STUDIES ON THE INTERMEDIATES INVOLVED IN HEMICELLULOSE BIOSYNTHESIS

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B.S., Kansas State University, 1970

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1972

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INTRODUCTION

Hemicelluloses are mixtures of predominantly neutral sugar heteroglycans and are common components of the cell walls of land plants. They may have some function connected with the wall properties of plant cells since compositional differences occur in hemicellulose during differentiation of plant cells. The precise mechanisms involved in hemicellulose biosynthesis are unknown. However, the participation of sugar nucleotides and glycosyltransferases in some type of membrane complex has been demonstrated.

Golgi dictyosomes apparently play an important role in hemicellulose biosynthesis but the exact nature of this role is unknown. Conflicting reports on the possible location of glycosyltransferases with this organelle in plant cells have appeared in the literature. Unequivocal evidence for the location and nature of intermediates involved in heteroglycan biosynthesis is lacking.

Recently, improved methods for the isolation of subcellular components from plant cells have become available. This study will be concerned with the chemical nature and distribution of component substances in these particulate fractions that are thought to be important in the biosynthesis of hemicellulose. It is hoped that this information will help to clarify the overall process of plant cell wall polysaccharide biosynthesis.

LITERATURE SURVEY

I. The Chemistry of Hemicellulose and Its Role in the Cell Wall.

The noncellulosic wall substances of plants consist of pectin and hemicellulose, of which the latter predominates (1). Both these substances consist mostly of heteropolysaccharides and although homopolymers do exist in each class, they are the exception. The classical definition of these two materials is based on solubility properties (2). Pectins are usually water soluble and consist of galacturonic acid containing polymers, while hemicelluloses may only be extracted by alkaline solutions and are made up of predominantly neutral sugars. Hemicellulose may be further subdivided into fractions A and B (1). Hemicellulose A precipitates upon neutralization of the alkaline extract and hemicellulose B, still dissolved, is precipitated from the solution by the addition of ethanol.

As would be expected, the above classification is clumsy due to overlapping solubility properties of polysaccharides in each fraction. Recent advances in the field now permit a classification of hemicellulose polymers based on their chemical constituents. Thus three classes of heteropolysaccharides can be supposed to comprise the hemicellulose group, polymers based on 1) a xylose backbone, 2) a mannose backbone and 3) a galactose backbone. This last group is sometimes isolated with the water soluble pectic substances, but they will be considered here as hemicellulose since they are neutral sugar polymers.

The general structure of xylans is that of a (1 4) linked xylose backbone with a degree of polymerization (DP) of approximately 150 to 200 units. Short terminal side chains are attached to the backbone (3) and true xylans, containing no side chains of other sugars, are very rare. In the

Gramineae family, xylans comprise the bulk of the hemicellulose present and are characterized by single L-arabinofuranose residues linked to the backbone through an (1 3) glycosidic bond (4). Some xylans have been isolated which contain arabinose side chains which are non-terminal (1). In this case, the arabinose is linked to the main chain and also via a (1 2) bond to the reducing end of another xylan chain. Occasionally, xylans are found in grasses which contain glucuronic acid or 4-O-methyl glucuronic acid residues linked through an (1 2) glycosidic bond. Xylose itself is sometimes attached to the backbone by a (1 2) link. Also, galactose is sometimes detected in purified xylan preparations (3), but its location in the molecule is unknown.

Wood xylans are characterized by the presence of glucuronic acid or its 4-O-methyl derivative as the principle side chain component, in addition to arabinose (3). Often the acid moiety is acetylated (4). Some wood xylans have been isolated which contain arabinose side chains with a DP of 2 to 6 units.

Polymers based on a (1 4) linked mannose backbone comprise the second group of hemicellulose molecules. True mannans are occasionally found as food reserves in seeds and nuts (4). Glucomannans are simply copolymers of glucose and mannose, in the ratio of 1:2 respectively, that are linked by a (1 4) glycosidic bond and having a DP of about 200. Softwood glucomannans also have traces of galactose residues that are linked to the copolymer backbone through an (1 6) bond. Glucomannans are the characteristic hemicellulose component of all woods, but they are also found in grassy plants. These copolymers have been shown to be true heteroglycans rather than mixtures of closely related mannans and glucans (3).

Galactoglucomannans are found only in gymnosperms and are simply a

glucomannan copolymer backbone with galactose side chains joined by a (1 6) glycosidic bond. These hemicelluloses have a DP of around 100 with a ratio of galactose, glucose and mannose of 1:1:3 respectively. Occasionally, glucose and galactose are also found as side chains of the backbone (3). It has been noted that once these galactoglucomannans are isolated from the plant cell wall, they may be water soluble.

Macromolecules consisting of a galactose backbone comprise the third group of hemicellulose molecules (1). The D-galactopyranose residues are linked through a (1 3) glycosidic bond. In larch wood, each galactose has a side chain of either a galactose or an arabinose oligosaccharide linked to the backbone by a (1 6) bond, thus making a highly branched arabinogalactan or galactan molecule. These polysaccharides are common in all land plants, and are especially abundant in the pine family.

The role of hemicellulose in plant cell walls may best be understood by studying the specific nature and placement of the hemicellulose molecule in the wall and also its relationships with the other components present in the cell wall. The cell walls of most land plants typically consist of cellulose microfibrils embedded in an amorphous arrangement of hemicellulose and pectin (2). Lignin may also be deposited around the complex especially in woods. Protein, both enzymatic and non-enzymatic in nature, is present also (5). The non-enzymatic protein, extensin, and its possible relationship to the matrix substances of the wall will be discussed later in this section. The primary function of the plant cell wall is to provide a supporting skeleton for the plant and to counteract the osmotic pressure produced by the cellular components on the plasmalemma. The wall may also have some function in disease resistance (5).

It has been shown that the composition of the hemicellulose of cells in various states of differentiation is very different, and that these differences are precisely and genetically controlled (6). The sugar composition of the various hemicelluloses is not the result of random effects. For example, in maize root cap cells (7), the hemicellulose synthesized there has a high percentage of fucose and galactose and is not found in other parts of the plant. In the same plant (8), small but precise changes occur between the hemicellulose of meristematic cells and the slower growing cells just behind them with respect to the relative amounts of arabinose and xylose in the hemicellulose polymers. From these observations, it could be predicted that changes in the composition of the hemicellulose molecules of the wall may alter the physical properties and functions of the plant cell wall (5).

Hemicellulose is usually found in the primary and secondary walls of the plant cell. Pectin, cellulose and hemicellulose are all found in the primary wall layer but during biosynthesis of the secondary wall, which is primarily cellulose, the thickness of the wall and the chemical composition of the hemicellulose that is laid down changes (2). No pectin is deposited in the secondary wall layer. The exact physical location and relationship of hemicellulose with the other substances of the wall remains unknown. To clarify the exact role of specific hemicellulose molecules in the wall of the plant cell, it will be necessary to elucidate the above facts.

Recently (9), it was unequivocally shown that plant cell walls contain a protein that is rich in hydroxyproline residues. Further studies indicated that this protein, called extensin, plays an important role in the structure, growth and differentiation of the cell wall. It may serve to cross-link some of the polysaccharides of the wall to form a sort of network similar

to the proteoglycan structure present in bacterial membranes. Cellulose microfibrils, however, do not appear to be chemically associated with this protein. After extraction of the cell wall with alkali (9), this protein can be shown to have oligosaccharides of galactose and arabinose attached to the hydroxyl group of the hydroxyproline residues of the extensin. A polysaccharide-protein complex has been isolated from the cytoplasm of sycamore leaf suspension cultures (10). The purified complex consisted of 95% polysaccharide and 5% protein and was postulated to represent the precursor of the polysaccharide-extensin cell wall complex.

It has been proposed (10) that the protein-polysaccharide network of the cell wall has a role in wall expansion and differentiation but precise mechanisms of the nature of this role remain unknown. According to the above hypothesis of cell wall structure, cleavage of certain base labile glycosidic bonds several sugar units away from the protein-polysaccharide linkage would release the hemicellulose molecules (10). This would help to explain the anomaly of extracted hemicellulose molecules being soluble in water but requiring alkaline conditions for extraction. Thus, while specific details are almost entirely lacking, a general outline for the role of cell wall heteroglycans, specifically hemicellulose, is emerging.

II. The Chemistry and Control of Hemicellulose Biosynthesis.

In Figure I, the metabolic transformations believed to be involved in precursor formation for plant cell polysaccharide biosynthesis are shown. In vivo, the two principle carbon sources appear to be glucose and sucrose (11, 12). However, diphosphonucleotides of other sugars are also produced via the appropriate pyrophosphorylase and thus may also serve as carbon sources.

Most of the enzymes responsible for the interconversions of the sugar nucleotides have been demonstrated (13, 14, 15). The epimerases, dehydrogenases and decarboxylases are all quite specific for only one type of sugar nucleotide, and they require that the sugar be esterified to a certain nucleotide (14). Although UDPG has been shown to predominate in most plant tissues, GDPG and ADPG were present in low but significant quantities after short term incubation with U-^{14}C-glucose (16).

The involvement of sugar nucleotides as intermediates in polysaccharide biosynthesis in general was postulated by Leloir as a result of his studies on UDPG (13, 17). The sugar nucleotide serves as a "high energy" intermediate in the biosynthetic process so that the transferase reaction will be thermodynamically favorable (14). Indeed, the free energy of hydrolysis of UDPG is -7700 cal/mole.

The existence of the branch of the pathway shown in Figure I, involving myo-inositol, was first postulated by Loewus (18, 19). He observed that certain sugar residues of hemicellulose and pectin of parsley leaves became labeled after incubation of the leaves with ^{14}C-myo-inositol. Also, ^{14}C labeled glucuronic acid supplied to the leaves gave a similar labeling pattern. Later (20), using myo-inositol labeled in a specific position, it was established that the 1-6 bond of the myo-inositol ring was cleaved and subsequently oxidized to glucuronic acid. Preliminary evidence (20) for the cyclization of glucose-6-phosphate to form myo-inositol-1-phosphate was later confirmed with the isolation of an enzyme system that catalyzed this reaction (21). Additional evidence has been obtained showing that myo-inositol and glucuronic acid follow a common metabolic pathway to the acid and pentose units of pectin and hemicellulose of Zea mays root tips (22). Apparently, once

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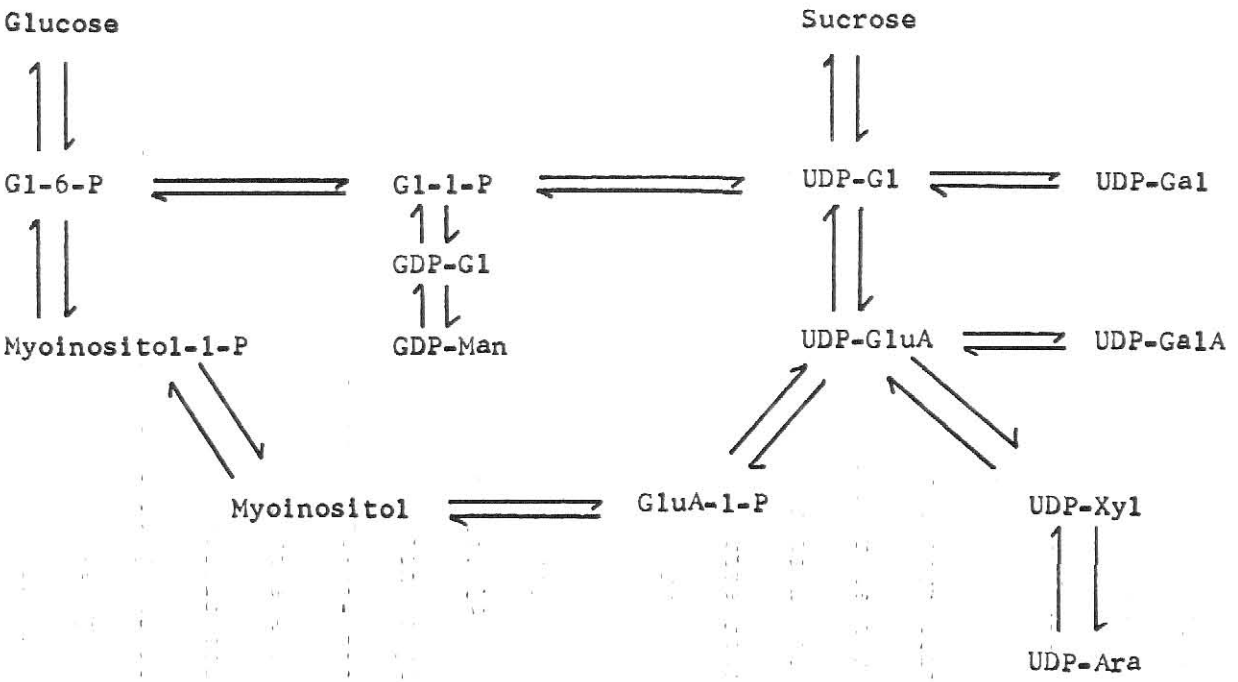


Figure I.

Sugar transformations involved in precursor formation in plant cell heteroglycan biosynthesis.

myo-inositol is formed, it cannot be converted back to glucose-6-phosphate. The site of the metabolic transformations involving myo-inositol in the cell is unknown. As will be shown later, other sugar nucleotide transformations appear to occur in a particulate fraction of the cell.

Additional evidence for the existence of the pathways presented in Figure I is provided by labeled sugar uptake studies using seedlings of various grassy plants. When ^{14}C-L-arabinose is supplied to corn root tips over a period of one to two hours, a xylan containing radioactive arabinose and xylose residues could be isolated (23). The radioactivity of ^{14}C-glucuronic acid or ^{14}C-galacturonic acid was incorporated by mung bean shoots into the pentoses and uronic acid units of hemicellulose (24). When ^{14}C-mannose was supplied to corn roots (25), only the L-galactose, mannose and fucose residues of extracted hemicellulose were labeled.

These studies indicate the existence of a pool of sugar nucleotide precursors. The mannose obviously did not equilibrate with the hexose phosphate pool but was immediately epimerized or dehydroxylated and incorporated into polysaccharide after esterification to a nucleoside. In the above studies with roots, it was noted that little activity found its way into protein or lipid over short periods of time. This would indicate that once the glycosyl residue was esterified to the diphosphonucleoside, it was committed to polysaccharide biosynthesis, and that roots are particularly suited to the in vivo study of polysaccharide biosynthesis because of their exclusive, extremely rapid heteropolymer biosynthetic capability.

The involvement of lipid intermediates in heteropolymer biosynthesis in bacteria is well documented (26, 27, 28). In the biosynthesis of the O-antigen portion of the lipopolysaccharide coat of Salmonella bacteria, the

trisaccharide repeating units are polymerized on a lipid carrier and then transferred to the growing chain by a transglycosylation step to the reducing end of the polymer. The peptidoglycan network of the E. coli membrane is synthesized in a similar manner (29). The existence of lipid intermediates in some animal polysaccharide biosynthesis has also been demonstrated (30).

Early evidence (31) for the possibility of the involvement of lipid intermediates in plant cell wall polysaccharide biosynthesis is now being confirmed. Using a particulate fraction from a plant cell preparation that will synthesize a (1 4) glucan from UDP-^{14}C, Villemez and co-workers have extracted two radioactive glucose containing compounds with lipid solvents (32). The distribution of these compounds in a series of particulate fractions separated by centrifugation is similar to that of the glucan synthetase activity. Starch grains containing the enzymes necessary for starch synthesis (33), yield a labeled steryl glucoside and a labeled acyl steryl glucoside after incubation with labeled glucose.

Kauss was able to extract a radioactive mannolipid from a particulate preparation from mung bean seedlings after incubation with UDP-^{14}C-mannose (34). It was postulated that the lipid portion was polyisoprenoid in nature, similar to the bacterial lipid involved in heteroglycan biosynthesis. The mannolipid compound had some transfer potential since the ^{14}C-radioactivity present in it could be chased by the addition of "cold" UDP-mannose to the incubate. Working with the same preparation, Villemez has isolated a mannolipid, confirming Kauss' work, and also a glycoprotein containing a labeled oligosaccharide of mannose (35, 36). The participation of the glycoprotein in hemicellulose biosynthesis was not demonstrated. Indeed, none of the intermediates mentioned above has been positively shown to directly participate

in hemicellulose biosynthesis.

A proposed cytoplasmic precursor to the cell wall of pollen tubes of Tradescantia has recently been isolated and partially characterized as a UDP-polysaccharide complex (37). This however is the only demonstration in the literature of such a nucleotide polysaccharide complex and its proposal as a cell wall precursor is also novel.

