1975) demonstrated that a water-insoluble, alkali-soluble polymer of glucose (presumed to be β(1-4) linked on the basis of the observations of Glaser (1958)) which was synthesised from UDPG by a cell-free enzyme preparation from *A. xylinum*, was adsorbed onto preformed cellulose. However unless the preparation was dried, the polymer could be solubilised with only relatively mild alkali (1 N NaOH).

The previous argument and the fact that sugar residues from the sugar nucleotides could be incorporated into both α-cellulose (i.e. produced by a hydrogen-bond-breaking extraction in 24% KOH) and U-cellulose (i.e. produced by a drastic hydrolytic extraction), strongly suggest that the insoluble products are crystalline and microfibrillar in nature. Even a prolonged extraction with the Updegraff reagent (6 times longer than the extraction period originally used by Updegraff (1969)) does not solubilise a large proportion of the products synthesised from 100 μM GDP-[U-^{14}C]-G, 100 μM GDP-[U-^{14}C]-G plus 100 μM GDPM, 100 μM GDP-[U-^{14}C]-M and 1 μM UDP-[U-^{14}C]-G. (In this chapter, for sake of clarity, concentrations in the range 100-104 μM GDP-[U-^{14}C]-G have been referred to as 100 μM.) This hypothesis is further supported by the observation that both glucomannan and mannan are known to be able to form crystalline structures. There is also a precedent for a fibrillar β(1-3) glucan which is highly resistant to extraction, as Herth *et al* (1974) found such a molecule, which was not solubilised by 20% NaOH, in the cell walls of pollen tubes from *L. longiflorum*.

Mannan microfibrils are the skeletal components of the algal families, the Codiaceae and Dasycladaceae (Preston, 1974). Crystalline mannan can exist at two polymorphs, mannan I which is found in nature, and mannan II which is produced from the native polymorph by swelling in alkali or recrystallization in an analogous manner to cellulose II

(Chanzy *et al*, 1982). Less is known of the crystallization behaviour of glucomannans although it is thought that glucose isomorphically replaces mannose in a mannan type crystalline structure. As the glucose:mannose ratio is increased, the perfection of the glucomannan crystals is decreased (Chanzy *et al*, 1982). It is possible that mannose could be incorporated into a glucan crystalline lattice in a similar manner, thus accounting for the increased susceptibility of the products synthesised from GDP-[U-^{14}C]-G to extraction by the Updegraff reagent when GDPM was also present in the incubation media.

If these purportedly crystalline mannose-containing polymers are autonomous, insoluble after extraction with Updegraff reagent in their own right and have no relevance to cellulose biosynthesis, then they must be considered as artefacts of the *in vitro* conditions existing in the particulate enzyme preparations. The mannans of higher plants are not found in a crystalline state (with the exception of mannan found in the endosperm of ivory nut) (Preston, 1974) and, as far as the author is aware, there are no reports of autonomous crystalline glucomannans in the cell walls of higher plants.

Thus the possibility is raised that crystalline, microfibrillar, mannose-containing polymers synthesised by the particulate enzyme preparation may not be artefacts but rather represent the synthesis of bona-fide cellulose microfibrils, the mannose-containing polymers being integral to the microfibrillar structure. This is further supported by the fact that it is difficult to envisage how the conditions required for the production of artefactual crystalline products could exist in the enzyme preparation during the experimental procedures. It is conceivable that the chloroform:methanol (3:2 v/v) extraction could cause precipitation and association of the newly synthesised polymers, resulting in a crystalline structure. However, it was shown that the omission of this extraction prior to extracting the

water-insoluble products in the Updegraff reagent had no effect upon the results obtained. This implies that the crystalline products must be formed in an aqueous environment. It is unlikely that the polymers could form an autonomous, artefactual, crystalline product (i.e. produced purely by a physical association of the polymers) in such an environment. Supporting evidence for this statement comes from the work of Kjosbakken and Colvin (1975). These researchers found that a non-microfibrillar, water-insoluble polymer of glucose (presumed to be $\beta(1\text{-}4)$ linked on the basis of the observations of Glaser (1958)) synthesised from UDPG by a cell-free enzyme preparation from *A. xylinum* could only form microfibrillar material when precipitated from aqueous, alkaline solution by ethanol. The resulting microfibrils were soluble in relatively mild alkali (1 N NaOH).

