CHARACTERIZATION OF APIOGALACTURONANS FORMED BY A CELL-FREE SYSTEM FROM LEMNA MINOR

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
LEONARD MASCARO, JR.
1975

This is to certify that the

thesis entitled

CHARACTERIZATION OF APIOGALACTURONANS FORMED BY A CELL-FREE SYSTEM FROM LEMNA MINOR

presented by

Leonard Mascaro, Jr.

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Date February 11, 1975

O-7639

ABSTRACT

CHARACTERIZATION OF APIOGALACTURONANS
FORMED BY A CELL-FREE SYSTEM
FROM LEMNA MINOR

By

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The products formed from UDP-D-apiose and UDP-D-galacturonic acid in a cell-free system from Lemna minor have been characterized. Characterization experiments have established that the products are apiogalacturonans.

UDP-D-[U-^{14}C]Apiose (containing some UDP-D-[U-^{14}C]xylose) and UDP-D-[U-^{14}C]galacturonic acid were incubated with a particulate enzyme preparation from L. minor. At the end of the incubation the reaction mixtures were extracted with methanol and water. The radioactive material synthesized from UDP-D-[U-^{14}C]-apiose and UDP-D-[U-^{14}C]galacturonic acid and remaining in the insoluble residue is referred to as D-[U-^{14}C]apiose product and D-[U-^{14}C]galacturonic acid product, respectively. Based on radioactivity measurements extraction of the products with ammonium oxalate solubilized 87% of the D-[U-^{14}C]apiose product and 91% of the

D-[U-^{14}C]galacturonic acid product. The radioactive materials that were solubilized by ammonium oxalate treatment are referred to as D-[U-^{14}C]apiose solubilized product and D-[U-^{14}C]galacturonic acid solubilized product. Fungal pectinase could hydrolyze both solubilized products. Hydrolysis of the D-[U-^{14}C]apiose solubilized product at pH 1 showed that 75% of the radioactivity was present in D-[U-^{14}C]apiose and 25% in D-[U-^{14}C]xylose (25%). The D-[U-^{14}C]galacturonic acid solubilized product was found to contain its radioactivity in D-[U-^{14}C]-galacturonic acid and a small amount (less than 6%) in D-[U-^{14}C]xylose.

D-[U-^{14}C]Apiose solubilized product released D-[U-^{14}C]apiose and [U-^{14}C]apiobiose when hydrolyzed at pH 4. A similar release of apiobiose has been reported for authentic apiogalacturonans. When [U-^{14}C]apiobiose was isolated from the D-[U-^{14}C]apiose product, reduced by $NaBH_4$ treatment, and hydrolyzed, the radioactivity was found equally in D-[U-^{14}C]apiose and D-[U-^{14}C]apiitol. This indicates that both moieties of the apiobiosyl side chains were synthesized <u>in vitro</u>.

Five fractions were obtained when D-[U-^{14}C]-galacturonic acid was chromatographed on a DEAE-Sephadex column. The fraction which eluted with 0.25 M NaCl (fraction D) contained 40% of the recovered

radioactivity. When similarly chromatographed on the DEAE-Sephadex column, the D-[U-^{14}C]apiose solubilized product was also recovered in 5 fractions that eluted in the same positions of the gradient as seen for the D-[U-^{14}C]galacturonic acid product. The fractions were labelled, in order of elution, A through E. The fractions eluted from the column in order of increasing D-[U-^{14}C]-apiose content. Fraction D which eluted with 0.25 M NaCl contained 21% of the recovered radioactivity. Analysis of this fraction showed that 88% of its radio-activity was contained in D-[U-^{14}C]apiose and the remainder in D-[U-^{14}C]xylose. Hydrolysis of fraction D at pH 4 released 63% of the D-[U-^{14}C]apiose, the majority as [U-^{14}C]apiobiose.

D-[U-^{14}C]Apiose solubilized product was also synthesized in the presence of exogenous UDP-D-galac-turonic acid and was chromatographed on the DEAE-Sephadex column. The chromatogram showed that there was increased synthesis of the more acidic products. Fraction D which eluted with 0.25 M NaCl contained 53% of the radioactivity recovered from the column. The amount of radioactivity in Fraction D contained in D-[U-^{14}C]apiose was 95% with the rest contained in D-[U-^{14}C]xylose. Hydrolysis at pH 4 released 81% of the D-[U-^{14}C]apiose in Fraction D.

Gel chromatography of D-[U-^{14}C]apiose product and D-[U-^{14}C]galacturonic acid product showed that both

contained molecules of different sizes. Both products eluted from a Bio-gel P-300 column over its entire fractionation range. Chromatography on a Bio-gel P-100 column showed that the D-[U-^{14}C]galacturonic acid solubilized product contained molecules of smaller size than did the D-[U-^{14}C]apiose solubilized product. Dialysis in water or chromatography on DEAE-Sephadex caused a decrease in the amount of high molecular weight material in the solubilized products as determined by gel-chromatography.

Large, medium, and small molecular weight components of the D-[U-^{14}C]apiose solubilized product were isolated by chromatography on Bio-gel P-300 and were found not to vary significantly in D-[U-^{14}C]apiose and D-[U-^{14}C]xylose content. Addition of UDP-D-galacturonic acid to the reaction mixture used to synthesize D-[U-^{14}C]-apiose solubilized product resulted in an increase in the size of the small molecular weight components. As D-[U-^{14}C]galacturonic acid product was synthesized for increasing lengths of time the size of the product was also increased. On the other hand, as the length of incubation used to synthesize D-[U-^{14}C]apiose product was increased the percent of radioactivity found in the large molecular weight component decreased.

These results show that the particulate enzyme preparation from L. minor synthesizes a product from

UDP-D-apiose and UDP-D-galacturonic acid which has a structure similar to the apiogalacturonans of the cell wall of L. minor. The data obtained from the gel chromatography experiments are consistent with a mechanism of synthesis where D-apiose side chains are added after formation of the polygalacturonic backbone.

CHARACTERIZATION OF APIOGALACTURONANS FORMED BY A CELL-FREE SYSTEM FROM *LEMNA MINOR*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1975

To Kathryn

and my parents

ACKNOWLEDGMENTS

I would like to thank Dr. Paul K. Kindel for his guidance and advice during the course of this research project. He has served both as a colleague and a friend.

I would also like to thank Dr. Y. T. Pan and Edwin Leinbach for their comradeship and help. Mr. Leinbach prepared the UDP[U-^{14}C]GalUA used in this work. The technical assistance of Mrs. Beth Laine and Mrs. Linda Bissett is also appreciated.

I thank the members of my guidance committee, Drs. Loran Bieber, William Wells, Derek Lamport, Deborah Delmer, and Clarence Suelter for the time that they have spent reviewing my work. Drs. John Speck and A. Kivilaan have also given me much advice on polysaccharide chemistry.

I also especially thank my wife, Kathryn, for the loving encouragement and sound scientific advice that she has given me.

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BSA -- bovine serum albumin

GDPGlc -- guanosine 5'-(α-D-glucopyranosyl pyrophosphate)

GDPMan -- guanosine 5'-(α-D-mannopyranosyl pyrophosphate)

UDPApi -- uridine 5'-(α-D-apio-D-furanosyl pyrophosphate)

UDPAra -- uridine 5'-(α-L-arabinopyranosyl pyrophosphate)

UDPGalUA -- uridine 5'-(α-D-galactopyranosyluronic acid pyrophosphate)

UDPGlc -- uridine 5'-(α-D-glucopyranosyl pyrophosphate)

UDPGlcUA -- uridine 5'-(α-D-glucopyranosyluronic acid pyrophosphate)

UDPXyl -- uridine 5'-(α-D-xylopyranosyl pyrophosphate)

UTP -- uridine triphosphate

V_t -- total volume

V_o -- void volume

INTRODUCTION

Cell-free preparations from a number of plants have been prepared which contain glycosyl transferase activities that are believed to be involved in cell wall synthesis. Recently, a particulate enzyme preparation was isolated from Lemna minor (duckweed) that incorporated radioactivite sugars from UDP[U-^{14}C]Api and UDP[U-^{14}C]-GalUA into products which were insoluble in methanol and water.

The cell wall of L. minor contains large quantities of apiogalacturonans. If the products synthesized in vitro with the cell-free system from L. minor have a structure similar to authentic apiogalacturonans then this will confirm that the particulate enzyme preparation contains an enzymatic system capable of synthesizing an actual cell wall polysaccharide.

The goal of this research was to characterize the products obtained when UDP[U-^{14}C]Api and UDP[U-^{14}C]-GalUA were incubated with particulate enzyme preparation from L. minor and to determine whether the products synthesized in vitro are the same as authentic

apiogalacturonan. A second goal was to gain insight into the mechanism of cell wall synthesis by studying the structure of the products synthesized under different reaction conditions. Some of these data have been presented previously (1).

LITERATURE REVIEW

The Plant Cell Wall

Components of the Cell Wall

Plant cells contain a cell wall exterior to the plasmalemma. The cell wall is important for the structure and growth of the cell. This wall is composed of lignin, carbohydrate, and protein.

Extraction of the cell walls of higher plants with such divalent metal chelating agents as ammonium oxalate, sodium hexametaphosphate, or EDTA results in the release of a polysaccharide fraction rich in D-galacturonic acid from the cell wall (2). This material has been termed pectin and is a complex mixture of acidic and neutral polysaccharides. Such neutral sugars as D-galactose, L-arabinose, D-xylose, L-rhamnose, and L-fucose have been found in pectin (2). D-Galacturonic acid is present in the cell wall as an α-1,4-galacturonan with small amounts of L-rhamnose contained in the backbone (2, 3). The galacturonic acid resudues are often found esterified at carbon atom 6 with methanol (3). Some of the galacturonans as isolated in pectin are free of neutral sugars while other galacturonans contain large

amounts of neutral sugars bound as side chains to the uronic acid backbone. A neutral polysaccharide found in pectin is a β-1,4-D-galactan with side chains of L-arabinose. This arabinogalactan can occur in pectin as a pure polysaccharide or as neutral blocks connected to galacturnans (3, 4). Other neutral polysacchrides isolated from pectin are D-galactan and L-arabinan (2).

Those polysacchrides in the cell wall which are neither pectins nor the β-1,4-glucan, cellulose, are known as hemicelluloses (5). These polysacchrides are not structurally related to cellulose. Hemicellulose polysaccharides are generally solubilized from the cell wall by alkali extraction and the following polysacchride structures are representative of them: β-1,4-xylans with side chains of L-arabinose and 4-O-methyl glucuronic acid, β-1,4-mannans which occur alone or with side chains of D-glucose or D-galactose, and β-1,3-galactans with L-arabinose side chains (5).

Several different proteins are contained in plant cell walls. These proteins are involved in the metabolism of the wall and as structural components. The cell walls of Zea mays and Avena sativa both contain proteins capable of autolytically degrading the wall (6, 7). A hydroxyproline-rich protein was found in the primary cell wall of sycamore and bean cells grown in suspension culture (8). This protein was given the

name "extensin" by Lamport and was postulated to be a structural component in the wall which acts to orientate the matrix polysacchrides and control cell elongation (9). During cell elongation there is need for an increase in the plasticity of the cell wall and extensin may function by allowing the cellulose microfibrils to slide over one another (9). The hydroxyproline and serine residues of extensin are attached O-glycosidically to L-arabinose and D-galactose, respectively, and therefore extensin seems to be linked to arabinogalactan (10, 11, 12).