The classic example of the process of assembly of sugars to form a polysaccharide is that of starch or glycogen synthesis (38, 39). The chains grow by the addition of glucose from its nucleotide ester by an (1 4) glycosidic bond to the non-reducing end of the polymer. The process is catalyzed by a specific glucosyltransferase.

Heteroglycan biosynthesis appears to be more complicated. The only case in which the assembly process for such a macromolecule is clear is the above mentioned bacterial O-antigen heteropolymer synthesizing system. The transfer of different sugars from the nucleoside diphospho-sugar to the growing oligosaccharide-lipid complex is mediated by glycosyltransferases that are highly specific for both the sugar being transferred and the acceptor sugar on the growing oligomer. The oligomer repeating unit is then added to the non-reducing end of the growing heteropolymer by another specific transglycosylase (27). Multiglycosyltransferase enzyme systems involved in glycoprotein and glycolipid synthesis in animal cells have also been described (40).

If plants synthesize heteropolysaccharides in the same manner as bacterial or animal cells, a number of specific transferases should be found. However, two problems have hampered success in this field. First, the plant cell transferases' inherent instability, and the inability to solublize the active

transferases from the particulate polysaccharide synthetase systems has made their study difficult. Second, little exact knowledge is available on the molecular architecture of any one hemicellulose molecule. It is unknown whether there is a specific repeating unit in it or even whether a specific transferase is actually required.

The control of hemicellulose biosynthesis appears to be mediated at several levels. By studying Figure I, it can be observed (4) that modification of the operation of the myo-inositol branch of the pathway can be used to control the levels of uronic acids and pentoses present relative to that of the hexoses. Also, the myo-inositol pathway negates the effect of feedback control by UDP-xylose on UDPG dehydrogenase (15) by completely bypassing this step. Of course, control of the synthesis of the various epimerases and other sugar nucleotide modifying enzymes will have an effect on the supply of certain sugars (41, 42, 43). Also, product and feedback inhibition of the glycosyltransferases serve as other points of control of the system (44).

Auxins are well known plant growth regulators (45) and have been shown to cause changes in the sugar composition of polysaccharides of oat coleoptile cell walls. Recent evidence (46) suggests that at least one of the effects of indoleacetic acid is to modify either the activity or supply of glycosyltransferases. Indoleacetic acid did not appear to affect UDPG pyrophosphorylase, nucleosidediphosphokinase or UDPG dehydrogenase in this plant.

The precise molecular mechanisms for the control of hemicellulose compositions and biosynthesis however, must await further progress in the knowledge of the structural nature of hemicellulose and the nature of the glycosyltransferases.

III. Properties of In Vitro Hemicellulose Synthetase Systems.

A number of particulate systems, from homogenates of various plant tissues, have been isolated which will synthesize hemicellulosic and pectic substances from sugar nucleotides (11). Seedlings of various plants were usually used as the source of enzyme. Typically, the plant tissue was ground in sand with a phosphate buffer, various salts, sucrose and bovine serum albumin (BSA), strained and then centrifuged at 4° C. The particles sedimenting between 4,000 and 30,000 x g were used as the synthetase system. The identity or cytoplasmic origin of the particles containing the polysaccharide synthetase is the subject of much controversy (10) and will be dealt with in a later section of this review.

Early workers, using asparagus shoots or immature corn cobs as enzyme sources (47, 48) were able to demonstrate the transfer of xylose from UDP-^{14}C-xylose to a growing macromolecular acceptor present in the isolated particles. The acceptor was later partially identified as a xylan. Some radioactive arabinose was detected in an HCl hydrolyzate of the preparation indicating the presence of a xylosyl epimerase in the particles. Xylose-1-phosphate could not function as a donor (47), demonstrating the absolute requirement of the glycosyltransferase for sugar nucleotide donors. The system also requires the uridine base in the sugar nucleotide. Product inhibition of the glycosyltransferase activity by UMP and UDP was also shown (44). In addition, AMP and GMP inhibited the enzyme although to a much lesser extent. The product of the transglycosylation reaction was partially characterized and shown to be similar in composition to authentic immature corn cob xylan isolated by conventional techniques.

A particulate preparation from mung bean seedlings has been shown to

contain an enzyme capable of transferring the mannose from GSP-^{14}C-mannose to a product partially characterized as a glucomannan. Other nucleoside bases would not function as donors (49, 50). Upon HCl hydrolysis of the enzymatic product, some labeled glucose was detected, again demonstrating the presence of an epimerase in the particles. This same preparation (51) will synthesize a glucomannan from GSP-^{14}C-mannose and GDP-^{14}C-glucose; but the mannonucleotide is only utilized in the presence of the glucose donor, indicating some sort of strict specificity of the transferase system to form the glucomannan. This system will also utilize UDP-^{14}C-galactose to form a galactan. The galactosyl transferase (52) activity in this preparation appears to be a function of the age of the seedlings from which it is extracted, indicating that either the specificity or supply of the transferase is altered during growth.

Studies have been performed on the metabolism of UDP-glucuronic acid in the particulate preparation obtained from immature corn cobs mentioned previously. Besides being incorporated into endogeneous hemicellulose (53), the radioactive glucuronic acid was transformed to UDP-xylose, UDP-arabinose and UDP-galacturonic acid since they were present in the preparation after incubation with UDP-^{14}C-glucuronic acid. Therefore, decarboxylation and epimerization enzymes were associated with the particles.

It has also been shown that the methyl group, attached to the 4-hydroxy group of the glucuronic acid unit in some hemicelluloses, is donated by S-adenosyl methionine (SAM) (54). Nucleotide bound 4-O-methyl glucuronic acid would not serve as an effective donor to hemicellulose, indicating that the SAM may donate its methyl group to a macromolecular acceptor. It was subsequently shown (55) in P. aureus preparations, that the mode of biosynthesis

of the 4-O-methyl glucuronic acid unit of hemicellulose is the same regardless of the type of polysaccharide to which the glucuronic acid is attached. In this particular instance, it was shown to be a galactan; in the previous case, it was a xylan.

Pectic materials appear to be synthesized by the same preparations that have been shown to contain the enzymes necessary for hemicellulose biosynthesis. Thus, polygalacturonic acid is synthesized from UDP-galacturonic acid by particulate preparations from mung bean seedlings (56, 57). The introduction of methyl ester groups to the pectin is accomplished by the use of SAM as the methyl donor (58).

Kauss has demonstrated that the methyl esterified pectin is synthesized inside a lipid membrane bound vesicle (59). This is based on two observations. First, the methyl transferase will not esterify exogeneous polygalacturonic acid. Second, when methyl esterase was present in the incubate, no differences in the amount of incorporation of ^{14}C-methyl-SAM into endogeneous macromolecular product were observed over that of the control incubate. If the integrity of the particles was disturbed by Triton X-100, sodium dodecyl sulfate or phospholipase A, the methyl ester previously synthesized was desterified by the methyl esterase in the incubate. Although shown only for the pectin synthetase, it may be inferred (59) that other synthetase systems responsible for cell wall polysaccharide biosynthesis may be associated with and dependent on some type of lipid membrane complex.

The particulate systems mentioned above are all characterized by their instability at room temperature over short periods of time. Even at -20° C., 60-70% of the transferase activity is lost in five days (44). Also, it is not possible to solublize the glycosyltransferase activity by using such

Triton X-100, lipid solvents or by sonication.

IV. Biochemistry and Role of Golgi Apparatus in Hemicellulose Biosynthesis.

The existence of the golgi dictyosome complex and the details of its structure have only recently been confirmed by the use of the electron microscope (EM) (60, 61, 62). The structure appears in the cell as made up of stacks of flattened, highly fenestrated disks called cisternae. Projecting from the central portion of the cisternae are highly branched, anastomosing tubules (60) and is called the mature face of the golgi.

The golgi apparatus (GA) and its associated structures are found in almost every type of eukaryotic cell, but they appear to be especially active in cells possessing a specialized secretory function (62). In general, the role of the golgi complex seems to be that of secretion or packaging of cellular products (61, 63). Based on the histochemical staining reactions of the golgi, other investigators (64) prefer a generalized function of endomembrane transformation for this organelle. The forming face of the GA, next to the endoplasmic reticulum (ER), stains similar to the ER while the mature face stains like the plasma membrane (PM) of the cell.

To further characterize the function of the golgi, enzyme and chemical composition studies of the isolated organelle have been made. Classically, (61, 65) histochemical studies have revealed the presence of various phosphohydrolases, but little else. The success of enzymatic studies on isolated fractions of GA depends largely on the purity of the membrane preparation. Gluteraldehyde stabilization is necessary (66) for the effective isolation of GA as intact structure by conventional means from plant tissue. But since gluteraldehyde could give extraneous results by nonspecifically binding exogeneous protein to the structure (67), its use is precluded for

enzymatic studies. This situation has hampered progress in the field but some work has been done. Little work has been done on the chemical and enzymatic composition of plant cell GA but it may be (68) that some of the results from animal tissue GA studies are applicable to plant cell GA as well, since GA of animal cells are relatively easy to isolate in significant amounts.

Morre' and Mollenhauer (69, 70, 71, 72) have done most of the work of relating the enzymatic composition to the purity of subcellular fractions obtained from rat liver tissue using discontinuous sucrose gradients. Assaying four subcellular fractions, which included the GA, ER, PM and mitochondria, for 22 different phosphohydrolases and 3 oxidoreductases, they found that the phosphohydrolytic activity was concentrated two to three times in the GA over that of the homogenate, but it was not exclusively located in the GA. Using the EM, they concluded that the golgi band was contaminated to the extent of about 20%.

Also using a discontinuous sucrose gradient, Lelavanthi et al (73) isolated a golgi band from rat liver. This fraction was contaminated with 2% of the glucose-6-phosphatase of the ER and 12% of the AMPase of the supernatant on a protein basis. For the first time a marker enzyme was tentatively assigned to the GA fraction. It contained approximately 90% of the UDP-galactose: N-acetyl glucosamine transferase activity of the homgenate. This same enzyme has been shown to be exclusively localized in isolated GA by other workers (74, 75) and its significance will be discussed later.

Recently, the purity of subcellular fractions from wheat root tip cells was made on the basis of several enzymatic activities, among them were

sucrose synthetase and UDPG hydrolase (76). These comparisons, along with EM observations showed that the GA fraction was purified to approximately the same extent as those prepared from rat liver discussed above. However, little glycosyltransferase activity was located in this fraction.

Some recent chemical and enzymological studies have indicated that the GA of rat liver is well equipped for endomembrane transformation functions (77, 78). The relative amounts of lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine were constant among the GA, ER, and PM preparations. However, the GA was intermediate in its content of sphingomyelin (it being much more concentrated in the PM) and in phosphatidylcholine, which was much more concentrated in the GA and ER than in the PM. In later work (75) it was shown that sphingomyelin is required for optimum 5'-nucleotidase activity of the PM.

There were also distinctive differences in the fatty acid composition of the phosphatidylserine, phosphatidylinositol and lysophosphatidylcholine among the three subcellular fractions with the GA always being intermediate with respect to the frequency of occurrence of each type of fatty acid. There was always a significant increase in the unsaturated fatty acid composition of the GA over that of the ER indicating some type of selective incorporation or exchange of these fatty acids in the golgi. Studies (79) of the effects of extracting the lipid of isolated fractions on the enzymatic activities of that fraction showed that the activity was reduced or lost; however, it could be partially restored upon addition of the extracted lipid.

Enzymes involved in phospholipid metabolism in animal cells and their association with various subcellular fractions have been studied. Van Golde et al (80) found that among others, acyl CoA: 1, 2-diacyl-sn-glycerol

acyltransferase was located only in the ER and rough ER. the golgi possessed most of the phospholipase A_1 and A_2. Choline kinase and phosphatidylcholine-cytidyl transferase were present in golgi preparations from rat liver (81) with the latter enzyme having the higher specific activity. It was concluded that the GA possesses the capability to introduce changes in the fatty acid composition of the phospholipids present and were also capable of de novo synthesis of lecithin and perhaps other phospholipids.

The distribution of lecithin biosynthetic enzymes in onion stem sub-cellular fractions has been reported (68, 82). The phosphoryl choline:cytidyl transferase activity in the organelles of these cells is predominantly located in the golgi, however significant amounts of this enzyme were also detected in the ER. Using in vivo uptake of lipid precursors (83), it has been shown that golgi were labeled more quickly and extensively than other cell fractions. This would seem to implicate this organelle in some manner to phospholipid metabolism. This is important since the enzymatic activities of many membrane systems are dependent on lipids and also because the membrane structure itself is dependent on phospholipids (4).

The fact that GA, ER, PM and mitochondria of rat liver cells have unique protein compositions was demonstrated by the use of disc gel electrophoresis. Some of the proteins were present in all fractions, but each had several proteins unique to it (84).

Also, ubiquinone and vitamin K (85) have been detected in rat liver golgi preparations. It appears to be concentrated there and then packaged for secretion.

Glycosyltransferase activity appears to be localized quite specifically in the golgi of a variety of animal cells. Uridine diphosphogalactose:

N-acetyl glucosamine transferase activity has been shown to be present in the golgi of mammary gland and liver tissues of rats (75, 74, 73, 86). Golgi have also been found to contain quite specific sugar nucleotide glycosyltransferases involved in glycoprotein biosynthesis (65, 75, 87, 88).

Unfortunately, no such consensus of opinion on the location of glycosyltransferase enzymes in plant cell organelles has yet emerged. Albersheim and his co-workers (89), using particulate preparations from finely chopped (razor blade) onion stems, demonstrated that the synthetase catalyzing the transfer of a variety of sugars from their nucleotides to a growing polysaccharide, was almost exclusively located in a very heavy particle. Examination of the fractions with the highest synthetase activity with the EM revealed no intact cells. It was concluded that the active particles were plasmalemma fragments. Just recently (76), these results were confirmed by centrifugation of a particulate preparation from wheat root tips on an essentially linear sucrose density gradient. Polysaccharide synthetase activity was localized almost exclusively in a very heavy particle. The golgi fraction isolated by this method had very little of the polysaccharide synthetase activity.

In direct contrast, Ray et al (90) found that particles from pea seedlings possessing a UDPG: glucan transferase activity sedimented on an isopycnic gradient to a density characteristic of golgi membranes. Good EM evidence is lacking however.

Recent investigations of crude preparations of golgi of plant cells indicate the possibility of the presence of a hemicellulose like substance. In these experiments, roots of wheat (16) or corn (91) were incubated with labeled glucose over a period of several hours, and a golgi preparation obtained. An acid hydrolyzate of the washed particulate preparation yielded

labeled sugars characteristic of hemicellulose.

Autoradiographic evidence for the role of golgi dictyosomes in animal cells has virtually established its function as the secretory and packaging organelle of the cell. Golgi also function as a place of assembly and modification of various materials. The goblet cell of rat liver secrets a mucous type of glycoprotein into the blood. Neutra and Leblond (92, 93) firmly established the role of golgi dictyosomes in this cell using pulse chase techniques. They followed the synthesis of the protein on the rough ER and its transport to the golgi for the addition of carbohydrate material. The product is blebbed off (94) the top or mature face of the dictyosome stack inside a secretory vesicle formed from the golgi membrane itself.

Subsequent autoradiographic evidence has implicated the golgi in the secretion of the mucous material forming the cell coat of intestinal cells (95), a part in mucopolysaccharide synthesis and secretion in chondrocytes (96), the addition of glucosamine to lipoprotein destined for secretion by liver cells (97, 98) and in the regeneration of the plasma membrane of amoeba (99). These studies have also shown that the golgi apparatus of certain algae are involved in scale formation (100).

Using pulse chase autoradiography, Northcote and Pickett-Heaps (101) showed, in the root cap cells of wheat seedlings, that within five minutes the label is taken up almost exclusively into the golgi and subsequently transported across the plasmalemma to the slime layer of the cell wall. Upon acid hydrolysis of the cell wall material, it was found that almost all of the label taken up by the root tips was present in sugars characteristic of cellulose and hemicellulose. In the meristem segment behind the root cap cells, little localization of the radioactivity was found in the golgi

although it was present in the wall after longer periods of incubation in the tritiated glucose. It was also noted that sometimes the polysaccharide was secreted from the root cap cells and not deposited at the wall (102).