How might such mannose-containing polysaccharides be integral to native cellulose microfibrils? The mannose residues could be present in the non-crystalline regions found at the surface and along the length of the microfibril (see chapter 1;B.2.3.).Thus some of the glucan chains which pass repeatedly between crystalline and non-crystalline regions of the microfibril may contain mannose in the non-crystalline regions. They would then in fact be glucomannans rather than pure glucans, albeit with a low proportion of mannose residues (at least for those plants with relatively "pure" cellulose). What proportion of the glucan chains are actually glucomannans is impossible to estimate, although it is not impossible that all cellulose molecules contain non-glucose residues, the amounts of which may vary according to the source of the cellulose. Such non-glucose residues are only removed by very strong acid treatments at high temperatures, which destroy the integrity of the microfibril, as indicated by the results of Dennis and Preston (1961). A drawback to this hypothesis is that GDP-$[U\text{-}^{14}C]$-M is a more efficient glycosyl donor for U-cellulose synthesis than GDP-$[U\text{-}^{14}C]$-G at the same concentration. This could be

explained if the mannosyl transferase activity was better preserved through tissue homogenisation, or is less limited by cofactor requirements. Another possibility is that *in vivo* a C_2 epimerase activity is present which acts at the polymer stage, converting most of the mannose to glucose within the glucomannan, and that this enzymic activity is lost *in vitro*. This epimerase enzyme could be located in the cellulose synthetase multi-enzyme complex along with the glucosyl and mannosyl transferases. Thus polymerisation, epimerisation and crystallisation could occur in quick succession. There is a precedent for post-polymerization modification of sugar residue configuration in the synthesis of alginate and dermatan sulphate (Aspinall, 1983).

If mannose-containing polymers are indeed part of the structure of cellulose microfibrils, then the kinetic studies on polysaccharide synthesis from GDPG and GDPM may be reconciled with cellulose biosynthesis. Villemez (1971) and Heller and Villemez (1972a) argued that glucomannan synthesis from GDPG declined and ceased due to depletion of the second substrate, GDPM. In this investigation, as in the earlier work, the addition of GDPM to the incubation media extended the period of incorporation from GDPG into the products. This concept can be extended to cellulose microfibril synthesis if one assumes tip synthesis by a large multi-enzyme complex, with the result that the interpretation of similar results to those obtained by Villemez and co-workers (Hinman & Villemez, 1975 and refs therein) leads to very different conclusions. If the microfibril is synthesised collectively as a unit by tip synthesis, all the glycan chains (of the order of 100 (Preston, 1974)) of one microfibril would have to be synthesised simultaneously and in a co-ordinated manner. Thus if, as postulated earlier, the microfibril contained glucomannan chains in addition to glucan chains, lack of the second substrate for glucomannan synthesis (GDPM) could bring the whole process to a halt.

The above hypothesis also offers an explanation for the lack of an increase in the initial rate of reaction from GDPG upon the addition of GDPM, a result obtained both in this investigation and by Villemez and co-workers (Hinman & Villemez, 1975 refs therein). These workers concluded from their results that the sole product from GDPG, regardless of the presence or absence of GDPM, was glucomannan, not cellulose. First, if the sole product from GDPG was indeed glucomannan, this does not exclude the possibility that the product was cellulosic in the light of this investigation. Secondly, if the microfibril is composed of both glucan and glucomannan and the microfibril is synthesised in a co-ordinated manner, no increase in the initial rate of reaction from GDPG could be expected even though two chemically distinct molecules are synthesised.

The question now arises - What purpose, if any, could there be for the presence of mannose residues in the cellulose microfibrils of higher plants? Barber (1982) reported that a pre-incubation of a solubilised enzyme preparation from *P. aureus* with GDPM resulted in the same degree of stimulation of product synthesis from GDPG as when both sugar-nucleotides were present simultaneously in the incubation. This result would suggest that the products of the mannosyl transferase are acting as priming molecules for the continued synthesis of glucan from GDPG. However, no support for this hypothesis was obtained in this investigation. It was demonstrated that GDPM had to be present simultaneously with GDPG for stimulation to be observed. It was noted that when both sugar-nucleotides were present in the incubation, the resulting water- and chloroform:methanol (3:2 v/v)-insoluble products were of an increased molecular weight, as judged by gel filtration on Sepharose CL-6B. This result suggests that the insertion of mannose residues into the glucan chain is required for continued chain elongation. However, it must be concluded that if the mannose-containing polysaccharides are integral parts of the structure of the cellulose microfibril, then the function of the mannose

residues can only be speculated upon. It is possible that the presence of mannose residues may provide "weak points" along the length of the microfibril. This could have a number of advantages :-

1) It offers a means of control over the tensile strength of the microfibril.

2) These "weak points" may provide sites at which microfibril degradation by enzymes could be initiated. This would be important in those processes where selective cell wall breakdown occurs, eg abscission and perhaps in cell wall extension, since Wong *et al* (1977) have proposed such a role for cellulases in this process.