Structure of the Cell Wall

The cell wall, as seen with the electron microscope, has an ordered structure. Figure 1 is a diagrammatic representation of the cell wall of a mature plant cell (13). The cell wall is divided into two major layers; the primary wall which is formed during cell growth and the secondary wall which is formed during cell differentiation (14). The period of cell differentiation with resultant formation of the secondary wall is referred to as secondary thickening. The primary wall consists of a loose, random network of cellulose microfibrils embedded in a matrix of pectins and hemicelluloses (14). The secondary wall is divided into three layers, each of which has a highly organized microfibril structure that is orientated differently in each of

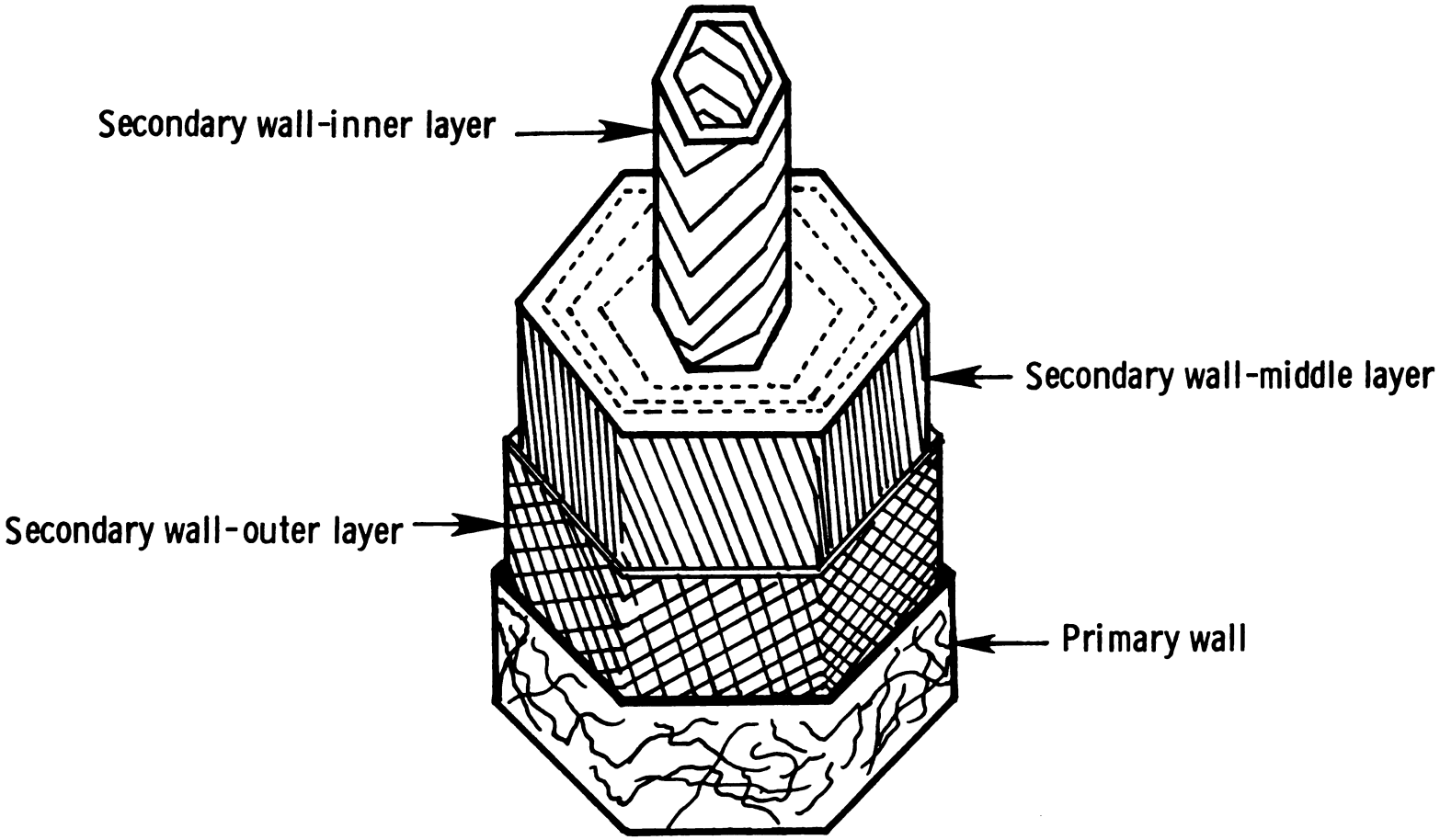


Figure 1. Diagrammatic representation of the cell wall (105).

the layers (14). Analysis of birch, ash, sycamore, and pine cell walls showed that the secondary wall contained cellulose, hemicellulose, and lignin, but not pectin (15).

The diameter of individual cellulose microfibrils is variable. Electron microscopy of negatively stained cellulose has shown that the individual microfibrils are made up of smaller, highly crystalline fibers having an average diameter of 35 $\mathring{A}$ (16). The arrangement of microfibrils in the polysaccharide matrix gives the cell wall the same increase in strength as seen in filament-wound reinforced plastic (17). The size of cellulose molecules isolated from primary and secondary walls differ. Primary wall cellulose in cotton balls has a nonuniform degree of polymerization of between 2,000 and 6,000 while secondary wall cellulose has a uniform degree of polymerization of 14,000 (M.W.=2.3 x 10^6) (18). Max-Figini has stated that there must be two different mechanisms of cellulose synthesis to account for the formation of these two cellulose species and that the uniform size of secondary wall cellulose suggests a template mechanism of synthesis (18).

Albersheim and co-workers have used chemical and enzymatic methods to degrade the extracellular and cell wall polysacchrides of suspension cultured sycamore cells (19, 20, 21). Characterization of the fragments obtained from cell wall degradation allowed these

investigators to postulate that the primary cell wall contained 4 major components (21). The first component consisted of elementary fibrils of cellulose; hydrogen bonded together to form microfibrils. The second component was a β-1, 4-glucan with side chains of xylose and fucosyl-galactose. The third component was a pectin consisting of a backbone of rhamnogalacturonan with side chains of arabinan and 4-linked galactan. The fourth component was a hydroxyproline-rich protein with arabinosyl tetrasacchride and 3, 6-linked galactan attached, respectively, to the hydroxyproline and serine residues of the protein. Albersheim and coworkers have also postulated the following arrangement for these four components in the primary wall (21). Xyloglucan is hydrogen bonded to the cellulose fibrils. This is a very tight bond because of the large number of available hydrogen-bonding sites and there is enough xyloglucan available in the wall to encapsulate all the cellulose fibrils in a monolayer of xyloglucan (20). The reducing ends of the xyloglucans are covalently bound to the galactan side chains of the pectin. The 3, 6-linked arabinogalactan side chains of the protein are in turn covalently bound to the pectin backbone. This tenative model of the primary wall indicates that all the components of the wall are tightly bound to form a single macromolecule. As Kleegstra et al. have

suggested, this model leads to interesting speculation about the mechanism of cell wall extension although other structural models for the primary wall could be derived from their data (21). Xyloglucans in cell suspension cultures of red kidney beans have been shown to have a nearly identical structure to the sycamore cell xyloglucan (22).

Morphology of Cell Wall Synthesis

Role of the Golgi Apparatus

The involvement of the golgi apparatus in plant cell wall synthesis can be deduced from electron micrographic studies of cell plate formation during cell division. The cell plate is formed by the fusion of small vesicles (23). These vesicles are derived from the golgi apparatus and their movement to the area of plate formation seems to be directed by microtubules (24, 25). Membranes from the coalescing vesicles constitute the plasmalemma of the new cell surface (26).

Further confirmation for the role of the golgo apparatus was obtained by autoradiographic studies. When intact root cells from wheat were soaked in a solution of [^{3}H]glucose for 5 min by Northcote and Pickett-Heaps the radioactivity in the cells was found to be localized in the cisternae of the golgi apparatus (27). Further treatment of the root cells for a short period with nonradioactive glucose caused the

radioactivity originally present in the golgi apparatus to be transferred to the golgi associated vesicles in the cytoplasm (27). Longer periods with nonradioactive glucose resulted in the localization of the radioactivity in the cell wall and slime layer (27). Similar autoradiographic evidence for the involvement of the golgi apparatus in cell wall synthesis was also obtained with sycamore seedlings (28).

Homogenization and fractionation of cells previously incubated with radioactive glucose have also shown that the resulting radioactive polysacchrides were localized in the golgi apparatus (29, 30, 27). The radioactive polysacchrides were identified as pectins and hemicelluloses (29, 30, 27). The absence of radioactivity in cellulose suggests that the golgi apparatus is not the site of synthesis for this glucan. I have previously cited data from the analysis of woody tissue (15) which stated that the secondary wall formed during cell differentiation did not contain pectin. However, in a fractionation experiment with differentiating maize cells soaked in radioactive glucose, radioactive pectin was found in the golgi apparatus (29). This indicates that some pectin is synthesized during synthesis of the secondary cell wall. Golgi apparatus were also the site of pectin synthesis in the elongation of Lilium longiflorum pollen tubes (31). Although the previously

discussed data all support the theory that the golgi apparatus is the site for synthesis of the plant cell wall matrix polysacchrides there have been other organelles proposed. For instance, Villemez et al. have used centrifugal fractionation to isolate the particles responsible for in vitro synthesis of a variety of polysacchrides in onion (32). Their data led them to state that the organelle responsible for polysacchride synthesis was of very large size and was most probably the plasma membrane.

The location of the polysaccharide synthesizing system in the golgi apparatus leads to a speculative theory about the control mechanism for cell wall synthesis at different stages of cell growth and differentiation. Northcote has postulated that since many of the enzymes responsible for the interconversion of sugars are membrane bound they may be contained in the golgi apparatus (33). Therefore, the golgi apparatus could control the type of polysacchride synthesized by controlling the synthesis of the appropriate precursors (33). Experimental evidence for this theory is not well documented although the appearance of UDP-glucose-4-epimerase activity in the green alga Acetabularia mediterranea is correlated with the development of the galactose-containing cap of this organism (34).

Orientation of Cellulose Microfibrils

The organelle responsible for cellulose synthesis and the mechanism for orientation of the microfibrils in the wall are not clearly understood. Microtubules are thought to have a role in cell wall and microfibril synthesis. In secondary thickening microtubules with the same orientation as the neighboring cellulose microfibrils are found adjacent to the cell wall (35, 36). This observation suggests that microtubules control the orientation of the microfibrils either by being the site of cellulose synthesis or by directing the matrix polysacchrides into the wall and thus directing the orientation of the developing microfibrils (36, 33).

Another organelle thought to be involved in the synthesis of cellulose microfibrils is a particle found attached to the plasmalemma. The 60 to 150 Å particle is clearly seen sculptured to the plasmalemma of freeze-etched yeast cells (37). Electron micrographs obtained by Moor and Muhlethaler indicated that the particles formed hexagonal arrangements on the plasmalemma and were penetrated by short fibrils, believed to be cellulose, which disappeared into the inner layer of the cell wall (38). In some cases the particles formed rows that have the same orientation as the adjacent microfibrils in the wall (39). These particles are presently thought to be the actual site of cellulose synthesis (33).

Although previously described data indicated that the golgi apparatus is not involved in cellulose synthesis, there is an important exception. The marine alga Pleurochrysis sherffelii has a cell wall composed of scales containing cellulose. An electron micrographic study of this alga show that the scale is completely synthesized in the organism's single golgi apparatus and then transported through the cytoplasm and deposited outside the cell by golgi derived vesicles (40). Therefore, in this organism cellulose is synthesized inside the golgi apparatus.