Working with highly differentiated plant cells where wall material deposition occurs at a very slow pace, it is possible to label the GA but the amount is always low (103, 104, 105). It has been proposed that golgi derived vesicles are involved in the biosynthesis of polysaccharides during transport to the cell wall and that once at the wall, the vesicles serve to insert the new wall material into the wall structure (106, 107, 108, 109). Since the golgi itself is seldom labeled in uptake studies on meristematic cells of roots, it may also be that the labeled glucose in these cells is taken up and incorporated into secretory vesicles at a slow rate, making it harder to "catch" on the autoradiograph (108).

Recently developed (106) histochemical methods for locating polysaccharide material, showed a progressively greater reactivity across the developing dictyosome stack which increases as the vesicles form from the golgi and move through the cytoplasm. These results could be explained if the vesicles contained a polysaccharide of very low molecular weight which is polymerized in the vesicles during transport to the cell plate or wall. These observations have been confirmed by other workers in a different plant (109).

The role of the golgi in the formation of the cell plate at mitosis is well documented (63, 110, 111). Microtubules also seem to be involved in promoting the direction of flow of the golgi derived vesicles after the first vesicles fuse to form the cell plate. However, golgi do not seem to be involved in the formation of cellulose microfibrils (112). Autoradiographic evidence indicates that cellulose is synthesized by an organized enzyme

system present on the outside of the plasmalemma of the cell. It has been proposed that these enzyme systems may be deposited at the plasmalemma by secretory vesicles derived from the golgi dictyosomes.

MATERIALS AND METHODS

MATERIALS

I. Wheat: Untreated wheat seeds, variety Shawnee, were obtained from the Kansas State University Agronomy Farm.

II. Radioactive Material: $KH_2{}^{32}PO_4$, specific activity of 500 mC/mM, was obtained from New England Nuclear, Boston, Massachusetts. U-^{14}C-glucose, specific activity of 240 mC/mM was obtained from Schwarz Bio-Research, Orangeburg, New York.

III. Enzymes: Hemicellulase, control number 7196, and pectinase, control number 6417, were obtained from Nutritional Biochemicals Co., Cleveland, Ohio. Pronase, B grade, 45,000 Pu/gr., lot number 46046, was obtained from Calbiochem, Los Angeles, California.

IV. Thin Layer Plates: Precoated silica gel thin layer plates, thickness 0.25 mm, were obtained from Brinkmann Instruments, Inc., Westbury, New York.

V. Chemicals: All chemicals used were of the highest reagent grade available.

METHODS

I. Preparation of Seedlings.

Wheat seeds were soaked in a 0.5% solution of formaldehyde for approximately 45 minutes. This was followed by a one to two hour washing procedure using distilled water. The seeds were then germinated at room temperature by placing the seeds in a single layer between two, 47 cm x 32 cm rust proof window screens. The screens were covered on both sides with wet Whatmann 3MM filter paper in a 48 cm x 33 cm x 2 cm tray with glass beads (3 mm diameter) between the bottom filter paper and screen. The entire assembly was covered by aluminum foil to prevent evaporation. Only the roots of the seedlings grew through the bottom screen and were easily removed by the use of a razor blade (76).

II. Radioactive Metabolite Incubation.

After 60-64 hours of germination, the wheat seed-screen apparatus was transferred to the incubation medium arrangement. This consisted of a Whatmann 3MM filter paper impregnated with the appropriate incubation medium, sandwiched between two sets of wheat seedling-screen assemblies with the roots in contact with the filter paper. The arrangement was placed in a tray and covered with aluminum foil. The Whatmann 3MM filter paper, cut to the size of the screens, had been soaked with approximately 40 ml of an incubation medium containing 1 mM potassium phosphate buffer, pH 6.5 and 10 mM glucose with 75-100 uC of U-^{14}C-glucose. Four sets of screens were usually used for each preparation. The incubation was allowed to proceed for two hours and the uptake of ^{14}C activity was about 50% in this time. For the $KH_2{}^{32}PO_4$ incubation, the glucose was omitted from the above incubation medium and a

total of 500 uC of ^{32}P was used. This incorporation was allowed to proceed for one hour.

III. Isolation of Particulate Fractions.

This isolation procedure was similar to that developed for wheat roots by Brown (76). The etiolated root tips (approximately 20-30 grams wet weight) were ground in approximately 25 ml of homogenization medium composed of 1% dextran, 1 mM calcium chloride, 75-100 mM gluteraldehyde, 0.5 M sucrose and stock buffer, which consisted of 0.0001 M manganese chloride and 0.1 M sodium phosphate buffer, pH 7.2. The roots were ground approximately 4 grams at a time in a glass-teflon homogenizer with a clearance of 0.026 inches. The homogenate was squeezed through several layers of cheesecloth into a beaker at 0-4° C. All operations of the isolation procedure were performed at 0-4° C.

The filtrate was then transferred to two 13 ml (1.4 cm x 8.9 cm) cellulose nitrate centrifuge tubes and spun for one hour at 5,000 rpm (4,800 x g) in a SW 41 rotor to remove debri and nuclei. All centrifugations were carried out in either a Beckmann L2-65 or L3-50 model ultracentrifuge. All sucrose solutions used in this isolation procedure were made with stock buffer. The supernatant was then transferred to another 13 ml cellulose nitrate tube and a 0.5 ml 1.8 M sucrose pad carefully layered underneath the supernatant. The tube was centrifuged for one hour at 15,600 rpm (43,500 x g). A band of particulate material was collected on top of the sucrose pad.

After removing and discarding the supernatant, the particulate material was removed and resuspended in 2 ml of stock buffer. Approximately 10 ml of 0.5 M sucrose solution was layered underneath the resuspended material and another 0.5 ml 1.8 M sucrose pad was layered underneath the entire solution. The tube was again centrifuged for one hour at 15,600 rpm (43,500

x g). The particulate band sedimenting on top of the sucrose pad was designated as "washed crude golgi".

This particulate material was then resuspended in approximately 1.5-2.0 ml of stock buffer and layered on top of a discontinuous sucrose gradient in a 6 ml (1.3 cm x 5.1 cm) cellulose nitrate centrifuge tube. The sucrose gradient consisted of 0.5 ml of 1.5 M sucrose, 1.0 ml of 1.25 M sucrose, 1.0 ml of 1.0 M sucrose and 1.0 ml of 0.5 M sucrose. The gradient was spun in a SW 50L rotor for three hours at 35,600 rpm (102,000 x g). The four particulate bands that resulted on the now linear sucrose density gradient were designated as top, middle, bottom and pellet.

Work on the cytoplasmic origin of each band using the electron microscope has been reported (76). The top band consisted of golgi dictyosomes; the middle band was made up of microsomes with some dictyosomal contamination; the bottom band contained mitochondria and dense vesicles; and the pellet contained some mitochondria, heavy lysosomes and numerous unidentified dense fragments. These fragments were postulated to be derived from the plasmalemma of the cell.

IV. Scanning of Sucrose Density Gradient for Radioactivity.

Studies on the incorporation of ^{14}C or ^{32}P into the particulate fractions isolated were carried out by placing the gradient centrifuge tube into a plexiglass eluting device and piercing the bottom of the tube. Water was pumped into the top of the elution device at 0.25 ml per minute using an ISCO density gradient fractionator. One-tenth ml fractions were manually collected on glass fiber filter discs, thoroughly dried under a heat lamp and their radioactivity measured.

V. Chemical Extraction of Particulate Fractions.

For preliminary chemical characterization of the radioactivity of the bands obtained on the density gradient, the bands were carefully pipetted off the gradient and resuspended in 4 to 5 ml of stock buffer and pelleted in 6 ml cellulose nitrate centrifuge tubes. The pellet was transferred to a 5 ml glass Sorvall centrifuge tube. Each band, excluding the pellet was treated as follows. A 0.5 ml 1.8 M sucrose pad was layered underneath the resuspended particulate bands and centrifuged in a SW 50L rotor for one-half hour at 40,000 rpm (160,000 x g). The sedimented material was removed and placed in a 5 ml glass Sorvall centrifuge tube.

All bands were extracted at 0-4° C. A 2.25 ml mixture of methanol, chloroform and formic acid (12:5:0.04 v/v) (113) was added and the tubes shaken for 30-40 minutes. One-half ml of water and 0.3 ml chloroform were added to each tube, shaken immediately and spun for 10 minutes at 5,000 rpm in a SE-12 Sorvall rotor using a Sorvall centrifuge, to separate the layers. The aqueous and chloroform layers were then removed from the tube. The insoluble material remaining was treated with 0.05 M KOH for 20-30 minutes at 100° C. and vacuum filtered. The filtrate was neutralized with a small amount of concentrated KH_2PO_4 solution to a pH of around 7.0. All fractions were stored at -25° C. for later study.

In studies on the chemical identification of the radioactive substances in the particulate material, only the washed crude golgi was prepared and transferred to a 5 ml Sorvall centrifuge tube and resuspended in 4 ml of stock buffer. The tube was centrifuged in a SE-12 Sorvall rotor at 10,000 rpm for 30-40 minutes. The supernatant was removed, designated Supernatant III and frozen for further study.

The particulate material was extracted with 2.5 ml of methanol, chloroform and 0.2 M formic acid (12:5:3 v/v) (113) for 30-40 minutes. The mixture was centrifuged at 5,000 rpm for 10 minutes on a SE-12 rotor. The supernatant was made biphasic by the addition of 0.7 ml of water and 0.5 ml of chloroform. After the layers had separated, they were removed and stored at -25° C. for further investigation. The insoluble material that had sedimented in the centrifuge tube was treated for 20-30 minutes with 0.05 M KOH at 100° C. After removal of the remaining insoluble material by vacuum filtration, the filtrate was neutralized with KH_2PO_4 and stored at -25° C. for further use.

VI. Purification and Assay of Hemicellulase and Pectinase.

Hemicellulase and pectinase were purified according to the following procedure. Five grams of the enzyme were dissolved in a minimum amount of distilled water and precipitated by saturating the solution with ammonium sulfate at 0-4° C. The enzyme was collected by centrifugation in 25 ml plastic nylon centrifuge tubes in a SS-34 rotor at 10,000 rpm. The enzyme was redissolved, precipitated and collected as before. The enzyme was again redissolved in a minimum amount of water and dialyzed against 2 liters of sodium acetate buffer, pH 5.0 overnight.

The hemicellulase was checked for activity with a hemicellulose A and B preparation, polygalacturonic acid and denatured bovine serum albumin (BSA). The assay was performed as follows. The incubation mixture consisted of 0.5 ml of enzyme and 0.5 ml of substrate (0.5% solutions of hemicellulose or 1.2% solution of polygalacturonic acid). At 1, 3, 7, 12 and 24 hours, a small aliquot of the incubation mixture was spotted on a silica gel thin layer plate and developed for monosaccharides as described in the thin layer chromatography methods section. It was found that hemicellulase

released xylose, arabinose and some of the hexoses from hemicellulose. It released very little galacturonic acid from the polygalacturonic acid over the 24 hour incubation period.

After incubation of a 1% BSA solution with hemicellulase and separation of the high and low molecular weight products by Sephadex G-75 column chromatography, it was found that hemicellulase hydrolyzed 2-5% of the amino acids from the BSA as determined by a crude ninhydrin assay.

Pectinase was assayed for activity on polygalacturonic acid and on hemicellulose in the same manner described above for the hemicellulase assay. It was found that pectinase released a small amount of xylose and arabinose and very minor amounts of hexoses from hemicellulose at about one-third the rate of the hemicellulase. Pectinase released large amounts of galacturonic acid from polygalacturonic acid very quickly.

VII. Pronase Assay.

The activity of the pronase was checked by incubating 5 mg of denatured BSA with 1 mg pronase in 2 ml incubation mixture consisting of 1 mM potassium phosphate buffer, pH 8 and 0.02% sodium azide. The mixture was incubated at 37° C. for 50 hours. An increase in ninhydrin sensitive material was noted over the first 30-40 hours and it remained essentially constant thereafter.

VIII. Column Chromatography.

All Sephadex gel columns used in this study were prepared in the following manner. The beads were allowed to soak for 12 to 24 hours in a dilute salt solution at room temperature. After pouring off the fine material, a slurry was made and poured into the column with the aid of a glass rod. The column was then washed with either distilled water or 1 mM potassium phosphate

buffer, pH 8 for 12-24 hours thereafter. The following columns were prepared: an analytical 1.7 cm x 89 cm Sephadex G-25 column, an analytical 1.7 cm x 77 cm Sephadex G-10 column and a preparative 1.0 cm x 33 cm Sephadex G-75 column.

Fractions were collected from each column using an LKB Ultrorac Type 7000 fraction collector. The G-25 and G-10 columns were standardized using a solution of 0.5% each of glucose, maltose, raffinose, stachyose and dextran. The Sephadex G-75 column was standardized by eluting a solution of 0.5% each of glucose and dextran. The column effluents were monitored using a Pharmacia refractive index monitoring device. Figure II shows a representative output of the refractive index monitoring device of a five component separation used to standardize the G-25 column mentioned above.

IX. Thin Layer Chromatography.

The application and composition of each solvent used in this study for silica gel thin layer chromatography separations are shown in Table 1.

For separations of monosaccharides using Solvents I and II, the plates were prepared by first activating them at 120° C. for at least two hours and then dipping them in a 0.2 M Na_2HPO_4 for 5-8 seconds and dried. For other separations, the plates were prepared as above but omitting the 0.2 M Na_2HPO_4 dipping procedure.

The procedure for locating the radioactive material separated by the thin layer chromatography solvents was as follows. A 10-15 cm streak of the unknown radioactive material was applied 1.5 cm from the bottom of the plate using a capillary tube. One percent sugar standards were applied in 2 cm wide streaks to the rest of the plate and developed in the appropriate solvent. After drying, the area above the radioactive origin was divided into

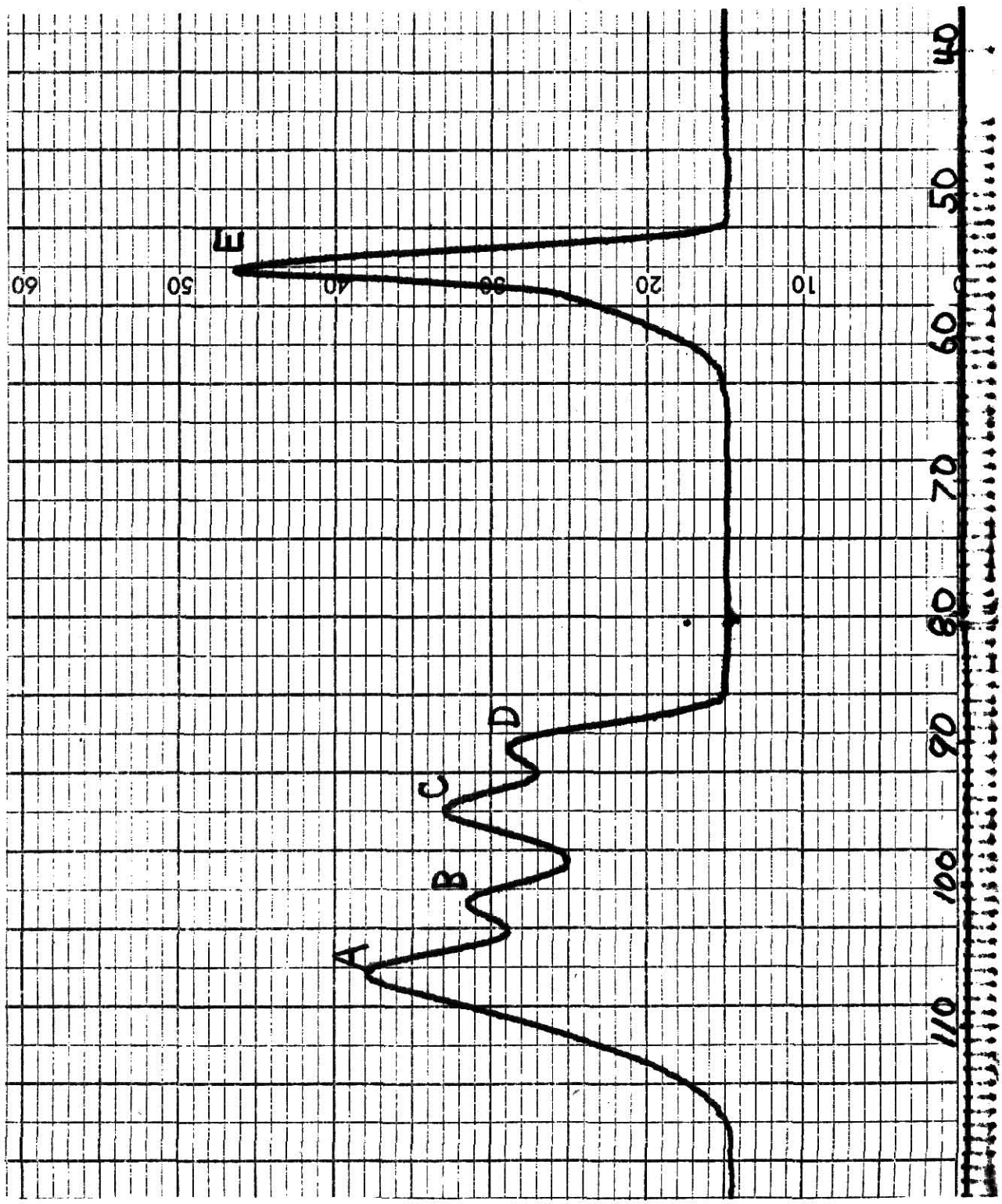
FIGURE II.