The preceding discussions would appear to be contradictory with the results obtained by a G.L.C. analysis of the alditol acetates produced from a total acid hydrolysate of the cellulosic fractions from the *P. aureus* enzyme preparations. In these studies only glucose and a number of spurious unidentified peaks were observed. As emphasised in sections 5.2 & 7.3 these results are suspect and further investigations on the chemical composition of the cellulosic fractions of *P. aureus* are continuing.

If subsequent G.L.C studies reveal that mannose is not a constituent of the *P. aureus* cellulose fractions there are a number of possible explanations:-

1) The glucosyl-transferase utilising GDPG activity in *P. aureus* enzyme preparations has no involvement in cellulose biosynthesis but rather, as suggested by Villemez and coworkers, the sole product synthesised from GDPG is non-cellulosic glucomannan.

2) The earlier arguments based on the possible presence of a C_2 epimerase acting at the polymer stage *in vivo* could

be extended to the extent where all the mannose residues in the synthesised glucomannan were epimerised to glucose after incorporation.

3) The effects of GDPM upon the synthesis of U-cellulose from GDPG are artefacts arising due to the *in vitro* preparation. It is thought that the glucosyl and the mannosyl transferases in *P. aureus* can use the same kind of acceptor molecules (Heller & Villemez, 1972a) and the results in this investigation would support this hypothesis. Thus, the destruction of cellular compartmentalisation brought about by tissue homogenisation might result in the two enzymic activities and their products being brought into contact, a situation that may not exist *in vivo*.

What is the substrate for the pure glucan of the microfibrils, such as exists in the cellulose crystallites obtained after severe acid degradation of the microfibril ? Results obtained in this investigation have not been able to distinguish between GDPG or UDPG as possible candidates. Evidence that pure glucan may be produced from GDPG is outlined below.

The synthesis of U-cellulose from 100 µM GDP-[U-^{14}C] -G ceases after 5 minutes and acetolysis of the products indicates the presence of β(1-4) linked glucose plus a smaller amount of mannosyl β(1-4) glucose oligosaccharide. The mannose found in the U-cellulose synthesised from 100 µM GDPG alone must come from endogenous sources, as there is no evidence from total acid hydrolysis studies for the presence of epimerase activity acting at the substrate level. When the concentration of GDP-[U-^{14}C] -G was increased to 1.0 mM, the synthesis of U-cellulose continued at an ever-decreasing rate for 30 minutes. This result suggests the presence of an additional GDPG-utilising glucosyl transferase which is only active at high concentrations. As it is likely that endogenous mannose sources are used up, at least in the latter period of the incubation, it would seem that this

enzyme is not dependent upon the presence of such mannose sources for sustained U-cellulose synthesis. Thus it is possible that pure glucan is synthesised, or at least long stretches of glucose residues within a glucomannan.

Further evidence for the presence of more than one glucosyl transferase activity using GDPG as a substrate, one of which synthesises pure glucan, comes from studies on the effects of E D T A upon the synthesis of U-cellulose from GDP-[U-^{14}C]-G. It was found that 10 mM E D T A totally inhibited the incorporation from 100 µM GDP-[U-^{14}C]-M and abolished the stimulation of U-cellulose synthesised from 100 µM GDP-[U-^{14}C]-G by 100 µM GDPM.

The synthesis of U-cellulose from GDPG was totally inhibited by E D T A at a substrate concentration of 1 µM and by 76% from 100 µM GDPG. This indicates the presence of two GDPG-utilising enzymes in the preparation, one of which is totally inactive in the presence of E D T A , possibly synthesising glucomannan, and another which is at least partially active in the presence of E D T A , and may produce pure glucan as the mannosyl transferase activity in the enzyme preparation is totally inactivated under these conditions. Furthermore, initial studies on the acetolysis product of U-cellulose synthesised from 100 µM GDP-[U-^{14}C]-G in incubations containing 10 mM E D T A did not reveal the presence of the mannosyl β(1-4) glucose oligosaccharide found with incubations containing GDP-[U-^{14}C]-G alone. Cellobiose was detected as usual. It is interesting to note that this enzyme appears to exhibit substrate activation. If so, this is the first report of a GDPG-utilising enzyme exhibiting such kinetics in the synthesis of polysaccharide.

There is some evidence to suggest that the putative glucan synthesised from GDPG may be the crystallites of the cellulose microfibril. A proportion of the U-cellulose synthesised from GDPG is not degraded by the total acid

hydrolysis conditions outlined in chapter 3 or by the conditions used by Dennis and Preston (1961) to obtain the pure glucan crystallites rodlets from the cellulose microfibrils of higher plants.