Biosynthesis of Cell Wall Polysacchrides

In vitro sugar nucleotides are the substrates for the enzymes synthesizing plant cell wall polysaccharides. The energy required to form a glycosidic bond requires that the monosacchrides be activated prior to incorporation into polysacchride (41). Nucleoside diphosphate sugars are excellent donors of sugars because of their high negative free energy of hydrolysis ($\Delta G°$). UDPGlc, for instance, has a $\Delta G°$ of hydrolysis equal to -7.600 kilocalories (41).

Synthesis of Glucans

The polysaccharide whose in vitro biosynthesis has been studied in the greatest detail is the β-1,4-

glucan, cellulose. Research in this area is often contradictory and is complicated by the fact that under certain conditions a β-1,3-glucan is also synthesized by particulate enzyme preparations.

In 1958 Feingold *et al.* first reported that particulate enzyme preparations from *Phaseolus aureus* (mung bean) could incorporate radioactivity from UDP-[U-^{14}C]Glc into a polysaccharide identified by partial acid hydrolysis as β-1,3-glucan (42). The β-1,3-glucan in plants is given the common name callose, but it is not a normal constituent of the plant cell wall (43). However, the synthesis of callose is known to be an important response to cell wounding and callose is a major component of sieve plates and pollen tubes (43). Although the synthesis of β-1,4-glucan from UDPGlc was not observed by Feingold *et al.* in *P. aureus* other researchers using approximately the same techniques have reported the simultaneous production of β-1,3- and β-1,4-glucan with particulate enzyme preparations from *P. aureus* and *Lupinus albus* (42, 44, 45). After publication of these conflicting experiments Hassid and coworkers undertook a careful study of the *P. aureus* and *L. albus* systems and announced that only β-1,3-glucan was synthesized from UDPGlc (46). To heighten the uncertainty of the identity of the UDPGlc product from *P. aureus* it should be noted that one of the original

reports identifying β-1,4-glucan as a product of UDPGlc came from Hassid's laboratory (44). A partial explanation for the discrepancy in the identity of the product synthesized from UDPGlc with P. aureus particulate enzyme preparations was proposed by Clark and Villemez. They reported once again that this enzyme preparation could synthesize a mixture of β-1,3- and β-1,4-glucans from UDPGlc but that the relative amounts of the two glucans produced was dependent on the temperature used to germinate the P. aureus seedlings (47). Although they could not explain why other researchers were unable to find evidence of β-1,4-glucan synthesis they felt that this may be the result of subtle differences in methods of enzyme preparation (47).

In contrast to the P. aureus system, the ability of particulate enzyme preparations from Avena sativa (oat) seedlings to synthesize β-1,3- and β-1,4-glucans from UDPGlc is well documented (48, 49). The concentration of UDPGlc in the reaction mixture determined whether production of β-1,3- or β-1,4-glucan predominated (49). The particulate enzyme preparation had UDPGlc Km's of 1.1×10^{-5} M and 6×10^{-5} M for the synthesis of β-1,4- and β-1,3-glucan, respectively (49). Particulate enzyme homogenates from wheat seedlings and Lilium multiflorum endosperm also exhibit a UDPGlc

concentration dependent synthesis of β-1,3- and β-1,4-glucans (50, 51).

Separate enzyme systems seem to be responsible for the synthesis of β-1,3- and β-1,4-glucans. Tsai and Hassid have "solubilized" the two activities by digitonin extraction and separated them from one another by absorption on hydroxylapatite gel and elution with phosphate buffer (51). Studies seeking to identify the organelles responsible for β-1,3- and β-1,4-glucan synthesis have provided additional evidence for the existence of separate enzyme systems. Ray et al. have used a combination of velocity and isopycnic density gradient centrifugation to isolate the enzymatic particle in pea seedling homogenates which are responsible for the synthesis of β-1,4-glucans from UDPGlc and GDPGlc (52). Electron micrographs showed that the isolated fraction with glucan synthetase activity contained condensed golgi dictyosomes and free dictyosomal membranes (52). An elegant study by Morré and co-workers showed that two organelles were responsible for glucan synthesis in onion stem: plasma membrane and golgi apparatus (53). Both of these organelles were able to synthesize β-1,3- and β-1,4-glucans but at 1.5 μM UDPGlc concentration the majority of the β-1,4-glucan synthesis occurred in the golgi dictyosome fraction and at 1 mM UDPGlc concentration the majority

of the β-1,3-glucan synthesis occurred in the plasma membrane fraction (53).

The discoveries by Morré and Ray that golgi membranes could synthesize β-1,4-glucan from UDPGlc seems to conflict with the previously discussed data on the absence of *in vivo* cellulose synthesis in golgi apparatus (29, 30, 27). Morré has stated that β-1,4-glucan synthesis in his golgi preparations may be part of a synthesis system for glycoproteins or hemicellulose polysaccharides (53). It is also possible that the golgi apparatus contained inactive cellulose synthesizing enzymes prior to their transport to the plasmalemma and the isolation of the golgi caused activation of these enzymes (53).

Plant particulate enzyme preparations can also use GDPGlc to synthesize glucans. A particulate enzyme preparation from *P. aureus* could incorporate D-glucose from GDPGlc into a polysaccharide component (54, 55). The polysaccharide was identified as cellulose since it released a series of β-1,4-glucan oligosaccharides after partial acid hydrolysis (55). The absolute identity of this material as cellulose, however, is unclear because the incorporation of D-glucose from GDPGlc in *P. aureus* was affected by the addition of GDPMan to the reaction mixture (56, 57). D-Mannose and D-glucose were both incorporated into an alkali insoluble material and

enzymatic degradation and acetolysis have shown that the material was a β-1,4-glucomannan (56). Addition of GDPMan to a reaction mixture containing GDP[U-^{14}C]Glc and a particulate enzyme preparation from P. aureus did not affect the rate of D-[U-^{14}C]glucose incorporation but did increase the total amount of incorporation (56, 58). The increase in total D-glucose incorporation as the result of the presence of GDPMan is believed to be caused by the synthesis of additional D-mannose-containing D-glucose acceptor. Addition of GDPGlc to a reaction mixture containing GDP[U-^{14}C]Man and the particulate enzyme preparation caused an increase in the rate of incorporation of D-[U-^{14}C]mannose and a decrease in the total amount of D-[U-^{14}C]mannose incorporated (58). It should be noted that in order to measure the initial rate of D-glucose and D-mannose incorporation the reaction mixture was extracted with lipid solvents in order to remove glycoproteins that were also synthesized (58). Synthesis of β-1,4-glucans with GDPGlc has also been reported with particulate enzyme preparations from L. albus and pea seedlings (51, 52). The L. albus preparation also contained UDPGlc-2-epimerase activity and D-mannose was incorporated from the resulting GDPMan (52). In the case of P. aureus Villemez has stated that GDPGlc is the substrate for the synthesis of a gluco-mannan rather than cellulose (58).

The role of UDPGlc and GDPGlc in cellulose synthesis was also studied in cotton. Barber and Hassid reported that a particulate enzyme preparation prepared from 4- to 8-day-old cotton balls could synthesize cellulose from GDPGlc and the extent of D-glucose incorporation was stimulated by the presence of GDPMan (59). Enzyme prepared from older balls was inactive (59). As previously mentioned it has been proposed that cellulose in the primary and secondary walls is synthesized by different mechanisms (18). Marx-Figini, in interpreting Barber's and Hassid's experiment, felt that the β-1, 4-glucan synthesized from GDPGlc in cotton represented synthesis of primary wall cellulose (60). He stated that Barber and Hassid were probably unable to synthesize secondary cell wall cellulose in their enzyme preparation because the isolation of the enzyme would have destroyed the template mechanism necessary for synthesis of homologous secondary wall cellulose (18, 60). Later work by Delmer and Beasley with intact isolated cotton fibers showed that at the stage of growth when primary wall is produced the fibers incorporated D-glucose from GDPGlc into alkali insoluble glucan (61). As the fibers aged formation of the primary wall ceased and secondary wall synthesis began (61). During this same time period the fibers lost the ability to synthesize glucan from GDPGlc but gained the ability to synthesize glucolipids from

UDPGlc (61). Delmer and Beasley explained these results by suggesting that in cotton GDPGlc is used for synthesis of cellulose only in the primary wall (61). This theory leads to interesting speculation about the control of cellulose synthesis, but must be tempered by the knowledge that the identity of the β-1,4-glucans synthesized from UDPGlc and GDPGlc as cellulose in both the P. aureus and cotton systems is not positive. In fact, x-ray diffraction studies of glucans synthesized in vitro with a particulate enzyme preparation from P. aureus suggested that only low molecular weight oligosaccharides were synthesized from UDPGlc and GDPGlc (62).

The bacterium Acetobacterium xylinum can synthesize fibrils of β-1,4-glucan which are identical in structure to the cellulose fibrils found in plants (63). Fibrils are formed extracellularly and since they are easily isolated, cellulose synthesis in A. xylinum has been extensively studied (63, 64). Intact cells will incorporate radioactivity from D-[U-^{14}C]glucose into cellulose (65). Cell-free homogenates obtained from A. xylinum were able to synthesize cellulose fibrils when supplied with D-glucose and ATP. Later work with cell-free homogenate prepared by sonication showed that UDPGlc was a precursor of cellulose of A. xylinum synthesis (66).

Synthesis of Matrix Polysaccharides

A variety of researchers have investigated the ability of particulate enzyme preparations from different plants to incorporate monosaccharides from sugar nucleotides into suspected cell wall matrix polysaccharides. Although many different sugars have been investigated, little success has occurred beyond announcements that particular enzyme activities have been discovered. The inability of researchers to go beyond this point and to investigate the mechanism of cell wall polysaccharide synthesis attests to the complexity of this system.

Incubation of a particulate enzyme preparation from immature corn cobs with UDP[U-^{14}C]GlcUA resulted in the incorporation of radioactivity into a water-insoluble fraction (67). Extraction of the insoluble material with ammonium oxalate and alkali showed that the radioactivity was incorporated into both pectin and hemicellulose polysaccharides (67). Hydrolysis of the fractions followed by paper chromatography of the hydrolysates showed that the radioactivity was contained in D-xylose, L-arabinose, glucurono-galactose, glucurono-xylose, D-galacturonic acid, and D-glucuronic acid with the majority of the radioactivity contained in D-xylose (67). The particulate enzyme preparations must have contained UDPGlcUA carboxylyase (EC, 4.1.1.35), UDPGlcUA-4-epimerase (EC, 5.1.3.6),

and UDPAra-4-epimerase (EC, 4.1.3.5) activities to account for the presence of these radioactive sugars in the polysaccharides. The D-galactose residue in glucuronogalactose was not radioactive (67). Therefore, D-galactose must have been incorporated into the acceptor molecule prior to the preparation of the particulate enzyme.

As described previously the D-glucuronic acid residues in hemicellulose polysaccharides are often methylated (5). Methylation occurs by the enzymatic transfer of methyl groups from S-adenosyl-L-methionine to the glucuronic residues of previously synthesized hemicellulose (68).