Standardization of Sephadex G-25 Column

Approximately 2 mg each of glucose, maltose, raffinose, stachyose and dextran (molecular weight 200,000) were applied to a 1.7 cm x 89 cm Sephadex G-25 column and eluted with distilled water. The eluent was monitored with a Pharmacia refractive index monitoring device (most sensitive setting).

Standards:

A - glucose
B - maltose
C - raffinose
D - stachyose
E - dextran (molecular weight 200,000)


A
B
C
D
E
10
20
30
40
50
60
40
50
60
70
80
90
100
110

horizontal 1.0 cm strips and scraped off the plate into scintillation vials for assay of the radioactivity. The remainder of the plate was sprayed with diphenylamine-aniline spray (114) and noting the position of the reference sugars with respect to the 1.0 cm strips that had been removed.

TABLE I

Solvent composition and application used in silica gel thin layer chromatography separations.

Solvent no.	Composition	Application
I	acetone, water (9:1 v/v) (114)	monosaccharide separation, 3 ascents
II	n-butanol, methanol, 0.03 M boric acid (5:3:1 v/v) (114)	monosaccharide partial confirmation, 2 ascents
III	n-butanol, acetic acid, water (12:5:3 v/v) (114)	uronic acid separation, 1 ascent
IV	n-butanol, formic acid, water (33:50:17 v/v) (114)	oligosaccharide separation, 1 ascent
V	chloroform, methanol, water (65:25:2.5 v/v) (115)	lipid separation, 1 ascent

X. Paper Chromatography.

A 20 cm x 40 cm piece of Whatmann 3MM filter paper was spotted 2 cm from the bottom with the material to be separated along with 1% solutions of sugar knowns as standards. The chromatogram was developed with three ascents of the solvent: ethyl acetate, pyridine, water, (10:4:3 v/v) (116).

After drying, the sugars on the chromatogram were located using the silver nitrate technique (116).

XI. Preparation of Samples for Liquid Scintillation Counting.

A fluor of 0.5% PPO in toluene was used for counting on a Beckmann LS-200B Scintillation Counter. Fractions, or aliquots of fractions from column chromatography experiments, were prepared for counting by freeze drying the fraction in a counting vial containing a glass fiber filter. Silica gel scrapings from thin layer plates were put into the counting vial containing the fluor and counted. No quenching corrections were made.

XII. Recovery of Samples from Liquid Scintillation Counting Process.

Radioactive material could be recovered from the glass fiber filters by first washing the filter with toluene to remove the PPO. Then the filter was air dried and extracted with a small amount of distilled water for 20-30 minutes. The filters were removed by vacuum filtration and the filtrate freeze dried. Seventy to eighty percent of the original water soluble radioactive substances present could be recovered in this manner. Labeled macromolecular substances however could not be recovered effectively with this process.

Recovery of the water soluble radioactive material from silica gel scrapings was accomplished by first washing the material with toluene to remove the PPO and then air dried. A "mini-column" was constructed by tightly packing glass wool into the tip of a disposable pipet and inserting it into a rubber stopper. The assembly was then put into a small (10 ml) suction flask. A thick slurry of the dried silica gel was made with distilled water and applied to the "mini-column" with the vacuum being applied.

One to two ml of water wash was necessary to elute the labeled material from the silica gel. Labeled macromolecular substances could be recovered in this way, however its efficiency was limited to 50-60%.

XIII. Preliminary Examination of Labeled Substances in the Density Gradient Particulate Material.

Each fraction of each band was carefully assayed for radioactivity. Since total activity of the labeled KOH soluble material of the top and middle bands was low, these two were combined. This material was analyzed by introducing the hydrolyzate into a 1.7 cm x 89 cm Sephadex G-25 column and eluting with distilled water. One ml fractions were collected and assayed for radioactivity. The labeled KOH soluble material of the bottom and pellet bands was analyzed separately in the same manner.

The labeled lipid soluble substances of each band were investigated using silica gel thin layer chromatography and Solvent V. One cm strips were scraped off the plate and assayed for radioactivity. No studies were made on the labeled water soluble material or KOH insoluble portions of each band.

XIV. Analysis of Washed Crude Golgi Labeled KOH Soluble Substances.

The KOH hydrolyzate was applied to a Sephadex G-75 column and eluted with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected and 0.1 ml aliquots counted for radioactivity on glass fiber filters. The fractions corresponding to the void volume radioactivity peak were pooled and lyphilized and designated "Fraction I". This material was then treated with a hemicellulase-pectinase enzyme system for two hours and the reaction stopped by gentle heating on a steam bath. Then, the hydrolyzate was

introduced to the Sephadex G-75 column and eluted with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected and 0.2 ml aliquots assayed for radioactivity after drying on glass fiber filters.

Another "Fraction I" was prepared and treated with the hemicellulase-pectinase enzyme system for 24 hours. The hydrolyzate was then spotted on a silica gel thin layer plate, developed in Solvent I and scanned for radioactivity. Labeled material remaining at the origin was recovered and applied to another thin layer plate, developed in Solvent II and scanned for radioactivity.

Pronase was used to further characterize Fraction I. A 0.5 ml preparation of "Fraction I" was treated with 0.5 mg of pronase at 37° C. in a 1 mM potassium phosphate buffer, pH 8, and 0.02% sodium azide. After 56 hours, the hydrolyzate was gently heated over a steam bath to inactivate the enzyme. The hydrolyzate was then applied to a Sephadex G-75 column and eluted with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected and 0.5 ml aliquots assayed for radioactivity. The fractions corresponding to the void volume radioactivity peak were pooled, lyophilized and designated "Fraction II".

This material was then treated with the hemicellulase-pectinase enzyme system for 24 hours at room temperature. The hydrolyzate was streaked on a silica gel thin layer plate, developed in Solvent I and scanned for radioactivity. Alternatively, "Fraction II" was hydrolyzed with 1 N HCl at 100° C. for 3 hours, aspirated over KOH pellets at 7° C. under vacuum to dryness, and chromatographed. Chromatography was performed either on thin layer plates with Solvent I or on Whatmann 3MM filter paper using an ethyl acetate, pyridine, water solvent (10:4:3 v/v).

XV. Analysis of Supernatant III.

Supernatant III was analyzed by passing it through a Sephadex G-75 column with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected and assayed for radioactivity.

XVI. Analysis of Washed Crude Golgi Labeled Lipid Material.

The lipid extract was taken almost to dryness with a stream of dry nitrogen and 1 ml of 0.05 M methanolic KOH added at 55-60° C. The hydrolysis was allowed to proceed for about 2 hours with some addition of methanol to prevent drying. Then, the hydrolyzate was adjusted to approximately pH 3 with dilute acetic acid to a final volume of 3-4 ml. This solution was extracted twice with chloroform and then air dried to approximately 1 ml. Then it was passed through a Sephadex G-25 column with 1 mM potassium phosphate buffer, pH 8.

Two ml fractions were collected and assayed for radioactivity. The radioactive peak eluting first was recovered from the glass fiber filters, pooled and lyphilized. The material was then subjected to thin layer chromatography with Solvent IV and scanned. Alternatively, the material was applied to a Sephadex G-10 column and eluted with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected and counted.

XVII. Analysis of Washed Crude Golgi Labeled Aqueous Extract.

The labeled aqueous extract was applied to a Sephadex G-25 column and eluted with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected and counted for radioactivity.

RESULTS

I. Incorporation of Metabolites into Particulate Bands.

The results of incubation of the wheat seedlings in $H^{32}PO_4$ and its incorporation into the particulate bands on the density gradient are indicated in Figure IIIA. The peaks of radioactivity corresponded exactly with the presence of particulate material. The pellet was also labeled. Although each band contained ^{32}P, the radioactivity did not return to background levels between the bands and the top band appeared to trail.

Labeled phosphate was used in these preliminary studies because previous studies (116) have been done on the uptake of this material into wheat seedlings. Also, in this first experiment with ^{32}P (Figure IIIA), a homogenization medium of 0.5 M sucrose, 1 mM calcium chloride, 1% bovine serum albumin (BSA) and stock buffer was used. However, in order to minimize phosphohydrolase activity that might be present, and also to stabilize the structure of the membranes being isolated 75-100 mM gluteraldehyde was added to the homogenization medium and the 1% BSA replaced with 1% dextran. The density gradient profile obtained with the new homogenization medium is shown in Figure IIIA. The improved results are evident; there was no trailing from the top band and the radioactivity returned closer to background levels between bands.

Figure IIIB shows a density gradient scan of the particulate bands isolated after incubation of the wheat seedling roots with U-^{14}C-glucose. Incorporation of the ^{14}C radioactivity into each band is evident. The pellet was also labeled.

Labeled glucose was used to label the particulate bands isolated on the density gradient because of its known precursor potential for polysaccharide

biosynthesis in wheat root tips (101). If polysaccharide biosynthesis takes place in or on the intracellular membranes isolated, they would contain intermediates important in biosynthesis. Thus the labeling process had two purposes. First it provided a means by which compounds participating in polysaccharide biosynthesis would be specifically labeled; and secondly, it provided a way to trace the exceedingly small amounts of material handled in this investigation. The results depicted in Figure III indicate that the incubation methods developed in this study effectively label the isolated particulate material under investigation.

II. Distribution of Radioactivity in Bands.

The distribution of the radioactivity of each band was investigated by fractionating each one into four solubility classes: water soluble, lipid soluble, KOH soluble and KOH insoluble. Table II shows the results as an average of several isolations. Figures in parentheses represent calculations in which water soluble activity was omitted. This was done since this fraction might have contained activity which remained bound to the particulate material after washing, and was released after extraction with the lipid solvent. The fact that the percentage of radioactivity in this fraction decreased with additional washing on the density gradient, lends credence to this conclusion. Also, radioassay of the wash material from subsequent particulate washing showed that a total of 7000 cpm were removed. As will be shown later, most of this radioactivity was present was very low molecular weight material.

Inspection of the table indicates that the KOH soluble label was somewhat more concentrated in the pellet, although significant quantities were present in the other three bands as well. Likewise, the pellet had less radioactivity in the lipid fraction, percentage-wise, than did the other

TABLE II

Distribution of ^{14}C Activity in Bands as a Percentage of the Total Activity of the Band

BAND	TOTAL CPM	% WATER SOL.	% LIPID SOL.	% KOH SOL.	% KOH INSOL.
Top	3100 (2620)	15.2 (--)	47.8 (56.4)	17.7 (20.8)	19.2 (22.6)
Middle	4832 (4270)	12.7 (--)	46.7 (51.1)	22.5 (25.8)	20.1 (22.8)
Bottom	7280 (6440)	11.5 (--)	48.1 (54.6)	18.5 (20.8)	21.7 (24.6)
Pellet	9200 (8560)	7.4 (--)	32.8 (35.5)	31.8 (34.1)	28.1 (30.5)

All figures represent an average of three separate determinations, except for the top band, the figures of which represent an average of two separate determinations. Figures in parentheses represent percentages calculated omitting the water soluble activity from the total cpm of the band.

three bands. No other significant trends were evident.

III. Chemical Nature of the Labeled Substances in the Bands.

Preliminary chemical investigations on the nature of the label in the KOH soluble and lipid soluble material were carried out. If hemicellulose was present in the isolated bands, it would be found in the KOH soluble fraction. Also, if a lipid intermediate participated in the hemicellulose biosynthetic process in the membrane, it would probably be associated with the particulate material and be extracted into the lipid soluble fraction.

Sephadex G-25 chromatography of the KOH soluble material of each band was carried out (Figure IV). Every band appeared to contain only labeled material of high molecular weight (greater than 5000) and low molecular weight. The bottom and pellet bands had a much higher ratio of high to low molecular weight labeled material than did the top-middle band combination.

The results of thin layer chromatography scans of the lipid soluble labeled substances of each band are displayed in Table III. A major slow running peak and a minor fast running peak of ^{14}C labeled compounds were detected for each band in this solvent. No major differences in the relative amounts of these two components between the four bands were observed.

Since each band appeared to contain similar labeled constituents and also due to the very small amounts of material available, it was decided to perform further work on extracts of washed crude golgi material, which contained all of the bands, instead of the individual bands from the density gradient.

IV. Chemical Nature of Labeled Substances in Washed Crude Golgi.

A. KOH Soluble Fraction.

Sephadex G-75 chromatography of the KOH soluble fraction of washed crude

FIGURE III.

Sucrose Density Gradient Scans

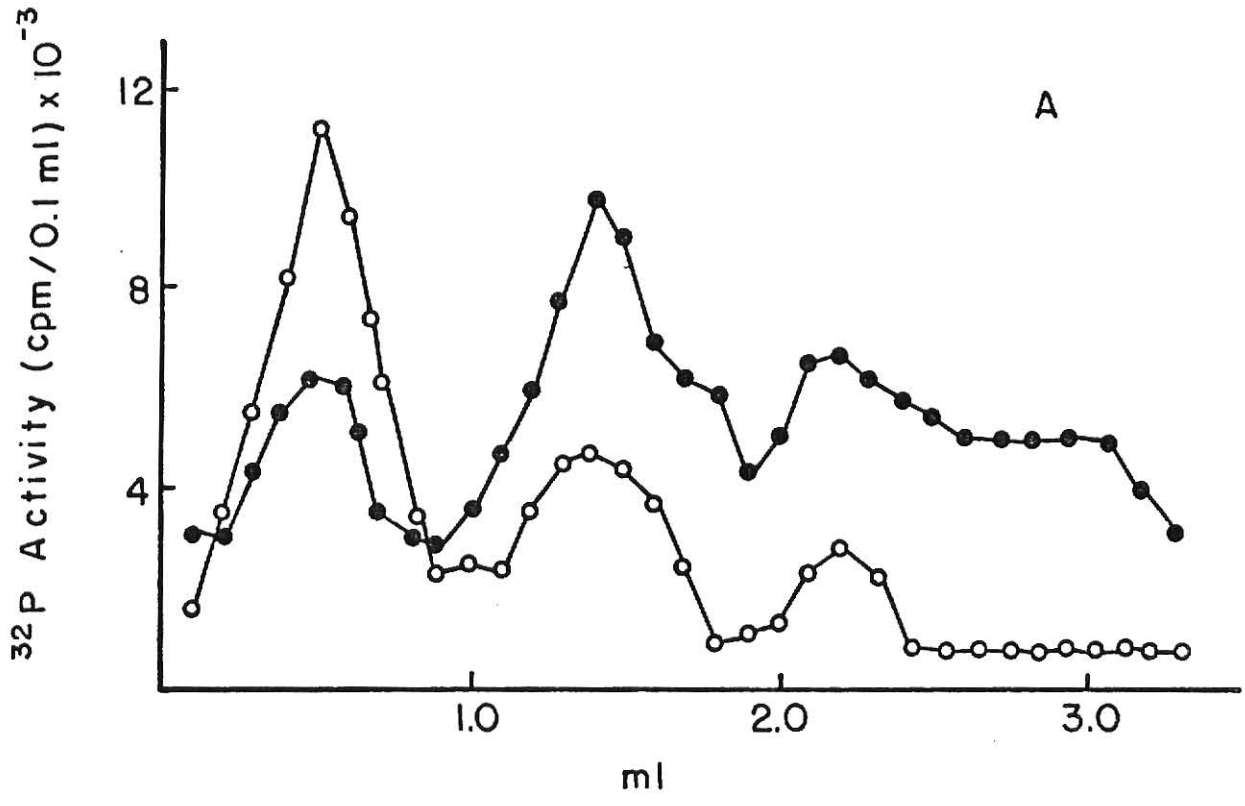
Wheat seedlings were incubated in radioactive metabolite and the subcellular fractions isolated on a density gradient. After piercing the bottom of the density gradient centrifuge tube, 0.1 ml fractions were collected on glass fiber filters, dried and counted.