In this investigation much higher concentrations of GDPG have been used than in earlier work. The initial reasons for this have been outlined in chapter 2. What are the possible reasons for the requirement of such high GDPG concentrations in order to observe pure glucan synthesis? If, as argued previously, the enzyme involved is substrate-activated, then the concentration of substrate required to activate the enzyme may be high. This could be due to a requirement for two GDPG molecules to bind simultaneously to the polymerase, each molecule of GDPG taking a different orientation and thereby permitting the ready formation of the two-fold screw axis found in the $\beta(1\text{-}4)$ glucan chains of cellulose. The formation of the two-fold screw axis of the cellulose chains must pose considerable steric problems for an insoluble enzyme and product, as is the case with cellulose synthetase and the microfibril.

Alternatively, if the GDPG-utilising glucosyl transferase involved in cellulose synthesis is not substrate activated, then it may have a high K_m value. However, in plant cells GDPG is found only at low levels. It is possible that the conformation of the cellulose synthetase is altered during enzyme preparation with the result that the K_m is markedly increased, or that intracellular compartmentalization results in localised high levels of GDPG in the vicinity of the cellulose synthetase. This could easily be envisaged if the GDPG-synthesising enzyme(s) was localised around, or even incorporated into, the cellulose synthetase enzyme complex.

UDPG is the favoured initial glucosyl donor for cellulose biosynthesis in the opinion of many researchers. However, only small amounts of glucose from UDPG (at the

concentrations reported for *in vitro* $\beta(1\text{-}4)$ glucan synthesis) could be incorporated into U-cellulose. This result is not unexpected for a multi-enzyme complex which synthesises the crystallites of the cellulose microfibril, as such an enzyme must co-ordinate both the synthesis and assembly (assuming tip synthesis) of the metastable cellulose I microfibril and as a result, it is probably particularly susceptible to physical disruption upon tissue homogenisation. In chapter 8 it was shown that the U-cellulose synthesised from 1 μM UDP-$[U\text{-}^{14}C]$-G contained $\beta(1\text{-}4)$ linked glucose and that this product was not the $\beta(1\text{-}4)$ glucan backbone component of a xyloglucan molecule. The presence of $\beta(1\text{-}3)$ linked glucose in the U-cellulose synthesised from 1 μM UDP-$[U\text{-}^{14}C]$-G prevents any firm conclusions as to the cellulosic nature of the $\beta(1\text{-}4)$ linked glucose, as the possibility that both of the types of glucosidic linkage are present in the same polymer cannot be ruled out.

Thus the results obtained during this investigation suggest that either or both of the sugar-nucleotides, GDPG and UDPG, could be the initial glucose donors for cellulose biosynthesis in *P. aureus*. It is difficult to envisage much further progress in this field using crude tissue homogenates as an enzyme source combined with empirical definitions of cellulose based on purportedly specific extractions. However, it would be worthwhile to carry out a more detailed study of the effects of E D T A on the synthesis of U-cellulose from high concentrations of GDPG.

The major set-back to progress is the ambiguity in the exact chemical composition of native cellulose. Preconceptions as to the purity or homogeneity of cellulose may have rendered investigators blind to the possibility that the cellulose microfibril may contain structurally disparate regions, which are covalently linked. An alternative assay for cellulose which would overcome this problem would be

the use of X-ray diffraction to detect the synthesis of native cellulose I. A positive result with this technique would be conclusive. However, the synthesis of large amounts of cellulose in the native cellulose structure may require the use of alternative *in vitro* preparations in which the integrity of the cellulose synthetase complex is better preserved. Such a system may be produced by gentle osmotic down shock of protoplasts to produce plasma-membrane ghosts. This system also would overcome the major problem of having to synthesise the large amounts of product required to detect an increase in the quantity of cellulose over and above the cellulose already present in enzyme preparations.

Alternatively, it might be worthwhile applying the methods used in the successful demonstration of *in vitro* α-chitin microfibril biosynthesis by chitosomes, particularly in view of the fact that Giddings *et al* (1980) visualised possible cellulose synthetase complexes, identical to those observed on the plasma-membrane, golgi-derived vesicles of *Micrasterias*. These vesicles may thus be analogous to chitosomes, containing a cellulose synthetase zymogen. The criteria used in this project could then be applied in the identification of the products synthesised from possible precursors.