Incorporation of D-xylose and L-arabinose from their respective uridine sugar nucleotides into hemicellulose was demonstrated with particulate enzyme preparations from immature corn cobs and Zea mays seedlings (69, 70). The product was soluble in alkali and identification of the oligosaccharides obtained after partial acid hydrolysis showed that the product consisted of a D-xylan backbone with L-arabinose side chains (69, 70). Another matrix polysaccharide synthesized with P. aureus seedling particulate enzyme was D-galactan. The substrate was UDPgalactose and the resulting water soluble polysaccharide had a molecular weight greater than 4,600 (70).

Particulate enzyme preparations from P. aureus and tomatoe could synthesize polygalacturonic acid when supplied with UDPGalUA (72, 73). Since UDP-methylgalacturonic acid will not function in this synthesis, it is assumed that methylation of D-galacturonic acid occurs after synthesis of the polysaccharide (72). The enzymatic introduction of methyl ester groups into pectin was demonstrated in P. aureus with S-adenosyl-L-methionine as the substrate (74). Methylation occurred with endogenous pectin but not with exogenous pectin that was present in the reaction mixture (75). This suggests that the location of the esterification system is within a membrane-bound organelle. Studies by Kauss et al. have shown that pectin methyl esterase when added to the particulate enzyme preparation would not degrade methylesterified pectin synthesized in vitro unless the lipid membrane of the particulate enzyme was disrupted by treatment with detergent or phospholipase A (73).

The structure of radioactive polygalacturonic acid synthesized from UDP[^{14}C]GalUA was investigated by enzymatic degradation with exopolygalacturonate transeliminase (76). This enzyme degrades polygalacturonic acid from the reducing end (77). When the product synthesized in vitro was treated with this enzyme, radioactivity was released solely as unsaturated digalacturonic acid (76). The amount of unsaturated digalacturonic acid released

was proportional to length of treatment (76). Since synthesis of polysaccharides by glycosylation occurs from the nonreducing end, the results of the transeliminase treatment indicate that the polygalacturonic acid was labelled throughout the chain and incorporation of galacturonic acid did not occur by the addition of a few residues to the ends of preformed chains.

Possible Role of Lipid Intermediate

The role of polyisoprenol sugar intermediates in the synthesis of components of the bacterial cell wall is well established (78). The possibility that similar lipid intermediates may function in plant cell wall synthesis has been investigated by several research groups.

Cell-free preparations from A. xylinum incorporated radioactivity from UDP[^{14}C]Glc into lipid material (79). Radioactive sugar was released from this material by treatment in 0.01 N H_2SO_4 at 100°C or in 0.1 N LiOH at room temperature (79). The radioactive products released from the glycolipid were identified as glucose, cellobiose, and possibly other higher intermediates (79). This suggests that in this organism a polyprenoidpyrophosphate may be an intermediate in cellulose synthesis.

Jung and Tanner have shown that the synthesis of yeast "mannan" from GDPMan proceeds through the high

energy lipid intermediate, dolichol monophosphate mannoside (80). The identity of the lipid as dolichol was verified by mass spectroscopy (80). Yeast "mannan" proved to be a heterogeneous glycoprotein and the mannolipid acted only to transfer a mannosyl residue to the protein (81). The rest of the mannose residues were added directly to the mannan from GDPMan without the intervention of dolichol monophosphate (81).

Several researchers have demonstrated the existence of dolichol isoprenoid compounds in higher plants. Both Kauss and Villemez have isolated a mannosyl lipid from a reaction mixture containing particulate enzyme preparation from P. aureus and GDP[U-^{14}C]Man (82, 83). The lipid was tentatively identified as an isoprenoid by a preliminary mass spectral analysis and the fact that in vivo studies showed that the lipid was synthesized from 5-[^{3}H]-DL-mevalonic acid (82, 84). The mannosyl lipid had a high transfer potential as evidenced by the fact that the sugar was released by acid hydrolysis at pH 2 (84). In addition, the incorporation of mannose into lipid could be reversed by addition of GDP but not GMP to the reaction mixture (84). Villemez, when studying the synthesis of the mannosyl lipid from GDPMan, isolated a low molecular weight membrane-bound protein from P. aureus which contained D-mannose oligosaccharides (84). A similar glycoprotein was also isolated when

the particulate homogenate was incubated with GDP[U-^{14}C]-Glc (84). He postulated that this glycoprotein may also be an intermediate in cell wall polysaccharide synthesis and could obviate the need for a primer polysaccharide (84).

Storm and Hassid were unable to demonstrate the transfer of D-mannose from endogenous mannosyl lipid to polysaccharide in P. aureus particulate enzyme preparations (85). This experiment indicated that the mannosyl lipid is not an intermediate in cell wall polysaccharide synthesis. Particulate enzyme preparations from P. aureus did transfer D-mannose from GDPMan to the exogenous lipids phytol phosphate, phytanol phosphate, dolichol monophosphate, and betulaprenol phosphate (86, 87). Since addition of these exogenous lipids allow for the synthesis of larger quantities of glycolipids, it is hoped that this will help in elucidating the possible role of glycosyl isoprenoids in plant cell wall synthesis.

Particulate enzyme preparations from cotton fibers have also shown evidence of containing lipid intermediates. Radioactive D-glucose and D-mannose were incorporated into an acid lipid fraction when UDPGlc and GDPMan, respectively, were incubated with particulate enzyme (88). The synthesis of the glycosyl lipids was reversed by the addition of UDP and GDP to the reaction mixture (88). Isolated glycosyl lipids were labile to

acid hydrolysis at pH 2 (88). The free lipid chromatographed with an R_f similar to ficoprenyl phosphate and the natural acceptor in the particulate enzyme preparation could be replaced by ficoprenyl phosphate (88).

Solubilization of Polysaccharide Synthesizing System

The inability of researchers to solubilize the plant cell wall polysaccharide synthesizing systems has hampered research efforts in understanding the mechanism of this complex process. The partial "solubilization" of the UDPGlc and GDPGlc dependent glucan synthesizing activities in *P. aureus*, *A. sativa*, and *L. albus* by extraction with digitonin has been reported (89, 57, 90). However, the procedure does not result in complete solubilization and there are still particles contained in the "solubilized" supernatant (89). Digitonin, therefore, probably did not cause a true release of particulate enzyme from membrane complexes. Heller and Villemez have reported the solubilization of the glucomannan synthesizing system in *P. aureus* by the use of Triton X-100 extraction (91). The solubilized enzyme activity could transfer sugar moieties from GDPGlc and GDPMan to suspected polysaccharides, but could not use UDPGlc, UDPGal, UDPXyl, UDPArab, or UDPGlcUA (91). Solubilization with Triton X-100 resulted in a 3.5 increase in the specific activity of the GDPMan activity and the

activity remained in solution after centrifugation at 300,000 x g for 40 min (91). The Trition solubilized activity was not purified further.

D-Apiose

Chemistry

In 1901 Vongerichten discovered that apiin, a flavonoid glycoside found in parsley, contained a previously unknown pentose sugar which he named apiose (92). Vongerichten later reported that apiose has a branched chain structure (118). The structure of the naturally occurring apiose was finally elucidated as 3-C-hydroxymethyl-aldehydo-D-glycero-tetrose (94). The Fisher (I) and Haworth (II) structures of D-apiose are shown. Structure II is one of 4 possible cyclic isomers of D-apiose. A detailed description of the chemistry of D-apiose is found elsewhere (95).

Occurrence in Nature

The first example of the occurrence of D-apiose in compounds other than a flavonoid glycoside was reported by Bell et al. These results suggested that D-apiose was a constituent of the polysaccharide fraction of the marine alga Posidonia australis (96). Besides P. australis the following plants have also been shown to possess polysaccharides containing D-apiose: Zostera marina (97, 98, 99), Lemna gibba

(100), Z. nana (101), Z. pacifica and Phyllospadix (98), and L. minor (101).

All reports on the occurrence of D-apiose in nature have been limited to members of the plant kingdom. However, D-apiose is a common constituent of plants. Duff has surveyed 175 plants and found that D-apiose was contained in the acid hydrolysates of at least 60% of the plants tested (101). According to Duff, one of the plants with the highest content of D-apiose was L. minor (duckweed) (101). D-apiose was localized in the cell wall of L. minor (101).

Identification of Apiogalacturonans in L. minor

Beck (102) and Hart and Kindel (103) extracted cell wall material from L. minor with ammonium oxalate and isolated a family of polysaccharides containing D-apiose and D-galacturonic acid. Both groups were able to further fractionate the polysaccharides after ammonium oxalate extraction. Beck isolated two fractions containing D-apiose and D-galacturonic acid, one of which also contained D-xylose and D-galactose (103). Hart and Kindel used a combination of NaCl fractionation and DEAE-Sephadex chromatography to fractionate their material into a series of polysaccharides containing D-apiose and D-galacturonic acid (102). The content of D-apiose in these polysaccharides varied from 7.9

to 38.1% by weight (102). The fractions were reported to contain only D-apiose and D-galacturonic acid although the techniques used by these workers were specific only for these two sugars (102). Hart and Kindel did report, however, that in analyzing the D-apiose content of the polysaccharides one and sometimes two faint spots not corresponding to D-apiose were found when the acid hydrolysate of the polysaccharide fractions were chromatographed on paper (102). The identities of these spots were not investigated.

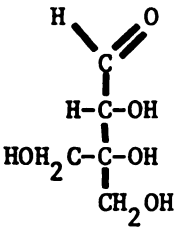
The following information on the isolation and partial characterization of the polysaccharides containing D-apiose isolated from L. minor was obtained from Hart and Kindel (102). When they extracted cell wall preparations from L. minor with 0.5% ammonium oxalate, 14% of the wall material was solubilized. This solubilized material contained 20% of the D-apiose originally present in the cell wall. After fractionation of the solubilized material, all the polysaccharides isolated were of the same general type: D-galacturonans with side chains of D-apiobiose. The sensitivity of the apiogalacturonans to pectinase degradation was found to be dependent on the D-apiose content of the polysaccharides. Those apiogalacturonans of low D-apiose content and insoluble in 1 M NaCl were degraded by pectinase, while those of high D-apiose content and soluble in 1 M NaCl were not

degraded. Resistant apiogalacturonans could be degraded by pectinase if the D-apiobiose side chains were first removed from the polysaccharide by mild acid hydrolysis. Formaldehyde release after periodate oxidation of apiogalacturonans showed that about 50% of the D-apiose molecules were substituted at either the 3 or 3' position.

Mild acid hydrolysis of the ammonium oxalate extracted material resulted in the release of a disaccharide from the polysaccharide which was identified as apiobiose (III) (104). Purified apiogalacturonan fraction IIa (as defined by Hart and Kindel [102]) was used to study the release of apiose from apiogalacturonan. Hydrolysis of fraction IIa at pH 4 for 3 hr at 100°C resulted in near total release of the D-apiose in the polysaccharide as apiobiose (104). The rate of D-apiose release from polysaccharide was pH dependent. The rate declined steadily from pH 3.5 to almost zero at pH 6.5 (104). These results led Hart and Kindel to propose that the structure of apiogalacturonans (IV) in L. minor consisted of a backbone of α-1, 4-linked polygalacturonic acid with side chains of apiobiose (102, 104).