A. Seedlings incubated in $H^{32}PO_4$ =

Bands isolated without gluteraldehyde in homogenization medium.

Bands isolated with gluteraldehyde in homogenization medium.

B. Seedlings incubated with U-^{14}C-glucose and isolated with gluteraldehyde in homogenization medium.


^{32}P Activity (cpm/0.1 ml) x 10^{-3}
12
8
4
A
1.0
2.0
3.0
ml

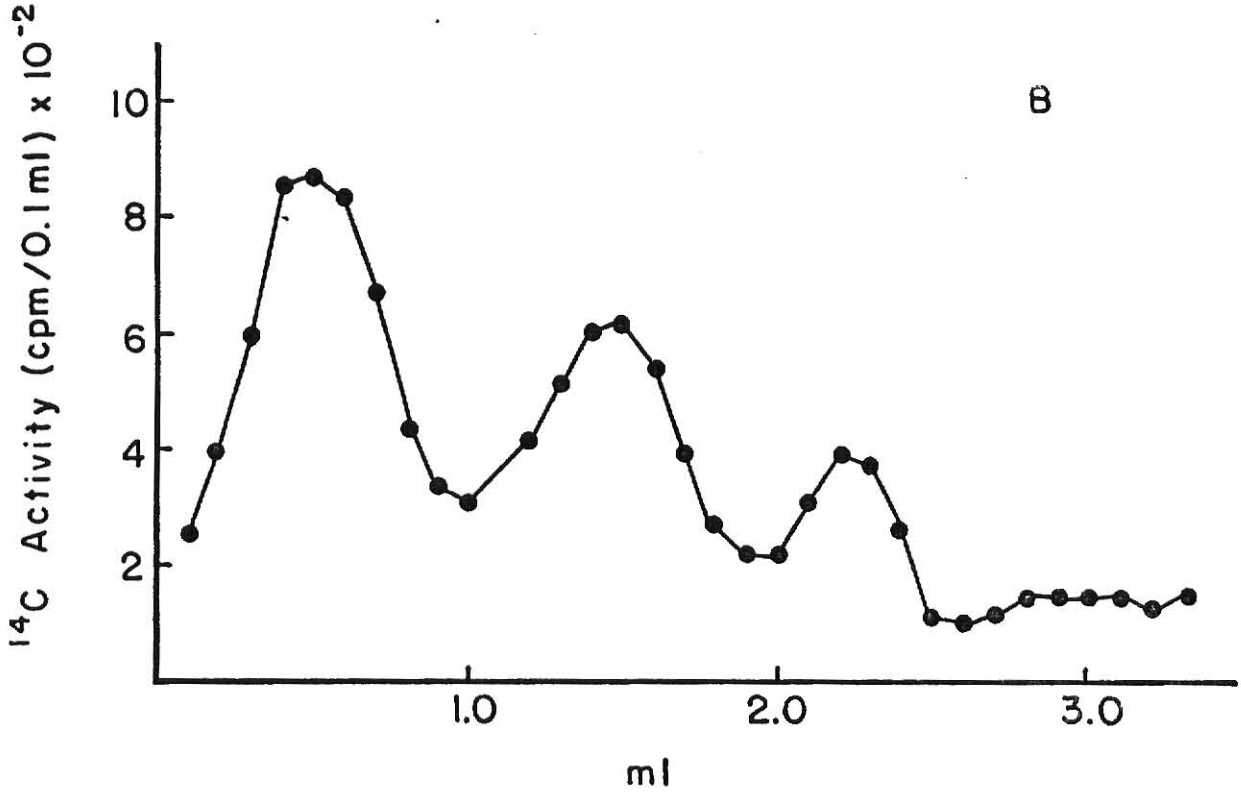

^{14}C Activity (cpm/0.1 ml) x 10^{-2}
10
8
6
4
2
B
1.0
2.0
3.0
ml

FIGURE IV.

Fractionation of KOH Soluble Components
of Density Gradient Bands on Sephadex G-25

The KOH soluble fraction of each band was eluted with distilled water on a 1.7 cm x 89 cm Sephadex G-25 column. Two ml fractions were collected, lyophilized and counted.

T+M - KOH soluble fraction of combined top and middle band.
B - KOH soluble fraction of bottom band.
P - KOH soluble fraction of pellet.

V_O - void volume
V_G - glucose elution volume

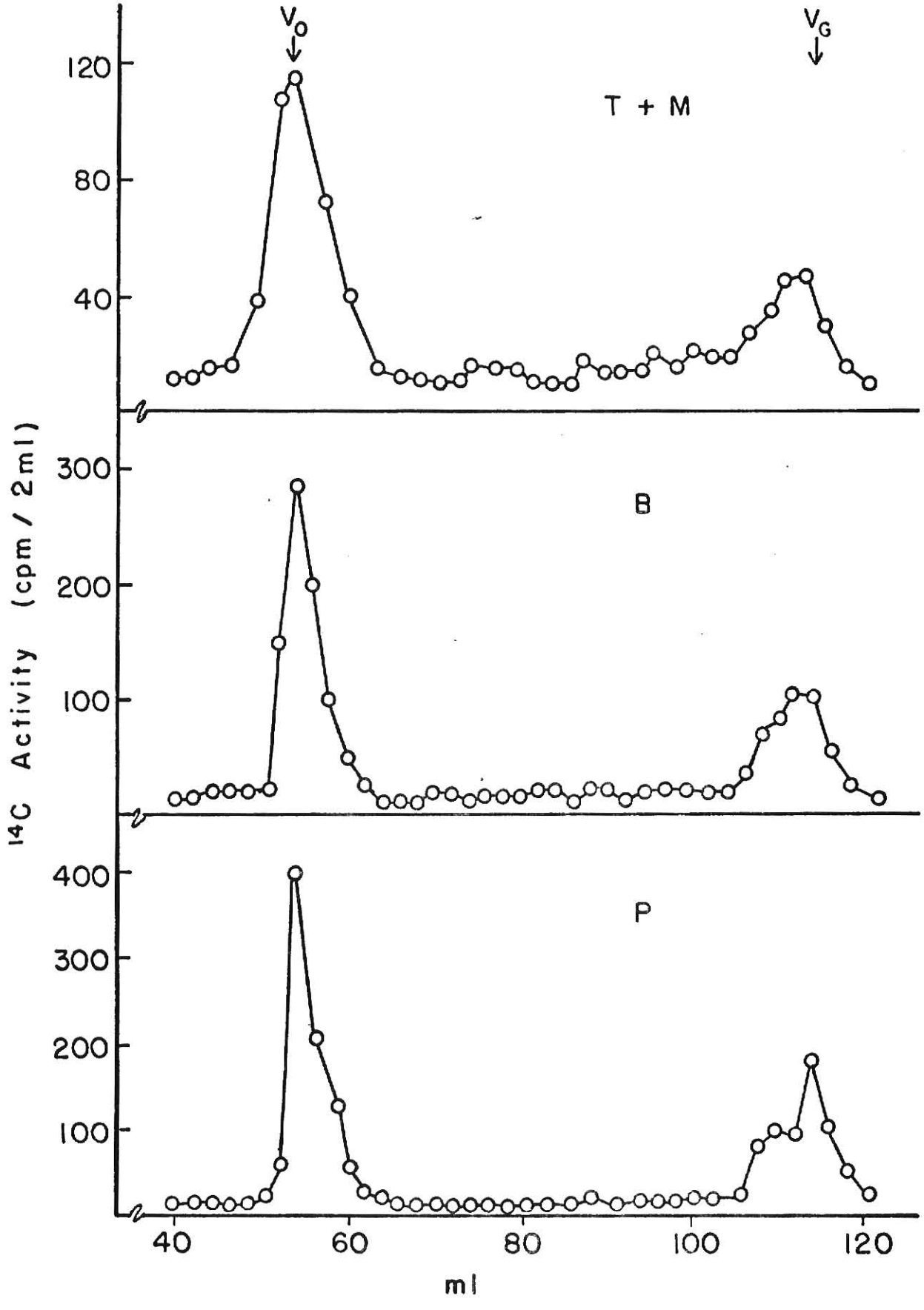

V_O
V_G
T + M
B
P
120
80
40
300
200
100
400
300
200
100
40
60
80
100
120
ml
^{14}C Activity (cpm / 2ml)

TABLE III

Thin Layer Chromatogram Scans:
Lipid Extract of Each Band

cm	top (cpm)	middle (cpm)	bottom (cpm)	pellet (cpm)
origin	24	36	30	37
1	18	26	40	24
2	222	221	83	36
3	57	207	528	515
4	41	81	104	104
5	20	15	74	98
6	24	37	45	45
7	12	20	16	33
8	20	46	36	30
9	16	27	62	62
10	16	25	22	21
11	65	109	112	129
12	82	73	110	113
13	34	36	76	80
14	14	3	13	14
15	18	8	12	1

The lipid fraction of each band was chromatographed on silica gel thin layer plates using solvent V in one ascent. One cm strips were scraped off the plate and counted.

golgi is shown in Figure V. The results indicated that most of the labeled material consisted of a high molecular weight fraction having a molecular weight greater than 50,000. This material was designated "Fraction I".

This material appeared to be intimately associated with the particular washed crude golgi as evidenced by G-75 chromatography of Supernatant III, shown in Figure VI. Very little high molecular weight material was lost in this last wash of the particulate crude golgi preparation at a centrifugal force of around 10,000 x g.

A series of experiments were performed to ascertain the chemical nature of the "Fraction I". Sephadex G-75 chromatography of a hemicellulase-pectinase enzyme treated "Fraction I" is shown in Figure VII. These enzymes exhibit specificity against glycosidic bonds. Labeled low molecular weight substances were evidently released by the action of these enzymes. A thin layer chromatogram scan of the hydrolyzate is shown in Figure VIII. Labeled substances migrating similar to galactose, arabinose, xylose, perhaps glucose and an unidentified fast running compound were present in the hydrolyzate. All are characteristic hemicellulose sugars.

Glucuronic acid moved as the lactone and free acid on the thin layer plate in Solvent I. The free acid barely moved from the origin and the lactone migrated near the solvent front. Rechromatography of the labeled fast running compound after overnight treatment with 2 N NH_4OH did not reveal a change in its migration characteristics, therefore it was probably not glucurono-lactone.

Recovery of the origin labeled substances and rechromatography in a uronic acid moving solvent (Solvent III) did not reveal the presence of any uronic acid-like labeled material. Apparently, either no uronic acid-like labeled material is present in "Fraction I" or the enzyme system was not

capable of releasing uronic acids from the "Fraction I" unknown.

The failure of the hemicellulase-pectinase enzyme system to release some of the labeled material from "Fraction I" as low molecular weight material over a long incubation period was an indication that a part of "Fraction I" was not polysaccharide. A "Fraction I" preparation was treated with pronase, a broad spectrum proteolytic enzyme. Sephadex G-75 chromatography of the hydrolyzate is shown in Figure IX. Approximately 40% of the labeled "Fraction I" was released as low molecular weight material by the action of pronase. The labeled, pronase resistant, high molecular weight material eluting at the void volume was designated "Fraction II".

The results of hemicellulase-pectinase treatment of "Fraction II" and subsequent thin layer chromatography are shown in the chromatogram scan in Figure X. Again, radioactive material migrating similar to galactose, arabinose, xylose perhaps glucose, and an unknown fast running compound was released by the enzyme system. The origin material was still present but in a much smaller quantity. Rechromatography of the labeled origin substance in a uronic acid moving solvent (Solvent III) again failed to reveal the presence of any uronic acids. Hydrolysis of "Fraction II" with 1 N HCl and subsequent thin layer chromatography yielded a chromatogram scan very similar to the one produced by the hemicellulase-pectinase hydrolyzate of "Fraction II".

Labeled material migrating like arabinose and galactose were rechromatographed using a different solvent (Solvent II) as partial proof of the presence of these monosaccharides. The labeled material again migrated exactly as the arabinose and galactose standards.

To further investigate the nature of the "Fraction II" labeled material,

the following experiment was performed. An-butanol extracted washed crude golgi preparation was washed with distilled water at room temperature, and then centrifuged. The butanol was used to extract the lipid material of the particulate preparation. The supernatant was treated with pronase and subjected to G-75 chromatography. The labeled void volume material was pooled and designated as "water soluble Fraction II". The pellet from the centrifugation was extracted with KOH as previously described, treated with pronase, and subjected to G-75 chromatography. The labeled void volume material was pooled and designated as "water insoluble Fraction II". Both fractions were hydrolyzed with 1 N HCl, subjected to paper chromatography and developed in silver nitrate as described in the Methods section. Figure XI shows the results. Glucose, galactose, arabinose, possibly xylose and a slow moving reducing compound were released from water soluble Fraction II. Glucose, galactose, arabinose and xylose were released from water insoluble Fraction II. No radioactivity measurements were made on the chromatogram. The results show that there were reducing sugars present in the high molecular weight fraction hydrolyzates and that some of the Fraction II material was water soluble after lipid extraction of the washed crude golgi preparation.

B. Lipid Soluble Material

Studies on the labeled lipid soluble substances from the washed crude golgi were aimed at detecting the presence of an intermediate participating in hemicellulose biosynthesis. Lipid intermediates so far encountered in animal (30) or bacterial cells (26, 27) have had the structure, saccharide-pyrophosphate-lipid. Dankert (26) employed basic hydrolysis to split the pyrophosphate linkage to the 1,2 cyclic phosphate of the oligosaccharide. The lipid fraction of this study was similarly treated (see Methods section).

Sephadex G-25 chromatography of the lipid KOH hydrolyzate is shown in Figure XII. The peak corresponding to G_3 was pooled and designated "G-3". To obtain a more precise molecular weight and to detect any size heterogeneity, the G-3 was subjected to Sephadex G-10 chromatography. The results indicated that the molecular weight was around 560 based on glucose oligosaccharide series standards. Also, only one peak was obtained. The G-3 was again recovered and subjected to thin layer chromatography using an oligosaccharide moving solvent (Solvent IV). It is clear from Figure XIII that the G-3 migrates in a manner similar to oligosaccharides. The above results do not however demonstrate conclusively that the material is homogeneous.

The possibility existed that the labeled G-3 material was not really derived from lipid material but was simply distributing between the chloroform and water phases in the initial lipid extraction. To investigate this, the aqueous extracts of several washed crude golgi preparations were pooled and chromatographed on a Sephadex G-25 column. The results are shown in the Figure XII. No material corresponding to the G-3 fraction was detected. Therefore, the labeled G-3 substance was truly esterified in some manner to the lipid material present in the membraneous material that was isolated. Due to lack of material, no other tests were made on the G-3 to ascertain its chemical nature.

FIGURE V.

Fractionation of KOH Soluble Components
of Washed Crude Golgi on Sephadex G-75

The KOH soluble fraction of the lipid extracted washed crude golgi was eluted on a 1 cm x 33 cm Sephadex G-75 column with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected, lyophilized and counted.

