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GLUCAN SYNTHESIS IN SOYBEAN CELLS: PROPERTIES OF THE ENZYMES INVOLVED IN DEPOSITION OF GLUCANS DURING CELL WALL REGENERATION

presented by

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has been accepted towards fulfillment of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date 26 JAN 81

O-7639

GLUCAN SYNTHESIS IN SOYBEAN CELLS: PROPERTIES OF THE ENZYMES INVOLVED IN DEPOSITION OF GLUCANS DURING CELL WALL REGENERATION

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A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1981

ABSTRACT

GLUCAN SYNTHESIS IN SOYBEAN CELLS: PROPERTIES OF THE ENZYMES INVOLVED IN DEPOSITION OF GLUCANS DURING CELL WALL REGENERATION.

By

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The early events in cell wall regeneration have been examined, using protoplasts derived from liquid suspension cultures of Glycine max. Without a detectable lag, supplied ^{14}C-glucose is incorporated into acetic and nitric acid-resistant material. This material has been identified chemically as cellulose by several independent techniques. Cytological observations indicate that there are no cellulose microfibrils present on the surface of soybean protoplasts, fixed in glutaraldehyde immediately after isolation. However after incubating protoplasts for 1 h in cell wall regeneration medium, numerous microfibrils are visible on the outer surface of the plasma membrane. These indicate that the supplied ^{14}C-glucose is incorporated into new cellulose microfibrils, not pre-existing fibrils.

During the first three h of wall regeneration, protoplasts also synthesize a wide range of polysaccharides and polysaccharide-containing polymers which qualitatively resemble the

pectic and hemicellulosic polymers found in the cell walls and recovered from the spent culture medium of cultured soybean cells.

While no appreciable quantities of 3-β-glucan are found in the cell walls or extracellular polysacharides of cultured soybean cells, protoplasts and intact cells utilize exogenous UDP-[^{14}C]-glucose to synthesize 3-β-glucan.

An attempt was made to isolate from soybean protoplasts a preparation of plasma membrane vesicles in which the topology was unaltered (i.e. periplasmic face remained facing outward. These vesicles were isolated by affinity chromatography on concanavalin A Sepharose. These vesicles utilize supplied UDP-glucose to synthesize 3-β-glucan, suggesting that the 3-β-glucan synthetase can accept substrate from the periplasmic surface of the cell. In addition, the role of the plasma membrane in modulation of enzyme activity is discussed. Results are described wherein the activity of the 3-β-glucan synthetase in membrane vesicles, derived from protoplasts, is stimulated by the induction of a transmembrane potential across the vesicles. Under conditions where protoplasts were competent to synthesize cellulose from glucose, UDP-glucose supplied to the periplasmic surface of the plasma membrane did not serve as an effective substrate of the cellulose synthetase.
These results are discussed in relation to various models for the orientation of glucan synthetases in the plasma membrane.

As a first step in the purification and characterization of the glucan synthetases of soybean cells, procedures were developed to prepare soluble forms of the enzymes. Over 75% of the glucan synthetases of a crude membrane preparation are solubilized by a 15

min. treatment with 30 mM cholate at pH 7.8. Enzyme activity is stimulated and stabilized by high concentrations of glycerol. The glucan synthesized by the soluble enzyme preparation were analyzed by methylation analyses; the results indicate that at least two UDP-glucose: glucan synthetases, producing 3-linked and 4-linked glucosyl residues are present in the soluble enzyme preparation.

ACKNOWLEDGEMENTS

I would like to express my graditude to Dr. Deborah P. Delmer for her time and effort expended on my behalf during my sojourn in the land of carbohydrate chemistry. I am most thankful for advise and moral support to: Dr. Peter S. Carlson, who with Dr. Delmer served as my joint thesis advisors; Dr. Andrew Hanson and my other committee members Drs. Arnold Revzin and Allan Morris.

My colleagues have taught me alot more than how to run a particular assay. I am particularly grateful to: Dr. Tony Bacic, who was a good friend, editor and motivating force while I wrote this thesis. I would also like to thank Carl Kulow, Kathy Johnson and Barbara Mitchell for their help during many stages of my research.

In addition I would like to thank: Dr. David Montezinos, for guiding me through some crucial cytological experiments; Dr. Larry Fowke for providing me with detailed instructions for preparing samples for electron microscopy, Dr. Gerhard Weber for sending me the soybean culture SB-1, Dr. Shelagh Ferguson-Miller for advise on membrane protein purification and Charles Caldwell for sharing his expertise on membrane proteins and affinity chromatography.

There is not enough room here for me to thank adequately, all of my friends who helped me attain this goal: my friends and colleagues at the Plant Research Laboratory and of 306 PBL, Dr. Stan Hattman, who provided inspiration and encouragement along with my many friends from

the University of Rochester; my other Lansing friends, and my family, who let me pursue my dreams. All of you made this volume possible.

This research was supported under Contract No. DE-AC02-7GER01338 for the U.S. Department of Energy. These funds are gratefully acknowledged.

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LIST OF ABBREVIATIONS

AN reagent	acetic acid nitric acid reagent
AN resistant	not hydrolyzed by acetic and nitric acid.
AN soluble	solubilized by AN reagent
BTP	1,3-bis [tris (hydroxymethyl-methylamino-propane)]
conA	concanavalin A
DCB	2,6 dichlorobenzonitrile
DTT	dithiothreitol
ECM	extracellular material: ethanol precipitable-polymers from protoplast culture medium
GC/MS	combined gas liquid chromatography and mass spectrometry
GLC	gas liquid chromatography
GDP-glucose	guanosine diphosphate glucose
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
MES	2[N-morpholino]ethane sulfonic acid
nig	nigericin
NP-40	noniondet P-40
PEG	polyethylene glycol
POPOP	p-bis [2-(5-phenyloxazolyl]-benzene
PPO	2,5 diphenyloxazole
PMSF	paramethylsulfinyl fluoride

TES	N-tris [hydroxymethyl] methyl-2-aminoethane sulfonic acid
UDP•glucose	uridine diphosphate glucose
Ψ	water potential
val	valinomycin

Introduction

Cell walls define the plant's shape, and capacity for growth, and act as a barrier to its external environment (Albersheim, 1976). Despite its importance as a major plant organelle, our understanding of the cell wall at a biochemical level is relatively limited (Darvill et al., 1980). Increasingly sophisticated analytical techniques are gradually revealing the complexity and dynamic nature of the covalent structure of polysaccharides and other macromolecular components of the cell wall (Darvill et al., 1980). Although our understanding of the chemistry of cell wall components is poor, even less is known about how these polymers are synthesized and subsequently secreted and assembled in the wall. Cell wall composition changes during different stages of development (Aspinall, 1973; Meinert and Delmer, 1977); it can also change in response to environmental stress (Boffey and Northcote, 1975; McNairn, 1972; Smith and McCully, 1977). Changes in cell wall composition could reflect changes in substrate availability, modulation of enzyme activity or altered transcription/translation of polysaccharide synthesizing enzymes. Although many theories concerning the dynamic nature of the plant cell wall have been formulated an overall understanding of the underlying processes involved in wall assembly is lacking (Delmer, 1977).

The composition of plant cell walls will be considered briefly as a framework for a discussion of cell wall regeneration and cell wall

biosynthesis. Cell wall regeneration provides advantages as a model system to elucidate cell wall structure and assembly: The individual components of the regenerating wall tend to be loosely associated and provide a more convenient material for some cytological and synthetic studies than the native cell wall (Willison 1976). The biosynthesis of cell wall polysaccharides will be reviewed, emphasizing particularly the problems involved in studying the synthesis of these polymers and the regulation of those polysaccharide-synthesizing enzymes which are associated with the plasma membrane .

A. Cell Wall Composition

The plant cell wall may be convienently described as a cellulosic lattice work bound together with an amorphous matrix of pectic and hemicellulosic polysaccharides (Rees, 1972). Cell wall composition has been extensively reviewed by Albersheim (1976); Aspinall (1973); Beck and Wieczorek (1977); Darvill et al. (1980); Delmer (1977); and Wilkie (1979).

Protein (especially glycoprotein) is part of this cell wall matrix material and accounts for generally less that 10% of the cell wall by weight (Albersheim, 1976). Lignin, a complex polymer of cinnamyl alcohols, contributes rigidity to the wall and is generally found in the secondary cell wall, i.e. the cell wall of nondividing, fully differentiated cells (Albersheim, 1976).

Native cellulose consists of linear 4-β-glucan chains which form extensive intra- and inter-molecular hydrogen bonds resulting in a crystalline fibrillar structure. Estimates of the degree of polymerization of the glucan-chains range from 2000 to more than 10,000

(Delmer, 1977). Cellulose usually represents from 10 to 50% of the dry weight of the cell wall (Albersheim, 1976; Delmer, 1977).

The pectic and hemicellulosic polysaccharides of monocots differ significantly from those found in dicots. The primary cell wall of monocots is composed of less than 9% by weight pectic polysaccharides (Darvill et al., 1980). These include arabinogalactan and a galacturonosyl- containing polymer. The pectic polysaccharides of dicots have been studied more extensively; they represent up to 30% of the weight of the cell wall. The major classes of dicot pectic polysaccharides are: rhamnogalacturonans, arabinogalactans, arabanans, galactans, and homogalacturonans. Little is known about the secondary, tertiary and quaternary structure of these polymers.

Hemicellulosic polysaccharides of dicots account for 20 to 30% of the cell wall. The principal hemicellulosic polysaccharides are xyloglucans, a linear backbone of 4-β-glucan with xylosyl substituents at the C-6 position. Some of the xylosyl residues are linked to other heterosaccharides. Glucomannans are primarily chains of 4-β-glucosyl and mannosyl residues. The ratio of mannose to glucose (ca. 3:1) may vary between species and the degree of polymerization ranges from 100 to 400 (Beck and Wieczorek, 1977). They are generally considered secondary wall components, but may be present in small amounts in the primary cell wall of some dicots and monocots (Beck and Wieczorek, 1977).

Hemicelluloses account for a greater proportion of the cell wall in monocots and are comprised of two major classes of polysaccharides: heteroxylans and mixed linkage β-glucans. Heteroxylans have a backbone of 4-β-linked D-xylopyranosyl residues substituted mainly with

arabinofuranosyl residues. Mixed linkage 3,4-β-glucans are widely distributed in the cell walls of monocots. These are linear molecules; the distribution of 4 and 3-linked residues varies between individual polymers (ratio 4- to 3-linked: 2 to 5 fold; Wilkie, 1979). There have been two reports of mixed-linkage β-glucans in dicots: a water soluble glucan isolated from three day old hypocotyls of Phaseolus (Bachala and Franz, 1974), and the product of an in vitro synthesizing system from a cell free extract of Pisum sativum (Brett and Northcote, 1975). However, these polysaccharides have not been isolated from other dicots, and are not generally considered to be constituent of the dicot cell wall (Darvill et al., 1980).

In addition to the types of polysaccharides described above, 3-β-glucans (callose) are found in the cell walls of some tissues, both in dicots and monocots. 3-β-glucan has been found in the cell walls of pollen, pollen tubes and developing cotton fibers (Darvill et al., 1980; Heslop-Harrison, 1968). It is also found surrounding plasmodesmata and is rapidly synthesized in response to wounding and stress (Brett, 1978; Hughes and Gunning, 1980; McNarin, 1972; Smith and McCully, 1977). The degree of polymerization of these glucans has not been determined. No fibrillar structure or other gross organization has been attributed to the 3-β-glucan in vivo (Heslop-Harrison, 1968); however, it is worthwhile to note that a particulate glucan synthetase preparation derived from Phytophthora produced fibrillar 3-β-glucan in vitro (Wang and Bartnicki-Garcia, 1977).

Liquid suspension grown cells have provided a convenient source of fairly uniform material for studies on the composition of the primary cell wall (Albersheim, 1976; Darvill et al., 1980). In addition, such

cells in liquid culture, secrete polysaccharides and glycoproteins into the culture medium; some of these materials are similar to cell wall polymers (Albersheim, 1976).

B. Cell Wall Regeneration

Analysis of cell wall biogenesis may be facilitated by an examination of the process of cell wall regeneration by protoplasts (Willison 1976; Willison and Klein, 1981). Polysaccharide-degrading enzymes are used to strip the cell wall away from the plant protoplast; under the appropriate cultural conditions they begin to synthesize a new cell wall. Here the processes of biosynthesis and secretion are separable, to some extent, from cell wall assembly.

One framework in which to consider cell wall regeneration is by comparison with the composition and structure of the native cell wall. Has the composition of cell wall polymers been altered? Are the individual constituents present in the same proportions in the regenerating wall and in the native cell wall? Is there coordination in the synthesis of individual wall polymers during regeneration, or does the process of removing the cell wall perturb the synthesis of some wall components but not others? How do the various polysaccharides and proteins interact to form a cell wall? Thus far there are no definitive answers to these questions. Nevertheless, characterization of the process of wall regeneration should provide useful insights about the organization and function of the cell walls of higher plants because it furnishes a mechanism to elucidate cell wall synthesis and assembly.

1. Chemical evidence for the synthesis of cellulose fibrils. The deposition of fibril-like material on the surface of isolated protoplasts is one of the characteristics of cell wall regeneration and has been an important experimental system in cytological studies of cellulose synthesis. However, caution should be exercised before equating visualization of fibrils with the presence of newly-synthesized cellulose, i.e. crystalline 4-β-glucan. (Meyer and Herth, 1977; Willison 1976). Unbranched polysaccharides, particularly homopolymers, form fibril-like structures in vitro (e.g. 4-β-mannan, 4-β-xylan of angiosperms (Preston, 1979; 3-β-glucan of fungi, Wang and Bartnicki-Garcia, 1976) Also, some of the noncellulosic components of the cell wall may exist as fibrils in situ (Preston, 1979). In many systems the regenerating wall will fluoresce when treated with Calcofluor White ST; however Calcofluor binding is not specific for 4-β-glucans and Calcofluor will stain the cell wall of a number of microorganisms which do not contain cellulose in their walls (Darken, 1961). Other criteria used as evidence that the regenerating wall contains crystalline cellulose are not unequivocal: Cellulase digestibility is not a rigorous test for the presence of cellulose because most available cellulase preparations contain other polysaccharide-degrading enzymes (Anderson and Ray, 1978). Similarly although alkali insolubility was the basis of the classical method for isolating 'pure' α-cellulose, it is now well established that other cell wall polysaccharides are insoluble in hot alkali (Delmer, 1977). Nevertheless, in previous reports on the chemical composition of the regenerating cell wall, (Asamizu et al., 1977; Asamizu and Nishi, 1980; Hanke and Northcote, 1974), one or the other of these procedures

was used as the sole means to demonstrate the presence of cellulose in the regenerating cell wall.

In another study Vinca rosea protoplasts were cultured for two days. The regenerating wall was fully soluble in 24% KOH, indicating that it did not contain cellulose (Takeuchi and Komamine, 1978).

Potentially, chemical inhibitors of cellulose synthesis could be used to verify the relationship between cellulose synthesis and fibril deposition. Coumarin (100μM) and 2,6-dichlorobenzonitrile (DCB) (10μM) specifically inhibit cellulose synthesis in cultured cotton ovules. (Montezinos and Delmer, 1980). At higher concentrations these compounds also inhibit the synthesis of other cell wall polysaccharides.

In an ultrastructural study, coumarin (0.5-1.5mM) inhibited fibril deposition on the surface of tobacco mesophyll protoplasts although after four days an aberrant wall was formed (Burgess and Linstead, 1977). Meyer and Herth, (1978) reported that coumarin (1mM) and DCB (5μM) totally inhibited cell wall formation by tobacco mesophyll protoplasts. Thus while these observations indicate that DCB or coumarin can certainly inhibit cellulose synthesis or fibril deposition, these compounds also seem to affect either the synthesis or assembly of other cell wall components, and the observations, described above, alone do not provide conclusive evidence that the fibrils are composed of cellulose.

Although the fibril-like material visualized on the surface of protoplasts in many other systems is most likely cellulose, more careful characterization of this material is advisable before general

conclusions can be drawn about the occurrence and secondary structure of cellulose in the regenerating cell wall.

2. Kinetics of cellulose synthesis by cultured protoplasts. The onset of wall regeneration by protoplasts may vary according to the source of the material and cultural practices (Willison, 1976). Burgess et al. (1978) reported that there was a lag of hours or days before fibrils could be detected on the surface of protoplasts isolated from tobacco mesophyll cells and Antirrhinum leaves; they suggested that during this lag period the cellulose-synthesizing machinery was reassembled. Grout (1977) presented similar data for tobacco mesophyll protoplasts. In other systems, e.g. protoplasts isolated from cell cultures of Vicia hajastanna, fibril deposition was observed within minutes after the protoplasts were removed from wall-degrading medium (Williamson et al., 1977).

Asamizu et al. (1977), reported that the cellulose content of carrot protoplast cultures gradually increased over eight days to a maximum level 50% lower than that of cultured cells. In a second paper, Asamizu and Nishi (1980) reported that when carrot protoplasts were supplied ^{14}C-glucose, label accumulated in an alkali-insoluble α-cellulose fraction within five hours after the protoplasts were isolated. As noted previously alkali insolubility alone is insufficient proof of the presence of cellulose (Delmer, 1977). Thus while Burgess et al. (1978) have proposed that freshly-isolated protoplasts are in general not competent to synthesize cellulose, there is evidence in some systems that there is no lag in microfibril biosynthesis during wall regeneration.

3. Other cell wall constituents. There have been a limited number of studies partially characterizing, on a biochemical level, the non-cellulosic components of the regenerating cell wall: soybean, Hanke and Norhtcote (1974); carrot, Asamizu and Nishi, (1980); and Vinca rosea: Takeuchi and Komamine, (1978); Tanaka and Uchida, (1979). The composition of the regenerating wall differed between and within the same experimental systems, but direct comparison between studies must be made with caution. Hanke and Northcote (1974), and Takeuchi and Komamine (1978), contrasted the composition and structure of the regenerating wall to that of the native cell wall. One dilemma is immediately apparent. What constitutes the regenerating cell wall? Conventional methods for the isolation of cell walls from intact cells depend on the huge size difference between the cytoplasmic organelles and pieces of the cell wall. Cell wall fragments may approach the dimensions of intact cells, so that they sediment rapidly at low centrifugal force, and are retained by coarse filters. During the early stages of wall regeneration, wall fragments are probably small and loosely organized. Growing cellulose chains are presumably anchored to the plasma membrane (Mueller et al., 1980, Giddings, et al., 1980). However, during the early stages of wall regeneration in the absense of a preexisting cellulosic network, much of the newly-synthesized cell wall material may float away from the surface of the protoplasts (Hanke and Northcote, 1974; Prat and Roland, 1971). Furthermore, cultured plant cells are known to excrete polysaccharides and glycoproteins into the culture medium, a characteristic likely to be shared by protoplasts. Thus during the early stages of wall regeneration it is difficult to separate cytoplasm and cell wall, and

at the same time distinctions between the true cell wall constituents and extracellular materials are blurred. In addition, cell wall regeneration may not proceed synchronously; in any case the regenerating cell wall is likely to be a dynamic organelle. Thus, perhaps it is premature to build detailed models of the structure of the regenerating wall.

Hanke and Northcote (1974) have studied cell wall formation by soybean protoplasts. Freshly-isolated protoplasts from suspension culture cells were supplied radioactive glucose in order to monitor newly-synthesized cell components after 20 or 40 hours in culture. After 20 hours in culture, protoplasts were not yet osmotically stable; the material retained on a sintered glass filter was designated the washed protoplast fraction, and the dialyzed filtrate was taken to represent both secreted material and cytoplasmic polymers. However, after 40 hours of culture the protoplasts did not lyse when collected on the glass filter. Therefore the filtrate consisted only of extracellular material. Comparisons were made between the two washed protoplast fractions obtained at the end of the culture periods, and also the corresponding dialyzed filtrates, although it is unlikely that these represented the same cytological cell fractions. Following 20 hours of culture, radioactivity from supplied glucose was incorporated into starch, protein, and 'cellulose' (determined by digestion with a crude cellulase preparation, obtained from the commercial product, Onozuka cellulase R 1500). One type of pectic polysaccharide was identified in the dialyzed filtrate; it was characterized on the basis of its electrophoretic mobility as a weakly acid pectinic acid, and contained some galacturonic acid. After 40 hours of culture, two

additional pectins were isolated from the dialyzed medium fraction. All three pectic polysaccharides had electrophoretic characteristics similar to pectins isolated from the parent soybean callus, which also contains additional classes of pectins. The three pectins were not found associated with the washed protoplast fractions after either 20 or 40 hours of culture and the authors interpreted this to mean that wall regeneration by soybean protoplasts was aberrant because of the absence of synthesis of some pectins associated with the lack of assembly of other pectins into the developing wall.

Cultural conditions required for the isolation of protoplasts may also affect the synthesis of cell wall polymers. Boffey and Northcote, (1975), studied pectin synthesis during the formation of a cell wall by plasmolyzed tobacco cells. Plasmolysis had altered the synthesis of some pectic polymers when compared to unplasmolyzed tissues. Incorporation of supplied ^{14}C-glucose into galacturonosyl and rhamnosyl residues of the bulk pectin fraction decreased, while incorporation into arabinosyl residues was not affected or was only slightly enhanced. This was interpreted to indicate that the synthesis of individual pectic polymers is separately regulated.

Asamizu and Nishi, (1980) examined the pattern of incorporation of ^{14}C-glucose and ^{3}H-myoinositol by carrot protoplasts into polysaccharide-containing polymers. In one set of experiments protoplasts were labeled for five hours after varying periods of culture (during which the wall was forming). The labeled protoplast samples were mixed with cultured carrot cells and then homogenized. Cell walls were isolated by conventional procedures using centrifugation and multiple washing steps. The walls were fractionated

by extraction into pectin, hemicellulose and an alkali-insoluble 'α-cellulose' fraction. As noted previously, alkali insolubility is not a unique property of cellulose. In samples from protoplasts cultured for a total of 5, 22, 46, 70 or 100 hours, radioactivity was found in all three classical cell wall fractions; however the proportion of radioactivity in pectin, hemicellulose or 'α-cellulose' fractions continuously changed. It is not clear whether the nascent cell wall from protoplasts cultured for only short periods, was isolated by these procedures. The overall neutral sugar composition of the regenerating walls from protoplasts cultured for 24 hours or seven days differed from that of the cell walls from seven day old cultured cells. Here a similar comparison of the extracellular, 80% ethanol-precipitable polymers from protoplasts also differed from that of material isolated from the medium of cultured cells. After 24 hours in culture, with continuous labeling from ^{14}C-glucose, the distribution of radioactivity in neutral sugar residues was determined from the three cell wall fractions. Arabinosyl and glucosyl residues were the predominant radioactive sugars in the pectic fraction, (although glucose is not normally found in pectic polysaccharides from native cell walls). Radioactive glucosyl, arabinosyl and galactosyl residues were found in the hemicellulose fraction; both galactosyl and glucosyl residues contained appreciable amounts of label in the 'α-cellulose' fraction. This labeling pattern is generally similar to that obtained with carrot cells in suspension culture (Asamizu and Nishi, 1979). Thus these reports indicate that all of the classical components of the cell wall are synthesized during wall regeneration by carrot protoplasts. However, the data presented do not discriminate

between the possibilities that specific pectic or hemicellulosic polysaccharides are absent during some stages of wall regeneration and/or that the synthesis of specific pectic or hemicellulosic polymers is not coordinated during wall regeneration as compared to during growth of the native cell wall.

Takeuchi and Komamine (1978) examined the regenerating wall and extracellular polysaccharides from Vinca rosea protoplasts, isolated after three days of culture. Protoplasts were collected from the culture medium, washed, broken by homogenization, and the wall was isolated by conventional techniques. The regenerated wall of Vinca protoplasts was fully soluble in 24% KOH; thus it did not contain any crystalline cellulose. The native cell wall of Vinca does contain cellulose, pectins and hemicelluloses (Takeuchi and Komamine, 1978). Instead the regenerated wall was composed of 3- and 4-linked glucans which are also present in the native cell wall of Vinca. They also examined extracellular polysaccharides from protoplasts and these were found to be similar to those in the medium of liquid suspension cultured cells; these polysaccharides included polyuronides and 3,6-linked arabinogalactan. However, these studies were not detailed enough to ascertain if specific cell wall pectins or hemicelluloses were not synthesized by protoplasts or were not assembled into the developing cell wall.

C. Cell Wall Biosynthesis

While cell wall regeneration provides one framework in which to examine wall biogenesis, a complementary approach involves elucidation of the enzymology of polysaccharide biosynthesis. Thus far only

limited information is available on this aspect of cell wall biology, (Delmer, 1977; Darvill et al., 1980).

Sugar nucleotides act as high energy intermediates, either directly, or possibly via lipid/and or protein-linked intermediates, in the synthesis of cell wall polysaccharides (Delmer, 1977; Karr, 1976). It is believed that pectic polysaccharides and hemicelluloses (with the exception of 3-β-glucan) are synthesized in the Golgi system (Ray et al., 1976; Robinson, 1977).

In cell fractionation studies, an enzyme which utilizes UDP-glucose, to produce a 4-β-glucan, is considered a tentative marker enzyme for the Golgi system (Ray et al., 1969). The enzyme, glucan synthetase I^1, is believed to synthesize the backbone of the xyloglucan polysaccharide and a second enzyme (a UDP-xylose: polysaccharide transferase) assembles the side chains (Quail, 1979; Ray, 1980).

Cytological observations suggest that the individual glucan chains of cellulose are synthesized at the plasma membrane and concomitantly assembled into microfibrils (Brown and Montezinos, 1976; Mueller et al., 1976). Glucan synthetase II, the 3-β-glucan synthetase (E.C.2.4.1.34) is also believed to be localized at the plasma membrane and is considered to be a provisional marker enzyme for the plasma membrane (Quail, 1979).

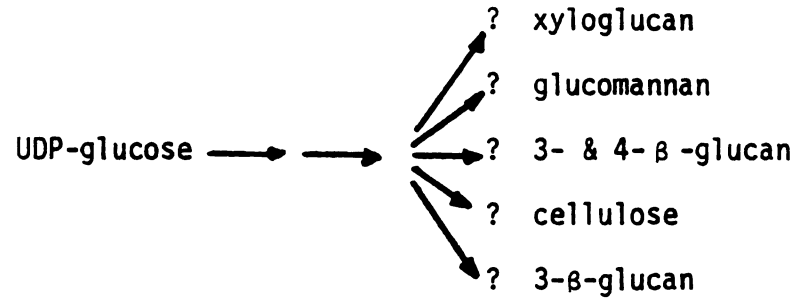
Crude membrane preparations derived from a variety of species, have been used to study the synthesis of polysaccharides in vitro. One difficulty in studying glucan synthesis in vitro is that the only method of detection is the incorporation of radioactively-labeled glucose, from the nucleotide sugar, into an insoluble product

(Villemez, 1973). Delmer (1977) noted that far too often these insoluble products have not been adequately characterized, thus resulting in erroneous interpretations. Earlier studies claimed *in vitro* cellulose biosynthesis with membrane preparations from *Phaseolus*, or other species; these utilized either GDP-glucose or UDP-glucose to synthesize alkali-insoluble products (Barber *et al.*, 1964; Brummond and Gibbons, 1965; Elbein *et al.*, 1963; Franz and Meir, 1969). In most instances these results have been reinterpreted: GDP-glucose incorporation is stimulated by GDP-mannose (Villemez, 1973), suggesting that this probably represents the synthesis of glucomannan. UDP-glucose is utilized as a substrate for the synthesis of xyloglucan, 3-β-glucan, and/or mixed linkage glucans (in monocots). (Delmer, 1977; Ray, 1980; Smith and Stone, 1973).