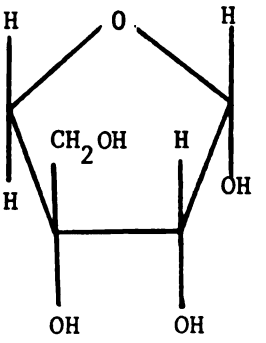
UDPApi

D-apiose is synthesized enzymatically in plants by the conversion of UDPGlcUA to UDPApi and CO_2 (105, 106). The enzyme responsible for this reaction has been



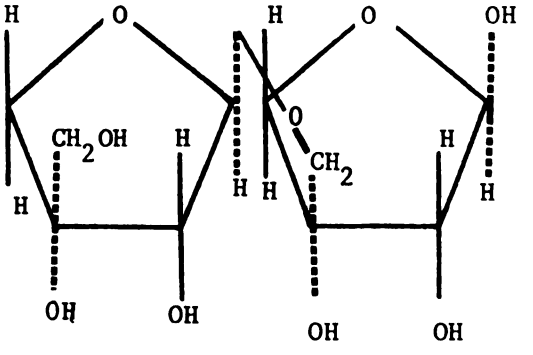
(I)

D-apiose



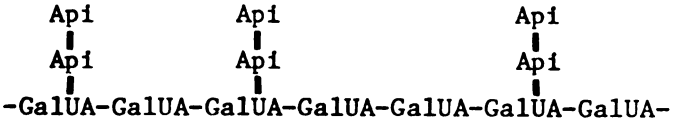
(II)

D-apiosylfuranose



(III)

apiobiose



(IV)

apiogalacturonan

List of structures. The dotted lines indicate undetermined stereochemistry.

partially purified from L. minor and has been given the common names UDPGlcUA cyclase and D-apiose synthetase (93, 107, 108). UDPApi was found to be an extremely labile compound as demonstrated by its half-life of 97.2 min when stored at pH 8.0 and 25°C (106). However, under proper conditions UDPApi can be stored for long periods of time. When stored at pH 6.4 and -20°C 94% of the UDPApi was still intact after 120 days (108).

In Vitro Synthesis of Apiogalacturonans

In vitro synthesis of apiogalacturonans in L. minor should, for a number of reasons, be an excellent system for studying cell wall polysaccharide synthesis.

1. Apiogalacturonans are presumably of physiological significance because of the large quantity contained in the cell wall of L. minor.
2. Because of the large quantities of apiogalacturonan in L. minor cell walls the apiogalacturonan synthesizing system should be easy to detect.
3. We can compare the structure of authentic apiogalacturonans with in vitro synthesized D-apiose containing compounds.
4. Procedures for characterizing apiogalacturonans are already developed.

5. Apiogalacturonans can be solubilized by relatively mild treatment.

From previous discussion of the mechanism of cell wall polysaccharides synthesis one would expect that sugar nucleotides containing D-apiose and D-galacturonic acid would be the precursors of apiogalacturonan. Kindel has reported that radioactivity from UDP[^{14}C]Api was incorporated into material insoluble in water and methanol when the sugar nucleotide was incubated with a particulate enzyme preparation from L. minor (109). This material was solubilized when extracted with 0.5% ammonium oxalate (109). Although UDP[^{14}C]Xyl was also present in the reaction mixture, Kindel did not observe D-[U-^{14}C]Xyl incorporated into the methanol and water insoluble material (109). Hydrolysis of the material at pH 4 resulted in the release of [U-^{14}C]apiobiose side chains thus suggesting that D-[U-^{14}C]apiose was incorporated into apiogalacturonans or a compound of similar structure (107).

The incorporation of D-apiose with the particulate enzyme preparation from L. minor has been further investigated by Pan and Kindel (110 and unpublished results). They found that the enzyme responsible for the transfer of D-apiose from UDPApi has a K_m of 4.9 μM and maximal activity at pH 5.7. They have also found that the particulate enzyme preparation from L. minor could

transfer D-[U-^{14}C]-xylose from UDP[U-^{14}C]Xyl to a methanol and water insoluble material. This activity has a K_m of 12.7 μM and maximal activity at pH 5.7. Leinbach and Kindel have found that particulate enzyme preparation from L. minor can also incorporate D-[U-^{14}C]galacturonic acid from UDP[U-^{14}C]GalUA into a methanol and water insoluble material (119). This activity exhibited a K_m of 8 μM and maximal activity at pH 6.2.

EXPERIMENTAL PROCEDURES

Materials

DEAE-Sephadex A-25 (particle size 40-120 μ, capacity: 3.5 ± 0.5 mequivalents/g) was purchased from Pharmacia Fine Chemicals. Bio-Gel P-30 (100-200 mesh), Bio-Gel P-100 (50-150 mesh), and Bio-Gel P-300 (50-100 mesh) were obtained from Bio-Rad Laboratories. UDPXyl and UTP were purchased from Schwarz/Mann and P-L Biochemicals, respectively. UDPGlcUA, UDPGalUA, and fungal pectinase were purchased from Sigma Chemical Co. The pectinase was partially purified by the manufacturer who stated it still contained several other enzymes. UDP-[U-^{14}C]GlcUA (190 mCi/mmole), UDP[U-^{14}C]Xyl (156 mCi/mmole), and D-[U-^{14}C]galactose-1-P (214 mCi/mmole) were obtained from New England Nuclear Corp. UDP[U-^{14}C]GalUA was synthesized from D-[U-^{14}C]galactose-1-P and UTP by the method of Feingold *et al.* (59). D-Apiose, apiobiose, D-apiitol, [^{14}C]22° sodium chloride soluble apiogalacturonan fraction, [^{14}C]60° sodium chloride soluble apiogalacturonan fraction and 22° sodium chloride insoluble apiogalacturonan fraction were prepared by Hart and

Kindel (102, 104). The specific activity of the [^{14}C] apiogalacturonan fractions ranged from 14,000 to 16,000 dpm per mg.

UDP[U-^{14}C]Api was enzymatically synthesized from UDP[U-^{14}C]GlcUA (108, Pan and Kindel, unpublished experiments). From the data of 11 individual preparations of UDP[U-^{14}C]Api, the percent of the total radioactivity in the final solution present in UDP[U-^{14}C]Api, UDP[U-^{14}C]Xyl, and UDP[U-^{14}C]GlcUA ranged from 61 to 72, 24 to 40, and 0 to 8%, respectively. UDP[U-^{14}C]Xyl was simultaneously synthesized with UDP[U-^{14}C]Api because of the presence of UDPGlcUA carboxy-lyase I activity in the UDPGlcUA cyclase preparations. The mixture of the three radioactive sugar nucleotides will be referred to as "UDP-[U-^{14}C]Api." In the present paper, values of radioactivity and sugar nucleotide content reported for "UDP[U-^{14}C]Api" are the sum of the total amounts contained in UDP[U-^{14}C]Api, UDP[U-^{14}C]Xyl, and UDP[U-^{14}C]-GlcUA. UDP[U-^{14}C]Api solution was stored frozen at -90°C.

General Methods

Radioactivity was detected on paper chromatograms with a Packard radiochromatogram scanner, model 7201 (Packard Instrument Co. Inc.). All other radioactivity measurements were made with a Packard Tri-Carb liquid-scintillation counter, model 3310, with one of the following scintillation solutions: (A) Bray's solution

(111); (B) 5.5 g 2,5-diphenyloxazole and 0.1 g 1,4-bis [2-(4-methyl-5-phenyloxazolyl)]-benzene in 667 ml of reagent grade toluene and 333 ml of Triton X-100; (C) 4.0 g 2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene in 1 liter of reagent grade toluene. Aqueous solutions of radioactive material (1.0 to 1.5 ml) were counted in 10 ml of scintillation solution A or B. Radioactive compounds on paper were counted directly by complete immersion of the paper in scintillation solution C. The counting effeciencies with scintillation solutions A, B, and C were 77, 81, and 63%, respectively.

Solutions were concentrated under reduced pressure by rotary evaporation at temperatures below 30°C. Gel chromatography was performed with columns prepared as recommended by the manufacturer; elution was performed at room temperature with descending flow. Dialysis was performed with tubing that had been freshly prepared by heating 30 min at 100°C. Means and mean deviation were reported for experimental values based on more than one determination.

Culture of L. Minor

L. minor was grown as described by Kindel and Watson (108). The plants used in the preparation of the particulate enzyme preparation were grown on 4 liters of inorganic medium in small plastic pans (29 x 33 cm).

After the fronds had multiplied to the point of covering the surface of the medium (18 to 22 days) L. minor were removed as needed.

Paper Chromatography

Descending paper chromatography was used and was performed with washed Whatmann No. 3MM paper. Washing was done with 0.1 M citric acid followed by water. Chromatography was performed at 22°C and the following solvents were used: (A) ethyl acetate - H_2O - acetic acid - formic acid (18:4:3:1, by vol), (B) n-propanol - ethyl acetate - H_2O (85:10:5, by vol), (C) isopropanol - H_2O (9:1, v/v), (D) H_2O saturated butanol, (E) ethyl acetate - acetic acid - pyridine - H_2O (5:1:5:3, by vol). Nonradioactive sugars were detected on chromatograms by spraying with aniline hydrogen pthalate (112) or by using the $AgNO_3$ dip method (113).

DEAE-Sephadex Column Chromatography

Columns of defined DEAE-Sephadex A-25 were treated with 3 bed volumes of 0.05 M phosphate buffer, pH 7.7, before the sample was applied. Samples were dialyzed in 0.05 M sodium phosphate buffer, pH 7.7, for 2 hr with one buffer change midway through the dialysis, before being applied to the column. The rate of sample application was equal to the rate used to operate the column. After the sample was applied the column was

treated with additional buffer followed by a step gradient of 0.1 to 0.3 M NaCl in 0.05 M sodium phosphate buffer, pH 7.7. Except for Figure 10 the salt gradient described on the figures indicates the first column fraction that each step of the gradient was applied. The column was operated at 4°C.

Isolation of Particulate Enzyme Preparation

L. minor was collected on 4 layers of cheesecloth, washed thoroughly with distilled water, freed of excess water with absorbent paper, and weighed. The remaining steps were performed at 4°C. The fronds were ground to a smooth consistency (for 1 to 2 min) with a mortar and pestle in 0.05 M sodium phosphate buffer, pH 7.3, containing 1% BSA (wt/vol) and 1 mM $MgCl_2$. Two ml of buffer were used for every 1 g wet weight of plant material. The resulting homogenate was filtered through 4 layers of cheesecloth and the filtrate was centrifuged for 10 min at 480 g. The precipitate was discarded and the supernatant solution was centrifuged for 8 min at 34,800 g. The supernatant solution was discarded and the precipitate was gently resuspended in 0.1 M sodium phosphate buffer, pH 6.0, containing 1% BSA (wt/vol) and 10% sucrose (wt/vol) with a Ten Broeck glass homogenizer. For every 10g wet weight of L. minor used, 0.5 ml of the buffer was used for resuspension.

This was called the particulate enzyme preparation. The final pH of the particulate enzyme preparation was 6.1.

Biosynthesis of Radioactive Product and Solubilized Product

Radioactive product which was insoluble in methanol and water was formed when "UDP[U-^{14}C]Api," UDP[U-^{14}C]GalUA, UDP[U-^{14}C]GlcUA, or UDP[U-^{14}C]Xyl was incubated with particulate enzyme preparation. A reaction mixture was prepared which contained 50 µl of particulate enzyme preparation and either 0.17 or 0.33 nmoles "UDP[U-^{14}C]Api" (60,000 to 120,000 dpm), 0.05 to 0.10 nmole of UDP[U-^{14}C]GalUA (25,000 to 36,000 dpm), 0.1 to 0.3 nmole of UDP[U-^{14}C]GlcUA(55,000 to 124,000 dpm) or 0.08 nmole of UDP[U-^{14}C]Xyl (91,200 dpm). Eleven different preparations of "UDP[^{14}C]Api" were used in the experiments reported in the present paper. The proportions of the three sugar nucleotides present in the solutions of "UDP[U-^{14}C]Api" were stated earlier in this section. In specified experiments UDPGalUA, UDPXyl, or UDPGlcUA was added to the reaction mixtures containing "UDP[U-^{14}C]Api." The volume of the reaction mixtures containing "UDP[U-^{14}C]Api" or UDP[U-^{14}C]GalUA were 70 µl. The volume of the other reaction mixtures will be stated in the appropriate places in the Results.