REFERENCES

1. Albersheim P., Nevins D.J., English P.D. and Karr A. (1967) Carbohyd. Res. 5, 340-345

2. Aloni Y. and Benziman M. (1982) in "Cellulose and other Natural Polymer Systems", chapter 17 pp 341-361, ed. Brown R.M. Jnr., Plenum Press, New York and London

3. Aloni Y., Delmer D.P. and Benziman M. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6448-6452

4. Anderson R.L. and Ray P.M. (1978) Plant Physiol. 61, 723-730

5. Aspinall G.O. (1982) in "The Polysaccharides", Vol 1 chapter 1 pp 1-18, ed. Aspinall G.O. Academic Press, London

6. Bacic A. and Delmer D.P. (1981) Planta 152, 346-351

7. Barber G.A. (1982) Archiv. Biochem. Biophys. 215, 253-259

8. Barber G.A., Elbein A.D. and Hassid W.Z. (1964) J. Biol. Chem. 239, 4056-4069

9. Barber G.A. and Hassid W.Z. (1964) Biochim. Biophys. Acta. 86, 397-399

10. Barber G.A. and Hassid W.Z. (1965) Nature 207, 295-296

11. Batra K.K. and Hassid W.Z. (1969) Plant Physicl. 44, 755-758

12. Behrens N.H., Parodi A.J. and Leloir L.F. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 2857-2860

13. Benziman M., Haigler C.H., Brown R.M.Jnr., White A.R. and Cooper K.M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 6678-6682

14. Blackwell J. (1982) in "Cellulose and other Natural Polymer Systems", chapter 20 pp 403-428, ed Brown R.M. Jnr., Plenum Press, New York and London

15. Boothby D. (1972) Planta 103, 310-318

16. Bowles D.J. and Northcote D.H. (1972) Biochem J. 130, 1113-1145

17. Brett C.T. (1978) Plant Physiol. (1978)62, 377-382

18. Brett C.T. (1981a) J. Exp. Bot. 32, 1067-1077

19. Brett C.T. (1981b) Techniques in Carbohydrate Metabolism, B307 pp 1-11, Elsevier/North Holland Scientific Publishers Ltd.

20. Brett C.T. and Northcote D.H. (1975) Biochem J. 148, 107-117

21. Brown R.M. Jnr., Franke W.W., Kleinig H., Falk H. and Sitte P. (1970) J. Cell Biol. 45, 246-271

22. Brown R.M. Jnr. and Montezinos D. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 143-147

23. Brown R.M. Jnr., Willison J.H.M. and Richardson C.L. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4565-4569

24. Brummond D.O. and Gibbons A.P. (1964) Biochem. Biophys. Res. Commun. 17, 156-159

+

25. Burgess J. (1979) Nature 278,212

+ Buchala A.J. and Franz G. (1974) Phytochemistry 13, 1887-1889

26. Carpita N.C. and Delmer D.P. (1980) Plant Physiol., 66, 911-916

27. Carpita N.C. and Delmer D.P. (1981) J. Biol. Chem. 256, 308-315

28. Chambers J. and Elbein A.D. (1970) Archiv. Biochem. Biophys. 138, 620-631

29. Chanzy H.D., Grosenaud A., Joseleau J.P., Dube M. and Marchessault R.H. (1982) Biopolymers 21, 301-319

30. Chao H.Y. and Maclachlan G.A. (1978) Plant Physiol. 61, 943-948

31. Clark A.F. and Villemez C.L. (1972) Plant Physiol. 50, 371-374

32. Colvin J.R. (1959) Nature 183, 1135-1136

33. Colvin J.R. (1961) Can. J. Biochem. Physiol. 39, 1921-1926

34. Colvin J.R. (1976) J. Polym. Sci. Polym. Chem. Educ. 14, 2377-2382

35. Colvin J.R. (1980a) in "The Biochemistry of Plants" Vol 3 Carbohydrates: Structure and function, chapter 14 pp 544-567, ed Preiss J., Academic Press, London