Although indirect evidence favors UDP-glucose as the most probable substrate for cellulose biosynthesis *in vivo*, there is no convincing evidence of *in vitro* synthesis of crystalline cellulose in systems derived from higher plants (Darvill *et al.*, 1980; Delmer, 1977). Carpita and Delmer (in press) have speculated that there is some crucial role of plasma membrane integrity required for cellulose biosynthesis which would be largely destroyed during membrane isolation.

Another consideration in the study of polysaccharide biosynthesis is, the extent to which compartmentation controls the types and complexity of polysaccharide synthesized *in vivo* (Villemez, 1973). Most of the work on polysaccharide synthesizing systems from higher plants have utilized crude particulate systems. The same nucleotide sugar, uridine diphosphate glucose serves as substrate, for more then

one polysaccharide-synthesizing enzyme. Furthermore it is difficult to identify the products of such in vitro systems and distinguish which polysaccharide-synthesizing system they arise from in vivo.



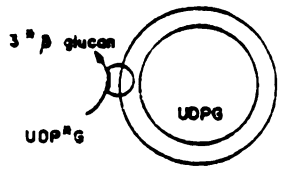
None of the cell wall polysaccharide-synthesizing enzymes from a higher plant have been isolated and extensively characterized.

D. Regulation of Biosynthesis

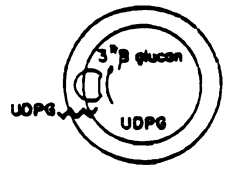
The rates of synthesis of cell wall polysaccharides change in response to various factors, including: 1) plant growth factors (e.g. indole acetic acid, Evans, in press; Spencer et al., 1971); 2) plasmolysis (Boffey and Northcote, 1975); 3) stage of development (Meinert and Delmer, 1975); 4) wounding (e.g. instaneous induction of 3-β-glucan synthesis: Brett, 1978); 5) temperature stress (Smith and McCully, 1977). Some information is available about the underlying mechanisms which may regulate the synthesis of cell wall polysaccharide: For example, during development of the cotton fiber, the cellular concentration of UDP-glucose increases, concomitant with the maximum rate of cellulose biosynthesis. (Carpita and Delmer, in press). Indirect evidence suggests a role for IAA-induced

transcriptional/translational control of polysaccharide-synthesizing enzymes during indole acetic acid-induced growth (Evans, in press). As indicated previously, the 3-β-glucan synthetase and cellulose synthetase are influenced by membrane integrity, disrupting the plasma membrane activates the 3-β-glucan synthetase while eliminating cellulose synthesis (Carpita and Delmer, in press). How is the activity of the 3-β-glucan synthetase rapidly modulated? The orientation of the 3-β-glucan synthetase within the plasma membrane, periplasmic versus cytoplasmic, could control the utilization of endogenous substrate (UDP-glucose) and thereby regulated the synthesis of glucan. Three models illustrated below suggest how the orientation of the enzyme within the plasma membrane, might regulate enzyme activity.

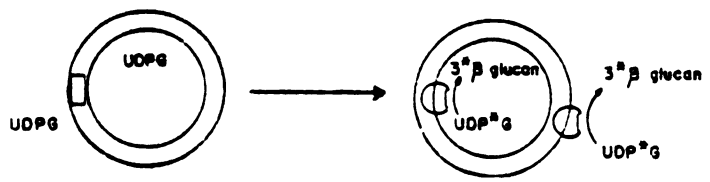
A.



B.



C.



The first model proposed by Hughes and Gunning (1979) shows the active site of the enzyme oriented toward the periplasmic face of the plasma membrane. Supplied UDP-[^{14}C]-glucose it utilized to synthesize 3-β-glucan. Cytoplasmic pools of UDP-glucose do not serve as substrate, except when the cell is wounded or stressed, allowing the UDP-glucose to leak thru the plasma membrane to the enzyme. In the second model, favored by Mueller and MacLachlan (1980) the plasma membrane is slightly permeable to the supplied UDP-[^{14}C]-glucose; as the nucleotide sugar crosses the plasma membrane of the protoplast or intact cell, it is immediately utilized by the 3-β-glucan synthetase which has a high affinity for the nucleotide sugar. However, this model does not explain how the activity of the enzyme is regulated such that the cytoplasmic pools of UDP-glucose are not utilized for the synthesis of 3-β-glucan. The third model, suggested from a report by Christopher Brett (1978), implies that the 3-β-glucan synthetase is normally in some latant form, perhaps buried within the plasma membrane. At certain stages of development or particularly in response to stress or wounding, the enzyme is activated.

This thesis addresses questions pertaining to cell wall biogenesis. The first chapter investigates cell wall regeneration by soybean protoplasts as a model for cell wall synthesis, secretion and assembly. The second chapter deals with preliminary studies to isolate polysaccharide-synthesizing enzymes, in order that the biochemistry of these enzymes may be better characterized. Finally chapter three examines how one of these enzymes, the 3-β-glucan synthetase may be regulated <u>in situ</u>, both as a function of its orientation within the

plasma membrane, and in response to perturbations in its physical environment in the membrane.

MATERIALS AND METHODS

Chemicals and reagents

D-[(U)-^{14}C]-glucose and UDP-[(U)-^{14}C]-glucose were obtained from New England Nuclear (Boston, MA, USA) or from ICN Pharmaceuticals (Irvine, CA, USA). A Streptomyces cellulase preparation and a 3-β-endoglucanase preparation from Rhizpous were the generous gifts of Dr. E.T. Reese, (U.S. Army Laboratories, Natick, MA, USA). Porcine pancreatic α-amylase (Type I-A), Commassie Brilliant Blue G, concanavalin A (IV), cyanogen bromide activated Sepharose 6B, Sepharose 2B, Reactive Blue Agarose, bovine serum albumin, valinomycin, cholic acid and Ficoll 400 were purchased from the Sigma Chemical Corp. (St Louis, MO, USA). Ficoll was purified before use as described in Boller and Kende (1979). Cholic acid was recrystallized twice from 70% ethanol. Nigericin was obtained from Eli Lilly and Co. (Greenfield, IN, USA). The herbicide DCB, (casoron), was a gift from Thompson-Hayward Chemical Co., (Kansas City, KA, USA). Ultragel ACA 34 was purchased from LKB Instruments (Rockville, MD, USA). Sephadex G-25 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, USA). Commassie Blue protein reagent and Bio-gel P-6 were purchased from BioRad Laboratories, (Richmond, CA, USA). Polyethylene glycol 4000 (PEG 4000, "Baker" grade) was obtained from T.T. Baker Chemical Co., (Phillipsburg, NJ, USA). Pentaerythritol is produced by Pfaltz-Bauer (Stamford, CN, USA). All other reagents were of analytical grade.

Double distilled, deionized water was used for all solutions. However, on at least four occasions over the last three years, the liquid-suspension cultures gradually deteriorated: two and three day old cell cultures were noticeably brown, indicating that the cells were producing phenolic compounds. The accumulation of phenolic compounds is usually associated with the stationary growth phase or stress (Hahlbrock and Griesebach, 1979). Simultaneously protoplast yields would decline. This was usually correlated with an accumulation of volatile amines in the double distilled H_2O supply used to prepare culture medium. Cotton ovule, Dictyostellium and mammalian cell cultures are adversely affected as the volatile amines accumulate (Ken Poff, Justin McCormick, personal communications).

The amine problem was circumvented by changing the ion exchange system of the primary distilled H_2O supply at frequent intervals: These ion exchange cartridges gradually deteriorate then leach quaternary amines in the distilled H_2O; furthermore, when the cartridges detoriate to this stage the system no longer removes morpholine, a volatile amine and an antiscale agent in the steam supply, from the distilled water.

Cell wall degrading enzymes were partially purified, as described by Gamborg (1975), with minor modifications. Cellulase R-10 (Onozuka; Kinki Yakult, Mfg Co., Ltd. Nishinomiya, Japan), Macerase (Calbiochem, Irvine, CA, USA) or Cellulysin (Calbiochem, were each dissolved in cold water, (10 to 15 g/100 ml) and clarified by centrifugation. The supernatant was applied to a Sephadex G-25 column (90 x 5.5 cm) and eluted with water. Fractions were monitored for protein with Bradford's reagent (Bradford, 1976); those which contained protein were

retained and lyophilized. The resulting material (ca. 10 to 40% by weight of the commercial preparation) was used in protoplast isolations.

Cell Cultures

A liquid suspension of cells of Glycine max L. Merr cv. Mandarin, designated SB-1 (derived from root tissues), was obtained from Dr. Gerhard Weber, (University of Utah, Salt Lake City, UT, USA). Cultures were maintained in 53 ml of 1B5 medium, (Gamborg, 1975) in 125 ml Erlenmeyer flasks, at 27°C in the dark on a gyratory shaker at 125 rpm. The cells were subcultured during log phase growth by a 1 to 3 dilution at 3-4 day intervals. The cell line was also maintained as a callus culture on medium solidified with agar; calli were subcultured monthly. On at least three separate occasions calli were used to start fresh suspension cultures.

Protoplast Isolation

All manipulations were performed under sterile conditions in a laminar flow hood. Two day old suspension cultures were transferred to conical tubes and allowed to settle under gravity. The cells were washed twice with a solution containing B5 salts and vitamins (Constabel, 1975). One volume of 3% (w/v) Cellulysin (or Cellulase) and 1% (w/v) Macerase in 10% (w/v) sorbitol, pH 5.5 in water, was added to the cells. The suspension of cells was transferred to a Petri dish (100 x 20 mm), and was incubated for 2.5 h on a reciprocal shaker (ca. 30 cycles min^{-1}), at 27°C, in the dark.

The suspension was filtered through Nitex Cloth (linear pore size, 48 μm; Tabler, Ernst and Traber, Inc. Des Plaines, IL, USA), then protoplasts and cell debris were collected by centrifugation (8 min, 120 x g in a clinical centrifuge). The protoplasts were purified by flotation upward through a ficoll gradient in a manner similar to that described by Boller and Kende (1979): First the pellet containing protoplasts and debris was resuspended in 6 to 7 ml of 19% Ficoll (w/w in wall regeneration medium, see below), and placed in the bottom of a 12 ml centrifuge tube. This suspension was overlain with 2 ml each of 16% and 13% ficoll and finally with wall regeneration medium. The gradient was centrifuged for 20 min at 170 x g, at room temperature in a clinical centrifuge. Protoplasts were collected from the interface between 13% ficoll and wall regeneration medium.

At this stage, the preparation appeared to contain only protoplasts; no intact cells or wall debris could be detected by routine light microscopy. Damaged and lysed protoplasts were trapped in the denser Ficoll layers. Typically 20 ml packed cell volume yielded betwen 1×10^7 and 4×10^7 protoplasts. Protoplasts were washed three times with wall regeneration medium and collected each time by centrifugation (3 min, 120 x g). Protoplasts were resuspended at final concentrations between 3×10^5 and 2×10^6 ml^{-1} in wall regeneration medium supplemented with the appropriate labeled sugar; 3 to 5 ml samples of protoplast suspension were transferred to individual plastic Petri dishes (60 x 15 mm) for incubation.

Protoplast wall regeneration medium is that described by Constabel (1975) for soybeans, except glucose was omitted and replaced with an equimolar amount of sorbitol.

To increase the reproducibility of protoplast yields to a level adequate for routine experiments, the procedure for protoplast isolation was eventually modified as follows: i) Cell suspensions were subcultured 18 to 24 h prior to use. ii) The wall degrading medium was altered to include 30mM Na·MES, pH 5.5, and 50mM mercaptoethanolamine (Tom Boller, personal communication). This modified protocol was used to prepare protoplasts for the experiments described in the Section A and B3 of Chapter One and for all of the experiments in Chapter Three.

Measurement of Water Potential

The water potential of protoplast culture medium was measured on a Wescor Dewpoint Hygrometer (Wescor, Inc., Logan, UT, USA) which had been calibrated with standard solutions of sucrose (Nelsen et al., 1978). The water potentials of various dilutions of a concentrated PEG 4000 solution were determined; these data was used to calculate the appropriate dilutions of the PEG 4000 stock solution to prepare protoplast media at -11, -13.5, -16 bars (see Table 1-1). Similar measurements and calculations were performed to prepare the sorbitol-based medium.

Isolation of Polysaccharides and Glycoproteins from Cell Walls and Spent Medium of Liquid Suspension Cultures

Two day old suspension-cultured cells were collected on a sintered glass filter. Three and one half volumes of ethanol were added to the spent medium (filtrate) and the mixture was chilled at 4°C overnight. The precipitate, containing polysaccharide and protein, was washed four times with 70% (v/v) ethanol. This material resembles the secreted

extracellular polysaccharide (SEPS) of cultured sycamore maple and secreted arabino-galactan protein described by Lamport (1977, 1978).

Cells were washed extensively with a solution of B5 salts (at equivalent concentration to that in the culture medium; ca 500 ml per 20 g. cells). The cells were resuspended in cold 100 mM potassium phosphate buffer, pH 7, with 4 mM sodium metabisulfite and disrupted at 4°C by three, 2 min cycles of sonication with a Biosonic VI sonifer (Bronwill Scientific, Rochester, NY, USA). Cell walls were prepared as described by Talmadge et al. (1973). Following the final acetone wash, the pellets were washed in 50% methanol three times and once in water. Walls were resuspended in 5 ml of 50 mM potassium phosphate buffer, pH 6.8, (ca. 10 mg ml^{-1}) with 100 U α-amylase per ml of suspension and incubated with 50 µl of toluene for 25 h at 30°C in a gyratory shaker (ca. 125 rpm). The walls were washed with water three times and once with acetone, lyophilized, and stored in a vacuum desicator.

Isolation of Newly-Synthesized Polymers

For the time course experiments (Chapter one; Figs. 1 thru 4), incubations were terminated by the addition of 4 volumes of ethanol to give a final ethanol concentration of 80% (v/v). In other experiments protoplasts were collected by centrifugation (3 min, 120 x g); and were washed four times with cold wall regeneration medium then resuspended in 80% ethanol. This was designated the protoplast fraction. The first supernatant (spent wall regeneration medium), was brought to 80% ethanol and the precipitate, consisting primarily of polysaccharide and protein, was collected by centrifugation (10 min, 10,000 x g). This was designated the extracellular fraction (ECM). The various

precipitates were washed four times with 80% ethanol and with chloroform methanol (1:1, v/v). To measure the radioactivity in soluble cytoplasmic pools, washed protoplasts were disrupted in 80% ethanol and chilled overnight. The precipitate was collected by centrifugation and the first supernatant was retained: this constituted the 80% ethanol-soluble cytoplasmic contents.

Cellulose Content

The cellulose content of cell walls was determined by the method described by Updegraff (1969).

Determination of the Radioactivity in Crystalline Cellulose

Carrier cellulose (ca. 0.5 mg) was added to the ethanol precipitate and the sample was boiled in 2 ml of acetic-nitric acid reagent (AN reagent, Updegraff, 1969) for 60 to 90 min. Water (3 ml) was added to reduce the viscosity and the insoluble material was collected by centrifugation (10 min, 10,000 x g). The supernatant was carefully removed and the pellet was washed four times with 5 to 10 ml of water. For direct determination of radioactivity the pellet was dissolved in 10 ml Biosolve (Beckman Scientific, Irvine, CA, USA) or ACS (Amersham, Arlington Heights, IL, USA) scintillation cocktail.

Neutral Sugar Analyses

The neutral sugar content of cell walls and the distribution of radioactivity in neutral sugar residues of the ethanol-precipitable material was determined after hydrolysis in 2N Trifluoroacetic acid at 120°C for 90 min. Crystalline cellulose is not readily hydrolyzed by

this procedure. Neutral sugars were reduced and acetylated by the procedure of Albersheim et al. (1967). Following acetylation, sugar derivatives were redissolved in dichloromethane and analyzed by GLC. Separations were performed in a Hewlett Packard model 8350 gas chromatograph (Avondale, PA, USA) equipped with a variable effluent stream splitter and integrator. Nitrogen, was used as a carrier gas (22 ml min^{-1}). The glass columns (180 x 0.2 cm i.d. configuration 8), were packed with Supelco Support SP 2340 (Bellefonte, PA, USA). The alditol acetates were separated with temperature programming at 6° min^{-1}, from 140°C to 225°C. Acetylated sugar derivatives of the cell wall were identified by relative retention time. Radioactivity in samples derived from protoplasts was identified by coelution with alditol acetates of a standard sugar mixture: 50% of the effluent stream went to the flame ionization detector, and the remainder was diverted to a heated collection port. The alditol acetates condensed in Uniform Drop Size Pipets (Scientific Products, Romulus, MI, USA) fitted to the collection port; the condensed material was rinsed into vials with toluene-based scintillation cocktail (4.2 g PPO, 52.5 mg POPOP l^{-1}) and radioactivity was determined by scintillation counting.

Methylation Analyses

The linkages of (radioactive) neutral sugar residues were determined by methylation analysis according to the method of Hakamori (1964), as described by Maltby et al., (1979) with the following modifications: After methylation samples were transferred to screw cap culture tubes (15 x 150 mm, with Teflon lined caps). Chloroform:

methanol, (2:1, v/v, 7 ml) was added to each, then 10 ml of water were added to extract unreacted methyliodide. The extraction was repeated seven times; each time the upper phase of methanol and water was removed, but any insoluble material at the interface and lower layer were retained. After the extractions, the lower layer and interface were transferred to a conical microflex tube, (Kontes, Vineland, NJ, USA), and dried under nitrogen. After acetylation, the remaining acetic anhydride was evaporated under nitrogen as an azeotrope with toluene, and the gelatinous residue was washed once with toluene. Dichloromethane, 0.5 ml, was added to each sample; the suspension was resuspended and centrifuged, in a bench top centrifuge (1 min, 625 x g) and the supernatant transferred to a clean vial. Chromatography was done on a Varian Aerograph GLC, (model 2100, Louisville, KY, USA) equipped with an effluent stream splitter (fixed ratio 10:1, collector to flame ionization detector). Helium was used as the carrier gas (30 ml min^{-1}). Separations were performed on glass columns (180 cm x 0.2 cm i.d.), packed with Gas-Chrom Q, (100-120 mesh, Applied Science Laboratory Inc., State College, PA, USA) coated with a mixture of 0.2% poly(ethylene glycoladipate), 0.2% poly(ethylene glycol succinate) and 0.4% silicone XE-1150. The alditol acetates of permethylated sugars were separated with temperature programming (115°C, with a 5 min hold, then 4° min^{-1} to 190°C). Fractions were identified by coelution with known sugar derivatives and the effluent was collected in tips of Pasteur pipets (9 inch, Scientific Products, Romulus, MI, USA). The identify of permethylated sugar derivatives of the cell wall and extracellular polysacharrides was verified by combined gas chromatography-mass spectrometry of samples which were reduced with

sodium borodeuteride; analyses were performed on a Hewlett Packard gas chromatograph-mass spectrometer model 5985A at the MSU mass spectrometry facility. Radioactivity was determined as described above.

In some experiments separations were carried out on a Hewlett Packard model 8350 gas chromatograph, with nitrogen as a carrier gas (22 ml min^{-1}); the elution profile of standards was similar on both chromatographs.

Cell Counts

The average number of cells per 0.5 ml packed cell volume was determined as follows. Duplicate 10 ml aliquots of cell suspensions were allowed to settle under gravity in 15 ml graduated conical tubes. The supernatant was removed and the cells were resuspended in approximately 0.25 ml concentrated sulfuric acid (as suggested by Dr. R. Greisbach). The suspension was diluted to 10 ml with H_2O and cell clumps were disrupted by passage thru a syringe equipped with a 22 1/2 gauge needle. The acid treatment was repeated if necessary. The average cell number per ml of suspension was based on the average of four individual determinations using a hemacytometer. 0.5 ml packed cell volume contained approximately 1.1×10^7 cells.

Protein Determinations

The protein content of samples was determined using the method of Bradford (1976). Samples (0.1 ml), containing 10 to 100 µg of protein were mixed with 5 ml of Commassie Brilliant Blue G reagent and the

absorbance was monitored at a wavelength of 595 nm. Bovine serum albumin was used to prepare a standard curve.

1. Protein content of soybean protoplasts.

The protein content of protoplasts was measured based on a unit of 1 X 10^6 protoplasts (as determined by counting protoplasts on a hemocytometer), and was 0.35 mg per 10^6 protoplasts.

2. Samples in cholate buffers. Prior to assaying protein content, samples which contained cholate, were extracted with four to nine volumes of absolute ethanol at 70°C for 10 min. Cholate, which interferes with the protein assay, is soluble in hot ethanol. The precipitated protein was collected by centrifugation and washed with ethanol (1 X 1 ml). The precipitate was redissolved in 16.5% glycerol, 50 mM K·TES, pH 7.8, 400 mM sorbitol, 5 mM $MgSO_4$ and protein content was determined using the micro method described by Bradford, (1976). Bovine serum albumin, treated in a similar manner was used as a standard.

Preparation of Samples for Electron Microscopy

Protoplasts were cultured for the times indicated in the figure legends, then resuspended in 1% (w/v) glutaraldehyde in wall regeneration medium at room temperature as described in Fowke et al., (1975); Williamson et al., (1976). After 1-2 h the protoplasts were resuspended in 3% glutaraldehyde in the same medium and held overnight at 4°C. The samples were then washed in 0.05 M sodium phosphate buffer and postfixed 2 h in 1% osmium tetroxide in 0.05 M sodium phosphate, pH 6.6 at 4°C. After 2 washes in cold distilled water, samples were dehydrated by passage through a graded ethanol series followed by amyl

acetate. Samples were transferred to poly-L-lysine coated cover slips and allowed to dry as described by Mazia et al., (1975).

The samples were shadowed with platinum-carbon at a 45° angle and then carbon coated at a 90° angle in a Balzers BA 360 vacuum evaporator. The replicas thus formed were removed from the coverslips by slowly dipping them into 50% hydrofluoric acid, rinsed by transferring them through two changes of distilled water, and cleaned by floating them on sodium hypochlorite (bleach, available commercially) for 1-3 days. The cleaned replicas were mounted on uncoated 300 mesh copper grids and examined in a Siemens 1A electron microscope.

Preparation of Membrane Bound Glucan Synthetases from Soybean Cells

Unless otherwise stated, the protocol used for preparing soybean cell membranes was as summarized in Fig 2-4 and described below.

Suspension grown cells (2d) were collected by filtration, in a Buchner funnel lined with Miracloth (Chicopee Mills, Inc. Milltown, NJ, USA). The cells were washed extensively with ice cold H_2O, then weighed. The cells were then plasmolysed in ice cold buffer (1.5 vol. per g fresh weight cells): 50 mM K•TES, pH 7.2, 400 mM sorbitol, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF. The suspension (20 to 60 ml) was injected into the sample chamber of a Ribi Press (Sorvall Inc., Norwalk, CT, USA) and disrupted under pressure (Ca. 8000 PSI) while maintaining the temperature of the Ribi valve at 5-12°C. The brei was collected in a beaker on ice. The sample chamber and Ribi valve were flushed with buffer (10 ml) and this was pooled with the broken cells.

This treatment resulted in uniform cell breakage, but cell walls and other organelles remained recognizable under the light microscope.

The brei was filtered through Nitex cloth (linear pore size, 40 μm) to remove large wall fragments. The filtrate was centrifuged at 500 x g at 5°C for 5 min. The pellet consisting primarily of small wall fragments was discarded. The supernatant was centrifuged at 30,000 x g, at 5°C, for 45 min. The resulting pellet constituted the membrane bound glucan synthetase preparation.

Solubilization of the Glucan Synthetases

The membrane pellet was weighed and resuspended in ice cold buffer (50 mM K•TES, pH 7.8, 1 mM $MgSO_4$, 1 mM DTT, 1 mM PMSF) containing 30 mM BTP•cholate (at a ratio of 1 to 2 ml per 100 mg wet weight of pellet). The particulate suspension was disrupted in a Dounce homogenizer (10 strokes), followed by brief sonication (15 to 30 sec.) in a bath type sonifier. The suspension was stirred for 15 min. at 4°C to facilitate solubilization, then centrifuged at 30,000 x g, at 5°C, for 45 min. The resulting supernatant (second 30,000 x g supernatant) constitutes the solubilized glucan synthetase preparation.

Glucan Synthetase Assay

Reaction mixtures contained 150 to 190 μl of enzyme sample and 0.1 mM UDP-^{14}C-glucose in a final volume of 0.2 ml in disposable glass test tubes (13 x 100 mm). The specific activity of UDP-glucose was either 5μCi/μmole or 10 μCi/μmole. Unless otherwise stated incubations were for 20 min. at 25°C. Reactions were terminated by the addition of 0.6 ml of unlabeled UDP-glucose (1.2 mM) followed immediately by the

addition of 1.4 ml of absolute ethanol. A small amount of carrier cellulose was added to each tube (ca. 0.5 mg) and the samples were heated at 70°C for 15 min. to facilitate solubilization of lipids. Subsequently the samples were stored at -20°C for $\geq$ 3 h to aid precipitation of glucan products.

Insoluble products were collected on glass fiber filters (2 cm. Whatman GFA or 934 AH, Whatman, Inc., Clifton, NJ, USA) on a 12 port Millipore vacuum filtration unit (Millipore Corporation, Bedford, MA, USA). The samples were washed with ice cold 70% ethanol (10 ml x 4) and chloroform methanol, (1:1, v./v.; 10 ml x 1). The filters were transferred to glass scintillation vials, dried, and radioactivity determined by scintillation counting in toluene-based cocktail (42 g PPO, 52.5 mg POPOP l^{-1}).

When the composition of glucan products was to be further analyzed, the reactions were carried out in 15 ml Corex tubes (Corning Glass, Corning, NJ, USA). The ethanol-precipitated products were collected by centrifugation (10 min, at 10,000 x g) and washed sequentially with 70% ethanol (5 ml x 4) and chloroform:methanol (1:1, 5 ml x 1).