The reaction mixtures were incubated for 15 min at 25°C. At the end of the incubation period 1 ml of 75% aqueous methanol (v/v) containing 1% KCl (wt/vol) was

added to the mixture and the precipitate was collected by centrifugation at room temperature. The supernatant solution was discarded and the precipitate was then extracted at room temperature as follows: twice with 1 ml portions of 75% aqueous methanol containing 1% KCl, twice with 1 ml portions of methanol, and once with a 1 ml portion of water. The radioactive material retained in the residue remaining after these extractions is referred to as the product in this dissertation. In the experiments reported here, D-[U-^{14}C]apiose product, D-[U-^{14}C]galacturonic acid product, D-[U-^{14}C]glucuronic acid product, and D-[U-^{14}C]-xylose product all refer to the products synthesized from "UDP[U-^{14}C]Api," UDP[U-^{14}C]GalUA, UDP[U-^{14}C]GlcUA, and UDP[U-^{14}C]Xyl, respectively. When UDPGalUA, UDPGlcUA, or UDPXyl were added to the reaction mixture used to synthesize D-[U-^{14}C]apiose product it will be stated explicitly that the D-[U-^{14}C]apiose product was synthesized in the presence of a specific nonradioactive sugar nucleotide.

Radioactive material was solubilized from the product by suspending the product in freshly prepared 1% ammonium oxalate, pH 6.5, and incubating for 15 min at 50°C. After incubation the suspension was centrifuged and the supernatant solution was removed. This procedure was repeated 3 additional times. In the first

extraction 0.3 ml of ammonium oxalate solution was used while in each of the subsequent 3 extractions 0.1 ml of ammonium oxalate solution was used. The four extracts were combined. The radioactive material contained in the combined extracts is referred to as solubilized product in this dissertation. The same terminology used in the preceding paragraph is used to describe which radioactive sugar nucleotides were used to synthesize the solubilized product and whether nonradioactive sugar nucleotides were present in the reaction mixture, i.e., D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA. The amount of radioactivity in the residue remaining after the extractions with ammonium oxalate were completed was determined by adding 3 drops of 2% NaOH to the residue, heating the suspension briefly in a boiling water bath, applying the suspension to a 2 cm x 6 cm piece of chromatography paper, and then assaying the paper for radioactivity by using scintillation solution C. In almost all experiments reported the solubilized products from several reaction mixtures were combined and then used in the various experiments. Whenever the solution containing solubilized product was concentrated it was first dialyzed either in 0.05 M sodium phosphate buffer, pH 6.8 or 7.7, or in water.

Partial Acid Hydrolysis with Fuming HCl

Radioactive product was suspended in concentrated HCl to which an equal volume of fuming HCl (concentrated HCl saturated with HCl gas at 4°C) was added (42). The mixture was incubated at 27°C for 60 min. After incubation the mixture was diluted with 10 volumes of water and the HCl was removed by concentrating the sample to dryness at 40°C. The dilution and concentration was repeated 2 additional times. The resulting hydrolysis products were identified by paper chromatography.

Hydrolysis at pH 1

The radioactive sample in solution was placed in a 1 dram screw-cap vial. A quantity of 2 M trifluoroacetic acid equal to one-tenth the volume of the sample was then added to the vial. The pH of the resulting solution was tested with pH paper. If the pH was above 1.5 then additional 2 M trifluoroacetic acid was added until the pH was between 1 and 1.5. In this dissertation these conditions will be referred to as hydrolysis at pH 1. The solution was heated in an autoclave for 30 min at 121°C and 15 lb pressure, and it was then spotted on chromatography paper. The paper was developed in the appropriate solvent, scanned, and the radioactive areas were cut out and counted in scintillation solution C. Recovery of radioactive material on paper

chromatograms after hydrolysis at pH 1 was routinely between 85 and 90%.

Hydrolysis at pH 4

The radioactive sample in solution and an equal volume of 0.5 M sodium acetate buffer, pH 4.0, were placed in a 1 dram screw-cap vial. The vial was heated for 3.5 hr at 100°C and the resulting hydrolysate was spotted directly on chromatography paper. The paper was developed with solvent A, scanned, and the radioactive areas were cut out and counted in scintillation solution C. The high concentration of acetate buffer used in this procedure was necessary because of the inherent buffering capacity of some of the samples. Recovery of radioactive materials on paper chromatograms after hydrolysis at pH 4 was routinely between 85 and 95%.

Hydrolysis of Solubilized Product With Fungal Pectinase

Radioactive solubilized product was dialyzed in water at 4°C as described in the individual experiments in order to remove ammonium oxalate. Portions of the solution of dialyzed solubilized product were mixed with a quantity of 0.5 M sodium acetate buffer, pH 4.5, equal to one-tenth the volume of the portion. The concentration of pectinase in the 0.5 M acetate buffer was 20 mg/ml.

The solution was incubated at 37°C for the times stated in the individual experiments.

RESULTS

Biosynthesis of Product and Solubilized Product

Biosynthesis of D-[U-^{14}C]Apiose Product and Solubilized Product

When the procedure for the biosynthesis of radioactive product described in the Experimental Procedures was used with "UDP[U-^{14}C]Api," 35% of the total radioactivity in the assay was incorporated into D-[U-^{14}C]-apiose product (Table 1).

Much of the radioactivity in D-[U-^{14}C]apiose product was solubilized by treatment with ammonium oxalate. The temperature needed to solubilize the maximum amount of D-[U-^{14}C]apiose product was determined. Identically prepared samples of D-[U-^{14}C]apiose product, each containing 13,000 dpm, were extracted with 1% ammonium oxalate at 22°C, 30°C, 40°C, and 50°C. This was done by extracting each sample of D-[U-^{14}C]apiose product for 15 min with 0.3 ml of 1% ammonium oxalate followed by 3 additional 15 min extractions each with 0.1 ml of 1% ammonium oxalate at the appropriate temperature. The remainder of the procedure was the same as that described in the Experimental Procedures except that the temperature

TABLE 1. Biosynthesis of D-[U-^{14}C]Apiose, D-[U-^{14}C]Galacturonic Acid, D-[U-^{14}C]Glucuronic Acid, and D-[U-^{14}C]Xylose Products and Solubilized Products[a]

[U-^{14}C]sugar Nucleotide Used in the Reaction Mixture	Radioactivity Incorporated into Product (%)	Amount of Product Solubilized By Ammonium Oxalate Treatment[b] (%)
"UDP[U-^{14}C]Api"	35 ± 2 (6)	87 ± 2 (6)
UDP[U-^{14}C]GalUA	44 ± 6 (8)	91 ± 1 (8)
UDP[U-^{14}C]GlcUA	28 ± 3 (3)	62 ± 2 (3)
UDP[U-^{14}C]Xyl	41 (1)	48 (1)

[a]Product and solubilized product was synthesized from "UDP[U-^{14}C]Api," UDP[U-^{14}C]GalUA, UDP[U-^{14}C]GlcUA, and UDP[U-^{14}C]Xyl as described in the Experimental Procedures. For the synthesis of product from UDP[U-^{14}C]GlcUA and UDP[U-^{14}C]Xyl, the volumes of the reaction mixtures were 60 µl. The values in parenthesis represent the number of experiments performed.

[b]Corresponds to solubilized product.

was varied as indicated. Based on radioactivity measurements the following amounts of D-[U-^{14}C]apiose product were solubilized by ammonium oxalate treatment: 40% at 22°C, 50% at 30°C, 75% at 40°C, and 87% at 50°C. These results show that 1% ammonium oxalate readily solubilizes D-[U-^{14}C]apiose product and that the extent of solubilization is dependent on the temperature at which the extraction is performed.

Water will also solubilize D-[U-^{14}C]apiose product but not to the same extent as 1% ammonium oxalate. The procedure described in the preceding paragraph was used to extract the D-[U-^{14}C]apiose product except that the 1% ammonium oxalate was replaced by water. The amount of D-[U-^{14}C]apiose product solubilized was 10, 11, and 38% at extraction temperatures of 22°C, 30°C, and 50°C, respectively.

Completeness of solubilization at 50°C was measured by extracting D-[U-^{14}C]apiose product (25,000 dpm) with 0.3 ml of 1% ammonium oxalate followed by 5 additional extractions with 0.1 ml of 1% ammonium oxalate. Except for the 2 additional ammonium oxalate extractions the procedure is identical to the isolation of solubilized product described in the Experimental Procedures. The amount of radioactivity solubilized by each extraction was determined with scintillation solution B. The following amounts of radioactivity,

in order of extraction, were recovered in each extract: 18,184, 2,063, 1,704, 1,605, 759, and 421 dpm. These results indicate that 4 extractions at 50°C with 1% ammonium oxalate are adequate to isolate D-[U-^{14}C]apiose solubilized product.

Biosynthesis of D-[U-^{14}C]Galacturonic Acid, D-[U-^{14}C]Glucuronic Acid, and D-[U-^{14}C]Xylose Products and Solubilized Products

[U-^{14}C]GalUA, UDP[U-^{14}C]GlcUA, and UDP[U-^{14}C]Xyl were used to synthesize the respective products and solubilized products (Table 1). Radioactivity was incorporated into product from all the radioactive sugar nucleotides although UDP[U-^{14}C]GlcUA was not as efficient a donor as the other sugar nucleotides. The amount of radioactivity in the three solubilized products varied (Table 1). Based on radioactivity measurements D-[U-^{14}C]-galacturonic acid product was solubilized slightly better than D-[U-^{14}C]apiose product but both D-glucuronic acid and D-xylose products had significantly less of their radioactivity solubilized by ammonium oxalate treatment. The completeness of extraction of these three solubilized products was investigated and was found to be similar to the D-[U-^{14}C]apiose solubilized product. When the 4 solubilized products described in Table 1 were dialyzed at 4°C for 2 hr the recovery of radioactivity in non-diffusible material was greater than 75%.

Addition of UDPGalUA, UDPGlcUA, and UDPXyl to the Reaction Mixture Used to Synthesize D-[U-^{14}C]Apiose Product and Solubilized Product

The effect of adding UDPGalUA, UDPGlcUA, and UDPXyl to the reaction mixture used to synthesize D-[U-^{14}C]apiose product and solubilized product was determined (Table 2). These data demonstrate that of the three nonradioactive sugar nucleotides used, only UDPGalUA caused a significant increase in the incorporation of radioactivity into both D-[U-^{14}C]apiose product and solubilized product. Addition of UDPXyl to the reaction mixture caused a large decrease in the formation of D-[U-^{14}C]apiose solubilized product as only 32% of the D-[U-^{14}C]apiose product synthesized in the presence of UDPXyl was solubilized by ammonium oxalate treatment. Addition of UDPGlcUA resulted in no significant change in the incorporation of radioactivity into D-[U-^{14}C]-apiose product, although, the amount of product solubilized by ammonium oxalate treatment decreased to 81%. The results in Table 2 also show that almost all of each solubilized product was nondialyzable.