36. Colvin J.R. (1980b) Planta 149, 97-107

37. Colvin J.R. and Leppard G.G. (1977) Can. J. Microbiol. 23, 701-709

38. Colvin J.R., Snowden L.C. and Leppard G.G. (1977a) Can. J. Microbiol. 23, 790-797

39. Colvin J.R., Chené L., Snowden L.C. and Takai M. (1977b) Can. J. Biochem 55, 1057-1063

40. Cooper D. and Manley R.St.J. (1975a) Biochim. Biophys. Acta 381, 109-119

41. Cooper D. and Manley R.St.J. (1975b) Biochim. Biophys. Acta 381, 78-96

42. Cooper D. and Manley R.St.J. (1975c) Biochim. Biophys. Acta 381, 97-108

43. Couso R.O., Ielpi L., Garcia R.C. and Dankert M.A. (1980) Archiv. Biochem. Biophys. 204, 434-438

44. Currier H.B. (1957) Am. J. Botany 44, 478-488

45. Delmer D.P. (1972) J. Biol. Chem. 247, 3822-3828

46. Delmer D.P. (1977) in "Recent Advances in Phytochemistry" 11, 45-47, ed. Loewus F., New York and London

47. Delmer D.P., Beasley C.A. and Ordin L. (1974) Plant Physiol. 53, 149-153

48. Delmer D.P., Heineger U. and Kulow C. (1977) Plant Physiol. 59, 713-718

49. Dennis D.T. and Preston R.D. (1961) Nature 191, 667-668

50. Depierre J.W. and Dallinger G. (1975) Biochim. Biophys. Acta 415, 411-472

†

51. Dugger W.M. and Palmer R.L. (1980) Plant Physiol. 65, 266-273

† Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A. and Smith F. (1956) Analytical Chem. 28, 350-356

52. Elbein A.D. (1969) J. Biol. Chem. 244, 1608-1616

53. Elnaghy M.A. and Nordin P. (1966) Archiv. Biochem. Biophys. 113, 72-76

54. Feingold D.S., Neufield E.F. and Hassid W.Z. (1958) J. Biol. Chem. 233, 783-788

55. Flowers H.M., Batra K.K., Kemp J. and Hassid W.Z. (1968) Plant Physiol. 43, 1703-1709

56. Flowers H.M., Batra K.K., Kemp J. and Hassid W.Z. (1969) J. Biol. Chem. 244, 4969-4974

57. Fölch J., Lees M. and Sloane-Stanley G.H. (1957) J. Biol. Chem. 206, 497-509

58. Forsee W.T. and Elbein A.D. (1972) Biochem. Biophys. Res. Commun. 49, 930-939

59. Forsee W.T. and Elbein A.D. (1973) J. Biol. Chem. 248, 2858-2867

60. Franz G. (1976) Appl. Polymer Symposium 28, 611-621

61. Franz G. and Meier H. (1969) Phytochemistry 8, 579-583

62. Freeze H. and Loomis W.F. (1977) J. Biol. Chem. 252, 820-824

63. Freeze H. and Loomis W.F. (1978) Biochim. Biophys. Acta 539, 529-537

64. Garcia R.C., Recondo E. and Dankert M. (1974) Eur. J. Biochem. 43, 93-105

65. Gardner K.H. and Blackwell J. (1974) Biopolymers 13, 1975-1980

66. Giddings T.H. Jnr., Brower D.L. and Staehlin L.A. (1980) J. Cell. Biol. 84, 327-339

67. Ginnivan M.J., Woods J.L. and O'Callaghan J.R. (1977) J. Appl. Bacteriol. 43, 231-238

68. Glaser L. (1958) J. Biol. Chem. 232, 627-636

69. Grout B.W.W. (1975) Planta 123, 275-282

70. Haigler C.H. and Benziman M. (1982) in "Cellulose and other Natural Polymer Systems" chapter 14 pp 273-297, ed. Brown R.M. Jnr., Plenum Press, New York and London

71. Haigler C.H., Brown R.M. Jnr. and Benziman M. (1980) Science 210, 903-906

72. Haigler C.H., White A.R., Brown R.M. Jnr and Cooper K.M. (1982) J. Cell.Biol. 94, 64-69

73. Hara M., Umetsu N., Miyamoto C. and Tamari K. (1973) Plant and Cell Physiol. 14, 11-28

74. Harris P.J. and Northcote D.H. (1970) Biochem J. 120, 479-491

75. Hawker J.S., Osbun J.L., Ozaki H., Greenberg E. and Preiss J. (1974) Archiv. Biochem. Biophys. 160, 530-551

76. Heineger U. and Delmer D.P. (1977) Plant Physiol. 59, 719-723

77. Heineger U. and Franz G. (1980) Plant Sci. Lett. 17, 443-450

78. Heller J.S. and Villemez C.L. (1972a) Biochem. J. 129, 645-665

79. Heller J.S. and Villemez C.L. (1972b) Biochem J. 128, 243-252

80. Helaper J.P.F.G. (1979) Planta 144, 443-450

81. Hepler P.K. and Palevitz B.A. (1974) Plant Physiol. 25, 309-362

82. Herth W., Franke W.W. and Bittiger G. (1974) Cytobiologie 9, 344-367

83. Hinman M.B. and Villemez C.L. (1975) Plant Physiol. 56, 608-612

84. Hogetsu T., Shibaoka H. and Shimokoriyama M. (1974a) Plant and Cell Physiol. 15, 389-393

85. Hogetsu T., Shibaoka H. and Shimokoriyama M. (1974b) Plant and Cell Physiol. 15, 265-272

86. Hopp H.E., Romero P.A., Daleo G.R. and Pont Lezica R. (1978) Eur. J. Biochem. 84, 561-571

87. Huwyler H.R., Franz G.A. and Meier H. (1978) Plant Sci. Lett. 12, 55-62

88. Katō K. (1981) in "Encyclopedia of Plant Physiol" (new series) Vol. 13, Plant Carbohydrates II; Extracellular Carbohydrates, chapter 4 pp 29-44, ed. Tanner W. and Loewus F., Springer-Verlag, Berlin, Heidelberg and New York