Separation of Membrane Vesicles on ConA Sepharose

1. Preparation of concanavalin A Sepharose 6B. The affinity gel was prepared by the method of Gurd and Mahler (1974) with some modifications as follows (C. Caldwell, personal communication): ConA was dialysed overnight, at 4°C, against 100 mM sodium carbonate buffer, pH 8.4, with 200 mM α-methylmannoside. This buffer and all others used in subsequent steps contained: 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM

$CaCl_2$. Cyanogen bromide-activated Sepharose 6B was hydrated for 3 h in 1 mM HCl. The activated Sepharose was mixed with conA (ca. 25 mg lectin to 1 gram of gel) in 100 mM sodium carbonate buffer, pH 8.4, (10 ml buffer per gram gel), and incubated overnight at 4°C with gentle agitation. The coupling reaction was stopped by the addition of ethanolamine (final concentration 1 mM) and the mixture was incubated for one additional hour. The conA-Sepharose beads were washed exhaustively with: 1) 100 mM Na-carbonate buffer, pH 8.4, 100 mM NaCl, 750 ml 2) 100 mM Na•Acetate buffer, pH 4.0, 100 mM NaCl, 750 ml 3) water 750 ml and 4) 10 mM K•HEPES, pH 7.2, 5 mM $MgCl_2$, 1 mM Mn Cl_2, 10 mM $CaCl_2$, 400 mM sorbitol, 750 ml. The affinity gel was stored in this buffer (4) with 0.02% sodium azide at 4°C.

2. Preparation of membrane vesicles and affinity chromatography. Protoplasts (2-4 x 10^7) were disrupted by osmotic shock in 2 ml of ice cold buffer: 20 mM K•HEPES, pH 7.2, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 2 mM $MnCl_2$. After 2 min., the water potential of the solution was lowered by the dropwise addition of 2 ml of 800 mM sorbitol containing 2 mM DTT and 2 mM PMSF, with continuous mixing. An aliquot of the suspension was withdrawn to determine total glucan synthetase activity and the remainder was centrifuged at 10,000 x g for 15 min. The resulting membrane pellet was resuspended in a Dounce homogenizer (10 strokes), in 2 ml of column buffer containing: 10 mM K•HEPES, pH 7.2, 400 mM sorbitol, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM PMSF, 1 mM DTT. An aliquot was removed to determine glucan synthetase activity and the remainder (1.2 ml) was applied to a 2 ml conA Sepharose column. After 20 min, vesicles were eluted with 16 ml of column buffer, at a rate of 4 $ml \cdot hr^{-1}$. Subsequently, 1.2 ml of

buffer, supplemented with 200 mM α-methylmannoside, was applied to the column. After 10 min, the putative right-side-out plasma membrane vesicles were eluted with 10 ml of column buffer (with α-methylmannoside) at a rate of 6 $ml \cdot hr^{-1}$. Finally the buffer was supplemented with 30 mM BTP·cholate, (final pH 7.8) and residual protein and lipid were eluted from the column (10 ml of cholate buffer).

Fractions containing approximately 0.5 ml were collected and monitored for absorbance at 280 nm light. Fractions containing vesicles ($A_{280}>0.20$) were pooled (Fig. 3-2) and assayed for glucan synthetase activity.

Induction of Transmembrane Potentials Across Vesicles Derived From Soybean Protoplasts

Freshly isolated soybean protoplasts were disrupted by osmotic shock by resuspension in ice cold 10 mM BTP·SO_4, pH 7.4 (or BTP·MES, pH 7.2), 1 mM DTT, 3 mM $MgSO_4$. Membrane vesicles were formed by the gradual addition of six volumes of 60 mmolal PEG 4000 (in the same buffer) (Carpita and Delmer, in press). The vesicles were divided into aliquots (200 - 1500 μg protein per sample). Concentrated salt solutions and ionophores were added to the samples (see Fig. 3-4); these combinations of cations and anions with different relative permeability coefficients, are believed to induce transmembrane potentials across the vesicles (Bacic and Delmer, in press; Pressman, 1976).

UDP-glucose was supplied at 0.1 mM final concentration; the specific activity of the label and reaction times are indicated for each experiment.

Estimated Counting Efficiency of Radioactive Samples

Standard liquid scintillation quench curves, utilizing an external standard or H value (Beckman instruments), were used to determine counting efficiency.

The counting efficiency for samples of labeled polysaccharide on glass fiber filters cannot be determined by standard methods because the counting geometry for such samples is not known. For these samples, counting efficiency was estimated by spotting a standard amount of ^{14}C-glucose or ^{14}C-aspartate on the filters and then counting these samples in toluene-based scintillation cocktail. The estimated counting efficiency on Whatman GFA filters was 80%; that of Whatman 934AH filters was 63%.

CHAPTER 1. EARLY EVENTS IN CELL WALL REGENERATION

Results

A. Incorporation of ^{14}C-Glucose

In order to monitor the synthesis of polysaccharides during wall regeneration, protoplasts were cultured in the presence of ^{14}C-glucose at high specific activity and low concentration. Incorporation of label into total 80% ethanol-precipitable polymers is enhanced by decreasing the concentration of solute (sorbitol or PEG 4000), possibly because this increases the water potential of the culture medium (Table 1-1). However, at any given water potential the level of incorporation of [^{14}C]-glucose is influenced by the solute used (c.f. sorbitol with PEG-4000). The water potential and solute composition of the medium also affect the distribution of label incorporated into cellulose (see below) and other 80% ethanol-precipitable polymers. Other factors, including the general physiological state of the protoplasts and the density at which they are cultured may also influence the absolute magnitude of incorporation (c.f. the values for incorporation in PEG-based medium at -11 bars, Table 1-1, protoplast density 7 x 10^5 ml^{-1}, with that of 0 μMDCB, in Table 1-2, protoplast density 12 x 10^5 ml^{-1}; also see Rubin and Zallin, 1976). These factors varied between experiments; therefore, the composition of the culture medium and the protoplast density are noted for each experiment described in the following sections.

In order to conveniently monitor the synthesis of polysaccharides, individual samples generally contained at least 2 x 10^6 protoplasts. Thus, roughly 1 x 10^7 protoplasts are required for a small experiment. Under optimal conditions, 20 ml packed volume of cells yield 1 to 4 x 10^7 protoplasts. However, these yields are drastically reduced by any condition which adversely affects the growth rate of soybean cells: power failure, change in culture room temperature, and particularly the accumulation of amines in the water supply used to prepare culture medium. It is not feasible to scale up by increasing the amount of starting material; the cost of cell wall-degrading enzymes is prohibitive. Instead this problem was remedied by minor modifications in cultural procedures and rigorous precautions to maintain rapidly growing liquid suspension cultures. These procedures are described in more detail the Materials and Methods.

B. Cellulose Synthesis

1. Cellulose content of the cell wall of cultured soybean cells.

Cell walls were prepared from rapidly growing liquid suspension cells harvested one or two days after subculture. On a dry weight basis, the average cellulose content of one day old walls is 18% and that of two day old walls is 22%. These values are similar to the amount of cellulose found in the cell walls of cultured dicots (Darvill *et al.*, 1980; Albersheim, 1976).

Cellulose is a significant fraction of the cell wall; experiments described below examine cellulose synthesis as a component of cell wall regeneration.

Table 1-1 Protoplasts (2.1×10^6 in 3 ml) were resuspended in buffer:10mM Tris·MES, pH 5.5, 5mM $MgSO_4$, 0.5 mM $CaCl_2$, supplemented with sorbitol or PEG 4000, with a water potential as indicated [as determined from standard curves for appropriate dilutions of concentrated sorbitol (800 mmolal) and PEG 400 (100 mmolal)] in buffer. [^{14}C]-glucose was supplied to give a final concentration of 1mM, with a specific activity of 10 μCi/μmole. Incubations were carried out at room temperature for 1 h and were terminated by the addition of four volumes of ethanol.

Table 1-1

The effect of sorbitol and PEG concentration on the incorporation of glucose into cellulose and other ethanol-precipitable polymers by soybean protoplasts.

Osmotic Stabilizer	Water potential[a] (bars)	total non-cellulosic polymers[b]	average[c]	cellulose	average[c]	ratio (cellulose/total polymers)
	-19	3,430 3,410	3,420	67.0 65.9	64.4	.019
	-16	6,180 4,350	5,260	202 161	182	.034
sorbitol	-13.5	8,750 9,190	8,970	267 308	288	.032
	-11	14,000 11,700	12,800	372 302	337	.026
	-16	1,690 1,930	1,810	38.2 86.0	621	.034
PEG 4000	-13.5	2,300 2,150	2,220	115.0 90.2	103	.046
	-11	2,840 2,440	2,640	139 107	123	.046

[a]The concentrations of solute are expressed as the final water potential of the culture media.
[b]Values expressed as pmoles [^{14}C]-glucose incorporated per 10^6 protoplasts per h.
[c]of duplicates

2. Chemical evidence that cellulose is synthesized during the first hours of wall regeneration. Polymeric constituents of plant cells with the exception of cellulose, are degraded when treated at 100° for 30 to 60 min. with acetic acid, nitric acid and H_2O (8:2:1, v/v, AN reagent; Updegraff, 1969). Crystalline cellulose is believed to be resistant to AN reagent, although low molecular weight 4-β-glucan chains may be hydrolyzed under these conditions. The AN treatment was utilized in labeling studies with protoplasts to measure cellulose synthesis during regeneration. AN resistant material represents a very small fraction of the labeled material (Table 1-1). However, it was first necessary to confirm the validity of this procedure. The following analyses were performed on samples of labeled AN-resistant material derived from total 80% ethanol-precipitable polymers isolated from protoplasts which had been supplied [^{14}C]-glucose:

a) AN-resistant material was hydrolyzed and the resulting monosaccharides separated by descending paper chromatography (Saeman et al., 1963). More than 80% of the radioactivity in the hydrolysate in monosaccharides co-chromatographed with the glucose standard (Fig. 1-1).

b) Another sample was degraded overnight with a partially purified cellulose preparation from Streptomyces (as described in Heiniger and Delmer, 1977). Cellobiose and glucose in an approximate ratio of 3:1 were the predominant small radioactive saccharides released (Fig. 2). Cellobiose, a disaccharide, is the repeating unit of cellulose, thus confirming that the AN-resistant material contains cellulose. Free glucose is the product of an enzyme contaminant(s) in

Figure 1-1. Monosaccharide content of AN-resistant material.

AN-resistant material was prepared from total ethanol-precipitable polymers, derived from protoplasts which had been incubated with [^{14}C]-glucose for 18 h. The AN-resistant material was hydrolysed with sulfuric acid as described in Saemen *et al.* (1963). The resulting monosaccharides and appropriate standards were separated by descending paper chromatography, developed in ethyl acetate: pyridine: H_2O (8:2:1, v/v). The chromatogram was cut into 1 cm strips and the distribution of radioactivity determined by the scintillation counting. CB: cellobiose, gal: galactose, glu: glucose, ara: arabinose, xyl: xylose.

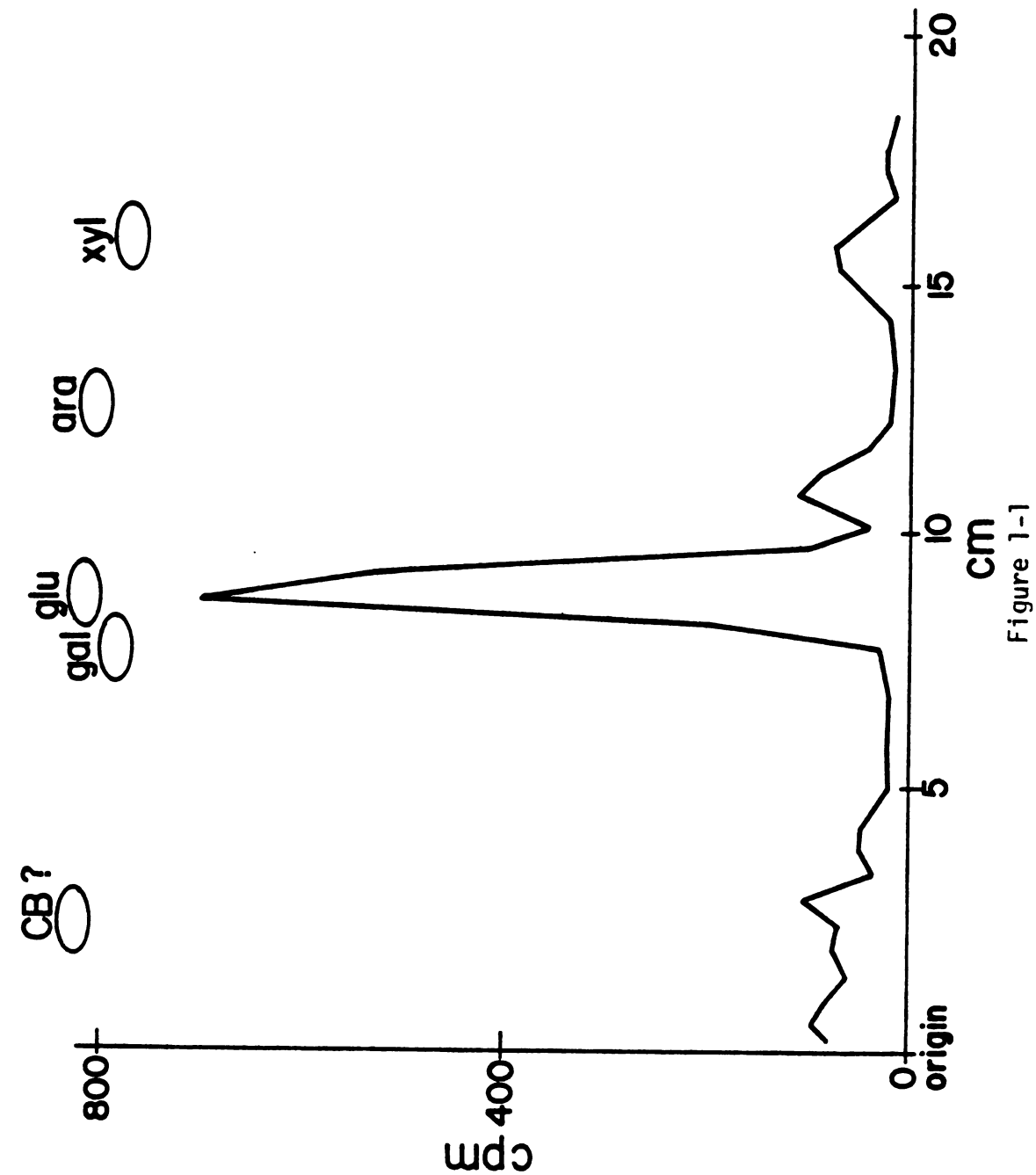


Figure 1-1

Figure 1-2. Saccharides released from AN-resistant material by cellulase digestion.

AN-resistant material was prepared from total ethanol-precipitable polymers, derived from protoplasts that had been incubated with [^{14}C]-glucose for 3 h. The AN-resistant material was digested with a cellulase preparation from Streptomyces [supplied by Dr. E.T. Reese; as described in Heiniger and Delmer (1977)]. The resulting saccharides and appropriate standards were separated by descending paper chromatography developed with n-propanol: ethyl acetate: H_2O (7:1:2, v/v). CB: cellobiose, LB: laminaribiose, glu: glucose.

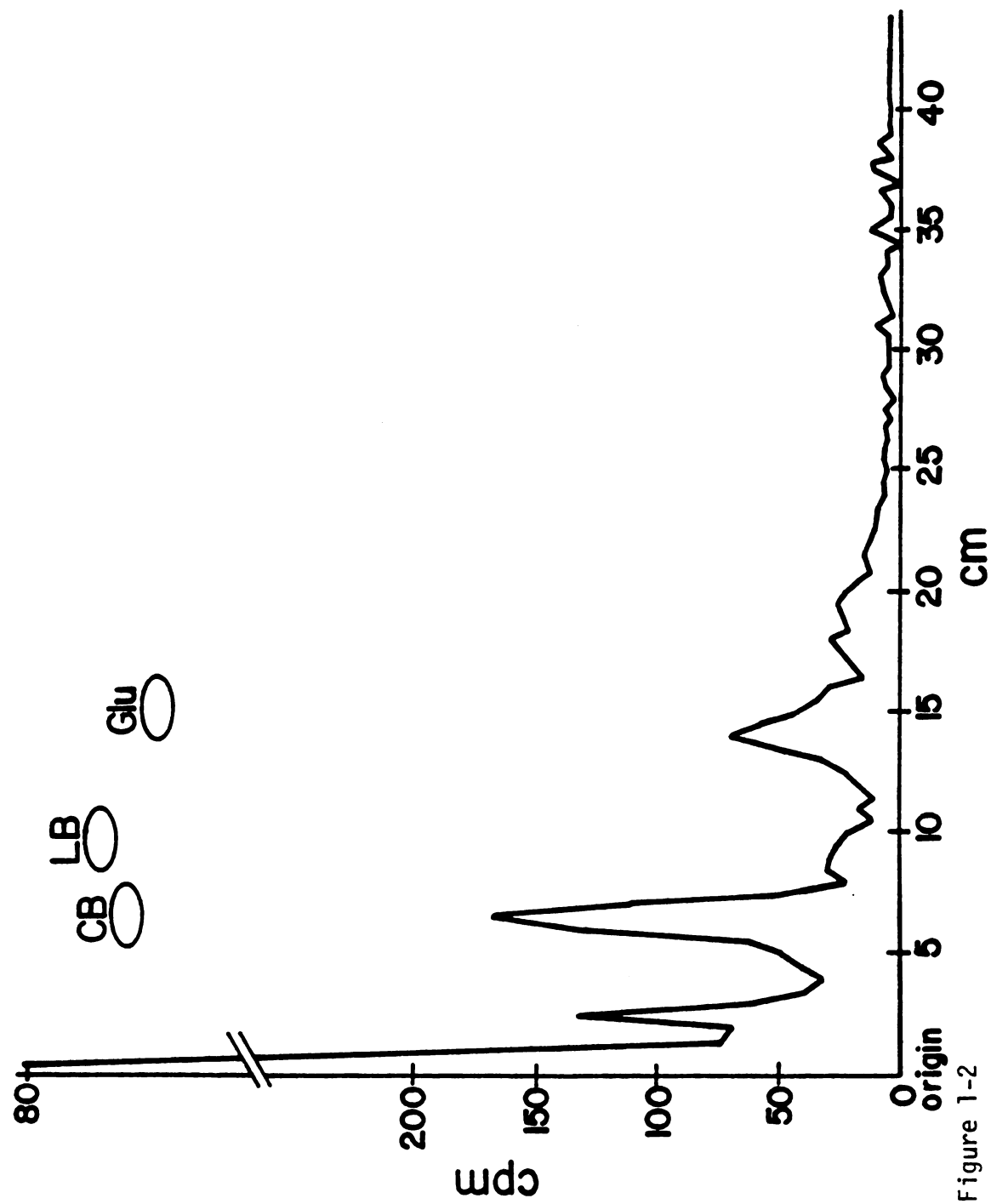


Figure 1-2

the Streptomyces preparation (i.e. a β-glucosidase acting on cellobiose) and may arise from cellulose or possibly other glucans.

AN-resistant material was subjected to methylation analyses (Fig. 1-3). Similar results were obtained whether samples were derived from protoplasts incubated with [^{14}C]-glucose for 1,3 or 21 hours. In most experiments, all of the radioactivity co-chromatographed with authentic 1,4,5 tri-O-acetyl-2,3,6-tri-O-methyl glucitol, i.e., 4-linked glucose. Small amounts of radioactivity were detected in derivatives with higher retention times; these can be attributed to undermethylated products of cellulose. In some experiments small amounts of radioactivity (less than 15%), co-chromatographed with 3-linked glucose (Fig. 1-3).

Thus, the labeled AN-resistant material is predominantly newly synthesized cellulose. Throughout the remaining text, the AN-resistant material will be referred to simply as cellulose.

3. Inhibition of cellulose synthesis by 2,6 dichlorobenzonitrile. DCB, at μmolar concentrations, has been shown to specifically inhibit cellulose synthesis in cotton fibers and other systems (Montezinos and Delmer, 1980). DCB (3.3 μM) also inhibits incorporation of ^{14}C-glucose into cellulose by soybean protoplasts, by 67% as compared to controls lacking DCB (Table 1-2). Higher concentrations of DCB also inhibit incorporation of glucose into total 80% ethanol precipitable polymers.

4. The time course of cellulose synthesis. Experiments were undertaken to ascertain whether or not there is a lag period in cellulose synthesis during wall regeneration, as has been suggested by Burgess and coworkers (1978). Protoplasts were isolated from rapidly

Figure 1-3. Methylation analysis of AN-resistant material

A. Gas liquid chromatogram of permethylated alditol acetates from a mixture of laminaribiose, cellobiose and cellulose. Peaks were identified by relative retention times. B. Elution profile of radioactivity of permethylated derivatives from acetic-nitric resistant material obtained from protoplasts which had been incubated for 1 h. C. Same as B, except the protoplasts were incubated for 3 h. D. Same as B, except the protoplasts were incubated for 21 h.

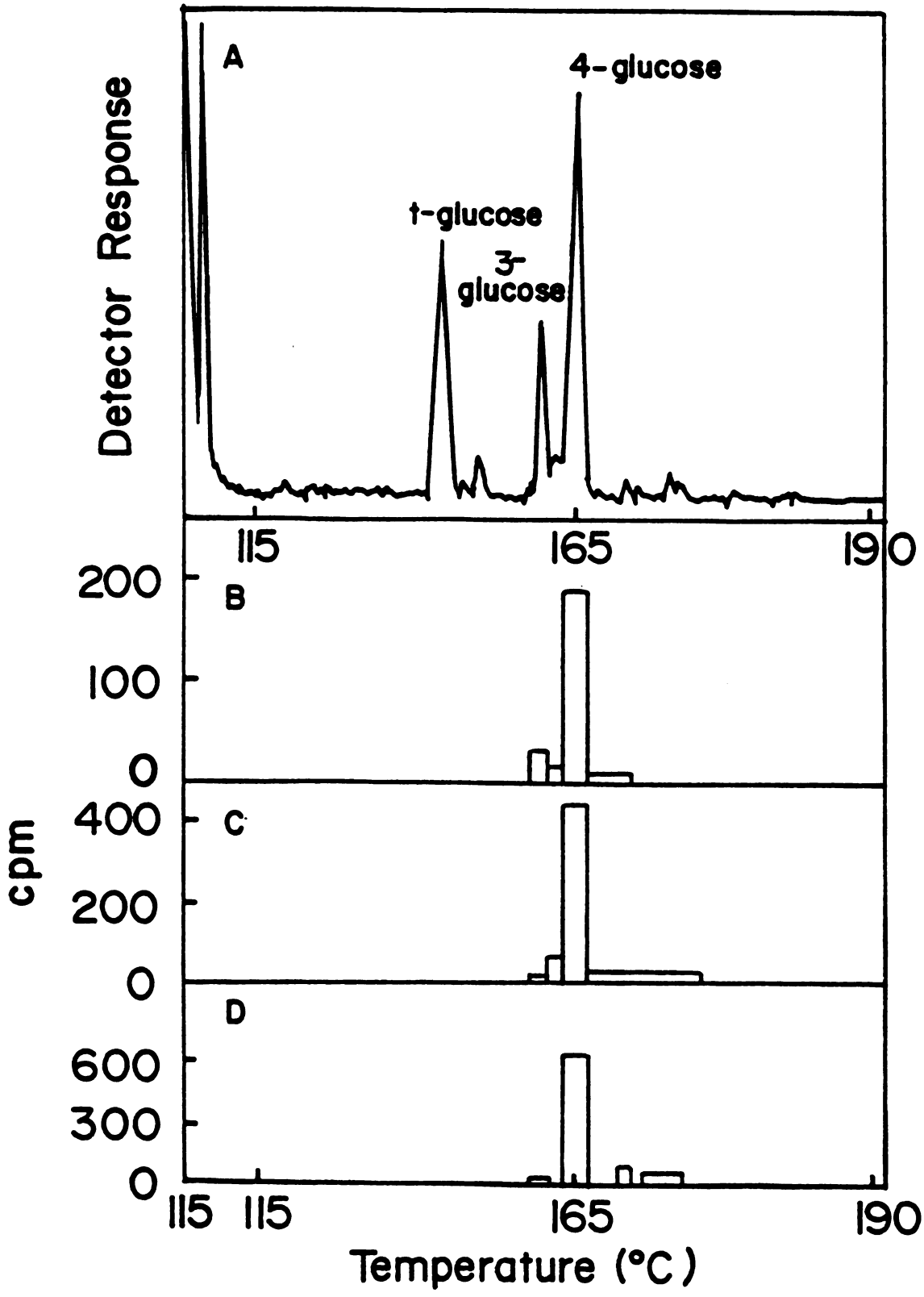

A
4-glucose
t-glucose
3-glucose
Detector Response
115
165
190
200
B
100
0
400
C
200
0
D
600
300
0
cpm
115
115
165
190
Temperature (°C)

Figure 1-3

Table 1-2 Protoplasts (3.6×10^6 in 3 ml) were resuspended in 10 mM Tris-MES, pH 5.5, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, supplemented with PEG 4000 to a final water potential equivalent to -11 bars (see table 1-1). DCB was supplied at the concentrations indicated. [^{14}C]-glucose was supplied to give a final concentration of 1 mM, with a specific activity of 10 μCi/μmole. Incubations were carried out at room temperature for 1 h, and terminated by the addition of four volumes of ethanol.

Table 1-2

The effect of DCB on the incorporation of glucose

[DCB] μM	total noncelloslic polymers [a]	average [b] polymers	cellulose[c]	average cellulose [b]	ratio cellulose / polymers	% inhibition of cellulose synthesis
	1,060		38.2			
0	933	906	35.9	34.1	0.038	-
	726		28.3			
3.3	961		13.6			
	978	844	11.6	11.4	0.014	67
	592		9.0			
10	432		5.1			
	728	633	5.9	6.4	0.010	81
	738		8.3			
33	407		4.2			
	832	674	9.2	7.0	0.010	80
	785		7.6			
100	757		8.1			
	360	563	3.3	5.7	0.010	85
	574		5.7			

[a] replicate samples
[b] average value for replicates
[c] pmoles [^{14}C]-glucose glucose incorporated in 1 h. per 1 x 10^6 protoplasts.

growing, 2 day old liquid suspension cultures of soybean cells. The protoplasts were resuspended in a complex culture medium which contained sorbitol as an osmotic protectant (modified from that described by Constabel, 1975; see Materials and Methods). The total water potential of the medium was approximately -19 bars. The final concentration of glucose in the medium was adjusted to try to provide: (i) an adequate carbon source to sustain development for the duration of each experiment and (ii) a specific activity of the supplied label sufficiently high to permit detection of newly synthesized polysaccharides after the tracer is diluted by the internal glucose pools in the protoplasts (as is indicated in the legends for Figs. 1-4, 5 and 6). Under these conditions, ^{14}C-glucose is incorporated into cellulose within 15 min and the amount of incorporation increases over 180 min (Fig. 1-4A). There is a concomitant increase in the ^{14}C content of total 80% ethanol-precipitable polymers (Fig. 1-4B). [^{14}C]-Glucose incorporation into cellulose continues to increase over 12 and 24 h (Fig. 1-5 and 6). The rate of incorporation increases in the 12 h time course and is roughly linear in the 24 h time course. These differences may reflect varying rates at which ^{14}C-glucose, at different supplied concentrations, accumulates in metabolic pools of the protoplast until it approaches a constant specific activity. These studies provide chemical evidence that cellulose is synthesized without an appreciable lag by soybean protoplasts during wall regeneration.