Partial Acid Hydrolysis of D-[U-^{14}C]Apiose Product

D-[U-^{14}C]Apiose product was subjected to partial acid hydrolysis. The hydrolysate was chromatographed on paper with Solvents A and B and the radioactive compounds on the chromatograms were located with a

TABLE 2. Synthesis of D-[U-^{14}C]Apiose Product and Solubilized Product in the Presence and Absence of Nonradioactive Sugar Nucleotides[a]

Fraction	Amount of Radioactivity Present in Each Fraction[b]			
	None (%)	UDPGalUA (%)	UDPGlcUA (%)	UDPXyl (%)
D-[U-^{14}C]Apiose Product	34.7 ± 2.0 (6)	41.7 ± 2.3 (3)	35.9 ± 0.7 (2)	35.8 ± 1.0 (2)
D-[U-^{14}C]Apiose Solubilized Product	30.3 ± 0.8 (6)	36.9 ± 0.8 (3)	26.4 ± 0.8 (2)	11.6 ± 3.6 (2)
D-[U-^{14}C]Apiose Solubilized Product that was Nondialyzable	26.2 ± 2.7 (6)	32.6 ± 0.6 (3)	24.6 ± 4.0 (2)	13.8 (1)

[a]D-[U-^{14}C]Apiose product and solubilized product were prepared as described in the Experimental Procedures except for the following changes. Three of the reaction mixtures contained either 4 nmoles of UDPGalUA, UDPGlcUA, or UDPXyl as indicated. The final volume of the reaction mixtures was 74 µl when UDPGalUA and UDPGlcUA were added and 75 µl when UDPXyl was added. Each solubilized product was dialyzed in 0.05 M sodium phosphate buffer, pH 7.7, at 4°C for 2 hr with 1 buffer change after 1 hr. The amount of radioactivity in each product was determined by measuring the amount of radioactivity in the solubilized product and in the insoluble residue remaining after the extractions with ammonium oxalate and summing these values.

[b]Individual values were calculated on the basis of the total radioactivity present in the UDP[U-^{14}C]Api solution. The numbers in parentheses designate the number of individual experiments that were performed and were used to calculate mean values.

radiochromatogram scanner (Figure 2). Three radioactive compounds were located, one of which was at the origin and is presumed to be unhydrolyzed product while the other two compounds had R_f values identical to D-xylose and D-apiose in the two solvents. This establishes that D-[U-^{14}C]xylose was incorporated into D-[U-^{14}C]apiose product from UDP[U-^{14}C]Xyl contained in the UDP[U-^{14}C]-Api solution.

Acid Hydrolysis at pH 1

Hydrolysis of D-[U-^{14}C]Apiose Solubilized Product

Partial acid hydrolysis cannot be used to quantitate the relative amounts of D-[U-^{14}C]apiose and D-[U-^{14}C]-xylose contained in D-[U-^{14}C]apiose product because of incomplete hydrolysis. Treatment of cell wall polysaccharides with 1 M trifluoroacetic acid for 1 hr and 121°C has been reported to result in complete hydrolysis (7). When tested in D-[U-^{14}C]apiose solubilized product, 1 M trifluoroacetic acid hydrolysis did result in complete hydrolysis as defined by the total release of radioactivity as monosaccharides. However, after paper chromatography only 71% of the radioactivity hydrolyzed was recovered on the chromatogram (Table 3). In order to optimize the recovery of radioactivity after hydrolysis with triflurоracetic acid samples of D-[U-^{14}C]apiose solubilized product were hydrolyzed with either 1.0 M,

Figure 2. Radiochromatogram scan of the products obtained from the partial acid hydrolysis of D-[U-^{14}C]apiose product. D-[U-^{14}C]apiose product was prepared as described in the Experimental Procedures except that 200 µl of particulate enzyme preparation and 2 µmoles of $MgCl_2$ were added to the reaction mixture which had a final volume of 245 µl. The D-[U-^{14}C]apiose product was subjected to partial acid hydrolysis as described in the Experimental Procedures and the hydrolysate was chromatographed on paper in Solvent A.

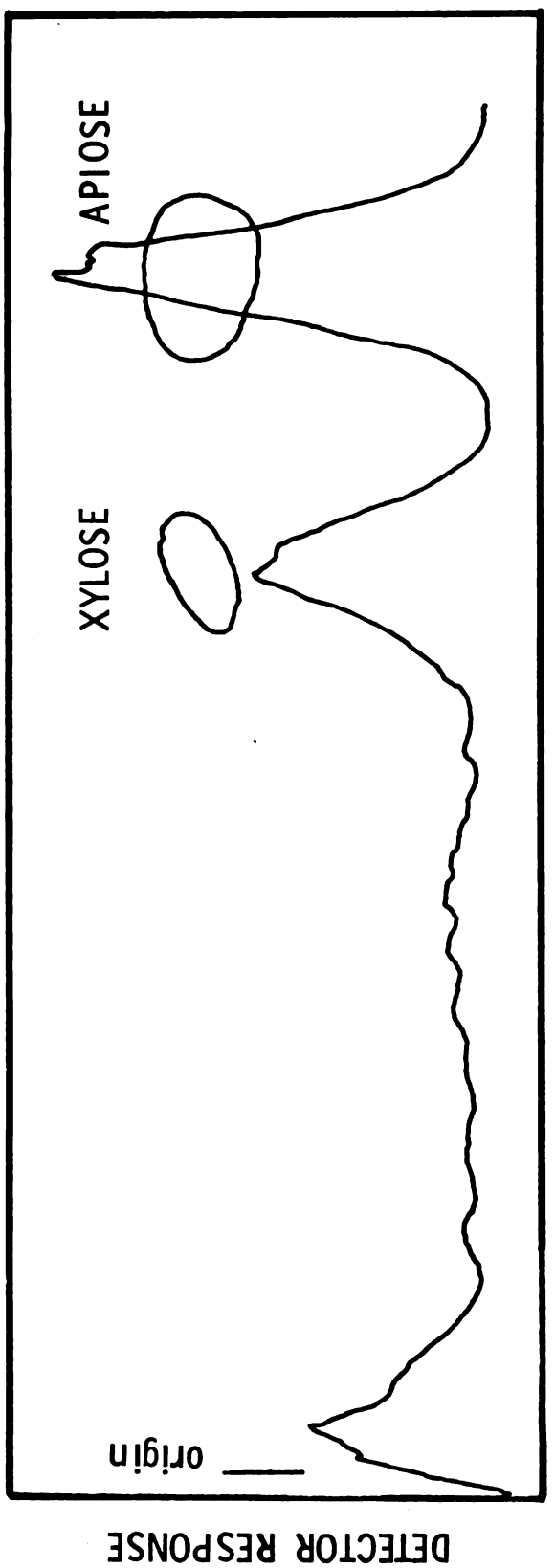


Figure 2

TABLE 3. Acid Hydrolysis of D-[U-^{14}C]Apiose Solubilized Product with Various Concentrations of Trifluoroacetic Acid[a]

Concentration of Trifluoroacetic Acid Used in the Hydrolysis (Molar)	Total Recovery of Radioactivity on Chromatogram (%)	Amount of Recovered Radioactivity Found		
		Origin (%)	Compound 1 (%)	Compound 2 (%)
1.0	71.4	2.0	27.8	70.3
0.1	88.2	2.5	24.9	72.6
0.05	86.0	3.6	25.7	70.7
0.01	86.2	25.0	3.9	69.2

[a]D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures and dialyzed at 4°C in H_2O for 2 hr with changes of H_2O after 30 and 60 min. Samples of dialysate (200 µl, 4000 dpm) were then placed in 1 dram screw-cap vials and 17 µl of 12.8 M, 10.5 µl of 2.0 M, 5.2 µl of 2.0 M, and 10.5 µl of 0.2 M trifluoroacetic acid were added to individual vials to obtain the desired final concentrations of 1.0, 0.1, 0.05, and 0.01 M, respectively. The vials were then placed in an autoclave and heated 1 hr at 121° and 15 pounds pressure. The remainder of the procedure was as described for acid hydrolysis at pH 1 in the Experimental Procedures. Paper chromatography was performed with Solvent A.

0.1 M, 0.05 M, or 0.1 M trifluoroacetic acid for 1 hr at 121°C. Two radioactive compounds were found when the hydrolysates were analyzed by paper chromatography (Table 3). The best recovery of radioactivity was obtained with a trifluoroacetic acid concentration of 0.1 M. For all four acid concentrations the percent of recovered radioactivity found in Compound 2 was nearly identical. The amount of recovered radioactivity found in Compound 1 was also nearly identical for acid concentrations of 1, 0.1, and 0.05 M. However, Compound 1 was not released when the D-[U-^{14}C]apiose solubilized product was hydrolyzed with 0.01 M trifluoroacetic acid. In another experiment hydrolysis times of 30 and 60 min with 0.1 M trifluoroacetic acid resulted in identical recoveries of radioactivity on the chromatograms and identical quantities of recovered radioactivity in Compounds 1 and 2. Therefore, I decided that optimum results would be obtained if solubilized products were hydrolyzed at pH 1 for 30 min at 121°C.

Samples of D-[U-^{14}C]apiose solubilized product were hydrolyzed at pH 1 as described in Experimental Procedures and the resulting hydrolysates were chromatographed in Solvents A - D. All four chromatograms showed that 98% of the recovered radioactivity was contained in two monosaccharides which had R_f values of D-apiose and D-xylose. Compounds 1 and 2 discussed

in the preceding paragraph, corresponded to D-xylose and D-apiose, respectively. Figure 3 shows the result of chromatographing the hydrolysate in Solvent A. These results established that the particulate enzyme preparation from L. minor can incorporate D-[U-^{14}C]apiose and D-[U-^{14}C]-xylose from their respective sugar nucleotides into D-[U-^{14}C]apiose solubilized product and hence into D-[U-^{14}C]apiose product. D-[U-^{14}C]Glucuronic acid was not detected in the D-[U-^{14}C]apiose solubilized product. The determination of the amounts of the D-[U-^{14}C]xylose and D-[U-^{14}C]apiose contained in the D-[U-^{14}C]apiose product will be discussed later.

Hydrolysis of D-[U-^{14}C]Galacturonic Acid, D-[U-^{14}C]Glucuronic Acid, and D-[U-^{14}C]Xylose Solubilized Products

Samples of D-[U-^{14}C]galacturonic acid, D-[U-^{14}C]-glucuronic acid, and D-[U-^{14}C]xylose solubilized products described in Table 1 were dialyzed for 2 hr in water at 4°C and hydrolyzed in 1 M trifluoroacetic acid for 30 min at 121°C and 15 pounds pressure in an autoclave. All procedures for the hydrolysis except for the 1 M acid concentration were identical to that described in the Experimental Procedures for hydrolysis at pH 1. Each sample contained 5,000 to 7,000 dpm. Paper chromatography was used to identify the radioactive hydrolysis

Figure 3. Radiochromatogram scan of the products obtained after hydrolysis at pH 1 of D-[U-^{14}C]apiose solubilized product. D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures and was dialyzed in water for 2 hr at 4°C with changes of water after 30 and 60 min. The dialyzed D-[U-^{14}C]apiose solubilized product (6,600 dpm) was hydrolyzed at pH 1 as described in the Experimental Procedures and the hydrolysate was chromatographed on paper in Solvent A. Recovery of radioactivity on the chromatograms was 97%.