89. Khan A.W. and Colvin J.R. (1961) Science 133, 2014-2015

90. King G.G.S. and Colvin J.R. (1976) Appl. Polymer Symposium 28, 623-626

91. Kjosbackken J. and Colvin J.R. (1973) in "Biogenesis of Plant Cell Wall Polysaccharides" pp 361-371, Ed. Loewus F., Academic Press, New York and London

92. Kjosbackken J. and Colvin J.R. (1975) Can. J. Microbiol. 21, 111-120

93. Larsen G.L. and Brummond D.O. (1974) Phytochemistry 13, 361-365

94. Lavintman N. and Cardini C.E. (1973) FEBS Lett. 29, 43-46

95. Leppard G.G., Snowden L.C. and Colvin J.R. (1975) Science 189, 1094-1095

96. Lloyd C. (1980) Nature 284, 596-597

97. Lui T.-Y. and Hassid W.Z. (1970) J. Biol. Chem. 245, 1922-1925

98. Maclachlan G.A. (1982) in "Cellulose and other Natural Polymer Systems" chapter 16 pp 327-339, ed. Brown R.M. Jnr., Plenum Press, New York and London

99. Maclachlan G.A., Dürr M. and Raymond Y. (1979) in "Methodological Surveys in Plant Biochemistry" 8B Plant Organelles pp 147-158, ed. Reid E., Wiley

100. Maltby D., Carpita N.C., Montezinos D., Kulow C. and Delmer D.P. (1979) Plant Physicl. 63, 1158-1164

101. Marx-Figini M. (1966) Nature 210, 754-755

102. Marx-Figini M. and Schulz G.V. (1966) Biochim. Biophys. Acta 112, 81-101

103. Meier H., Buchs L., Buchala A.J. and Honeywood T. (1981) Nature 289, 821-822

104. Meyer K.H. and Misch L. (1937) Helv. Chim. Acta 20, 232-244

105. Montezinos D. (1982) in "Cellulose and other Natural Polymer Systems", chapter 1 pp 3-21, ed. Brown R.M. Jnr., Plenum Press, New York and London