5. Deposition of fibrils on the surface of the plasma membrane. Protoplasts were fixed in glutaraldehyde immediately following the isolation procedure. Transmission electron microscopy of the protoplast indicates that the surface of the plasma membrane is devoid

Figure 1-4. Incorporation of [^{14}C]-glucose into cellulose and ethanol-precipitable polymers: short term.

A. cpm in cellulose B. cpm in noncellulosic polymers. Protoplasts (9×10^5 in 3 ml) were cultured for times indicated in wall regeneration medium supplemented with [^{14}C(U)]-glucose (2.5 μCi/μmole, final glucose concentration 0.75 mM). Each point is the average of duplicates.

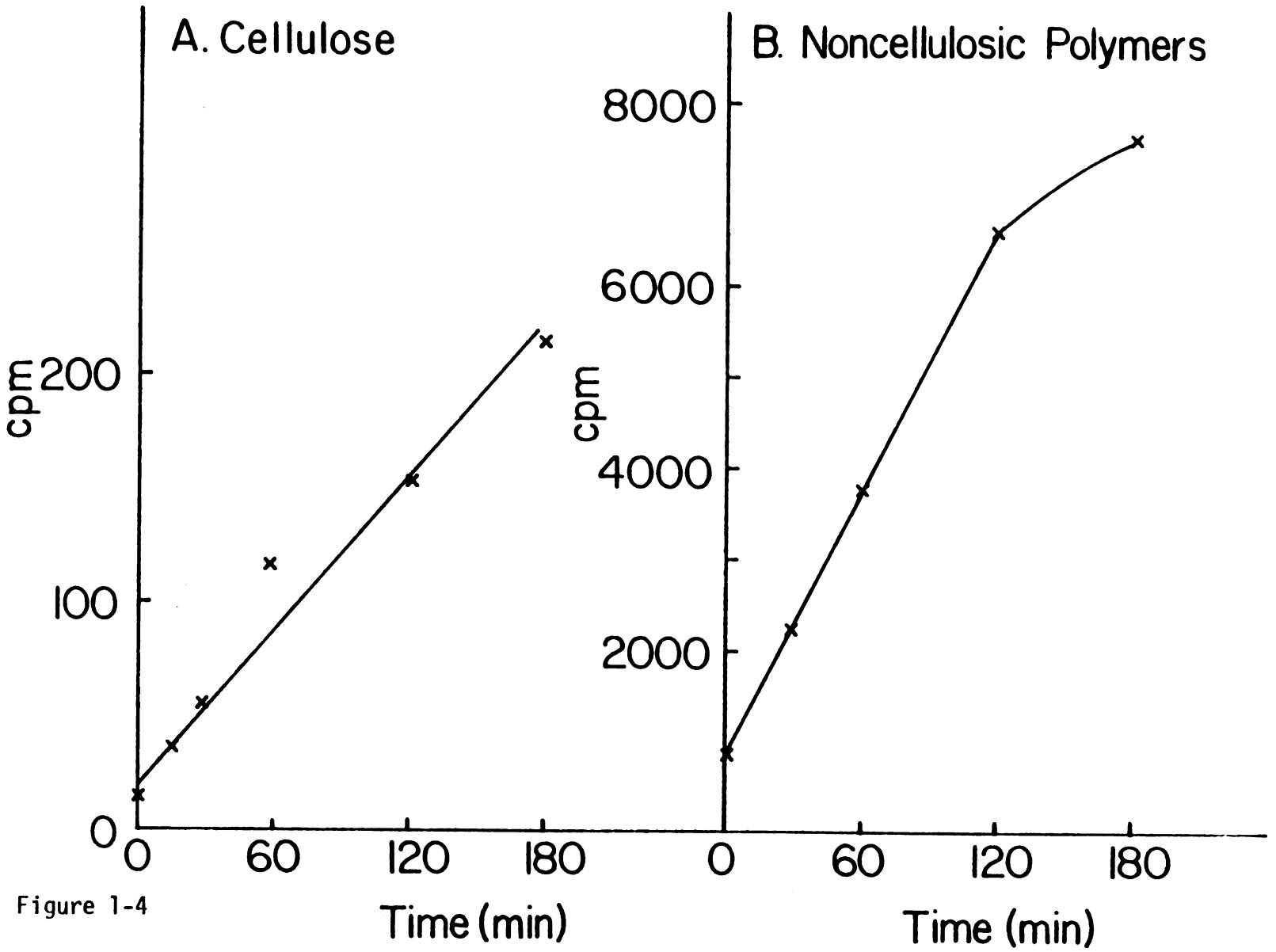

A. Cellulose
B. Noncellulosic Polymers
cpm
200
100
0
8000
6000
4000
2000
0
60
120
180
Time (min)

Figure 1-4

Figure 1-5. Incorporation of [^{14}C]-glucose into cellulose and other polymers: 12 h. time course.

A. cpm in cellulose B. cpm in noncellulosic polymers. Protoplasts (1.65 x 10^6 in 3 ml) were cultured for the times indicated in wall regeneration medium supplemented with [^{14}C(U)]-glucose (0.25 μCi/μmole, final glucose concentration 7.5 mM). Each point represents the average of duplicates.

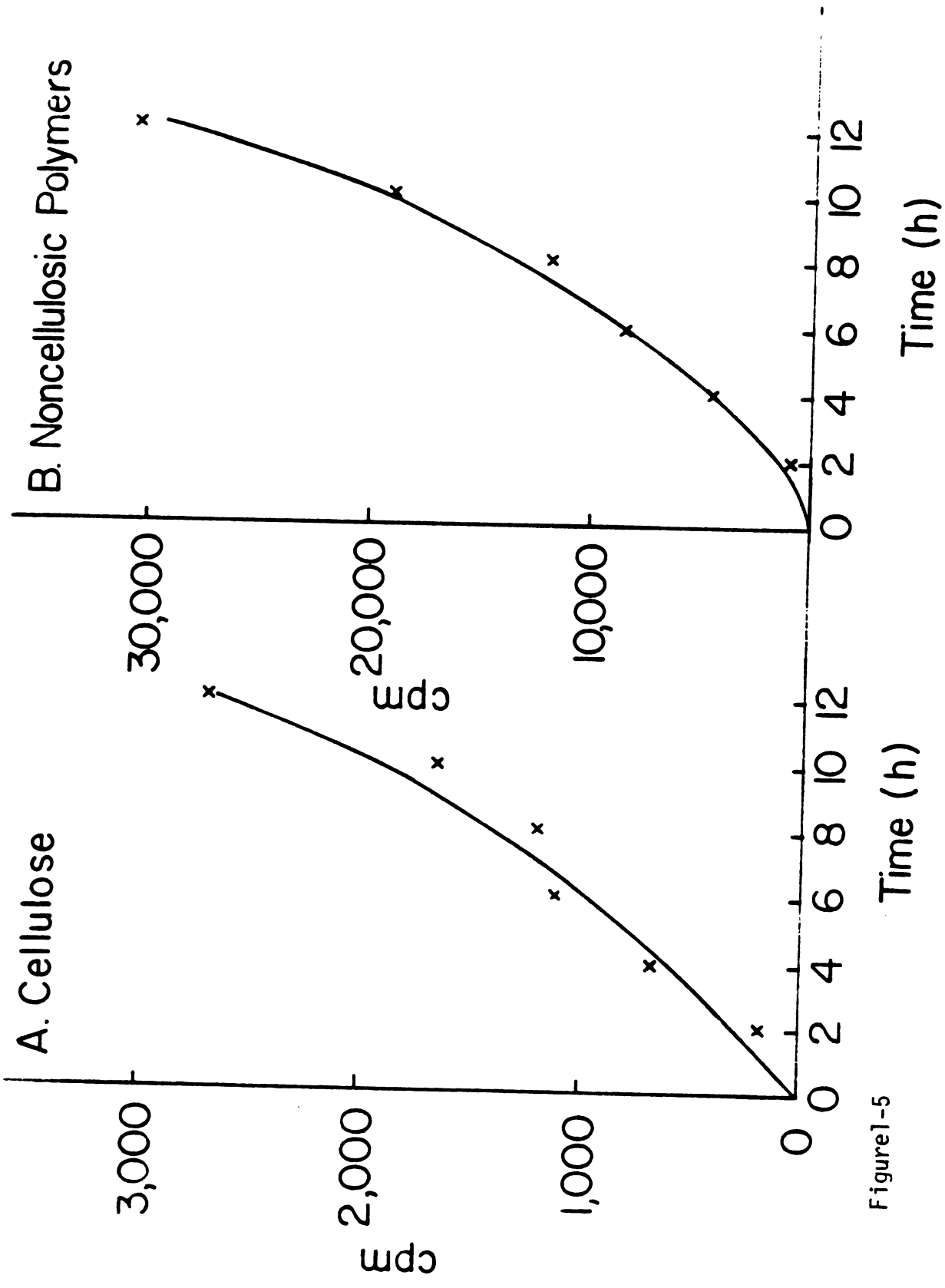

A. Cellulose
B. Noncellulosic Polymers
cpm
3,000
2,000
1,000
0
30,000
20,000
10,000
0 2 4 6 8 10 12
Time (h)

Figure1-5

Figure 1-6. Incorporation of [^{14}C]-glucose into cellulose: 24 h time course.

Protoplasts (4.6 x 10^6 in 5 ml) were cultured for the times indicated in wall regeneration medium supplemented with [^{14}C(U)]-glucose to give final concentration of 20 mM, at a specific activity of 0.1μCi/μmole.

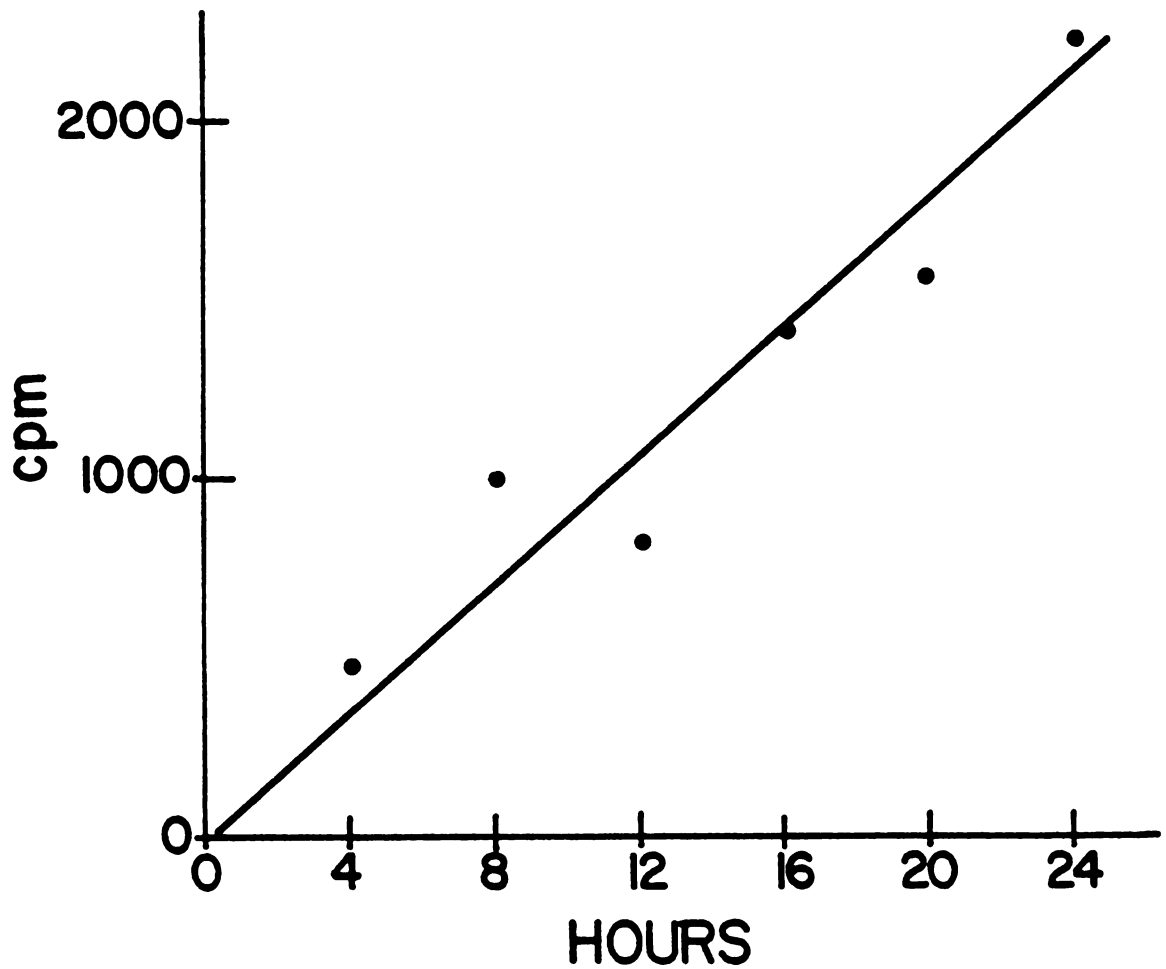


Figure 1-6

Figure 1-7. Time dependent deposition of fibrils on the surface of protoplasts. A. The surface of a protoplast fixed immediately after isolation (x28,000). B. The surface of a protoplast that was cultured for one hour (x31,000). C. Fibrils present on the surface of protoplasts that had been cultured for 3 h (x50,000).

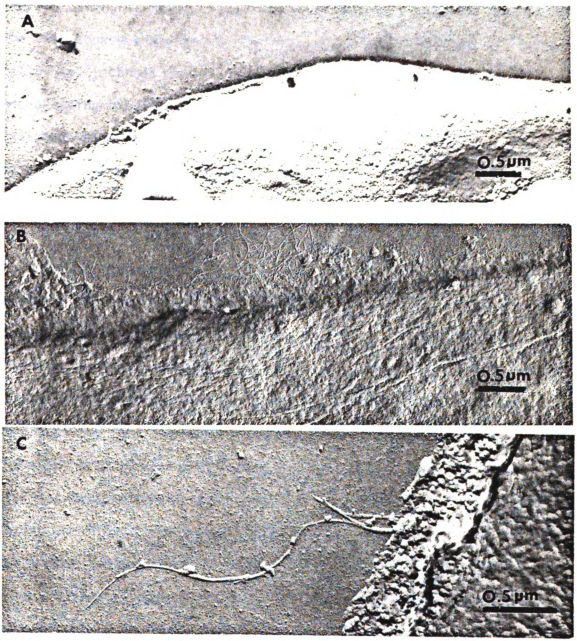


Figure 1-7

of wall material (Fig. 1-7A). Protoplasts fixed after one and three h in culture show numerous microfibrils on the surface of the plasma membrane (Figs. 1-7B and 7C). The distribution of fibrils on the surface of protoplasts is not uniform, possibly reflecting some loss of fibrils during fixation. These observations reinforce the results of the time course experiments (Section B4), i.e., that there is no lag in cellulose synthesis during wall regeneration. The absence of pre-existing fibrils on the protoplast surface (see Fig. 1-7A) indicates that the ^{14}C-glucose is incorporated into newly synthesized 4-β-glucan chains.

C. Synthesis of Other Cell Wall Components

For reasons discussed in the Introduction it is difficult to identify and isolate the cell wall during the initial stages of wall regeneration. Therefore ^{14}C-glucose labeled protoplasts were divided into two fractions, by collecting intact protoplasts (including that portion of the regenerating wall which adhered to the plasma membrane) and then isolating total ethanol-precipitable material from the spent protoplast culture medium (ECM). These fractions were utilized to examine the synthesis of non-cellulosic wall components during the first three of wall regeneration. For comparison, cell walls were prepared from one and two day old cultures of soybean cells grown in liquid suspension culture. Ethanol was added to the spent culture medium to precipitate polysaccharides and glycoproteins (secreted polymers).

1. Neutral sugars. The neutral sugar composition of soybean cells walls and extracellular polymers is presented in Table 1-3A.

This analysis gives the composite neutral sugar profile for all of the polysaccharides and glycoproteins in the fraction, except that of cellulose. The distribution of sugars is different between the two fractions: Rhamnose is present only in the cell wall, glucose accounts for the largest proportion of the neutral sugars in the wall. Xylose, followed by galactose are the predominant sugars of extracellular polymers.

Protoplasts were incubated in the presence of ^{14}C-glucose for 3 h and total 80% ethanol-precipitable polymers were isolated from the intact protoplasts and the spent culture medium. The distribution of label in the monosaccharide constituents of these polymers was determined by GLC as described in the Materials and Methods (Table 1-3B). During the 3 h of culture, ^{14}C-glucose is converted to various nucleotide pentoses and hexoses; these serve as substrates for the synthesis of polysaccharides and/or glycoproteins (the 80% ethanol-precipitable material). The distribution of label in the protoplast fraction is distinct from that of the ECM: arabinose, mannose, and glucose are the major components of the protoplast fraction, with smaller amounts of galactose, xylose, and rhamnose + fucose. Arabinose, galactose and glucose are the major components of the ECM, with smaller amounts of mannose, xylose and rhamnose + fucose. The distribution of ^{14}C label in newly-synthesized polymers in both the protoplast and ECM fractions also differs from the overall composition of the cell walls and excreted polymers of liquid suspension cells (c.f. Table 1-3A and 3B). These experiments demonstrate that protoplasts synthesize a variety of glycoconjugates during the first three h of wall regeneration.

Table 1-3-A Neutral sugar compositions of the cell walls and secreted polysaccharides were determined as described in the Materials and Methods. The alditol acetates were identified by comparison to the retention times of derivatives of standard sugar mixtures. Values are expressed mole% composition, after correction for differences in recovery for each sugar (based on derivatives of standard sugar mixtures, as described in Meinert and Delmer, 1977).

3-B The neutral sugar composition of newly-synthesized polysaccharides derived from protoplasts. Protoplasts were incubated for 3 h. with ^{14}C-glucose at a final concentration of 1 mM to give a specific activity of 10μCi/μmole. Samples were prepared as described in the Materials and Methods. Chromatography was performed on duplicate samples and expressed as the average mole % composition, after correction for differences in recovery between various sugars.

Table 1-3

Neutral sugar compositions

A. Of cell walls and secreted polymers from soybean cell cultures (mole %).

	cell walls		secreted polymers	
	1 day	2 day	1 day	2 day
rhamnose	9	9	-	-
fucose	4	3	2	2
arabinose	19	18	13	14
xylose	20	21	26	32
mannose	4	3	9	7
galactose	19	20	27	26
glucose	27	23	22	19

B. Distribution of radioactivity in the neutral sugar residues of newly synthesized-polymers from protoplasts.

	Protoplast fraction	ECM
rhamnose + fucose	7	8
arabinose	22	21
xylose	10	13
mannose	24	14
galactose	10	21
glucose	27	23

2. Methylation analysis. GLC of permethylated alditol acetates from cell walls of two day old liquid-suspension cells and the secreted polymers from the spent medium illustrate the spectrum of sugar linkages present (Figs. 1-8A and 1-8B). These derivatives are typical of the cell wall and secreted polymers synthesized by dicots. For example, i) Terminal-arabinosyl and 3,6-linked galactosyl residues are characteristic of soluble arabinogalactan hydroxyproline-rich proteins (Lamport, 1977; Clarke et al., 1979). These are predominant in the secreted polymers. ii) 4-linked glycosyl residues characteristic of cellulose and starch are prominent in the profile of the derivatized cell walls. iii) 4-6 linked glucosyl residues are characteristic of hemicellulosic polysaccharides. Uronic acid residues, characteristic of pectic polysaccharides are not identifed by this type of analysis.

Labeled polysaccharide-containing polymers from protoplasts and the ECM were subjected to similar methylation analyses as described in the Materials and Methods. Due to lack of suitable standards, these data have not been corrected for differences in recovery between individual derivatives. The elution profile of radioactivity indicates that a variety of pentosyl and hexosyl residues, i.e. sugar linkages, are present in the newly-synthesized polymers (Figs. 1-8B and 8D). These include: i) t-Arabinosyl and 3,6-linked galactosyl residues in the polymers derived from protoplasts may represent the synthesis of arabinogalactan proteins, destined for secretion into the extracellular medium. These residues are heavily labeled in the ECM fraction. Alternatively, all or part of the t-arabinosyl in the protoplast fraction may represent araban, a pectic polysaccharide commonly found in dicot cell walls. ii) t-Xylosyl and 4,6- linked glucosyl residues

Figure 1-8. Methylation analyses of soybean polysaccharides

A. GLC elution profile of permethylated derivatives of soybean cell walls. Cell walls were isolated from 2 day old suspension culture cells and derivatized as described in Materials and Methods. Peaks were identified by relative retention times, comparison with standards, and/or mass spectral analysis (data not shown). Peak numbers refer to the deduced linkage of glycosyl residues: 1:t-arabinosyl; 2:t-xylosyl; 3:1,5-arabinosyl; 4:t-galactosyl; 5:2- and 4-xylosyl; 6:3-glucosyl; 7:4-hexosyl; 8:6-galactosyl; 9:4,6-galactosyl 10:4,6-glucosyl + plasticizer; 11:3,6-galactosyl 12:inositol (internal standard). B. Elution profile of radioactivity of permethylated derivatives obtained from the whole protoplast fraction. Protoplasts (9.0×10^6 in 5 ml), were cultured for 3 h in wall regeneration medium supplemented with [^{14}C(U)]-glucose (10 μCi/μmole; final glucose concentration 1 mM). Protoplasts were collected by centrifugation and methylated. Chromatography was performed on duplicate samples, and fractions were identified by co-elution with unlabeled standards. The profile is typical of several similar experiments. C. GLC elution profile of permethylated derivatives of polymers secreted into the culture medium of cell suspensions. The material was isolated, derivatized and chromatographed as described in Materials and Methods. Peak identifications are the same in A. D. Elution profile of radioactivity in derivatives prepared from the ECM isolated from wall regeneration medium after 3 h of protoplast culture.

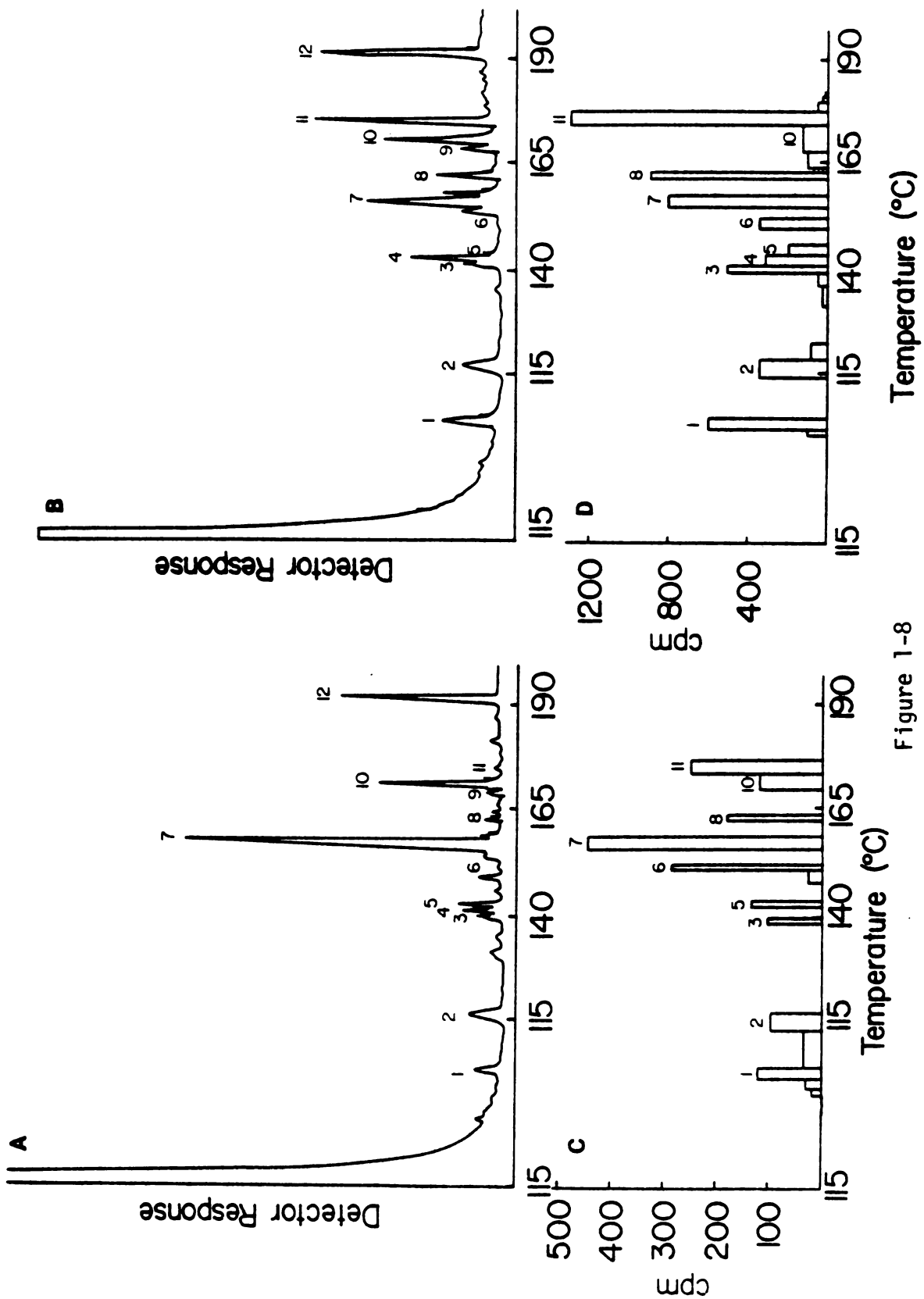

A
B
C
D
Detector Response
Detector Response
cpm
cpm
Temperature (°C)
Temperature (°C)
115
140
165
190
100
200
300
400
500
400
800
1200

Figure 1-8

in protoplast polymers may represent xyloglucan, a hemicellulosic polymer found in dicot cell walls (Albersheim, 1976; Darvill et al., 1980). iii) t-galactosyl, 4- and 6-linked Galactosyl residues may also indicate the presence of galactans (Darvill et al., 1980).