V_o - void volume

V_G - glucose elution volume

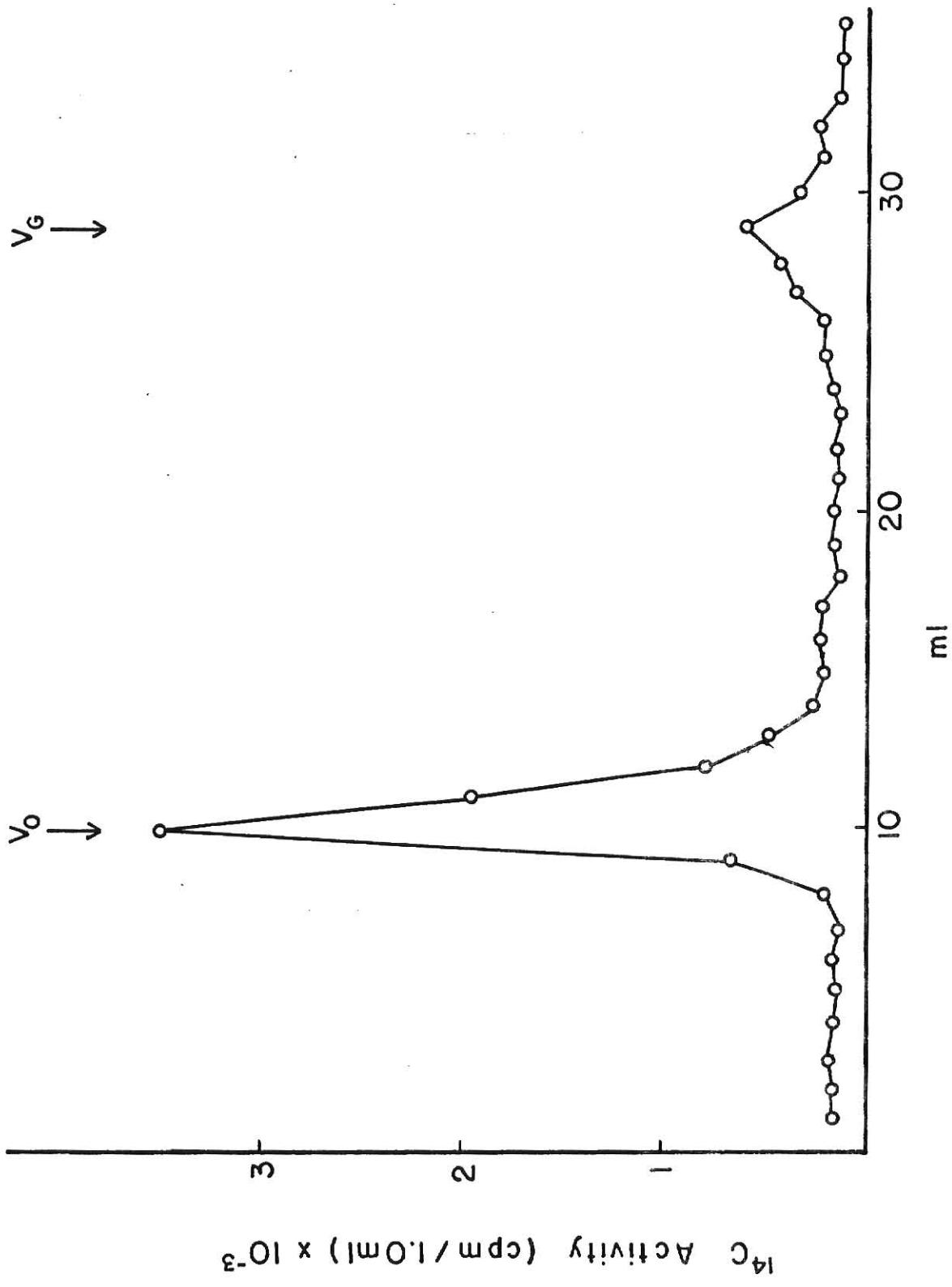

^{14}C Activity (cpm / 1.0 ml) x 10^{-3}
3
2
1
V_O
V_G
10
20
30
ml

FIGURE VI.

Fractionation of "Supernatant III"
of Washed Crude Golgi on Sephadex G-75

A washed crude golgi preparation was resuspended in stock buffer and centrifuged at 10,000 x g. The supernatant was eluted from a 1 cm x 33 cm Sephadex G-75 column with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected, lyophilized and counted.

V_o - void volume

V_G - glucose elution volume

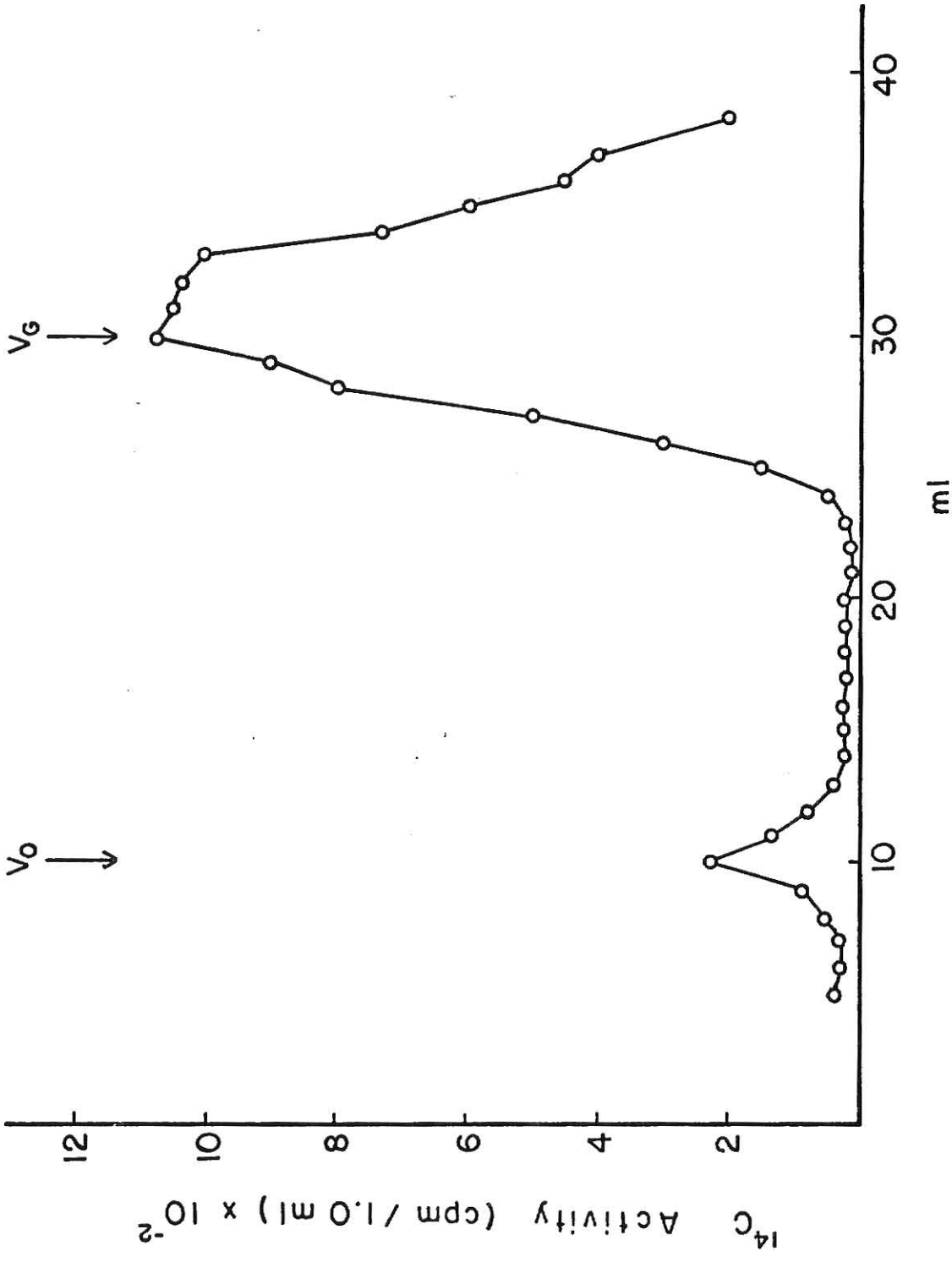

^{14}C Activity (cpm / 1.0 ml) x 10^{-2}
12
10
8
6
4
2
V_O
V_G
10
20
30
40
ml

FIGURE VII.

Fractionation of "Fraction I" Hemicellulase-Pectinase Hydrolyzate on Sephadex G-75

"Fraction I" was treated for 2 hours with the hemicellulase-pectinase enzyme system. The hydrolyzate was eluted on a 1 cm x 33 cm Sephadex G-75 column with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected, lyophilized and counted.

V_o - void volume

V_G - glucose elution volume

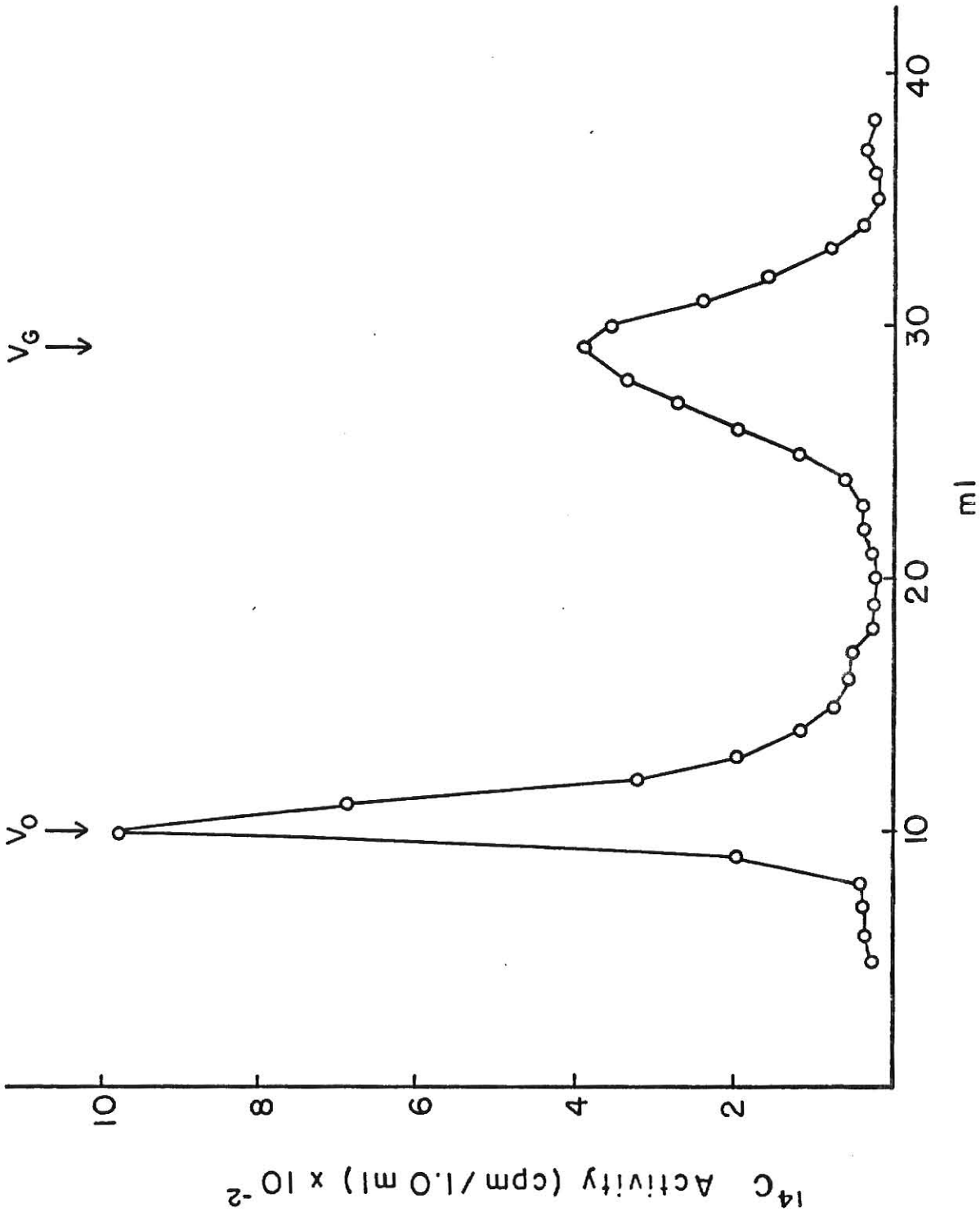

^{14}C Activity (cpm / 1.0 ml) x 10^{-2}
2
4
6
8
10
V_O
V_G
10
20
30
40
ml

FIGURE VIII.

Analysis of "Fraction I" Hemicellulase-Pectinase Hydrolyzate with Thin Layer Chromatography

"Fraction I" was treated for 24 hours with the hemicellulase-pectinase enzyme system. The hydrolyzate was streaked directly onto a silica gel thin layer plate and developed with three ascents of Solvent I. After drying, the plates were scanned by scraping off 1 cm strips and counting them.

Standards:

A - galactose
B - glucose
C - mannose
D - arabinose
E - xylose
F - glucurono-lactone

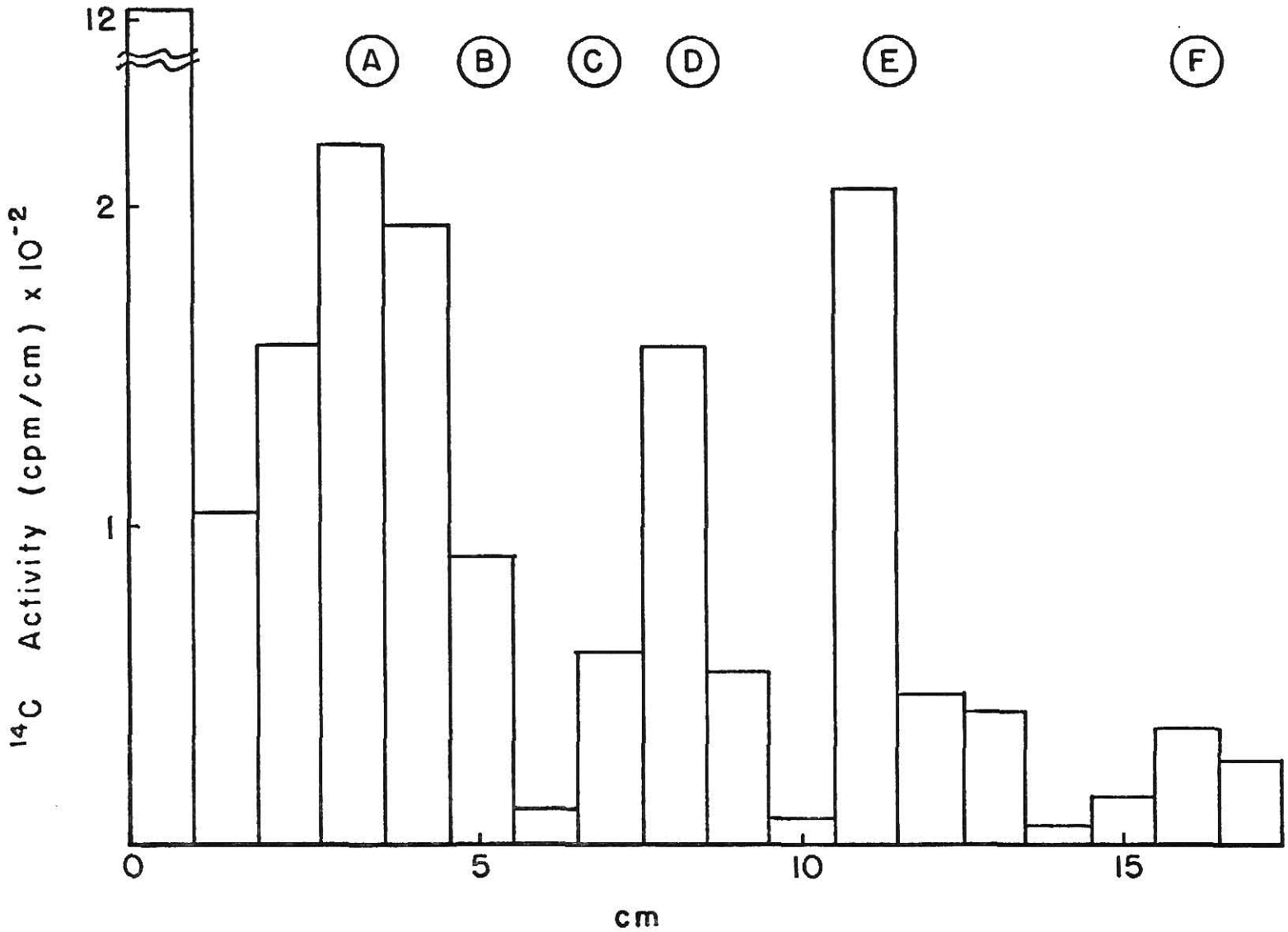

12
2
1
A
B
C
D
E
F
^{14}C Activity (cpm/cm) x 10^{-2}
0
5
10
15
cm

FIGURE IX.

Fractionation of "Fraction I"
Pronase Hydrolyzate on Sephadex G-75

"Fraction I" was treated for 56 hours at 37° C. with pronase and then eluted from a 1 cm x 33 cm Sephadex G-75 column with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected, lyophilized and counted.