106. Montezinos D. and Delmer D.P. (1980) Planta 148, 305-311

107. Mortimer D.C. (1967) Can. J. Botany 41, 995-1004

108. Mueller S.C. and Brown R.M. Jnr. (1980) J. Cell Biol. 84, 315-326

109. Mueller S.C. and Brown R.M. Jnr. (1982a) Planta 154, 489-500

110. Mueller S.C. and Brown R.M. Jnr. (1982b) Planta 154, 501-514

111. Mueller S.C., Brown R.M. Jnr. and Scott T.K. (1976) Science 194, 949-951

112. Mueller S.C. and Maclachlan G.A. (1980) Plant Physiol 65 (suppl.), 106

113. Nikaido H. and Hassid W.Z. (1971) Adv. Carbohyd. Chem. Biochem. 26, 351-483

114. Northcote D.H. (1969) Essays in Biochem. 5, 89-137

115. Northcote D.H. (1972) Ann. Rev. Plant Physiol. 23, 113-132

116. Nowak-Ossario M., Gruber E. and Schurz J. (1976) Protoplasma 88, 255-263

117. Ordin L. and Hall M.A. (1967) Plant Physiol. 42, 205-212

118. Ordin L. and Hall M.A. (1968) Plant Physiol. 43, 473-476

119. Peaud-Lenoël C. and Axelos M. (1968) Eur. J. Biochem. 4, 561-567

120. Peaud-Lenoël C. and Axelos M. (1970) FEBS Lett. 8, 224-228

121. Pickett-Heaps J.D. (1968) J. Cell Sci. 3, 55-64

122. Pont Lezica R., Brett C.T., Romero Martinez P. and Dankert M.A. (1975) Biochem. Biophys. Res. Commun. 66, 980-987

123. Preston R.D. (1974) The Physical Biology of Plant Cell Walls, Chapman and Hall, London

124. Ray P.M. (1973) Plant Physiol. 51, 609-614

125. Ray P.M. (1975) Plant Physiol. 56 (suppl), 16

126. Ray.P.M. (1979) in "Methodological Surveys in Biochemistry" 8B Plant Organelles pp 135-146, ed. Reid E., Wiley

127. Ray P.M. (1980) Biochim. Biophys. Acta 629, 431-444

128. Ray P.M., Eisenger W.R. and Robinson D.G. (1976) Ber. Dtsch. Bot. Gesell 89, 121-146

129. Ray P.M., Shininger T.L., Ray M.M. (1969) Proc. Natl. Acad. Sci. U.S.A. 64, 605-612

130. Raymond Y., Fincher G.B. and Maclachlan G.A. (1978) Plant Physiol. 61, 938-942

131. Robinson D.G. and Cummings W.R. (1976) Protoplasma 90, 483-495

132. Robinson D.G. and Quader H. (1981) J. Theor. Biol. 92, 483-495

133. Robinson D.G. and Ray P.M. (1977) Cytobiologie 15, 65-77

134. Sanderman H. (1977) FEBS Lett. 81, 294-298

135. Satoh S., Matsuda K. and Tamari K. (1976) Plant and Cell Physiol. 17, 1243-1254

136. Shafizadeh F. and McGinnis G.D. (1971) Adv. Carbohyd. Chem. Biochem. 26, 297-349

137. Shore G. and Maclachlan G.A. (1973) Biochim. Biophys. Acta 329, 271-282

138. Shore G. and Maclachlan G.A. (1975) J. Cell Biol. 64, 557-571

139. Shore G., Raymond Y. and Maclachlan G.A. (1975) Plant Physiol. 56, 34-38

140. Sloneker J.H. (1971) Analyt. Biochem. 43, 539-583

141. Smith M.M. and Stone B.A. (1973a) Biochim. Biophys. Acta 313, 72-94

142. Smith M.M. and Stone B.A. (1973b) Phytochemistry 12, 1361-1367

143. Spencer F.S. and Maclachlan G.A. (1972) Plant Physiol. 49, 58-63

144. Spencer F.S., Ziola B. and Maclachlan G.A. (1971) Can. J. Biochem. 49, 1326-1332

145. Stafford L.E. and Brummond D.O. (1970) Phytochemistry 9, 253-258

146. Stockell A. and Smith E.L. (1957) J. Biol. Chem. 227, 1-26

147. Stockman V.E. (1972) Biopolymers 11, 251-270

148. Storm D.L. and Hassid W.Z. (1972) Plant Physiol. 50, 473-476

149. Swissa M., Aloni Y, Weinhouse H. and Benziman M. (1980) J. Bacteriol. 143, 1142-1150

150. Tandecarz J.S. and Cardini C.E. (1978) Biochim. Biophys. Acta 543, 423-429

151. Thomas De.S., Smith J.E. and Stanley R.G. (1969) Can. J. Botany 47, 489-496

152. Timmell T.E. (1964) Adv. Carbohyd. Chem. 19, 247-302

153. Trevelyan W.E., Procter D.P. and Harrison J.S. (1950) Nature 166, 444-445

154. Tsai C.M. and Hassid W.Z. (1971) Plant Physiol. 47, 740-744

155. Tsai C.M. and Hassid W.Z. (1973) Plant Physicl. 51, 998-1001

156. Updegraff D.M. (1969) Anal. Biochem. 32, 420-424

157. Valent B.S. and Albersheim P. (1974) Plant Physiol. 54, 105-108

158. Villemez C.L. (1971) Biochem. J. 121, 151-157

159. Villemez C.L., Franz G. and Hassid W.Z. (1967) Plant Physiol. 42, 1219-1223

160. Villemez C.L. and Heller J.S. (1970) Nature 227, 80-81

161. Villemez C.L. and Hinman M.B. (1975) Plant Physiol. 56 (suppl.), 15

162. Waldron K.W. and Brett C.T. (1983) Biochem. J. 213, 115-122

163. Willison J.H.M. (1982) in "Cellulose and other Natural Polymer Systems" chapter 6 pp 105-125, ed. Brown R.M. Jnr., Plenum Press, New York and London

164. Willison J.H.M. and Brown R.M. Jnr. (1978) J. Cell Biol. 77, 103-119

165. Willison J.H.M., Brown R.M. Jnr. and Mueller S.C. (1980) J. Microscopy 118 (part 2), 177-186

166. Willison J.H.M. and Grout B.W.W. (1978) Planta 140, 57-58

167. Wise L.E. and Ratliff E.K. (1947) Analyt. Chem. 19, 459-462

168. Wong Y.S., Fincher G.B. and Maclachlan G.A. (1977) Science 195, 679-680

169. Wooding F.B.P. (1968) J. Cell Sci. 3, 71-80

170. Zaar K. (1976) J. Cell Biol. 80, 773-777