The cumulative proportion of radioactivity in each type of glycosyl residue (i.e., t-glucosyl plus 4-linked and 4,6-linked glycosyl residues) is generally consistent with the neutral sugar analyses (Section C-1). Again, the labeling patterns of polymers in the protoplast and ECM fraction are distinct and differ from the composition of the cell walls and secreted polymers. These data are insufficient to permit definitive identification of most cell wall and extracellular polysaccharides synthesized by protoplasts during the initial stges of wall regeneration: this requires separation of individual polymers and subsequent detailed analyses for each. Nonetheless, these results indicate that a wide range of complex polymers, including pectins and hemicelluloses, are synthesized during the first three hours of wall regeneration and these bear some similarities to the cell wall constituents and secreted polymers from liquid-suspension cells.

3. Synthesis of 3-β-glucan. No appreciable quantity of 3-linked glucan is detected by methylation analysis in the cell walls or excreted polymers of liquid-suspension soybean cells (Figs. 1-8A and 1-8B). However, protoplasts, fed ^{14}C-glucose, synthesize significant amounts of 3-linked glucan (Figs. 1-8B and 8D). This is most probably 3-β-glucan, a polymer whose synthesis is induced by wounding and which is also present in specialized tissues: e.g., pollen tubes, and cotton fibers (see Chapter 3, Darvill et al., 1980).

Discussion

The Early Events in Cell Wall Regeneration. Soybean protoplasts were cultured in the presence of ^{14}C-glucose to examine the synthesis of polysaccharides and glycoproteins during the initial stages of wall regeneration. The relative amount of glucose incorporated into polymeric material is influenced by the solute concentration and composition of the culture medium (Table 1-1). Cell elongation in whole plants is slowed by a drop in leaf water potential from -2 bars to -12 bars (for a review see Hsiao 1973). However it is difficult to acess whether these represent the same phenomenon, that is an inhibition of the synthesis of cell wall material, because protoplasts require a culture medium with a maximum water potential of at least -12 bars.

Cellulose Synthesis. Microfibril deposition on the surface of the plasma membrane is characteristic of the early stages of wall regeneration (Willison, 1976). In most previous studies these fibrils have been equated with crystalline cellulose, without rigorous characterization. Protoplasts, isolated from rapidly growing suspension cultures of soybean cells, were incubated with ^{14}C-glucose and this label was incorporated into ethanol-precipitable polymers. A very small proportion (<5%) of these newly-synthesized polymers are resistant to hydrolysis with AN reagent; this labeled AN-resistant material is predominantly newly-synthesized cellulose (Figs. 1-1, 2 and 3). Additional experiments demonstrate that soybean protoplasts begin to synthesize cellulose within 15 min after the protoplasts are transferred to culture medium (Fig. 1-4).

Cytological observations indicate that the surface of protoplasts, fixed in glutaraldehyde immediately after isolation, is devoid of microfibrils; after one or three hours of culture, numerous fibrils are visible (Figs. 1-7). The initial absence of microfibrils suggests that the labeled glucose is incorporated into new cellulose chains and not preexisting microfibrils. These findings indicate that the cellulose synthesizing machinery retains its capacity to synthesize cellulose during the isolation of protoplasts from these rapidly growing soybean cells. In contrast, Burgess and coworkers (1978) observed a "lag" period of eight h before protoplasts derived from leaf tissue of Nicotiana begin to synthesize microfibrils. They suggested that this "lag" period is a common feature of cell wall regeneration. It is more probable that this lag period is a function of cell type and perhaps cultural conditions: Protoplasts from slowly growing tissue or mesophyll cells may have to synthesize the cellulose synthetase de novo, prior to microfibril deposition. Low water potential and high salt concentration also inhibit wall regeneration (Herth and Meyer, 1978; Willison and Klein, 1981).

Other cell wall components. There have been a limited number of studies which partially characterized, at a biochemical level, the noncellulosic components of the regenerating cell wall (see Willison and Klein, 1981). Hanke and Northcote (1974) reported that during the initial 20 h of wall regeneration, soybean protoplasts utilized exogenous glucose primarily for the synthesis of starch, protein and cellulose. They suggested that little other wall material is synthesized during the first day of wall regeneration. In contrast, Asamizu

and Nishi (1980) reported that over the same time scale, carrot protoplasts synthesize pectins, hemicelluloses and cellulose.

In this study, soybean protoplasts, incubated for three h with tracer levels of glucose synthesize a variety of polysaccharide containing polymers (Table 3B, Figs. 8B and 8D).

The newly-synthesized polymers contain a variety of glycosyl residues (Table 1-3B and Figs. 1-8B and 8D). Of the neutral sugar residues in these polymers (excluding cellulose) only 30% of label in the protoplast fraction is found in glucosyl residues; the distribution of glucosyl residues in these polymers suggest that starch is not a major component of these newly-synthesized polymers. This non-cellulosic glucose may be derived from 3-β-glucan, xyloglucan and other cell wall polysaccharides. Alternatively, part of the glucosyl residues may represent cytoplasmic polysaccharides or polysaccharides synthesized within the cell but destined for secretion to the wall or culture medium. Substantial amounts of mannose in these samples represent a distinct difference in the composition of this soybean wall material as compared to that of the primary cell wall of other cultured cells which have been studied in detail (Darvill et al., 1980). Other t-pentosyl and glycosyl residues in the newly synthesized polysaccharides are characteristic of a variety of pectic and hemicellulosic polysaccharides and glycoproteins similar to those of the wall and secreted polymers of liquid-suspension cells. Additional work would be required to identify which of these newly synthesized polymers form the regenerating wall or how they are assembled and how their synthesis is regulated as compared to that of the cell wall

of suspension grown cells.

In contrast significant amounts of 3-β-glucan are synthesized by protoplasts during wall regeneration (Figs. 1-8B and 8D). Little or no 3-β-glucan is present in the secreted material or cell walls of suspension grown cells. This synthesis of 3-β-glucan may represent a wound phenomenon or may be part of a transient phase of wall regeneration.

The 3-β-glucan synthetase is believed to be a marker enzyme for the plasma membrane (Leonard and Hodges, 1980; Quail, 1979). In many tissues the activity of the enzyme is enhanced by wounding (Brett, 1978) or environmental stress (Smith and McCully, 1977); in other instances the enzyme is under developmental control (Maltby et al., 1979). The glucan-synthesizing enzymes of soybean cells are examined in the following chapters. Attention is focused mainly on the 3-β-glucan synthetase because of its importance in cell wall regeneration. In addition, the information accumulated about this enzyme suggests its isolation and characterization may provide a good starting point for understanding the enzymology of cell wall biosynthesis.

CHAPTER 2. SOLUBILIZATION OF β-GLUCAN SYNTHETASES

Results

The 3-β-glucan synthetase and cellulose synthetase are believed to be components of the plasma membrane (Leonard and Hodges, 1980; Quail, 1979). The synthesis of other non-cellulosic cell wall polysaccharides is believed to be associated with endomembrane systems (Ray et al., 1976). Thus far, none of these enzymes has been purified or fully characterized. As an initial step towards the isolation of the 3-β-glucan synthetase, experiments were undertaken to prepare a solubilized form of the enzyme and to define conditions to maintain enzyme activity for subsequent purification procedures.

A. Influence of pH and Sorbitol Concentration on Glucan Synthetase Activity in Crude Membrane Preparations.

When β-glucan synthesis, from UDP-glucose, is assayed in a buffered medium (pH 5.5) lacking an osmotic stabilizer, only very low amounts of product are detected (Fig. 2-1). Glucan synthesis is enhanced by including 400 mM sorbitol in the assay buffer at pH 5.5. If the pH is raised to 7.2 (by substituting K•TES for K•MES), in the presence of sorbitol, there is a further substantial increase in enzyme activity. Unless otherwise stated, subsequent assays for β-glucan synthetase activity utilized buffers at pH 7.2 and contained 400mM sorbitol.

Figure 2-1. Effect of pH and sorbitol concentration on glucan synthetase activity from a crude membrane fraction.

11 g of 2 day old cells were disrupted in 50 mM K•TES pH 7.2, 1 mM DTT, 10 mM $MgCl_2$; 400 mM sorbitol. The brei was filtered through two layers of nitex cloth (40 µm, average pore size) and the filtrate was centrifuged at 1,000 x g for 5 min to remove cell wall fragments. The supernatant was divided into 4 aliquots, these were centrifuged at 25,000 x g for 30 min. The resulting pellets (crude membrane preparation) were resuspended in 2 ml of one of the follow buffers: i) 50 mM K•MES, pH 5.5, 1 mM DTT, 5 mM $MgCl_2$; ii) same as (i) + 400 mM sorbitol; iii) 50 mM K•TES, pH 7.2, 1 mM DTT, 5 mM $MgCL_2$; or iv) same as (iii) + 400 mM sorbitol. Each reaction contained approximated 92 µg of protein.

UDP-glucose was supplied to give a final concentration of 0.1 mM, with a specific activity of 5 µCi/µmole and the reactions were incubated at 25°C. At intervals indicated, duplicate aliquots of 0.2 ml were removed and pipeted directly into 0.8 ml of 100% ethanol. Total glucan synthesis was determined as described in the Material and Methods section. Total radioactivity in individual samples ranged from 1,500 to 55,000 cpm.

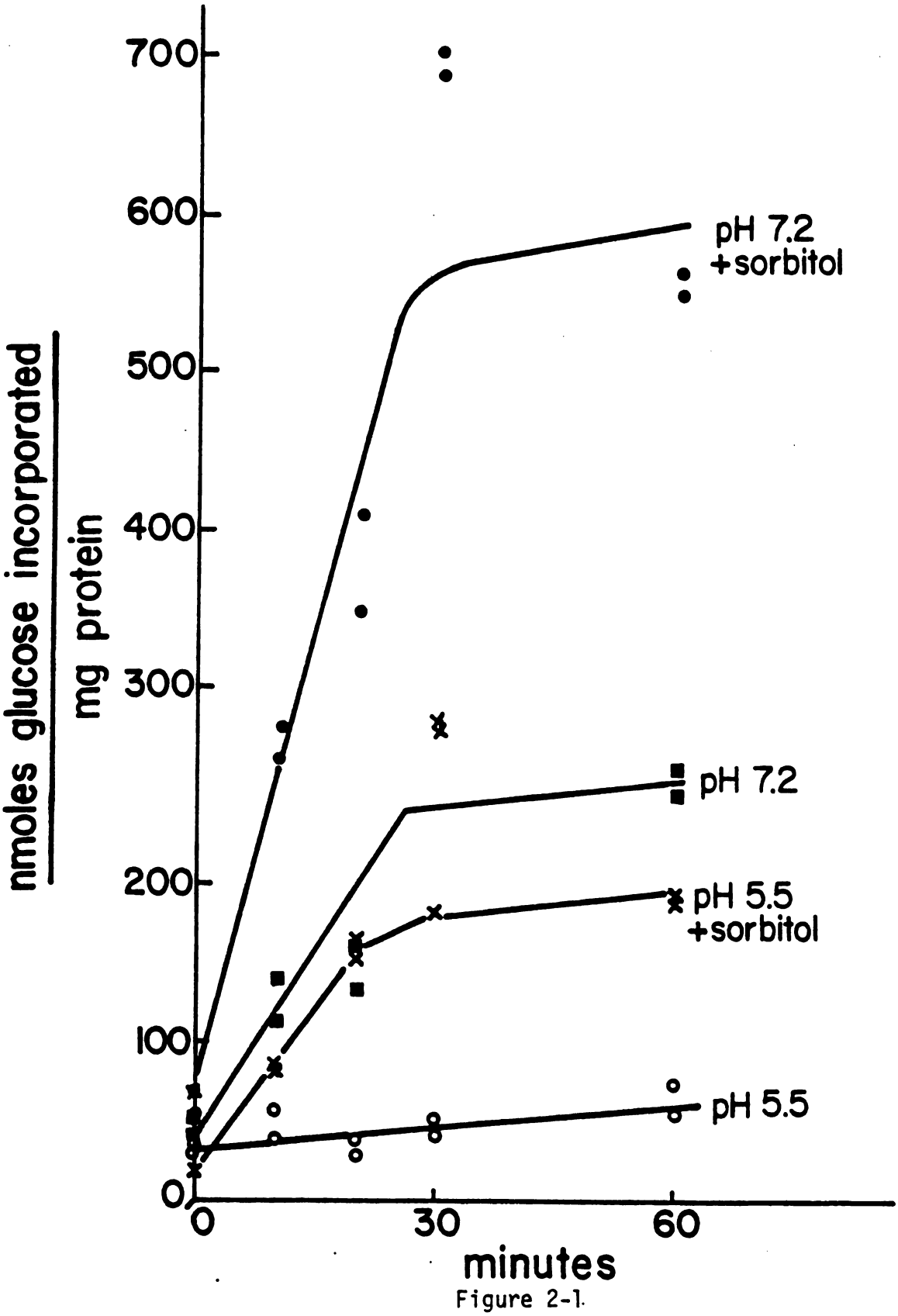

700
600
500
400
300
200
100
0
nmoles glucose incorporated
mg protein
pH 7.2
+sorbitol
pH 7.2
pH 5.5
+sorbitol
pH 5.5
0
30
60
minutes

Figure 2-1.

These crude membrane fractions probably contain plasma membrane and some endomembrane vesicles, both of which are likely sites of glucan synthesis (Leonard and Hodges, 1980; Ray, 1980; Quail, 1979). Thus, although the assay conditions would probably have favored a plasma membane localized 3-β-glucan synthetase (glucan synthetase II; high UDP-glucose), the measured glucan synthetase activity may represent the products of more than one enzyme (Quail, 1979).

B. Detergent Solubilization of Glucan Synthetases

Several detergents were screened for their effect on enzyme activity and their potential to solubilize the glucan synthetases. These included lipids, neutral detergents and one ionic detergent, cholate (Fig. 2-2). A crude membrane pellet was resuspended and stirred in buffer plus detergent; each detergent critical micelle concentration, and/or two to five times its critical micelle concentration. Ater 15 min. an aliquot was removed to measure total glucan synthetase activity (first 30,000 x g pellet). Half of the remaining sample was centrifuged at 30,000 x g and enzyme activity was measured in the resulting supernatant (solubilized fraction) and pellet (second 30,000 x g pellet, which was resuspended in assay buffer without detergent). The remainder of the sample was treated with detergent for an additional 130 min. and then centrifuged as described above.

Phosphatidyl choline, noniondet P-40 and octyl-β-glucoside treatments do not solubilize appreciable amounts of glucan synthetase (Fig. 2-2). Phosphatidyl choline and noniondet P-40 both slightly

Figure 2-2. Influence of detergents on glucan synthetase activity and distribution in membrane preparations.

21 g of 2 and 4 day old cells were disrupted as described in Figure 2-1. The crude membrane preparation was resuspended in 16 ml of buffer, 50 mM K·TES, pH 7.2, 1 mM DTT, 5 mM $MgCl_2$, 400 mM sorbitol, (at 0.58 mg of protein per ml) and divided into eight, 2 ml samples. Detergent was added at the concentrations indicated in the Figure; each sample was briefly sonicated in a bath type sonifier. After detergent treatment and centrifugation, the second membrane pellet was resuspended in buffer without detergent. Enzyme activity was assayed in duplicate, in 0.2 ml aliquots of each fraction, with 0.1 mM UDP-glucose (at 5 μCi/μmole); reactions were incubated for 20 min. Radioactivity in the labeled products ranged from 1,500 to 65,000 cpm. The average total activity for each fraction is indicated.

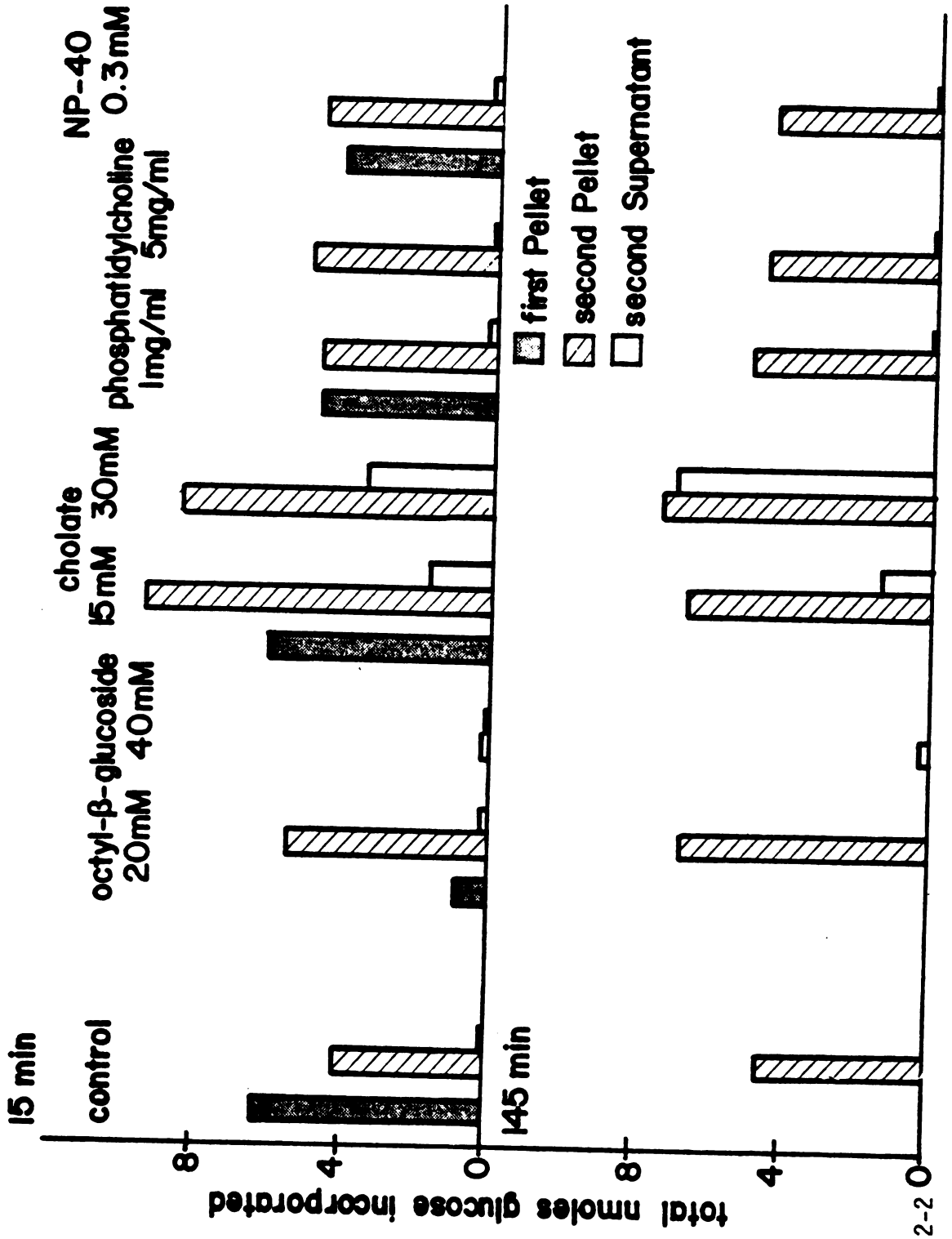

15 min
control
octyl-β-glucoside 20mM 40mM
cholate 15mM 30mM
phosphatidylcholine 1mg/ml 5mg/ml
NP-40 0.3mM
145 min
first Pellet
second Pellet
second Supernatant
total nmoles glucose incorporated
8
4
0
8
4
0

Figure 2-2

inhibit glucan synthesis (Fig. 2-2); Tween-20, digitonin and Triton X 100, other neutral detergents, also slightly inhibit enzyme activity (data not shown). This inhibition of glucan synthesis may result from disruption of a favored conformational state of the enzyme within the membrane (Helenius and Simons, 1975). Octyl-β-glucoside severely inhibits glucan synthesis in the first 30,000 x g pellet fraction; the β-glucosyl moiety may act as a competitive inhibitor as it bears some similarity to the substrate UDP-glucose. However when some of the detergent has been removed by centrifugation (e.g. after the initial treatment with 20 mM octyl β-glucoside) the second pellet fractions (assayed in buffer without detergent) exhibit at least as much glucan synthetase activity as the control samples (Fig. 2-2). Glucan synthesis is inhibited in all fractions after treatment with 40mM octyl-β-glucoside.

(Na) cholate, an ionic detergent, at pH 7.2 stimulates glucan synthesis (Fig. 2-2). This may represent increased substrate accessibility or altered enzyme conformation (Helemius and Simons 1975). Cholate (15mM) treatment solubilizes a small proportion of the total enzyme activity in 15 min. Solubilization is enhanced when the concentration of cholate is raised to 30mM and the detergent treatment is extended to 145 min; approximately 50% of the total glucan synthetase activity is solubilized.

By raising the pH of the buffer to 7.8 (the pK of cholic acid), approximately 80% of the glucan synthetase activity is solubilized after 15 min. of treatment with 30 mM K cholate (Fig. 2-3). (In this experiment, the second pellet fractions were assayed in the presence of (K) cholate). To optimize conditions for solubilization, the ionic

Figure 2-3. Effects of salt concentration on cholate-mediated solubilization.

9 g of 3 day old soybean cells were disrupted as described in Figure 2-1. The crude membrane preparation was resuspended in 8 ml of buffer containing 50 mM K·TES, pH 7.8, 1 mM DTT, 30 mM K·cholate, 5 mM $MgCl_2$. The membrane suspension was divided into 4 samples and KCl was added to each at the concentrations indicated. After the samples were separated into soluble fractions and a second membrane pellet; this pellet was resuspended in buffer with salt. Enzyme activity was determined in 0.2 ml aliquots of each fraction. UDP-glucose was supplied at a final concentration of 0.1 mM, (10 μCi/μmole); reactions were incubated for 20 mins. Radioactivity in the labeled ethanol-precipitable products ranged from 1,000 to 21,000 cpm after background (30 cpm) was subtracted.

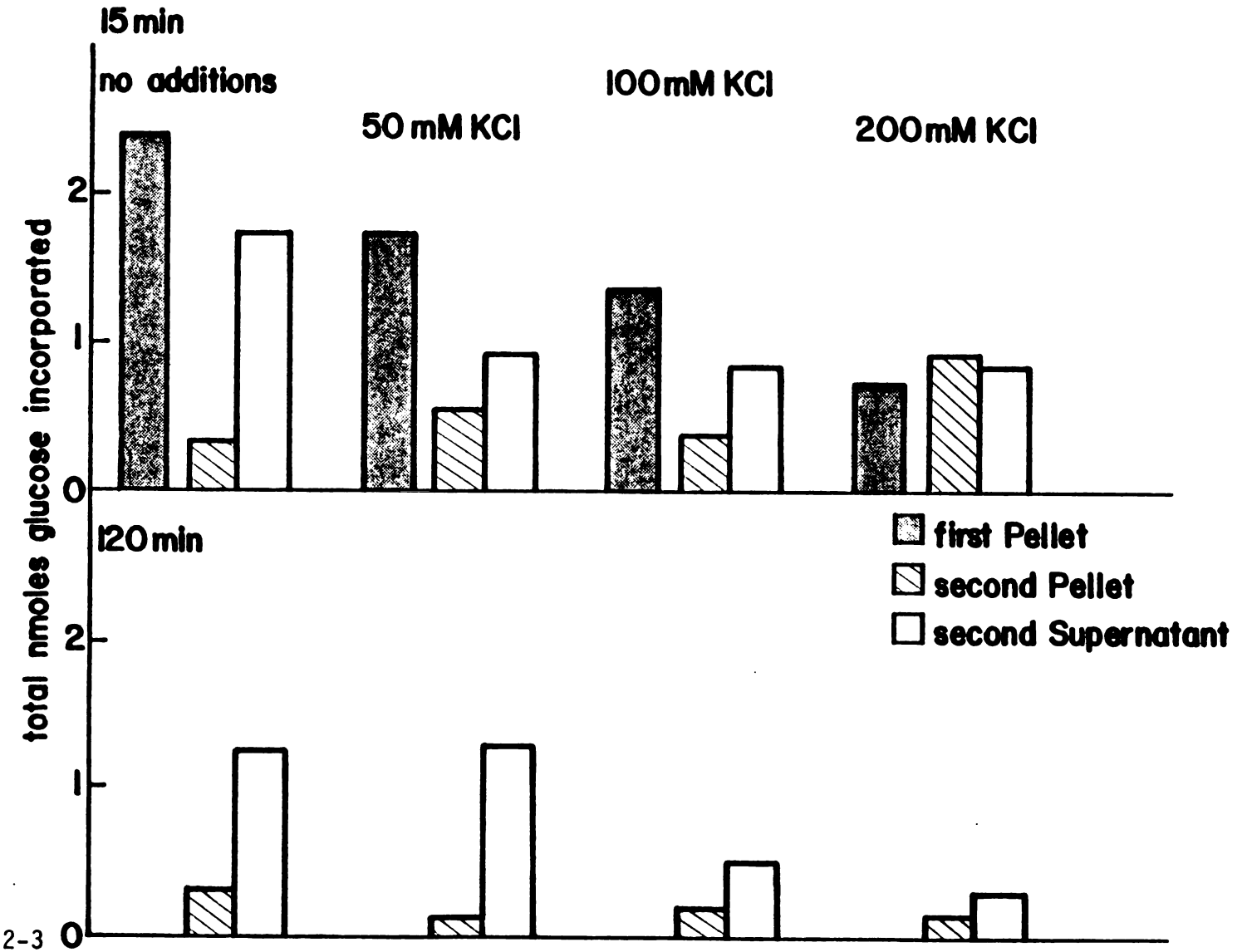

15 min
no additions
50 mM KCl
100 mM KCl
200 mM KCl
total nmoles glucose incorporated
2
1
0
120 min
2
1
0
first Pellet
second Pellet
second Supernatant

Figure 2-3

strength of the buffer was increased by varying the concentration of added KCl. However high salt concentrations do not enhance solubilization (Fig. 2-3) and final concentrations of KCl at $\geq$ 50 mM actually inhibit glucan synthesis. Removal of cholate from the soluble enzyme preparation, on a desalting column, results in loss of glucan synthetase activity and precipitation of the protein (data not shown).

The solubilized enzymes contain at least two glucan synthetase activities (these results are described at the end of section C). These experiments define conditions for preparing solubilized forms of membrane-associated glucan synthetases. Of the detergents screened only cholate solubilizes glucan synthetase activity. The solubilized glucan synthetase(s) is probably part of a micellar complex consisting of enzyme, cholate and, possibly lipid (Helenius and Simons, 1975). Removal of cholate from the solubilized enzyme preparation, by gel filtration chromatography (on Bio-gel P-6), resulted in the precipitation of protein and loss of glucan synthetase activity (data not shown). Pure cholate micelles, in contrast to many other detergents, are relatively small (MW 2,200 daltons; Helenius and Simons, 1975). Therefore the molecular weight of the mixed enzyme-cholate micelle would be expected to be similar to that of its constituent protein and thus within a range amenable to gel filtration chromatography even in the presence of detergent.