V_o - void volume

V_G - glucose elution volume

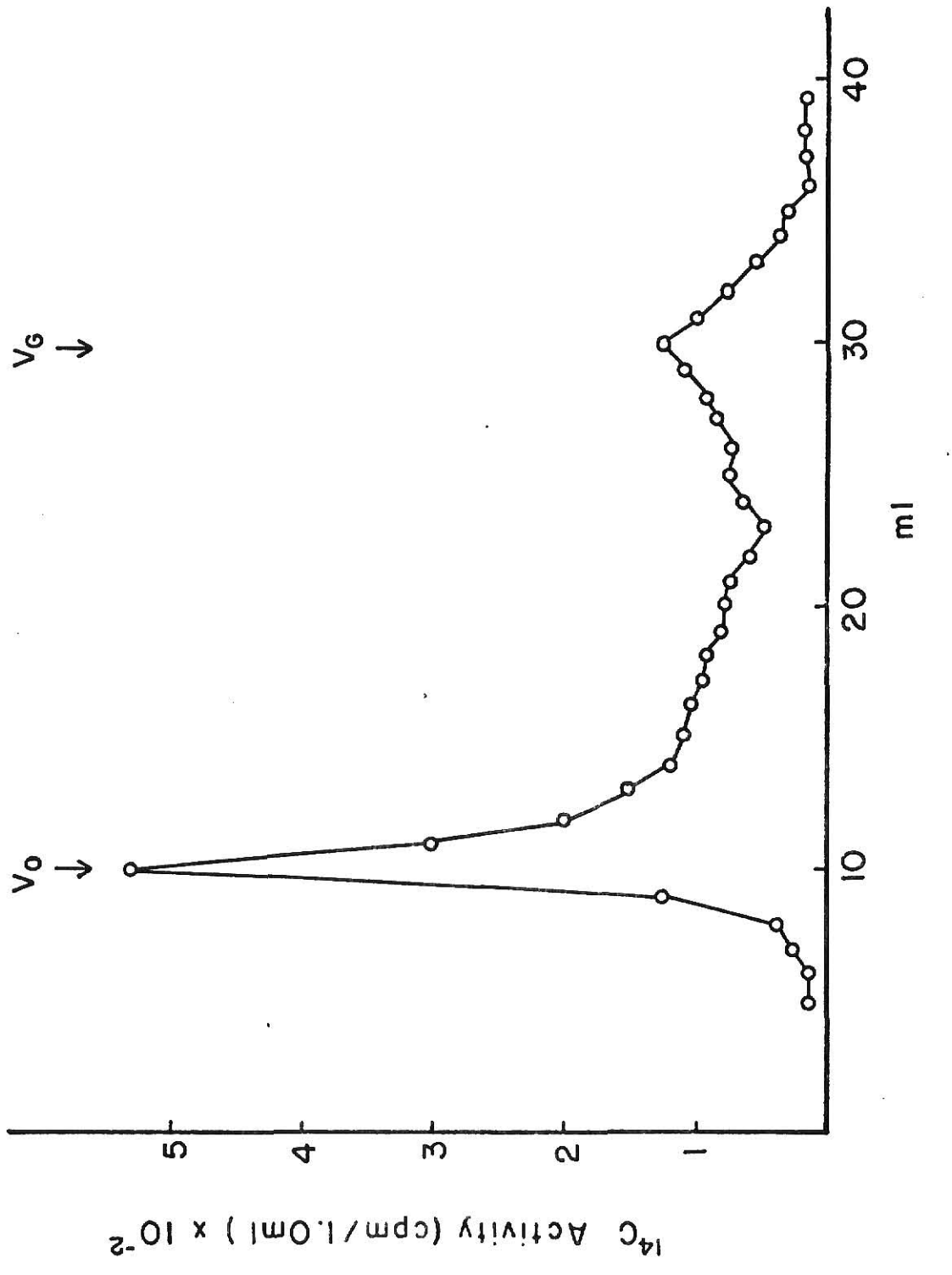

^{14}C Activity (cpm/1.0ml) x 10^{-2}
5
4
3
2
1
V_o
V_G
10
20
30
40
ml

FIGURE X.

Analysis of "Fraction II" Hemicellulase-Pectinase Hydrolyzate with Thin Layer Chromatography

"Fraction II" was treated with the hemicellulase-pectinase enzyme system for 24 hours and streaked directly onto a silica gel thin layer plate and developed with three ascents of Solvent I. The plate was scanned by scraping 1 cm strips off and counting them.

Standards:

A - galactose
B - glucose
C - mannose
D - arabinose
E - xylose
F - glucurono-lactone

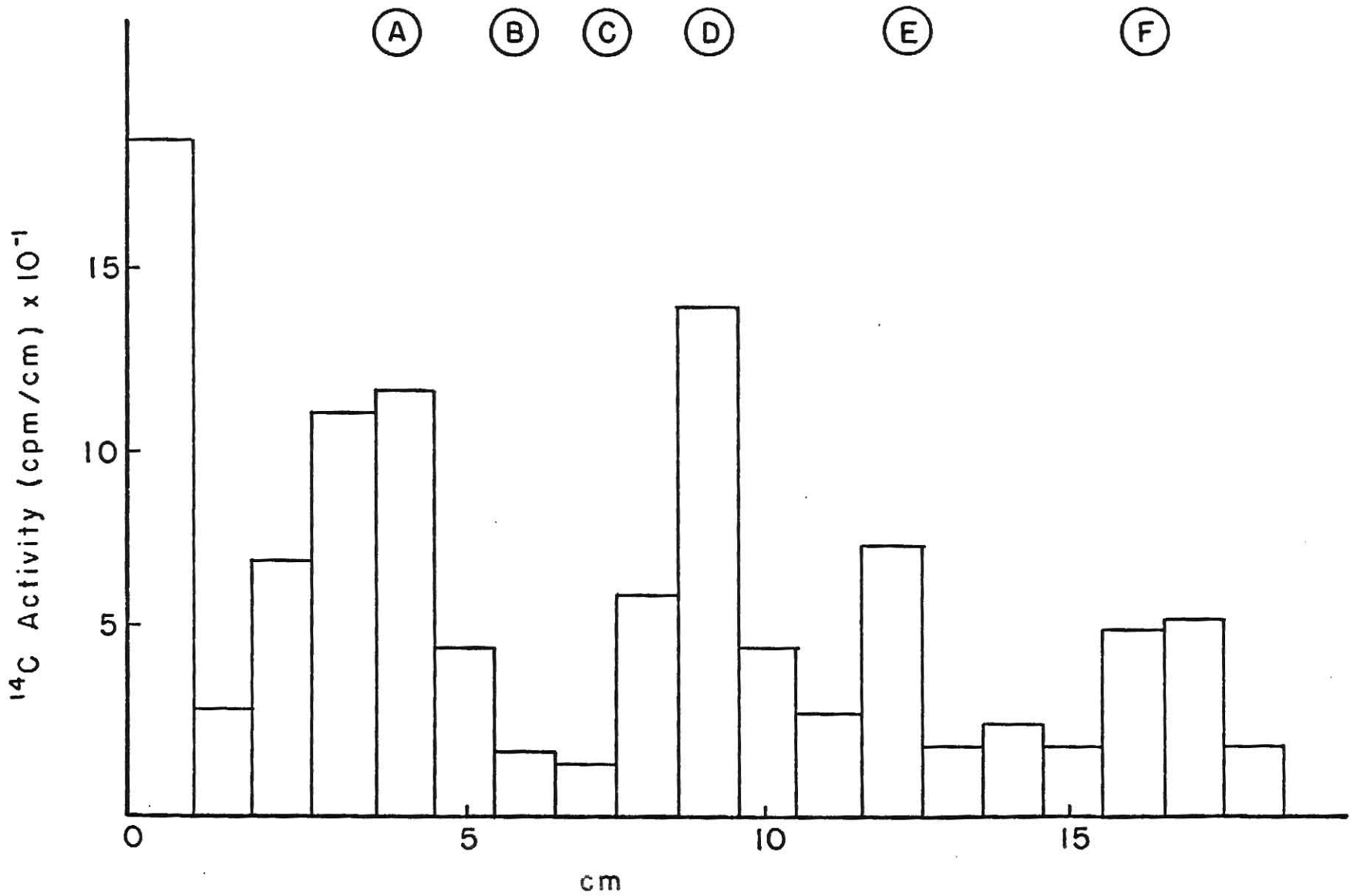

A
B
C
D
E
F
^{14}C Activity (cpm/cm) x 10^{-1}
15
10
5
0
5
10
15
cm

FIGURE XI.

Analysis of Water Soluble and Water Insoluble "Fraction II" Acid Hydrolyzates with Paper Chromatography

One normal HCl hydrolyzates of water soluble and water insoluble Fraction II were evaporated by aspiration over KOH pellets at 7° C. The hydrolyzates were spotted on a Whatmann 3MM filter paper and developed in the ethyl acetate, pyridine, water solvent as described in Methods. The entire chromatogram was developed in silver nitrate.

Standards:

A - mannose
B - galactose and fucose
C - arabinose
D - galactose, glucose, mannose, arabinose, xylose
E - glucuronic acid and glucurono-lactone
F - glucose and xylose

Hydrolyzates:

IIa - water soluble Fraction II
IIb - water insoluble Fraction II

ILLEGIBLE DOCUMENT

THE FOLLOWING DOCUMENT(S) IS OF POOR LEGIBILITY IN THE ORIGINAL

THIS IS THE BEST COPY AVAILABLE


A
B
IIA
IIB
C
D
E
F

FIGURE XII.

Fractionation of KOH Hydrolyzate
of Lipid Extract on Sephadex G-25

Material was eluted through a 1.7 cm x 89 cm Sephadex G-25 column with 1 mM potassium phosphate buffer, pH 8. One ml fractions were collected, lyophilized and counted.

o-o-o-o left hand ordinate. Lipid extract of washed crude golgi was hydrolyzed with methanolic KOH. The hydrolyzate was acidified and extracted twice with chloroform before column chromatography.

●-●-●-● right hand ordinate. Pooled aqueous wash of the lipid extracts of washed crude golgi.

V_o - void volume

G_4, G_3, G_2, G_1 - elution volumes of stachyose, raffinose, maltose and glucose respectively.

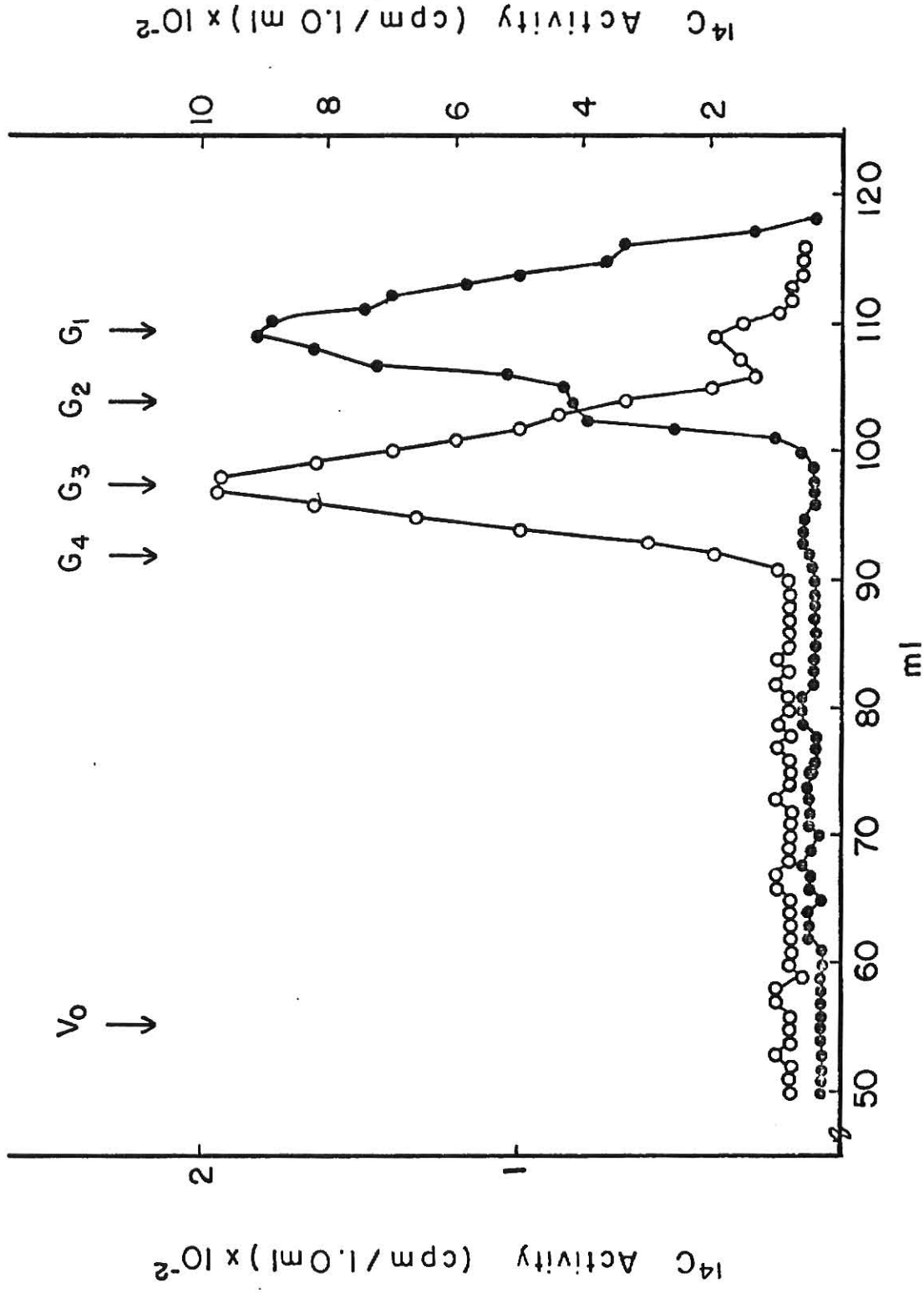

^{14}C Activity (cpm / 1.0 ml) x 10^{-2}
2
1
0
50
60
70
80
90
100
110
120
ml
V_o
G_4
G_3
G_2
G_1
2
4
6
8
10
^{14}C Activity (cpm / 1.0 ml) x 10^{-2}

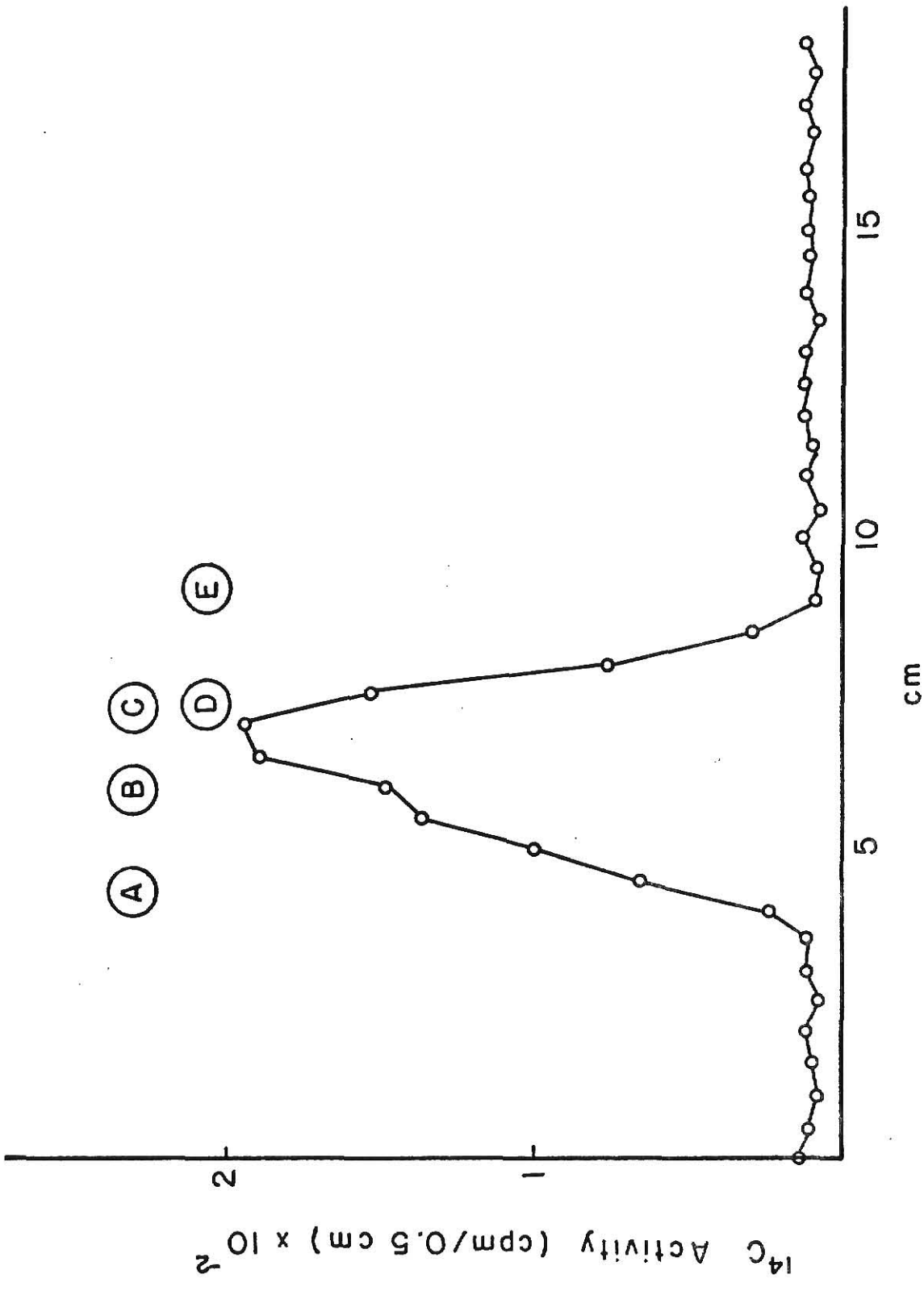
FIGURE XIII.