C. Preparation of Solubilized Glucan Synthetases.

1. General protocal. Paramethylsulfonylfluoride, (PMSF, 1mM) was routinely added to all buffers, immediately prior to use, to

Figure 2-4. General protocol for preparing solubilized glucan synthetases.

See Materials and Methods

Figure 2-4.

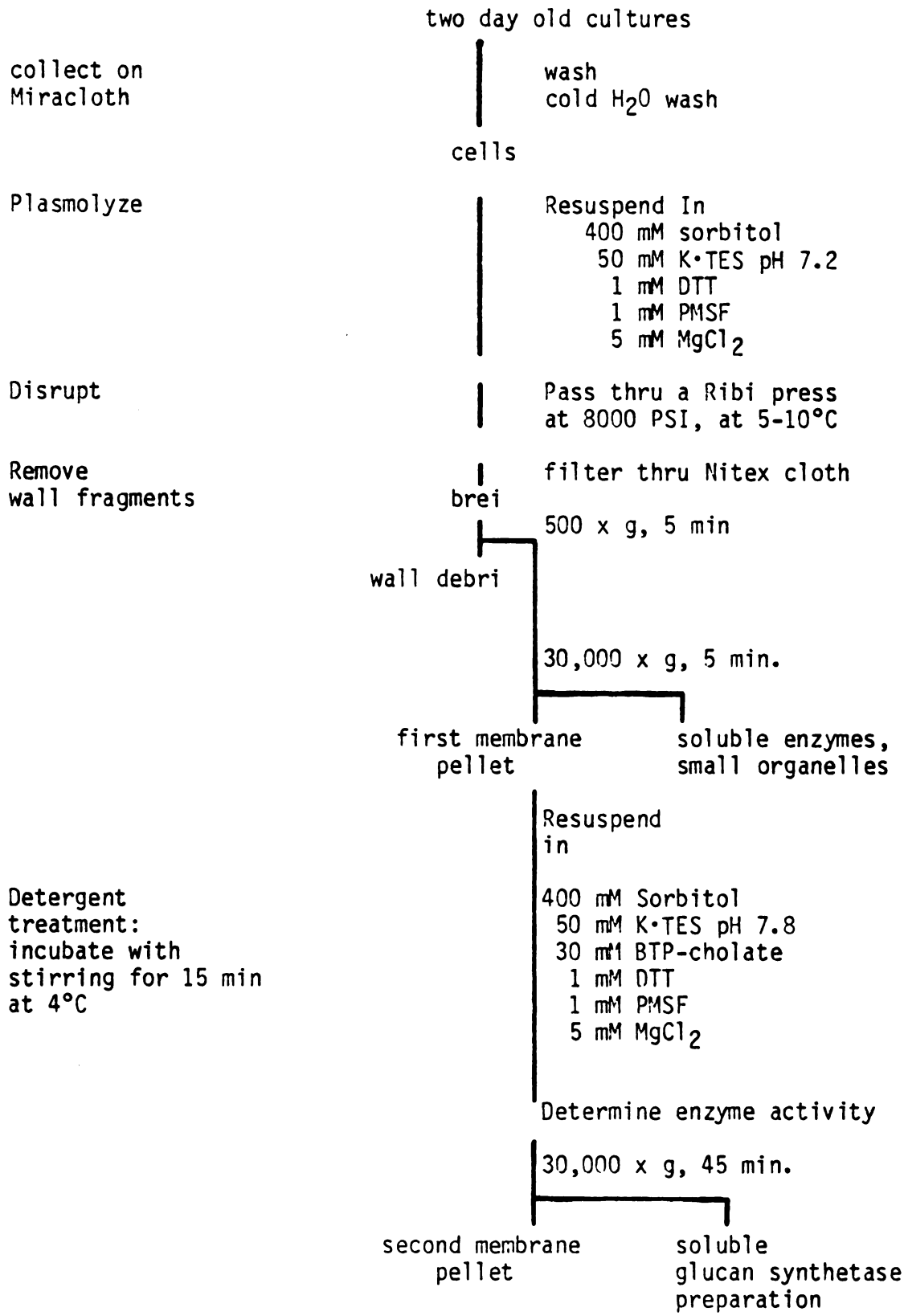

two day old cultures
collect on Miracloth
wash
cold H_2O wash
cells
Plasmolyze
Resuspend In
400 mM sorbitol
50 mM K•TES pH 7.2
1 mM DTT
1 mM PMSF
5 mM $MgCl_2$
Disrupt
Pass thru a Ribi press at 8000 PSI, at 5-10°C
Remove wall fragments
filter thru Nitex cloth
brei
500 x g, 5 min
wall debri
30,000 x g, 5 min.
first membrane pellet
soluble enzymes, small organelles
Resuspend in
400 mM Sorbitol
50 mM K•TES pH 7.8
30 mM BTP-cholate
1 mM DTT
1 mM PMSF
5 mM $MgCl_2$
Detergent treatment: incubate with stirring for 15 min at 4°C
Determine enzyme activity
30,000 x g, 45 min.
second membrane pellet
soluble glucan synthetase preparation

Table 2-1 17.8 g. of 2 day old soybean cells were disrupted in buffer. The crude membrane preparation weighed 744 mg. After detergent treatment, the second membrane pellet weighted 239 mg (lipid, protein and (?) cholate). Each fraction was assayed, in duplicate, for glucan synthetase activity; UDP-glucose was supplied at a final concentration of 0.1 mM to give a specific activity of 0.1 μCi/0.02 μmole. Enzyme activity was also measured after the addition of glycerol (16.5% ((v/v)) final concentration). Reactions were incubated for 20 min. Radioactivity in the reaction products ranged from 900 to 60,000 cpm.

Table 2-1

Preparation of soluble glucan synthetases

Fraction	Total protein (mg)	pellet weight (mg)	Total activity nmoles glucose incorporated (per 20 min)	+glycerol	specific activity nmoles per mg per min	+glycerol	ratio +/-glycerol
Total homogenate[a]	177		1,160	2,010	.455	.568	1.25
500xg pellet[a]	3.78	-	34.9	73.2	.462	.968	2.10
1st 30K supernatant[a]	127	744	61.3	255	.024	.100	4.16
1st 30K pellet[b]	6.44		221	3,600	1.72	28.0	16.3
2nd 30K pellet[b]	0.88	239	113	270	6.46	15.4	2.39
2nd 30K supernatant[b]	6.10		26.2	1,010	2.15	8.53	38.40

*[a] in 50 mM K-TES, pH 7.2, 5 mM $MgSO_4$, 400 mM sorbitol, 5mM DTT, 1 mM PMSF

**[b] in 50 mM K-TES, pH 7.8, 5 mM $MgSO_4$, 400 mM sorbitol, 30 mM BTP·cholate, 1 mM DTT, 1 mM PMSF

protect against serine proteases. PMSF did not inhibit β-glucan synthesis (data not shown). This suggests that serine residues are not involved in the active site(s) of this glucan synthetase.

The general protocol for preparing solubilized glucan synthetase(s) is illustrated in Fig. 2-4 and described in more detail in the Material and Methods Section. Table 2-1 summarizes the distribution of glucan synthetase activity in the various soluble and membrane pellet fractions. The proportion of the total protein from the first membrane pellet, which was solubilized by cholate, varied considerably (25 to 90%). Solubilization is enhanced if the ratio of cholate buffer to initial pellet weight is at least two ml per 100 mg. This illustrates that solubilization is not only a function of detergent concentration per se but is also dependent on the ratio of detergent to membrane (Helenius and Simons, 1975).

It should be noted that the total amount of enzyme activity in each fraction may be altered by a number of conditions which are varied between successive fractionation steps (Table 2-1). These include: 1) pH: Glucan synthetase activity was assayed at pH 7.2, for the total homogenate, 500 x g pellet and first 30,000 x g supernatant (soluble enzymes and small organelles). For the remaining fractions, glucan synthetase activity was determined at pH 7.8. 2) Cholate: Glucan synthetase activity was measured in the presence of 30 mM cholate in the first and second 30,000 x g pellet and the second 30,000 x g supernatant. Cholate stimulates glucan synthetase activity as compared to control samples without detergent (Fig. 2-2).

Only 12% of the glucan synthetase activity from the first 30,000 x g membrane pellet was recovered in the solubilized enzyme fraction (c.f. total activity: 221 nmoles glucose incorporated versus 26 nmoles incorporated). Four times as much glucan synthetase activity was found in the second membrane pellet (113 nmoles incorporated). This distribution of glucan synthetase activity contrasts with results obtained in previous experiments (Figs. 2-2 and 2-3). However, a possible explanation for this discrepancy is that the glucan synthetase activity is not proportional to protein concentration under these experimental conditions (as was observed in a preliminary experiment). In the experiment summarized in Figs. 2-2 and 2-3, the solubilized enzymes were assayed directly (at approx. 580 µg protein per ml). In the experiment summarized in Table 2-1, total glucan synthetase activity was determined from an aliquot of the solubilized enzyme fraction, which had been diluted (final protein concentration 94 µg protein per ml). The amount of membrane lipid present in each fraction under these assay conditions also varied. Davies (1977) has speculated that lipid concentration may influence the activity of membrane-associated aspartate kinase measured *in vitro*.

The addition of glycerol to the solubilized enzyme preparation results in a substantial stimulation of glucan synthesis (40 to 100 fold). Glycerol activation of a particulate glucan synthetase, derived from *Lupinus albus*, has been reported by Thomas and Stanley (1968). Enzyme activity, measured in the presence of glycerol, (16.5%, v/v, final concentration), is stimulated in all of the fractions (Table 2-2). The ratio of stimulation (+/- glycerol) generally increases during the fractionation and is highest for the solubilized glucan

synthetases (38 fold). The distribution of glucan synthetase activity between the second 30,000 x g pellet and the soluble fraction is now reversed: The soluble fraction has four times as much total activity as the pellet when both are assayed in the presence of glycerol. (Total activity in the soluble fraction: 1,010 nmoles glucose incorporated versus 270 nmoles in the pellet fraction.)

The solubilized enzyme fraction was stored with glycerol (final concentration 33%), then diluted 1:1 with cholate buffer prior to use. Enzyme activity is stable for at least four weeks at temperatures of -40°C and below. Enzyme activity is also stable in the presence of glycerol for at least 24 h at 4°C. In an effort to protect enzyme activity during subsequent purification, glycerol (final concentration 16.5%) was included in the cholate-based buffers.

2. Product analysis. The ethanol-precipitable in material synthesized by each fraction (from Table 2-1) was subjected to methylation analysis and the distribution of radioactivity in the products was examined. Table 2-2 details some preliminary results. In the products of the solubilized enzyme preparation more than 60% of the radioactivity is found in 3-linked glucosyl residues. The remainder is found primarily in 4-linked glucosyl residues and some t-glucosyl residues. Radioactivity in t-glucosyl residues probably represents chain terminations. This labeling pattern is similar to glucan synthesized in the presence of glycerol. The ratio of labeled 4-linked to 3-linked glucosyl residues may increase in the glucan synthesized by the total homogenate as compared to the solubilized enzyme preparation (data not shown). Since different linkages are usually formed by specific enzymes these results indicate that at least two UDP-glucosyl

Table 2-2 Samples of radioactive glucan(s) were prepared under standard assay conditions (200 µl of dilute enzyme preparation per sample, 0.1 µCi/0.02 µmole UDP-[^{14}C]-glucose, 20 min reaction time, less than 20 µg of protein per assay). The ethanol-precipitable products were permethylated and the derivatives separated by GLC as described in the material and methods. The reaction products for two separate enzyme preparations were analyzed. The total radioactivity in samples, before methylation ranged from 1,500 to 60,000 cpm. Each derivatized sample, was concentrated, as much as was feasible, to increase the actual level of radioactivity for a given GLC separation.

Table 2-2

Products of glucan synthetase(s)

solubilized glucan synthetases		cpm in each residue			
		t-glucosyl	3-linked glucosyl	4-linked glucosyl	ratio (3- to 4-linked products)
- glycerol	Preparation 1	-	66	40	1.6
	Preparation 2	20	574	150	3.8
+glcyerol	Preparation 1	10	240	68	3.5
	Preparation 2	26	1,150	580	2.0

transferases are present in the solubilized enzyme preparation: a 3-β-glucan synthetase and a 4-β-glucan synthetase. It is not yet possible to conclude whether the latter activity is associated with xyloglucan, glucomannan or cellulose biosynthesis.

D. Preliminary Experiments to Isolate Individual Glucan Synthetases.

1. Affinity Chromatography on Reactive Blue Agarose. The blue chromophore linked to this agarose matrix may be used as a general affinity ligand to purify enzymes which utilize nucleotides or nucleotide derivatives. In two separate experiments, a sample of the solubilized glucan synthetase was applied to a 3 ml column of Reactive Blue Agarose, equilibrated with cholate-glycerol buffer. The sample was held on the column for 45 min. then washed with five volumes of buffer. Subsequently the buffer was supplemented with UDP-glucose (final concentration 1mM) and the enzymes were eluted with 6 ml of buffer. Enzyme activity was monitored for 0.5 ml fractions; glucan synthetase activity eluted in the void fractions, but no enzyme activity was detected in fractions specifically eluted with UDP-glucose. Approximately 85% of the protein applied eluted in the void volume; the remaining 15% specifically eluted with UDP-glucose, but did not exhibit glucan synthetase activity. Thus this affinity procedure does not seem promising as a purification technique: The amount of protein, presumably enzymes other than the glucan synthetases, removed from the enzyme preparation (i.e. bound to the Reactive Blue Agarose) does not give a significant improvement in the specific activity of the sample.

2. Gel filtration chromatography. A sample of the cholate-solubilized glucan synthetase preparation was chromatographed on Ultragel ACA34 in buffer containing cholate and glycerol. The fractionation range for this gel matrix is approximately 20,000 to 350,000 daltons for globular proteins. All of the protein eluted in the void volume of the column, indicating that the protein-cholate micelles are in excess of 350,000 daltons (approximate molecular weight). This suggests the membrane bound enzymes are inherently quite large, perhaps part of multi-enzyme complexes. During the gel filtration procedure, enzyme activity decayed significantly. This may reflect losses of cofactors or glucan primers, retarded by the gel filtration column.

In addition gel filtration chromatography was performed on Sepharose 2B, which has a fractionation range of 200,000 to 2 x 10^6 daltons. In the initial experiment, no appreciable quantities of protein eluted from the 75 ml column. Sepharose adsorption of lipid and proteins has been observed previously (P. Kelly, personal communication; Klein and Delmer, unpublished results). However, if the same column is used repeatedly, eventually it becomes saturated with lipid and protein and begins to act as a gel filtration matrix. In a second experiment with the same column, the A_{280} elution profile exhibited two small absorbance peaks well within the included volume of the column, and two peaks of glucan synthetase activity were identified.

E. Other Properties of the Solubilized Enzyme Preparation.

Additional experiments examined the kinetics of UDP-glucose incorporation by the solubilized enzyme preparation. At a substrate concentration of 0.1mM UDP-glucose (the standard concentration for routine assays) the reaction exhibits roughly linear incorporation kinetics for at least 15 min (with 30 µg of protein per assay; Fig. 2-5). Between 20 and 60 min., incorporation proceeds but at a decreased rate. The ^{14}C-incorporation was highly variable at later times. Two linear rates may represent the different reaction kinetics of the multiple glucan synthetases in the soluble enzyme preparation; however, an alternative explanation is that the substrate concentration decreased significantly thus slowing the reaction.

The initial velocity was measured for substrate concentrations ranging from 1.85×10^{-6} M to 1×10^{-3} M (data not shown). The data was plotted using a variety of standard methods for kinetic analysis, however no meaningful values for the Michaelis constants, K_m and V_{max} were obtained. The enzymes appeared to exhibit substrate activation, as has been previously demonstrated for glucan synthetases in cotton (Delmer et al. 1977). In as much as there are at least two glucan synthetases in this preparation, it is perhaps premature to pursue such kinetic analysis.

Figure 2-5. Time course of product formation by a solubilized preparation of glucan synthetases.

0.2 ml samples of the solubilized enzyme preparation (containing 29.7 µg of protein) were incubated at 25°; UDP-[^{14}C]-glucose was supplied to give a final concentration of 0.1 mM, specific activity 5 µCi/µmole. The radioactivity in the ethanol precipitable products ranged from 100 cpm (zero time) to 42,000 cpm. Each time point is the average for duplicate samples.

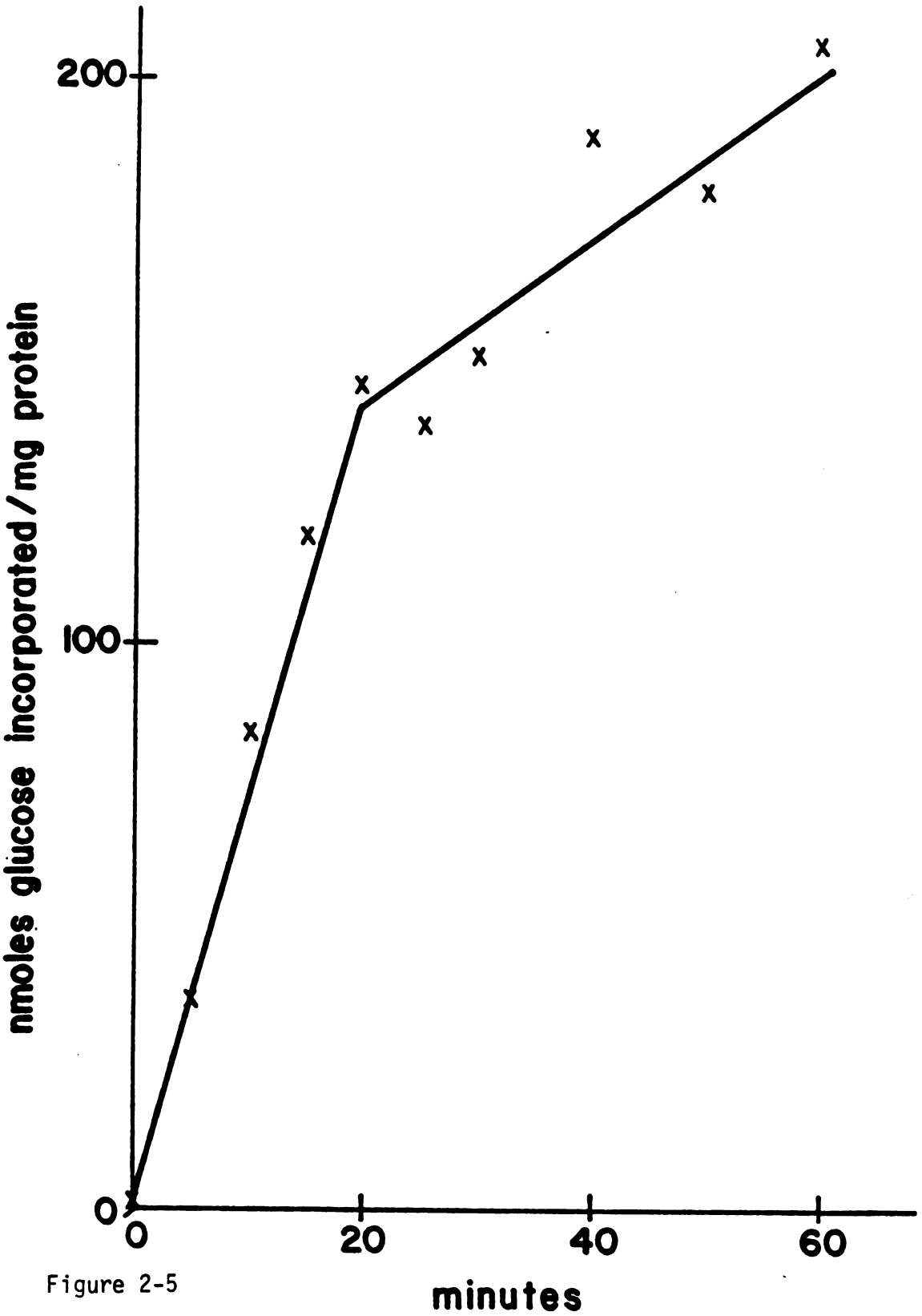

200
100
0
nmoles glucose incorporated/mg protein
0
20
40
60
minutes

Figure 2-5

Discussion

Glucose-containing polysaccharides represent a significant proportion of the extracellular polysaccharides produced by plant cells (see Chapter one).

Membrane preparations which utilize UDP-glucose to synthesize 3- and/or 4-β-glucans have been described for dicots (e.g. Phaseolus aureus, Gossypium, Lupinus, Pisum) and monocots (e.g. Avena, Lolium) (see ref. cited in Delmer, 1977; Darvill et al. 1980; Robinson, 1977). However the modulation of these enzymes, whether during cell wall development or regeneration is poorly understood (Delmer, 1977).

The UDP-glucose: β-glucan synthetases of soybean cells are primarily associated with a crude membrane preparation (500 to 30,000 x g pellet, Table 2-1). Enzyme activity requires 400 mM sorbitol, possibly as an osmotic stabilizer (Fig. 2-1). This osmotic effect has been observed in a particulate glucan synthetase system derived from Lupinus but is generally overlooked when the glucan synthetase activity is used to identify membrane systems on sucrose gradients (Ephritikhine et al., 1980). Total glucan synthesis in the soybean system is enhanced at alkaline pH; this suggests at least some of the enzymes involved may be localized in the cytoplasm which is normally alkaline.

Several detergents with different ionic properties were screened for their effect on enzyme activity and potential to solubilize the soybean glucan synthetases. Only cholate, an ionic detergent stimulates glucan synthetase activity in the crude membrane preparation and solubilizes appreciable amounts of glucan synthetase (Fig. 2-2). By raising the pH of the buffer system to 7.8, (the pKa of cholate),

75% of the enzyme is solubilized after 15 min of detergent treatment (Fig 2-3).

The level of glucan synthetase activity in the soluble enzyme preparation, appears to be dependent on the concentration of protein in the assay mixture i.e. enzyme activity is not a linear function of protein concentration. A similar effect was observed for the 3-β-glucan synthetase activity, in a particulate system derived from developing cotton fibers (Heiniger and Delmer, unpublished results). In contrast, the glucan synthetase system from Phaseolus exhibits a linear relation between total glucan synthetase activity and protein concentration (Chambers and Elbein, 1970).

When glycerol (final conc. 16.5%) is added to the solubilized enzyme preparation from soybean cells there is a dramatic stimulation of glucan synthesis (e.g. 40 fold, Table 2-1). Glucan synthetase activity recovered in this soluble preparation, measured at low protein concentrations, in the presence of glycerol, represents 80% of the total activity (cf. $\frac{\text{soluble}}{\text{soluble + pellet}}$).

The 3-β-glucan synthetase activity of Phaseolus rapidly decayed at 0°C (Feingold et al., 1958). However, I have observed that the solubilized glucan synthetase from soybean cells is stable in the presence of 33% glycerol for at least 24 h. at 4°C and for at least five weeks at temperatures below < 40°C.

Some reports have indicated that membrane preparations from Phaseolus (and a digitonin solubilized form), synthesized exclusively 3-β-glucans (Chambers and Elbein, 1970; Feingold et al., 1958; Flowers et al., 1968). In contrast, methylation analyses of the glucan products from the soybean system indicate that the soluble enzyme

preparation contains at least two glucan synthetase activities since both labeled 3-linked and 4-linked glucosyl residues are detected.

In preliminary experiments the ratio of 3- to 4-linked glucosyl residues in the soybean products varied considerably (1.5 to 3) but the distribution of label in the products was not influenced by the addition of glycerol to the reaction mixture. In contrast only the glucan products synthesized by particulate preparation from Lupinus in the presence of glycerol, are susceptible to 3-β-glucanase (from Rhizopus arrhizus (QM 1032); Thomas and Stanley, 1968).

Cholate-mediated solubilization and glycerol stabilization of soybean glucan synthetases provides convenient starting material for the isolation of the individual enzymes. This work is now in progress in collaboration with B. Mitchell in Dr. Delmer's laboratory. In addition we plan to characterize the reaction products: Are the 3- and 4-linked glucosyl residues part of the same polymer or two distinct polymers? Is the 4-β-glucan synthetase activity associated with xyloglucan synthesis or glucommannan synthesis (e.g. is the incorporation of glucose from UDP-glucose stimulated by GDP-mannose or UDP-xylose)? The next chapter will describe some interesting regulatory properties of the 3-β-glucan synthetase, in situ.

CHAPTER 3. PROPERTIES OF THE 3-β-GLUCAN SYNTHETASE *in situ*

Results

This chapter examines some properties of the 3-β-glucan synthetase *in situ*. The purpose of these studies is to: 1) examine the hypothesis that enzyme activity is controlled, in part, by the localization of the enzyme within the plasma membrane, with the enzyme's active site oriented either towards the periplasmic or cytoplasmic surface (as described in the introduction) and 2) identify other factors which may regulate enzyme activity.

A. Glucan Synthesis from Exogenous UDP-Glucose

3-β-glucan is not detected in appreciable quantities in the cell wall or excreted polymers derived from liquid-suspension cultured soybean cells (Figs. 3-1A and 1C). However when whole cells are incubated in the presence of UDP-[^{14}C]-glucose, the exogenous nucleotide sugar is incorporated into glucan (Table 3-1). This supports the idea that the 3-β-glucan synthetase may accept exogenous substrate but is somehow blocked from utilizing cytoplasmic UDP-glucose. Glucan synthesis is stimulated 3 to 4 fold by plasmolysis. Plasmolysis of intact cells mimics some properties of the physiological state of protoplasts (Boffey and Northcote, 1975). Protoplasts also utilize the supplied nucleotide sugar as a substrate

for glucan synthesis (Table 3-1). Total incorporation is similar to that of the normal (e.g. not plasmolyzed) cells perhaps because the protoplasts as opposed to the plasmolyzed cells have been purified to remove leaky or severely damaged cells (e.g. caused as a result of the initial plasmolysis). Total incorporation of UDP-glucose varied up to two fold between experiments and represented 0.5 to 1.0% of the supplied 3 UDP-glucose.

In several similar feeding experiments when protoplasts were supplied UDP-glucose, less than 0.05% of the label became associated with soluble cytoplasmic pools during the three h. of incubation. This suggests that the plasma membrane is not permeable to UDP-glucose and that the nucleotide sugar is not actively absorbed (also see Delmer et al., 1977). After 3 h. less than 1% of the supplied label, remaining in the cultured medium was degraded indicating that enzymes which hydrolyze nucleotide sugars are not secreted into the culture medium. This observation also shows that under these incubation conditions there is a low level of protoplast lysis, which would result in a release of these hydrolases into the culture medium.

Total incorporation of exogenous UDP-glucose is increased five fold when protoplasts are disrupted by osmotic shock, and then incubated in a medium with a water potential equivalent to that of the original protoplast culture medium (Table 3-2).

Protoplasts also synthesize significant quantities of 3-β-glucan from supplied ^{14}C- glucose (Figs. 1-7B and 7D). This most probably results from incorporation of [^{14}C]-gluose into endogenous UDP-glucose pools, after the supplied label had been absorbed (Carpita and Delmer, in press; Karr, 1976). From other experiments (not shown)

Figure 3-1. Identification of glucan synthesized from exogeneous UDP-glucose.

A and B. GLC elution profile of permethylated alditol acetate standards derived from cultured soybean cells. The elution temperature of the derivatives of 3-linked glucosyl residues is indicated (see Fig 1-8). C and D. The elution profile of radioactivity in labeled polymers derived from protoplasts which had been supplied UDP-[^{14}C]-glucose. A. Cell walls B. Secreted polymers C. Protoplast fraction D. ECM

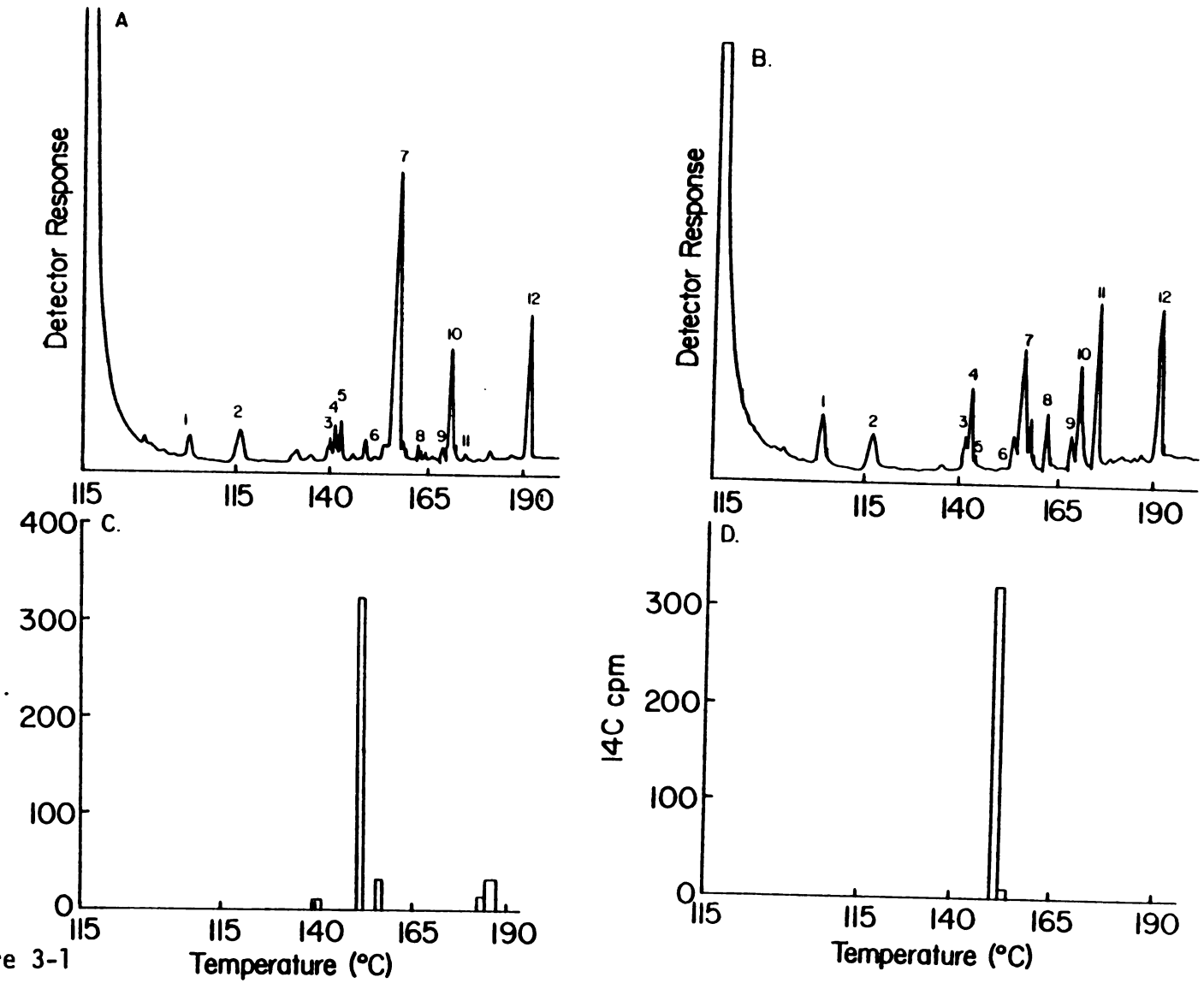

A
Detector Response
1
2
3
4
5
6
7
8
9
10
11
12
115
115
140
165
190
B.
Detector Response
1
2
3
4
5
6
7
8
9
10
11
12
115
115
140
165
190
C.
400
300
200
100
0
115
115
140
165
190
Temperature (°C)
D.
14C cpm
300
200
100
0
115
115
140
165
190
Temperature (°C)

Figure 3-1

it can be concluded that this synthesis of 3-β-glucan is not the result of de novo synthesis of the 3-β-glucan synthetase since it occurs without a detectable lag. Taken together the results with protoplasts also support the hypothesis that supplied UDP-glucose is probably utilized at the plasma membrane.

The glucan product synthesized under these conditions is primarily 3-β-glucan: 1) Methylation analysis of labeled material derived from protoplasts indicates that the glucose moiety from UDP-[^{14}C]-glucose is incorporated primarily into 3-linked glucosyl residues (Figs. 3-1B and 1D). 2) Samples of the labeled material are extensively degraded by a 3-β-glucanase preparation from Rhizopus suggesting that glucosyl residues are linked in the β configuration. A small proportion of the label, usually less than 10%, is found in 4-linked glucosyl residues. This 4-linked product is hydrolysed by AN reagent and therefore it is not crystalline cellulose. The 4-linked glycosyl residues may represent low molecular weight precursors to cellulose or other cell wall glucans. Thus, under conditions where protoplasts are competent to synthesize cellulose from ^{14}C-glucose (see chapter 1), UDP-glucose, supplied to the periplasmic surface of the plasma membrane, is not utilized effectively as a substrate for cellulose synthesis.

B. Orientation of the 3-β-Glucan Synthetase in the Plasma Membrane

The orientation of the active site of the 3-β-glucan synthetase in the plasma membrane, cytoplasmic versus periplasmic, may control utilization of endogenous versus supplied UDP-glucose. In order to examine the orientation of the enzyme in the plasma membrane more

Table 3-1 Samples of 2 day old cells contained 0.5 ml, packed cell volume (approximately 1.1 x 10^7 cells). The cells were washed twice and resuspended in 3 ml of protoplast culture medium described in Material and Methods, or the same medium made up without sorbitol. UDP-[^{14}C]-glucose was supplied to give a final concentration of 1 mM with a specific activity of 1 μCi/μmole. The samples were incubated at room temperature for 3 h.

Protoplasts were incubated under similar conditions. In the experiment shown, 9.4 x 10^6 protoplasts were resuspended in 5 ml of medium; UDP-[^{14}C]-glucose was supplied to give a final concentration of 1 mM, at a specific activity of 0.5 μCi/μmole. Similar results were obtained in at least four other experiments.

Table 3-1.

Utilization of exogenous UDP-[^{14}C]-glucose by whole cells and protoplasts.

	cpm incorporated in 3 hrs[a]	Total nmoles incorporated in 3 h 1 x 10^6 cells or protoplasts
Control Cells	80,000	3.70
	79,900	3.70
Plasmolyzed Cells	226,000	10.5
	352,000	16.3
Protoplasts	34,900	3.6

[a]Replicate samples

Table 3-2 Samples contained the equivalent of 2.5 x 10^6 protoplasts. In the controls, the protoplasts were resuspended in three ml of buffer containing 250 mmolal PEG 4000, 10 mM Tris-MES pH 5.5, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$ and 100 mM pentaerythritol. An equivalent number of protoplasts were first lysed, by osmotic shock, then mixed with a concentrated buffer solution so that the final water potential of the samples was equivalent to that of the control samples. UDP-[^{14}C]-glucose was supplied to give a final concentration of 0.1 mM, with a specific activity of 10μCi/μmole. Incubations were carried at room temperature for 1 h.

Table 3-2

Stimulation of glucan synthesis from exogenous UDP-[^{14}C]-glucose by controlled lysis of protoplasts.

	cpm incorporated over 1 h	Total nmoles per 1 X 10^6 protplasts
control	88,500[a]	1.8
	71,400[a]	1.4
lysed	403,000[a]	8.2
	346,000[a]	7.1

[a]replicate samples

closely, I attempted to isolate membrane vesicles of uniform topology ((1) membrane vesicles in which the topology remained unaltered; that is membrane vesicles with the periplasmic face remaining outward and (2) inverted vesicles), and examined how these vesicles utilized supplied UDP-glucose. The results are described below.

1. Isolation of putative right-side-out plasma membrane vesicles by affinity chromatography. A crude preparation of membrane vesicles was prepared from protoplasts and fractionated on a con A-Sepharose affinity column, as described in the Materials and Methods. Inside-out plasma membrane vesicles, (cytoplasmic face outward), other cellular organelles and excess right-side-out plasma membrane vesicles (periplasmic face outward) should not be retarded on the column. Right-side-out plasma membrane vesicles, with exposed glycosyl receptors, are expected to bind to the column (Brunner et al., 1977, Glimelius et al., 1978; Williamson et al., 1976; Zachowski and Paraf, 1974). Subsequently these may in principle be specifically eluted from the column with α-methylmannoside. The initial goal was to determine whether a membrane vesicle fraction could be isolated from protoplasts this way, and if so, whether the glucan synthetase in the putative right-side-out plasma membrane vesicles could directly utilize UDP-glucose.

The absorbance profile at 280 nm (protein + light scattering by vesicles) of a typical fractionation is shown in Fig. 3-2. The fractions were pooled as indicated and assayed for glucan synthesis from supplied UDP-glucose. The results are summarized in Table 3-3.

There is considerable loss (ca. 90%) of glucan synthetase activity during the fractionation procedure; this is presumably due to the

Figure 3-2. Separation of membrane vesicles by affinity chromatography on conA Sepharose.

Membrane vesicles were prepared from soybean protoplasts and separated on a 2 ml conA Sepharose column as described in the Material and Methods section. The elution profile for material which absorbs or refracts 280 nm light is shown. Fractions were pooled as indicated and assayed for glucan synthetase activity.

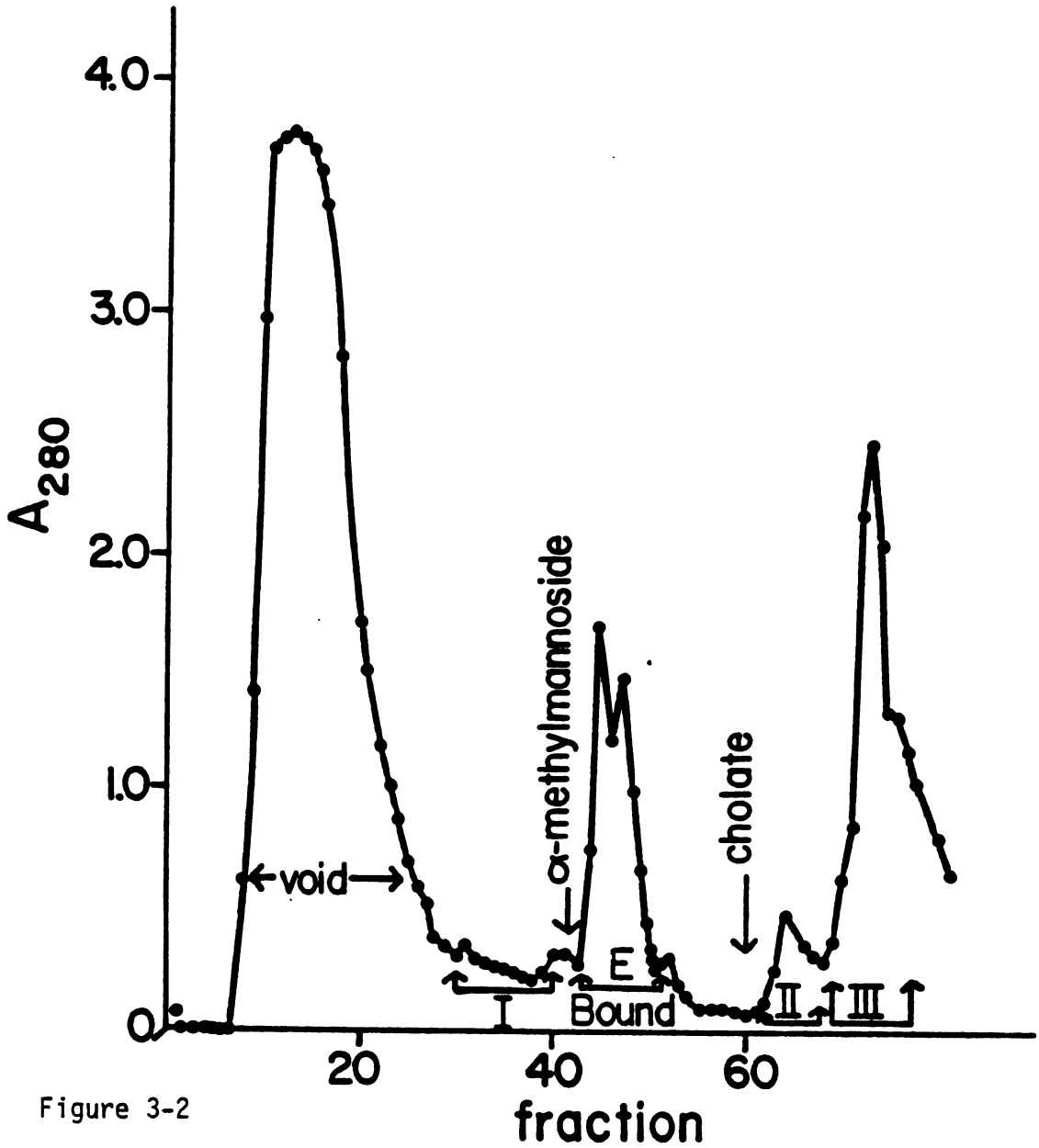

4.0
3.0
2.0
1.0
0
A_{280}
void
α-methylmannoside
cholate
E
Bound
I
II
III
20
40
60
fraction

Figure 3-2

Table 3-3 Membrane vesicles were separated on a conA Sepharose column as indicated in Fig. 3-2. The glucan synthetase activity in each fraction was assayed; UDP-[^{14}C]-glucose was supplied to give a final concentration of 0.1 mM, with a specific activity of 10μCi/μmole. Reactions were carried out at 25°C for 20 min. The total activity of the original protoplast lysate and the resulting crude membrane preparation are indicated.

Table 3-3

Glucan synthetase activity of vesicles separated by conA Sepharose chromatography

	Total protein (mg)	cpm glucose incorporated per standard assay	total activity nmoles incorporated in 20 min	Specific activity (nmoles per min per mg protein)
Total lysate	14.0	55,700[+]	417	29.8
10,000 x g supernatant	5.40	20,800[+]	33.3	6.18
10,000 x g pellet	7.78	82,000[+]	393	50.6
10,000 x g pellet	5.90*	5,430*	19.2	3.2
column fractions				
Void	2.60	1,820	6.7	2.6
conA bound	.080	4,000	6.2	77.5
I	0.041	730	1.1	27.5
II	<2μg	n.d.	n.d.	n.d.
III	0.08	n.d.	n.d.	n.d.

n.d. no data

[+]initial activity determined immediately after membrane preparation

*activity determined for an aliquot equivalent to that applied to the conA column; activity determined at the end of all chromatography steps (after approximately 7 hours at room temperature).

action of proteases and phenolics compounds in the membrane preparation and the long exposure to room temperature (5 h). In addition fractionations were carried out at room temperature to facilitate conA binding. The bound fraction, putative right-side-out vesicles, utilized UDP-glucose to synthesize ethanol-precipitable glucan indicating that the 3-β-glucan synthetase may accept substrate from the equivalent of the periplasmic face of the plasma membrane. The specific activity of glucan synthetase in the bound fraction is considerably higher than that of the total membrane preparation (measured after affinity chromatography) and void fraction. This is consistent with the prediction that right-side-out plasma membrane vesicles are preferentially bound to conA.

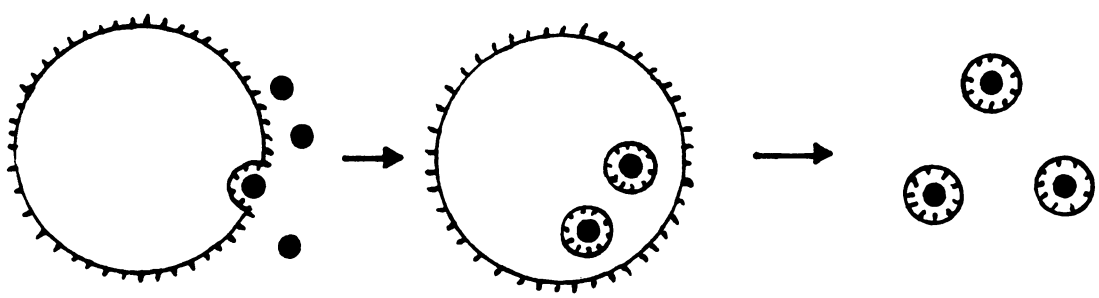
Fraction I exhibited significant glucan synthetase activity. These may represent tailing of the void fraction combined with a slow displacement of right-side-out vesicles from the column. These vesicles are quite large as compared to the size of conA-receptors embedded in the membrane; mechanical forces, including the flow of buffer down the column, may shear the vesicles off of the conA (Brunner et al., 1977).

Additional UV absorbing material was eluted from the column when cholate was added to the column buffer (fractions II and III). This may represent very large vesicles trapped at top of the column; cholate would disrupt these vesicles, allowing their constituents to be eluted. Fractions II and III had low and variable low amounts of protein and glucan synthetase activity and were not further characterized.

The principal observation - that the putative right-side-out vesicles synthesize glucan from supplied UDP-glucose - is consistent

with two models for the orientation of the active site of the 3-β-glucan synthetase in the plasma membrane: 1) The active site is on the periplasmic surface of the plasma membrane. 2) The enzyme, latent in vivo (i.e. buried in the plasma membrane) is activated during the preparation of the membrane vesicles and can then utilize supplied UDP-glucose. However these results do not exclude the possibility that the plasma membrane vesicles have become "leaky" to UDP-glucose, with the active site of the 3-β-glucan synthetase on the cytoplasmic face of the plasma membrane.

2. Isolation of putative inside-out plasma membrane vesicles. Experiments were performed to determine whether the 3-β-glucan synthetase could utilize UDP-glucose supplied to the equivalent of the cytoplasmic surface of the plasma membrane. Protoplasts, incubated with latex beads, phagocytize some of the beads; these are now coated with inside-out (cytoplasmic face out) plasma membrane (Hale et al., 1980).



The coated beads are readily isolated by differential centrifugation from lysed protoplasts. In two separate experiments, 3-β-glucan synthetase activity was not detected on the coated beads. This indicates that the 3-β-glucan synthetase cannot utilize UDP-glucose from the cytoplasmic surface; however these experiments are equivocal inasmuch as: i) Low amounts of proteins are isolated on the beads,

even under optimal conditions (Hale et al., 1980). It was not feasible to measure the amount of protein on the beads because the beads are coated with polyvinylpyrroledine which interferes with the protein assay and ii) Glucan synthesis may require concomitant translocation of the product to the periplasmic space; translocation may be blocked on the beads.

C. The Influence of Membrane Potentials on Glucan Synthesis.

An electrical potential of roughly 60 to 90 millivolts, negative with respect to the cytoplasm, exists across the plasma membrane of higher plant cells (Rubinstein, 1979). This membrane potential would probably be destroyed when the cell is disrupted. However Carpita and Delmer (in press) suggested that this transmembrane potential may be required to maintain an active cellulose synthetase complex. Recent work by Dr. Bacic in our lab showed that the activity of membrane-bound glucan synthetases from developing cotton fibers are stimulated four- to 12-fold by treatments believed to induce membrane potentials, which are positive with respect to the inside of the membrane vesicles. Some preliminary experiments were performed to examine this phenomenon in the soybean system to determine whether membrane potential is one factor involved in the regulation of the 3-β-glucan synthetase.

1. Influence of potential on glucan synthesis in membrane vesicles. Protoplasts, disrupted by osmotic shock, were mixed with buffered PEG 4000 to prepare a suspension of vesicular structures (observed by light microscopy). Membrane potentials were induced in these membrane vesicles by the use of combinations of anions and cations with different permeability coefficients. Thus a positive

potential (with respect to the inside of the vesicle) is established by the addition of K^+ (50mM final concentration) in the presence of a K^+ specific ionophore, valinomycin, in combination with a relatively impermeant anion MES or $SO_4^=$ (Higinbotham et al., 1967; Kasai and Komentani, 1979; Pressman, 1976). Valinomycin facilitates transport of K^+ down its concentration gradient. A negative potential, with respect to the inside of the vesicle, is induced by combining a relatively impermeant cation (e.g. Bis-tris-propane, BTP^+) with a relatively permeant anion, NO_3^- (Higinbotham et al., 1967).

Crude membrane vesicles utilize UDP-glucose as a substrate for glucan synthesis (Tables 3-4). A positive potential stimulates glucan synthetase activity by 2 fold (in similar experiments with KMES/val, the activity was stimulated by 50%). The negative potential also stimulates glucan synthesis although the magnitude of the stimulation is smaller.

Since the experiments were conducted with crude membrane vesicles it was not possible to measure directly the potentials. If the observed stimulation is due to a transmembrane potential, then replacing the relatively impermeant anion (MES, $SO_4^=$) with a more permeant species (SCN^-) should diminish or dissipate the potential and decrease glucan synthesis. Less enzyme activity is observed under these conditions (Table 3-4).

DCB, a specific inhibitor of cellulose synthesis, slightly stimulates glucan synthesis as compared to the control. However, the stimulation of glucan synthetase activity by the positive potential is not influenced by the addition of DCB (Table 3-4). Glucan synthesis increases slightly (21% c.f. controls) in the presence of ionophores,

Table 3-4 Protoplasts were lysed by dilution into cold, 10 mM BTP·SO_4, pH 7.4, 1 mM DTT, 3 mM $MgSO_4$. After three min, six volumes of buffered 60 mM PEG 4000 were gradually added to the lysate. The resulting suspension of vesicles was divided into 0.8 ml aliquots containing approximately 370 µg of protein and the appropriate salts and ionphores were added to each sample. UDP-[^{14}C]-glucose was supplied to give a final concentration of 0.1 mM, with a specific activity of 5 µCi/µmole (total reaction volume 1 ml). The reactions were incubated at room temperature for 15 min and terminated by the addition of 7 volumes of ethanol. Each assay was performed in duplicate.

Table 3-4

Effect of treatments designed to induce transmembrane potentials on β-glucan synthesis in vesicles derived from soybean membranes.

Treatment	cpm incorporated per assay	total activity nmoles in 15 min per mg protein	ratio of activity (treatment/control)	rate nmoles $min^{-1}mg^{-1}$
control	51,900	14.2		0.95
Val (5μM)	64,700	17.7	1.25	1.18
K_2SO_4 (25mM)	77,400	21.1	1.48	1.41
K_2SO_4/val	105,000	28.8	2.03	1.92
DCB (5μM)	64,300	17.6	1.24	1.17
K_2SO_4/DCB/ val	101,000	27.6	1.94	1.84
KSCN/val (25 mM)	63,500	17.4	1.26	1.16
BTP·NO_3 (25 mM)	78,300	21.3	1.50	1.42

[a]Average of duplicate samples

[b]Least significant difference at the 5% level (LSD 0.05) is 3.6.

Table 3-5 Samples contained 8 X 10^5 protoplasts (288 µg protein). The protoplasts were resuspended in 0.5 ml of buffer, pH 5.5, as indicated; the water potential of each treatment was held constant at the equivalent of 500 milliosmolar, by the addition for BTP•MES. UDP-glucose was added to each sample to give a final concentration of 0.1 mM with a specific activity of 4 µCi/µmole. The samples were incubated at room temperature for 20 min. Unlabeled UDP-glucose was added (final concentration 1.0 mM), followed immediately by seven volume of ethanol. Assays were performed in triplicate and the average incorporation is given, except for the KCl treatment, where only one determination was made.

The change in potential induced by each treatment was predicted based on ion transport rates determined for pea and oat root (Higinbotham et al.,1967), estimated relative permeability (Beck and Sacktor, 1975) and estimated intracellular concentrations of ions (N.E. Good, A. Hanson and P. Filner, personal communication).

Table 3-5

Influence of treatments designed to alter membrane potential on glucan synthesis *in vivo*

Treatment	Predicted change in membrane potential	total cpm in glucan products	total incorporation nmoles per mg protein in 20 min	ratio enzyme activity (treatment/control)
	K^+ flow			
BTP-MES (control)	none, no effect	1,920	1.20	-
BTP-MES/val	out, hyperpolarize	1,060	0.66	0.55
KCl 250 mM	in, no effect or depolarize	9,620	6.02	5.01
KCl/Val 250 mM	in, depolarize	2,490	1.56	1.30
KSCN 250 mM	in, depolarize or no effect	6,570	4.1	3.42
KSCN/Val	in, depolarize	5,460	3.42	2.85
K_2SO_4/Val [166 mM]	in, depolarize	2,740	1.71	1.42

without K^+ (Table 3-4). Valinomycin, and DCB are hydrophobic compounds which become associated with the membrane and thus may alter membrane environment resulting in a slight stimulation of glucan synthetase activity.

The glucan products, examined by methylation analysis, contain both 3-linked and 4-linked glycosyl residues in an approximate ratio of 10 to 1. A preliminary experiment indicates this distribution of products is not altered by the induction of a positive potential in the vesicle preparation. The glucan products are solubilized by AN reagent, thus the labeled 4-linked glucosyl residues do not represent crystalline cellulose.

2. Influence of membrane potential on 3-β-glucan synthesis, from exogenous UDP-glucose, in vivo. Experiments were designed to examine whether pertubation of the transmembrane potential of protoplasts would affect glucan synthesis from exogeneous UDP-glucose. Treatments were designed to establish K^+ flow in or out of the protoplasts by incubating protoplasts with valinomycin and/or in the presence of high levels of K^+ salts (Table 3-5). K^+ was combined with anions having different permeability coefficients; it is not clear as to whether these treatments may have other indirect effects on enzyme activity. When the membrane potential is hyperpolarized, glucan synthesis is inhibited. This effect is also observed when protoplasts are incubated in high concentrations of $BTP \cdot NO_3$ (e.g. hyperpolarized, data not shown). K^+ combined with the relatively impermeant anion $SO_4^=$, is expected to depolarize the protoplasts, this effect should be facilitated by valinomycin. Glucan synthesis is stimulated by this treatment (Table 3-5), however the degree of stimulation is higher when

K^+ is combined with a relatively more permeant anion (Cl^- or SCN^-). Furthermore the addition of valinomycin actually decreases glucan synthesis (cf. KSCN treatment ± val).