Analysis of Lipid Derived "G-3" Material
by Thin Layer Chromatography

The "G-3" material was recovered and streaked on a silica gel thin layer plate and developed in Solvent IV. The plate was scanned by scraping 0.5 cm strips off the plate and counting them.

Standards:

A - schardinger - dextrin or cycloheptaamylose
B - stachyose
C - maltose
D - aldotriuronic acid (4-O-methyl glucuronic acid-xylose-xylose)
E - glucose


^{14}C Activity (cpm/0.5 cm) x 10^{-2}
2
1
A
B
C
D
E
5
10
15
cm

DISCUSSION

The initial goal of this study was to determine the chemical composition of isolated membranes of plant tissue to determine whether or not they contained cell wall heteroglycan precursory substances. A method was available for the isolation of morphologically defined particulate material of reasonable purity, from wheat root cells (76). There was also preliminary evidence for the presence of labeled high molecular weight material in a crude golgi preparation after incubation of etiolated wheat root tips in U-^{14}C-glucose (16).

During the course of this study, a system was developed to study the chemistry of the isolated particles. A method of incubation of intact wheat seedlings in radiometabolite was developed which resulted in a definite improvement in the uptake of the labeled glucose compared to that of etiolated root tips. Gluteraldehyde was used in the homogenization medium to stabilize the membranes and to inactivate hydrolases. Phosphohydrolase is especially active in plant preparations (113) and is difficult to inactivate without damaging the preparation. However, the use of gluteraldehyde presented dangers in interpreting results because of its binding properties. Nevertheless, other investigators have used it during chemical studies of cytomembranes of plant tissues (83, 91). A major advantage of the system developed to study the chemistry of the isolated particles, was that extremely small amounts of material could be handled and studied with the aid of the radioactivity.

The existence of cell wall hemicellulose precursor molecules associated with plant cell cytomembranes having a polysaccharide synthesizing role has been postulated by a number of workers. A paper by Northcote and

Pickett-Heaps (101) demonstrated that golgi membranes of rapidly growing wheat root cap cells concentrated labeled glucose from an incubation medium and subsequently transported radioactivity across the plasmalemma via golgi derived vesicles. Since the labeled material was not extracted from the golgi and associated vesicles during preparation of the tissue for electron microscopy and autoradiography, they concluded that the radioactivity was located in a macromolecule, possibly a polysaccharide. Later, histochemical studies (106, 109) indicated that polysaccharide staining material was present in golgi derived vesicles; also the golgi dictyosome stack stained progressively heavier from the forming face to the mature face of the organelle.

Northcote and Harris (91) have recently performed a sulfuric acid hydrolysis on a golgi preparation from pea seedlings after incubation with ^{14}C-6-glucose. The hydrolysis products were typical labeled sugars of hemicellulose and pectin. However, the relative purity of their golgi preparation was not assessed.

The work reported in this thesis is the first to directly demonstrate the presence of hemicellulose polymers associated with plant intracellular membranes by in vivo labeling techniques. The labeled high molecular weight material appeared to be an integral part of the washed crude golgi particles. Nonspecific binding of hemicellulose alone to the particles by gluteraldehyde would seem remote since the relative proportion of sugars obtained by hydrolysis (Fig. XI) are different than shown by TLC (Fig. X). Also, previous (16) experiments in this laboratory, showing the presence of labeled macromolecules associated with a particulate preparation, was accomplished without the aid of gluteraldehyde.

At least some of the hemicellulose material was bound by a lipid membrane complex and released only after lipid extraction by washing with distilled water while the rest of it stayed firmly bound to the lipid and water insoluble material. This latter polysaccharide could only be released by treatment with hot dilute KOH. The water soluble polymer had a similar sugar composition, but had less xylose and arabinose than the hemicellulose released by the KOH treatment. Presumably a base labile linkage was cleaved to release this hemicellulose molecule.

Some of the labeled macromolecules in the KOH soluble fraction of the washed crude golgi were susceptible to pronase. This would indicate that a radioactive protein was present or perhaps that labeled, low molecular weight (less than 1000) sugar residues were attached to the protein. A radioactive glycoprotein has been demonstrated by Villemez (35) in a particulate in vitro polysaccharide synthetase system incubated with UDP-^{14}C-mannose. The radioactivity was present in a mannose oligomer attached to the protein, but its role in hemicellulose biosynthesis is unknown.

It is possible that the water insoluble hemicellulose polymers demonstrated in this study were present in the isolated cytomembranes in the form of a mucopolysaccharide. The KOH treatment may have released hemicellulose and glycoprotein or simply a protein. The labeled substances released by the pronase would then be low molecular weight peptides or glycopeptides and the pronase resistant, hemicellulase-pectinase susceptible labeled material would correspond to hemicellulose polymers.

This possibility is interesting in the light of recent findings by Lamport (9, 10) that a hydroxyproline containing protein may crosslink the hemicellulose molecules of the plant cell wall via the hydroxyl group of

the hydroxyproline residues. This protein-polysaccharide structure in the cell wall yields hemicellulose upon basic treatment. Presumably, there exists a base labile glycosidic bond several saccharide units away from the protein, since sugar oligomers remained attached to the protein. Little is known about the biosynthesis of this complex structure, however a proposed (10) cytoplasmic precursor has been isolated and is composed of 95% polysaccharide and 5% protein. Recently, however, Northcote has disputed this hemicellulose-extensin complex theory (117).

Previous suggestions (16) that a lipopolysaccharide, which functions in polysaccharide biosynthesis, may be associated with the washed crude golgi membranes isolated in this study have not been disproven. However, polysaccharide biosynthesis in this manner would involve growing chains of saccharide molecules attached to the lipid which would be soluble in lipid solvents. No intermediate molecular weight labeled molecules of this kind were observed in this study besides that of the possible oligosaccharide isolated in the lipid extract. Certainly, other interpretations of the data on the high molecular weight KOH soluble material are possible, but none as consistent with the literature as the mucopolysaccharide theory expressed above. Indeed, some of the hemicellulose is not covalently linked to any other molecule in the membranes. The proof lies in further investigation of the chemical properties of the labeled high molecular weight substances.

The labeled high molecular weight KOH soluble substances are more predominant in the pellet fraction of the material isolated on the sucrose density gradient. Earlier enzymatic studies (76) on this pellet material demonstrated the localization of a polysaccharide synthetase there. Studies with other <u>in vitro</u> polysaccharide synthetase systems (44, 53, 55) have shown

that the sugar is transferred to an undefined polysaccharide acceptor present in the isolated particles. It is likely that the hemicellulose demonstrated in this study is the corresponding polysaccharide acceptor of the synthetase particles isolated in the pellet of the density gradient. It is interesting to note, however, that labeled high molecular weight substances were present in every band of the density gradient.

The evidence presented in this study for the relatively small amount of labeled high molecular weight material in the golgi apparatus fraction seems to be in direct contrast to the proposed (101) function of the golgi as the site of polysaccharide biosynthesis. However, an examination of the recent literature suggests that the golgi derived vesicles may perform most of the polymerization function and also subsequent modification of the resulting heteropolymer (106, 109).

The golgi apparatus itself is probably responsible for the assembly of the complex enzyme system and raw materials needed for the biosynthesis of the cell wall structure. Perhaps some of the first transglycosylation reactions occur during the maturation of the dictyosome stack just before the secretory vesicle is blebbed off. From these observations one would expect only minor amounts of hemicellulose to be isolated with the golgi apparatus. Also, autoradiography studies (105) of meristematic cells of wheat roots show that radioactivity rarely accumulates in the golgi because of the transitory nature of the structure. Most of the cells used in this study consisted of meristematic cells, although a few were root cap cells.

Although further histochemical and enzymological clarification of the density gradient particulate material is needed, it is possible that some of the unidentified vesicles present in the bottom two bands represented golgi

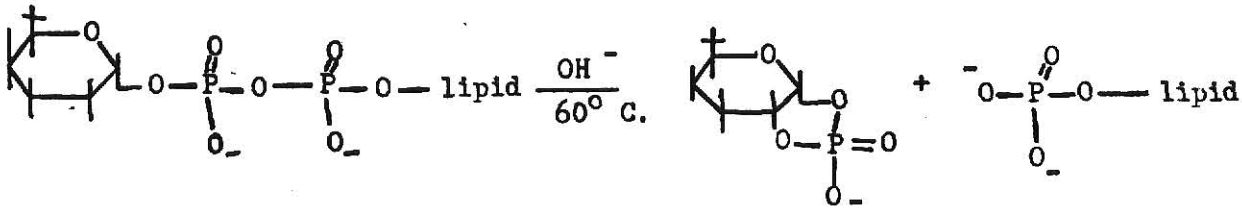
derived secretory vesicles. One would expect a cell wall precursory structure to sediment very fast in a density gradient since cell wall particles themselves would be dense structures. The possibility that some of the pellet material consisted of cell wall matrix particles seems remote because the heaviness of this material would cause it to sediment with the first 4000 x g centrifugation step.

The role of the membrane associated hemicellulose demonstrated in this thesis in hemicellulose biosynthesis was not directly investigated But based on observations reported in the recent literature, it is possible to assign a cell wall heteropolymer precursor role to this hemicellulose. It is probably similar to the polysaccharide acceptor present in other polysaccharide synthetase preparations. Also, it is likely to be responsible for the polysaccharide specific staining reactions displayed by golgi derived vesicles and the mature face of the dictyosome stack.

The experiments reported here indicate the possibility of a labeled oligosaccharide being attached to a lipid soluble substance in the lipid soluble extract of the washed crude golgi. If there is a lipid intermediate participating in plant cell wall heteroglycan biosynthesis in the isolated cytomembranes, this proposed oligosaccharide was the only candidate found. However, as yet there is no firm evidence that the material is saccharide in nature.

Other glycolipids of plants that have been proposed as intermediates in polysaccharide biosynthesis (32, 34) have only a monosaccharide residue. Assuming that the "G-3" is really an oligosaccharide, it would have at least three sugar units. There was little label at the glucose elution volume of the column upon which the "G-3" was isolated.

The small percentage of label associated with the "G-3" compared to the total activity of the lipid fraction is a bit puzzling. Some degradation of sugars could have occurred since reducing sugars are quite susceptible to base treatment. However, if the sugar of the hypothetical glycolipid intermediate was linked to the lipid by a pyrophosphate residue attached to carbon number one, little degradation would have taken place. Base treatment of it would have yielded the 1-phosphate derivative of the sugar which would be fairly stable under alkaline conditions.



On the other hand, if the saccharide were attached to the pyrophosphate by any other carbon, extensive degradation would have occurred upon release of the sugar phosphate because the hemiacetal function of the sugar was not protected.

Of course, it is probable that not all of the radioactivity of the lipid extract is associated with sugar substances. Golgi apparatus, and other smooth membranes of plant tissues are extremely active with respect to membrane lipid metabolism (83). This is the case especially in rapidly growing root tissues. Thus it is not too surprising to have found so much of the radioactivity in the lipid extracts of these fractions.

SUMMARY

Using methods developed in this study, intact wheat seedlings were incubated in U-^{14}C-glucose for two hours. A washed crude golgi preparation was obtained from the roots of the seedlings. After lipid extraction of the preparation with methanol, chloroform, 0.2 M formic acid (12:5:3 v/v), the insoluble residue was treated with 0.05 M KOH at 100° C. for 20 minutes. Sephadex G-75 column chromatography of this extract indicated the presence of a labeled high molecular weight (greater than 50,000) fraction.

Part of this material was degraded to low molecular weight substances by treatment with pronase. The remaining high molecular weight material was susceptible to a hemicellulase-pectinase enzyme preparation. Labeled products released from the pronase resistant substance by treatment with either 1 N HCl at 100° C., or the hemicellulase-pectinase enzyme system included xylose, arabinose, galactose and perhaps glucose. Thus some of the labeled macromolecules associated with the washed crude golgi preparation were hemicellulose polymers.

The labeled high molecular weight KOH soluble material was quite firmly attached to the washed crude golgi membranes. Some of the labeled macromolecules were soluble in water only after lipid extraction with butanol, while some were released from the lipid extracted material after KOH treatment of the remaining residue.

The labeled high molecular weight KOH soluble material appeared to be more concentrated in the pellet band over the other bands obtained by sucrose density gradient centrifugation of the washed crude golgi particles. It was postulated that the hemicellulose was associated with golgi derived vesicles present in the pellet and was functioning as a cell wall

polysaccharide precursor substance.

A water soluble labeled substance was released by treatment of the washed crude golgi lipid extract by the action of methanolic 0.05 M KOH at 60° C. for about 2 hours. Based on Sephadex G-25 and G-10 column chromatography, the substance had a molecular weight of approximately 550 with glucose series oligosaccharides as standards. The unknown also migrated similarly to oligosaccharides on silica gel thin layer plates. It was postulated that the partially identified "oligosaccharide" may exist as a lipid linked intermediate in cell wall polysaccharide biosynthesis.

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Philip Nordin, my supervising professor, for his advice, encouragement and most of all his patience during the course of this work.

I also wish to acknowledge my appreciation to my wife, Judy, whose patience and understanding helped to make this effort successful.

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STUDIES ON THE INTERMEDIATES INVOLVED IN HEMICELLULOSE BIOSYNTHESIS

by

ROBERT LAURENCE JILKA

B.S., Kansas State University, 1970

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1972

This study arose from the need to clarify the chemical nature and intracellular location of the intermediates involved in plant cell wall polysaccharide biosynthesis.

After germinating wheat seeds for 60-64 hours, they were incubated in U-^{14}C-glucose for 2 hours. The roots were homogenized and a "washed crude golgi" particulate fraction obtained by a series of centrifugations. After lipid extraction of this material, treatment with hot dilute KOH released a labeled high molecular weight substance (Fraction I). Exhaustive treatment of this fraction with pronase yielded pronase resistant, labeled high molecular weight material (Fraction II), and also labeled low molecular weight substances. Treatment of Fraction II with either 1 N HCl at 100° C. or a hemicellulase-pectinase enzyme preparation released low molecular weight labeled material. Silica gel thin layer chromatography of the hydrolyzate revealed the presence of labeled galactose, arabinose, xylose and perhaps glucose. Therefore, a hemicellulose type of polysaccharide was present in the particles.

The washed crude golgi could be rendered partially water soluble by vigorous agitation with 1-butanol. The washed crude golgi could now be further divided into water soluble and KOH soluble fractions. Each fraction was treated with pronase and the labeled high molecular weight pronase resistant portion isolated. Acid hydrolysis of these materials followed by paper chromatography demonstrated the presence of glucose, galactose, xylose and arabinose in both fractions.

After sucrose density gradient centrifugation of the washed crude golgi preparation, it was found that the labeled high molecular weight substances predominated in the pellet fraction. Little was present in the golgi band of the density gradient. It was postulated that the hemicellulose was

present in golgi derived vesicles and plasmalemma fragments present in the pellet fraction.

Upon methanolic KOH hydrolysis of the lipid extract of the washed crude golgi particles, a labeled water soluble substance was released that had some of the characteristics of an oligosaccharide. Sephadex column chromatography indicated that it had a molecular weight of around 560. Also, it migrated in a manner similar to authentic oligosaccharides when chromatographed on a silica gel thin layer plate.