The results from these in vivo experiments do not conform to a simple model by which transmembrane potential regulates 3-β-glucan synthetase activity: Total glucan synthesis does not reflect the predicted change in membrane potential. As indicated earlier the effects of these treatments on other aspects of cellular physiology may have indirect effects on 3-β-glucan synthetase activity. Nonetheless the imposition of a transmembrane potential on membrane vesicles from soybeans stimulates glucan synthesis. This implies that the transmembrane potential may have a regulatory function in the synthesis of 3-β-glucan.

Discussion

3-β-glucan is not found in appreciable quantities in the walls or excreted polymers from liquid suspension cultures of soybeans. However it is synthesized in significant amounts from exogeneous UDP-glucose during the early stages of wall regeneration and also by plasmolyzed cells. These observations may indicate that the plasma membrane has become "leaky" to UDP-glucose, thus the supplied nucleotide sugar diffuses to the active site of the 3-β-glucan synthetase on the cytoplasmic surface of the plasma membrane (see the models described in the Introduction). However, that model does not explain why under normal conditions the enzyme does not utilize endogenous UDP-glucose. What discriminates between these substrate pools *in vivo*? Alternatively if the active site of the enzyme is localized on the periplasmic surface of the plasma membrane, how is enzyme activity stimulated by protoplast lysis? Is some latent form of the enzyme activated by wounding (Brett, 1978)? Is the mechanism(s) of enzyme activation a function of membrane perturbation?

In order to elucidate the mechanism(s) of 3-β-glucan synthetase regulation *in vivo* experiments were untaken to examine the orientation of the enzyme in the plasma membrane.

Orientation of the 3-β-Glucan Synthetase. Hughes and Gunning (1980) have proposed that the active site of the 3-β-glucan synthetase is on the periplasmic surface of the plasma membrane. They have suggested that when a cell is wounded, i.e. the integrity of the plasma membrane is disrupted, cytoplasmic UDP-glucose diffuses to the active site of the enzyme and the resulting synthesis of 3-β-glucan forms a "plug" at

the damaged site. This model was developed from cytological observations of the formation of callose plugs around plasmodesmata.

Anderson and Ray (1978) reported that when UDP-glucose was supplied to pea stem sections, it was utilized primarily at cut edges for the synthesis of 3-β-glucan. Mueller and Machachlan (1980) prepared microautoradiograms of pea stem sections which had been incubated with labeled UDP-glucose; in these samples the plasma membrane was appressed to the cell wall and the periplasmic surface of the membane was not examined. Incorporation of UDP-glucose appeared to occur at the cytoplasmic surface. The resolution of this type of microautoradiograph is comparatively low and thus these results do not exclude alternative models for the orientation of the enzyme in the plasma membrane (e.g. both cytoplasmic and periplasmic orientation, Montezinos, personal communication).

Affinity chromatography on conA Sepharose was used to isolate putative right-side-out plasma membrane vesicles derived from soybean protoplasts. Glucan synthetase II (3-β-glucan synthetase) and a K^+-stimulated ATPase, are the only markers so far suggested for the plasma membrane of higher plants (Leonard and Hodges, 1980; Quail, 1979). The ATPase is fairly labile (C. Caldwell, personal communication). Mitochondria of mammalian cells are known to contain conA receptors (Bittiger, 1976); however, it seems unlikely that their counterparts in plants contain 3-β-glucan synthetase activity (Leonard and Hodges, 1980; Quail, 1979). In the absence of more refined techniques to characterize the vesicles fractionated on conA Sepharose as plasma membrane vesicles, tentative interpretation of these experiments provides some new information with which to evaluate

various models for the orientation of the 3-β-glucan synthetase in the plasma membrane: UDP-glucose supplied to the equivalent of the periplasmic surface of the plasma membrane serves as a substrate for glucan synthesis.

The Influence of Membrane Potential on 3-β-Glucan Synthetase Activity. When the integrity of the plasma membrane is disrupted, (i.e. during wounding) 3-β-glucan synthesis is stimulated. This may, in part, be the result of enzyme activation (Brett, 1978), e.g., the physical environment of membrane bound enzymes may influence enzyme activity (Dupont, 1979; Helenius and Simons, 1975).

Treatments designed to induce of a positive potential across soybean membrane vesicles derived from protoplasts, results in a 1.5 to 2 fold stimulation of glucan synthesis from supplied UDP-glucose. This potential may influence enzyme conformation within the membrane or alter the membrane environment (e.g. fluidity) thereby indirectly influencing enzyme activity (Dupont, 1979; Laane et al; 1979, Lelkes; 1979; Toci et al., 1980). Alternatively, the electrical gradient may facilitate the movement of the negatively charged substrate across the membrane to the active site of the enzyme.

This last interpretation is perhaps less likely: Imposition of a negative potential also results in a stimulation of glucan synthesis, albeit smaller than that of the positive potential.

These observations actually suggest a paradox: Membrane depolarization (i.e. protoplast lysis) results in a stimulation of 3-β-glucan synthetase activity. Treatments designed to impose a positive or negative potentials across the isolated membrane vesicles

also stimulates enzyme activity (i.e. membrane polarization). Both effects implicate membrane environment as a regulatory factor in 3-β-glucan synthesis. These are perhaps related to the mechanism by which mild temperature stress induces callose synthesis in corn (Smith and McCully, 1977). In addition elucidation of the mechanisms of 3-β-glucan synthetase regulation may help identify factors which regulate the other plasma membrane glucan synthetase, the cellulose synthetase (Delmer, 1977).

CONCLUDING REMARKS

Cell wall regeneration by soybean protoplasts provides a good model system for studying the many facets of cell wall biogenesis. In contrast to protoplasts isolated from leaf tissue of dicots and tissue cultures of monocots, soybean protoplasts rapidly begin to synthesize the fibrillar components of the primary cell wall, i.e. cellulose, when transferred to suitable culture medium. In addition, within three hours of wall regeneration, protoplasts synthesize and secrete other polysaccharides which qualitatively resemble components of the native cell wall and also polymers secreted into the culture medium by suspension cultures (see Chapter one). Because of the relative ease with which protoplasts can be disrupted (as compared to intact cells) it will be possible to isolate cell wall precursors from protoplasts, obtaining new information about their composition and complexity as well as insights into the assembly of these polymers into a cell wall.

At least two polysaccharide synthesizing enzymes are part of the plasma membrane and the regulation of their activity may be in part mediated through their localization in the membrane and by the fluidity of the plasma membrane (Chapter three). The exposed plasma membrane of protoplasts may be probed with sophisticated procedures developed for mammalian cells in order to examine how the plasma membrane modulates the activity of these enzymes. Further purification and

characterization of these enzymes will provide important information about the biosynthesis of cell wall polysaccharides.

The influence of membrane potential on enzyme activity can be examined in more detail using solubilized forms of the β-glucan synthetases. In reconstitution experiments the isolated enzymes may be inserted in to artificial lipid vesicles; these can be used to verify that the induced potentials actually influence enzyme activity.

FOOTNOTE

[1]Previously this enzyme and one that utilizes GDP-glucose have both been designated cellulose synthases (E.C. 2.4.1.12 and E.C. 2.4.1.29). These assignments are incorrect, (see below, Darvill et al., 1980; Delmer, 1977). Many of the other polysaccharide-synthesizing enzyme have not been assigned E.C. numbers).

REFERENCES

Albersheim, P. (1976) The primary cell wall. In "Plant Biochemistry" Bonner, J. and Varner, J.E., Academic Press, Inc., New York, NY, pp. 225-274.

Albersheim, P., Nevins, D.S., English, P.D. and Karr, A. (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohydrate Res. 5:340-345.

Anderson, R. and Ray, P. (1978) Labeling of the plasma membrane of pea cells by a surface localized glucan synthetase. Plant Physiol. 61:723-730.

Asamizu, T. and Nishi, A. (1979) Biosynthesis of cell wall polysaccharides in cultured carrot cells. Planta 146:49-54.

Asamizu, T., and Nishi, A. (1980) Regenerated cell wall of carrot protoplasts isolated from suspension-cultured cells. Physiol. Plant 48:207-212.

Asamizu, T., Tanake, K., Takabe, I. and Nishi, A. (1977) Change in the molecular size of cellulose during regeneration of cell wall on carrot protoplasts. Physiol. Plant 40:215-218.

Aspinall, G.O. (1973) Carbohydrate polymers of plant cell walls. In "The Biogenesis of Plant Cell Wall Polysaccharides" Loewus, F. ed., Academic Press, New York, NY pp. 515-536.

Barber, G.A. Elbein, A.D. and Hassid, W.Z. (1964) The synthesis of cellulose by enzyme systems from higher plants. J. Biol. Chem. 239:4056-4061.

Beck, E. and Wieczorek, J. (1977) Carbohydrate Metabolism. In "Progress in Botany" vol. 39, Ellenberg H., ed., Springer-Verlag, Berlin, W. Ger., pp. 62-82.

Beck, J.C. and Sacktor, B. (1975) Energetics of the Na^+-dependent transport of D-glucose in renal brush border membrane vesicles. J. Biol. Chem. 250:8674-8680.

Bittiger, H. (1976) Application of concanavalin A - Ferritin to subcellular fractions. In "Concanavalin A as a Tool" Bittiger, H. and Schnebli, H.P., eds., J. Wiley and Sons, New York, NY, pp. 117-122.

Boffey, S. and Northcote, D.H. (1975) Pectin synthesis during wall regeneration of plasmolyzed tobacco leaf cells. Biochem. J. 150:433-440.

Boller, T., and Kende, H. (1979) Hydrolytic enzymes in the central vacuole of plant cells. Plant Physiol. 63:1123-1132.

Bradford, M. (1976) A rapid and sensitive assay for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. 72:249-259.

Brett, C.T. (1978) Synthesis of β(1-3)glucan from extracellular uridine diphosphate glucose as a wound response in suspension-cultured soybean cells. Plant Physiol. 62:377-382.

Brett, C.T. and Northcote, D.H. (1975) The formation of oligoglucans linked to lipid during synthesis of β-glucan by characterized membrane fractions isolated from pea. Biochem. J. 148:107-117.

Brown, R.M. Jr. and Montezinos, D. (1976) Cellulose microfibrils: Visualization of biosynthetic and orienting complexes in association with the plasma membrane. Proc. Nat. Acad. Sci. USA 73:143-147.

Brunner, G., Ferber, E., Resch, K. (1977) Fractionation of membrane vesicles I. A separation method for different populations of membrane vesicles of thymocytes by affinity chromatography on conA-Sepharose. Anal. Biochem. 80:420-429.

Brummond, D.O. and Gibbons, A.P. (1964) The enzymatic synthesis of cellulose by the higher plant Lupinus albus. Biochem. Biophys. Res. Com. 17:156-159.

Buchala, A.J. and Franz, G. (1974) A hemicellulosic β-glucan from the hypocotyls of Phaseolus aureus. Phytochem. 13:1887-1889.

Burgess, J. and Linstead P.J., (1977) Coumarin inhibition of microfibril formation at the surface of cultured protoplasts. Planta 133:267-273.

Burgess, J., Linstead, P.J. and Bonsall V.E. (1978) Observations of the time course of wall development at the surface of isolated protoplasts. Planta 139:85-91.

Carpita, N. and Delmer, D.P., (in press), Concentration and metabolic turnover of UDP-glucose in developing cotton fibers. J. Biol. Chem.

Carpita, N. and Delmer, D.P. (in press) Protection of cellulose synthesis by polyethylene glycol in detached fibers in cotton. Plant Physiol.

Chambers, J. and Elbein A. (1970) Biosynthesis of glucan in mung bean seedlings: formation of β(1-4)-glucans from GDP-glucose and β(1-3)-glucans from UDP-glucose. Arch. Biochem. Biophys. 138:620-631.

Clarke, A.E., Anderson, R.L. and Stone, B.A. (1979) Form and function of arabinogalactans and arabinogalactan-proteins. Phytochem. 18:521-540.

Constabel, F. (1975) Isolation and culture of plant protoplasts. In. "Plant Tissue Culture Methods" Gamborg, O.L. and Wetter, L.R., ed. National Research Council of Canada, Saskatoon, Saskatchewan pp. 11-21.

Darken, M. (1961) Applications of fluorescent brighteners in biological systems. Science 133:1704-1705.

Darvill, A., McNeil, M., Albershem, P. and Delmer, D.P. (1980) The primary cell walls of flowering plants. In "The Plant Cell", Tolbert, N.E., ed. Academic Press, New York, NY pp. 163-182.

Davies, H.M. (1977) Regulation of amino acid biosynthesis in carrot tissue cultures. Ph.D. Thesis, University of London.

Delmer, D.P. (1977) The biosynthesis of cellulose and other plant cell wall polysaccharides. In "Recent Advances in Phytochemistry" Vol. 11, Loweus, F.A. and Runeckles, V.C., eds, Plenum Publishing Corp., New York, NY, pp. 45-77.

Delmer, D.P., Heiniger, U. and Kulow, C. (1977) UDP-glucose:glucan synthetase in developing cotton fibers I. Kinetic and physiological properties. Plant Physiol. 59:713-718.

Dupont, Y. (1979) Electrogenic calcium transport in the sarcoplasmic reticulum membrane. In "Cation Flux across Biomembranes" Mukohata, Y. and Packer, L., eds, Academic Press, New York, NY pp. 141-160.

Elbein, A.D., Barber, G.A. and Hassid, W.Z. (1964) Synthesis of cellulose by an enzyme system from a higher plant. J. Am. Chem. Soc. 86:309-310.

Ephritikhine, G., Lamant, A. and Heller, R. (1980) Influence of sucrose on the characterization of higher plant membranes by β-glucan synthetase activity and its relation to osmotic pressure. Plant Sci. Letters 19:55-64.

Evans, M. The mechanism of action of auxin in the promotion of cell elongation. Manuscript in preparation.

Feingold, D.S., Neufeld, E.F. and Hassid, W.Z. (1958) Synthesis of a β-1,3-linked glucan by extracts of Phaseolus aureus seedlings. J. Biol. Chem. 233:783-788.

Flowers, H.M., Batra, K., Kemp, J. and Hassid, W.Z. (1968) Biosynthesis of insoluble glucans from uridine-diphosphate-D-glucose with enzyme preparations from Phaseolus aureus and Lupinus albus. Plant Physiol. 43:1703-1709.

Fowke, L.C, Bech-Hansen, C.W., Gamborg, O.L. and Constabel, F. (1975) Electron-microscope observations of mitosis and cytokinesis in multinucleate protoplasts of soybean. J. Cell. Sci. 18:491-507.

Franz, G. and Meir, H. (1969) Biosynthesis of cellulose in growing cotton hairs. Phytochem. 8:579-583.

Gamborg, O.L. (1975) Callus and cell culture. In "Plant Tissue Culture Methods" Gamborg, O.L. and Wetter, L.R., National Research Council of Canada, Saskatoon, Saskatchewan, pp. 1-10.

Giddings, T.H. Jr., Bower, D.L. and Staehlin, L.A. (1980) Visualization of particle complexes in the plasma membrane of Micrasterias denticulata associated with the formation of cellulose fibrils in primary and secondary walls. J. Cell. Bio. 84:327-339.

Glimelius, K., Wallin, A. and Erikkson (1978) Concanavalin A improves the polyethylene glycol method of fusing plant protoplasts. Physiol. Plant 44:92-96.

Grout, B.W. (1975) Cellulose microfibril deposition at the plasmalemma surface of regenerating tobacco mesophyll protoplasts: a deep etch study. Planta 123:275-282.

Gurd, J.W. and Mahler, H.R. (1974) Fractionation of synaptic plasma membrane glycoprotein by lectin affinity chromatography. Biochem. 13:5193-5198.

Hahlbrock, K. and Grisebach, H. (1979) Enzymic controls in the biosynthesis of lignin and flavonoids. Ann. Rev. Plant Physiol. 30:105-131.

Hakamori, S. (1964) A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide J. Biochem. 55:205-208.

Hale, C.C. II, McEntire, K. and Roux, S.J. (1980) Analysis of proteins of membrane-enveloped beads phagocytized by oat protoplasts. Plant Physiol. Supplement 65:813.

Hanke, D.E. and Northcote, D.H. (1974) Cell wall formation by soybean callus protoplasts. J. Cell. Sci. 14:29-50.

Heiniger, H. and Delmer, D.P. (1977) UDP-glucose:glucan synthetase in developing cotton fibers II. Structure of the reaction product. Plant Physiol. 59:719-723.

Helenius, A. and Simons, K. (1975) Solubilization of membranes by detergents. Biochim. Biophys. Acta 415:29-79.

Herth, W. and Meyer, Y. (1977) Ultrastructural and chemical analysis of the wall fibrils synthesized by tobacco mesophyll protoplasts. Biologic Cellularie 30:33-40.

Heslop-Harrison, J. (1968) Pollen wall development. Science 168:230-237.

Higinbotham, J., Etherton, B. and Foster, R.J. (1967) Mineral ion contents and cell transmembrane electropotential of pea and oat seedlings tissue. Plant Physiol. 42:37-47.

Hughes, J.E. and Gunning, B.E.S. (1980) Gluteraldehyde-induced deposition of callose. Can. J. Bot. 58:250-258.

Karr, A. (1976) Cell wall biogenesis. In "Plant Biochemistry" 3rd edition, Bonner, J., and Varner, J.E., eds., Academic Press, New York, NY, pp. 405-427.

Kasai, M. and Komentani, T. (1979) Ionic permeability of sarcoplasmic reticulum membrane. In "Cation Flux across Biomembranes" Markohata, Y. and Packer, L., eds. Academic Press, New York, NY 167-177.

Laane, C., Krone, W., Konigs, W.N., Haaker, H. and Veeger C. (1979) The involvement of the membrane potential in nitrogen fixation by bacteroids of Rhizobium leguminsarum. FEBS Letters 103:328-332.

Lamport, D.T.A. (1977) Structure, biosynthesis and significance of cell wall glycoproteins. In "Recent Advances in Phytochemistry" vol. II, Loeweus, F.A. and Ruenckles, V.C., eds., Plenum Publishing Corp., New York, NY, pp. 79-115.

Lamport, D.T.A. (1978) Cell wall carbohydrates in relation to structure and function. In "Frontiers in Plant Tissue Culture" Thorpe, T.E., ed, International Association for Plant Tissue Culture, Calgary, Canada, pp. 235-244.

Lamport, D.T.A. (in press) Cell wall carbohydrates in relation to structure and function. In Encyclopedia of Plant Physiology, N.S. Vol. 3.

Lelkes, P.I. (1979) Potential dependent rigidity changes in lipid membrane vesicles. Biochem. Biophys. Res. Commun. 90:656-662.

Leonard, R. and Hodges, T. (1980) The plasma membrane. In "The Plant Cell", Tolbert, N.E., ed., Academic Press, New York, N.Y., pp. 163-182.

Maltby, D., Carpita, N.C., Montezinos, D. and Delmer, D.P. (1979) β-1,3-Glucan in developing cotton fibers: Structure, localization and relationship of synthesis to that of secondary wall cellulose. Plant Physiol. 63:1158-1164.

Mazia, D., Schatten, G. and Sale, W. (1975) Adhesion of cells of surfaces coated with polylysine. J. Cell. Biol. 66:198-200.

McNarin, R.B. (1972) Phloem translocation and heat-induced callose formation in Gossypium hirsutum L. Plant Physiol. 50:366-370.

Meinert, M. and Delmer, D.P. (1977) Changes in biochemical composition of the cell wall of the cotton fiber during development. Plant. Physiol. 59:1088-1097.

Meyer, Y. and Herth, W. (1978) Chemical inhibition of cell wall formation and cytokinesis but not of nuclear division in protoplasts of Nicotianna tabacum L. cultivated in vitro. Planta 142:253-262.

Montezinos, D. and Delmer, D.P. (1980) Characterization of inhibitors of cellulose synthesis in cotton fibers. Planta 128:303-311.

Mueller, S. and Brown, R.M. Jr. (1980) Evidence for an intramembrane component associated with a cellulose microfibril synthesizing complex in higher plants. J. Cell. Biol. 84:315-326.

Mueller, S., Brown, R.M. Jr. and Scott, T.K. (1976) Cellulosic microfibrils: Nascent stages of synthesis in a higher plant cell. Science 194:949-951.

Mueller, S.C. and Maclachlan, G.A. (1980) Plasma membrane only utilize UDP-GLC supplied to the protoplasmic surface. Plant Physiol. (supplement) 65:582.

Nelsen, C.E., Safir, G.R. and Hanson, A.D. (1978) Water potential in excised leaf tissue: comparison of a commerical dew point hygrometer and a thermocouple psychrometer on soybean, wheat and barley. Plant Physiol. 61:131-133.

Prat, R. and Roland, J.C. (1971) Etude ultrastructurale des premiers stades de néoformation dûne envelope par les protoplastes végétaux separes mécaniquement de leur paroi. C. R. Acad. Sci. (Paris) 273:165.

Pressman, B. (1976) Biological applications of ionophores. Ann. Rev. Biochem. 45:501-530.

Preston, R.D. (1979) Polysaccharide conformation and cell wall formation. Ann. Rev. Plant Physiol. 30:55-78.

Quail, P.H. (1979) Plant cell fractionation. Ann. Rev. Plant Physiol. 30:425-484.

Ray, P. (1980) Cooperative action of β-glucan synthetase and UDP-xylose xylosyl transferase of Golgi membranes in the synthesis of xyloglucan-like polysaccharide. Biochim. Biophys. Acta. 227:278-287.

Ray, P.M. Eisinger, W.R. and Robinson, D. (1976) Organelles involved in cell wall polysaccharide formation and transport in pea cells. Ber Deutsch Bot. Ges. 89:121-146.

Ray, P.M., Shininger, T.L. and Ray, M.D. (1969) Isolation of β-glucan synthetase particles from plant cells and identification with Golgi membranes. Proc. Nat. Acad. Sci. 64:605-612.

Rees, D.A. (1972) Shapely polysaccharides. Biochem. J. 126:257-273.

Robinson, D. (1977) Plant cell wall synthesis. In "Advances in Botanical Res." Vol. 5, Woolhouse, H. ed., Academic Press, NY, NY, pp. 89-150.

Rubin, G. and Zallin, M. (1976) Cell concentration as a factor in precursor incorporation by tobacco leaf protoplasts or separated cells. Planta 131:87-89.

Rubinstein, B. (1978) Use of lipophilic cations to measure the membrane potential of oat leaf protoplasts. Plant Physiol. 62:927-929.

Saeman, J.F., Moore, W.E., and Millett, M.A. (1963) Sugar units present. In "Methods in Carbohydrate Chemistry." Vol. III, Whistler, R., ed. Academic Press, New York, NY pp. 54-69.

Smith, M.M. and McCulley, M.E. (1977) Mild temperature "stress" and callose synthesis. Planta 136:65-70.

Smith, M.M. and Stone, B.A. (1973) β-Glucan synthesis by cell-free extracts from Lolium multiflorum endosperm. Biochem. Biophys. Acta 313:72-94.

Spencer, F.S., Shore, G., Ziola, B. and Machlachlan, G.A. (1972) Particulate glucan synthetase activity: generation and inactivation after treatments with indole acetic acid and cycloheximide. Arch. Biochem. Biophys. 152:311-317.

Takeuchi, Y. and Komamine, A. (1978) Composition of cell wall formed by protoplasts isolated from Vinca nosea suspension culture. Planta 140:227-232.

Taku, A., Hirsch, T. and Fan, D.P. (1980) Dissociation and reconstitution of membranes synthesizing the peptidoglycan of Escherichia coli: lipid dependence of the synthetic enzymes. J. Biol. Chem. 255:2848-2854.

Talmadge, K.W., Keegstra, K., Bauer, W.D. and Albersheim, P. (1973) The structure of plant cell walls I. Macromolecular components of the walls of suspension-cultured sycamore cells with a detailed analysis of pectic polysaccharides. Plant Physiol. 51:158-173.

Tanaka, M. and Uchida, T. (1979) Heterogenity of hydroxyproline-containing glycoprotein in protoplasts from a Vinca rosea suspension culture. Plant Cell Physiol. 20:1295-1296.

Thomas, D. des S., and Stanley, R.G. (1968) Glycerol activation of glucan synthesis in cell-free preparations from Lupinus albus. Biochem. Biophys. Res. Comm. 30:292-296.

Toci, R., Belaich, A. and Belaich, J.P. (1980) Influence of "Energization" on the binding of M protein with p-nitrophenyl α-D-galactopyranoside. J. Biol. Chem. 255:4603-4606.

Villemez, C.L. (1973) The relation of plant enzyme-catalyzed β-(1,4)-glucan synthesis to cellulose biosynthesis in vivo. In "Plant Carbohydrate Chemistry" Academic Press, New York, NY. pp. 183-189.

Wang, M.C. and Bartnicki-Garcia (1976) Synthesis of β-1,3-glucan microfibrils by a cell-free extract from Phytophthora cinnamomi. Arch. Biochem. Biophys. 175:351-354.

Wilkie, K.C.B. (1979) The hemicelluloses of grasses and cereals. Adv. Carb. Chem. Biochem. 36:215-264.

Williamson, F.A., Fowke, L.C., Constabel, F.C. and Gamborg, O.L. (1976) Labeling of concanavalin A sites on the plasma membrane of soybean protoplasts. Protoplasma 89:305-316.

Williamson, F.A., Fowke, L.C., Weber, G., Constabel, F. and Gamborg, O.L. (1977) Microfibril deposition on cultured protoplasts of Vicia hajastana. Protoplasma 91:213-218.

Willison, J.H.M. (1976) Synthesis of cell walls by higher plant protoplasts In "Microbial and Plant Protoplasts", Peberdy, J.F., Rose, H.H., Rogers, H.J., and Cocking, E.C., eds, Academic Press, London, pp. 283-298.

Willison, J.H.M. and Klein, A.S. (1981) Cell wall regeneration by protoplasts isolated from higher plants. In "Cellulose and other Natural Polymer Systems: Biogenesis, Structure and Degradation" Brown, R.M., Jr., ed., Plenum Press, New York, NY, (in press).

Updegraff, D.M. (1969) Semi-micro determination of cellulose in biological materials. Anal. Biochem. 32:420-424.

Zachowski, A. and Paraf, A. (1974) Use of concanavalin A polymer to isolate right-side-out vesicles of purified plasma membranes from eukaryotic cells. Biochem. Biophys. Res. Comm. 57:787-790.