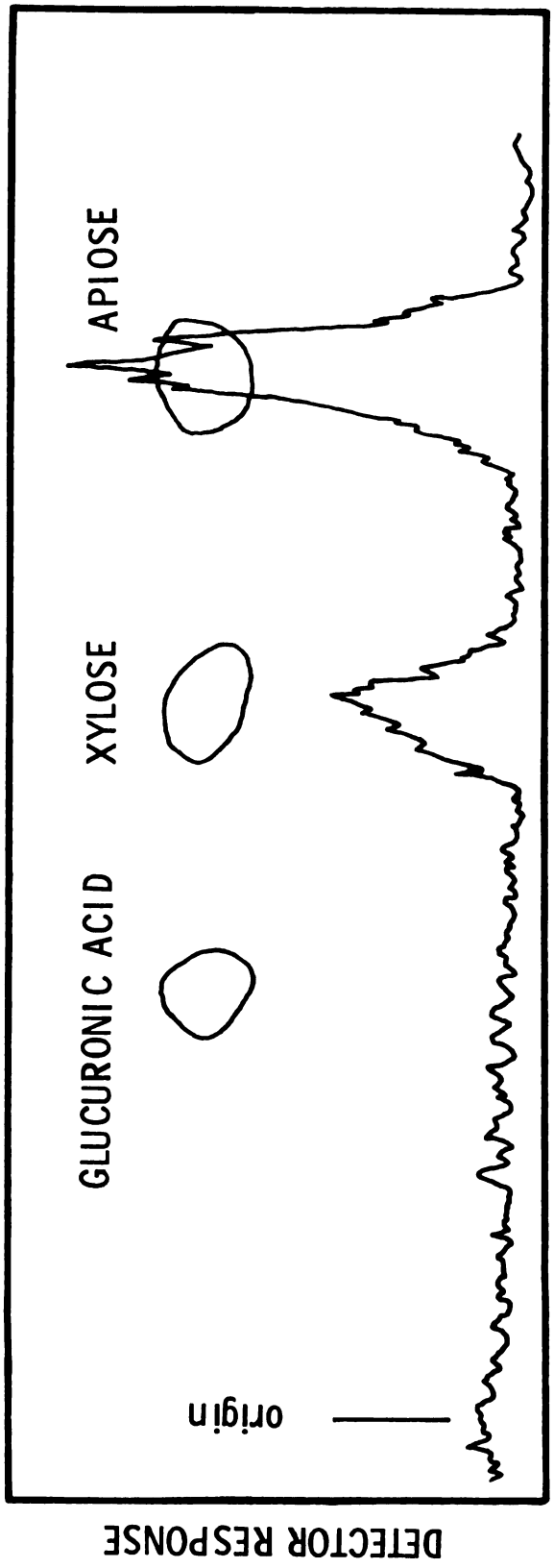

DETECTOR RESPONSE
origin
GLUCURONIC ACID
XYLOSE
APIOSE
DISTANCE

Figure 3

products. Recovery of starting radioactivity on the chromatograms ranged from 75 to 80%.

The hydrolysates obtained from D-[U-^{14}C]galacturonic acid solubilized product were chromatographed in solvents A and E. Four radioactive compounds were detected on the chromatograms. One compound contained 60% of the recovered radioactivity and had the same R_f as D-galacturonic acid. These 2 solvents resolve D-galacturonic acid and D-glucuronic acid. A second compound with an R_f of D-xylose in Solvent A contained less than 6% of the recovered radioactivity. The remainder of the recovered radioactivity was contained in two compounds which were not identified but presumably are oligosaccharides containing uronic acid as they did not move far from the origin.

The hydrolysates obtained from D-[U-^{14}C]glucuronic acid solubilized product were chromatographed in Solvents A, C, and E. Four radioactive compounds were detected on the chromatograms. Interestingly, one compound contained 64% of the recovered radioactivity and had the same R_f as D-xylose. Of further interest was that the second major radioactive compound, which contained 23% of the recovered radioactivity, had the same R_f as D-galacturonic acid rather than D-glucuronic acid. The two unidentified radioactive compounds with the smallest R_f values are again presumed to be oligosaccharides.

Chromatography in Solvent C of the hydrolysate obtained from D-[U-^{14}C]xylose solubilized product showed that the recovered radioactivity was contained in a single compound which had the same R_f as D-xylose. Solvent C is capable of resolving D-xylose and L-arabinose.

From these results we conclude that the particulate enzyme preparation from L. minor apparently contained UDPGlcUA carboxy-lyase and UDPGlcUA 4-epimerase activities that convert UDP[U-^{14}C]GlcUA to UDP[U-^{14}C]Xyl and UDP[U-^{14}C]GalUA. The particulate enzyme preparation either does not contain UDPAra 4-epimerase activity to convert UDP[U-^{14}C]Xyl to UDP[U-^{14}C]Ara or it cannot incorporate L-[U-^{14}C]arabinose into solubilized product.

Hydrolysis of Authentic Apiogalacturonan from Whole Plants

Since previously described experiments showed that the particulate enzyme preparation from L. minor incorporated D-[U-^{14}C]xylose into product we decided to hydrolyze authentic apiogalacturonans (isolated from whole plants) with trifluoroacetic acid in order to determine whether they contained D-xylose also. Two apiogalacturonan fractions, isolated by Hart and Kindel (104) [^{14}C]22°C sodium chloride soluble fraction (7,000 dpm) and [^{14}C]60°C sodium chloride soluble fraction (9,400 dpm), were hydrolyzed at pH 1 as

described in the Experimental Procedures. The hydrolysates were analyzed by paper chromatography in Solvent A. Recovery of radioactivity on each chromatogram was 85%. Two radioactive compounds were detected in the hydrolysate of the [^{14}C]22°C sodium chloride soluble apiogalacturonan fraction; one at the origin and the other with a R_f of D-apiose. The compound with a R_f of D-apiose contained 40% of the recovered radioactivity. Four radioactive compounds were detected on the chromatogram of the hydrolysate of the [^{14}C]60°C sodium chloride soluble apiogalacturonan fraction. One compound contained 26% of the recovered radioactivity and was located at the origin. Another compound contained 5% of the recovered radioactivity and had the same R_f as D-galacturonic acid. The two remaining compounds had the same R_f values as D-xylose and D-apiose and contained 27 and 41%, respectively, of the recovered radioactivity. The compound corresponding to D-xylose was eluted from the chromatogram and when re-chromatographed on paper in Solvent B it had the same R_f as D-xylose. Compounds with the R_f values of D-apiose and D-xylose were also identified when the hydrolysate from the [^{14}C]60°C sodium chloride soluble apiogalacturonan fraction was chromatographed in Solvents C and D. These results indicate that D-xylose was contained in the apiogalacturonan fraction isolated from intact plants at 60°C.

Hydrolysis of D-[U-^{14}C]Apiose Solubilized Product Synthesized in the Presence of Nonradioactive Sugar Nucleotides

D-[U-^{14}C]Apiose solubilized products synthesized in the absence and presence of UDPGalUA, UDPGlcUA, and UDPXyl were hydrolyzed at pH 1 and the hydrolysates were analyzed for D-[U-^{14}C]apiose and D-[U-^{14}C]xylose content (Table 4). These data show that the relative amounts of D-[U-^{14}C]apiose and D-[U-^{14}C]xylose incorporated into D-[U-^{14}C]apiose solubilized product were affected by the presence of nonradioactive sugar nucleotides in the reaction mixture. In addition to this data the data in Table 6 show that the percent of radioactivity contained in D-[U-^{14}C]apiose increased with increasing amounts of UDPGalUA present in the reaction mixture. A decrease in the percent of D-[U-^{14}C]xylose incorporated in the presence of UDPXyl is not surprising because of the resultant dilution of UDP[U-^{14}C]Xyl. Addition of UDPGlcUA to the reaction mixture would also cause the formation of UDPXyl in the reaction mixture because of the presence of UDPGlcUA carboxy-lyase activity in the particulate enzyme preparation.

TABLE 4. Hydrolysis at pH 1 of D-[U-^{14}C]Apiose Solubilized Product Synthesized in the Presence of Nonradioactive Sugar Nucleotides[a]

Sugar Nucleotide Added To Reaction Mixture	Radioactivity Recovered In D-[U-^{14}C]Apiose (%)	$\frac{\text{D-[U-}^{14}\text{C]Apiose}}{\text{D-[U-}^{14}\text{C]Xylose}}$
none (4)	75.2 ± 2.6	3
UDPGalUA (3)	88.3 ± 0.4	7.5
UDPGlcUA (2)	91.0 ± 0.0	10.1
UDPXyl (1)	89.2	8.1

[a]The dialyzed D-[U-^{14}C]apiose solubilized product and the dialyzed D-[U-^{14}C]apiose solubilized products synthesized in the presence of UDPGalUA, UDPGlcUA, and UDPXyl described in Table 2 were hydrolyzed at pH 1 as described in the Experimental Procedures. The hydrolysates were analyzed by paper chromatography with Solvent A. Radioactivity not recovered in D-[U-^{14}C]apiose was contained in D-[U-^{14}C]xylose. The values in parenthesis represent the number of times each experiment was performed.

Hydrolysis of D-[U-^{14}C]Apiose Solubilized Product at pH 4

Hydrolysis of D-[U-^{14}C]Apiose Solubilized Product Synthesized in the Absence and Presence of Nonradioactive Sugar Nucleotides

D-[U-^{14}C]Apiose solubilized product was hydrolyzed at pH 4 and the hydrolysate was chromatographed on paper (Figure 4). Three peaks were obtained. The largest peak was due to material at the origin of the chromatogram which we assumed is unhydrolyzed D-[U-^{14}C]apiose solubilized product. The other two peaks were due to D-[U-^{14}C]apiose and [U-^{14}C]apiobiose since they had the same R_f values as the authentic compounds on the chromatogram. No D-[U-^{14}C]xylose was detected on the chromatogram. The procedure would detect as little as 6% of the D-[U-^{14}C]xylose present in the D-[U-^{14}C]apiose solubilized product.

D-[U-^{14}C]Apiose solubilized products synthesized in the absence and presence of UDPGalUA, UDPGlcUA, and UDPXyl were hydrolyzed at pH 4 and the amounts of D-[U-^{14}C]apiose and [U-^{14}C]apiobiose released from each were determined (Table 5). These hydrolysis experiments demonstrate that the presence of nonradioactive sugar nucleotides during synthesis resulted in the formation of a solubilized product which released an increased amount of D-[U-^{14}C]apiose and [U-^{14}C]apiobiose when hydrolyzed at pH 4.

Figure 4. Radiochromatoram scan of the products obtained from the hydrolysis at pH 4 of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA. D-[U-^{14}C]Apiose solubilized product, synthesized in the presence of UDPGalUA, was prepared as described in the Experimental Procedures. The reaction mixtures contained 4 nmoles of UDPGalUA and had a final volume of 75 µl. The D-[U-^{14}C]apiose solubilized product was dialyzed in H_2O for 2 hr at 4°C with a change of water after 1 hr. The dialyzed D-[U-^{14}C]apiose solubilized product, synthesized in the presence of UDPGalUA, (200 µl, 10,000 dpm) was hydrolyzed at pH 4 as described in the Experimental Procedures except that the hydrolysis time was 3 hr. Recovery of radioactivity on the chromatogram was 79%.

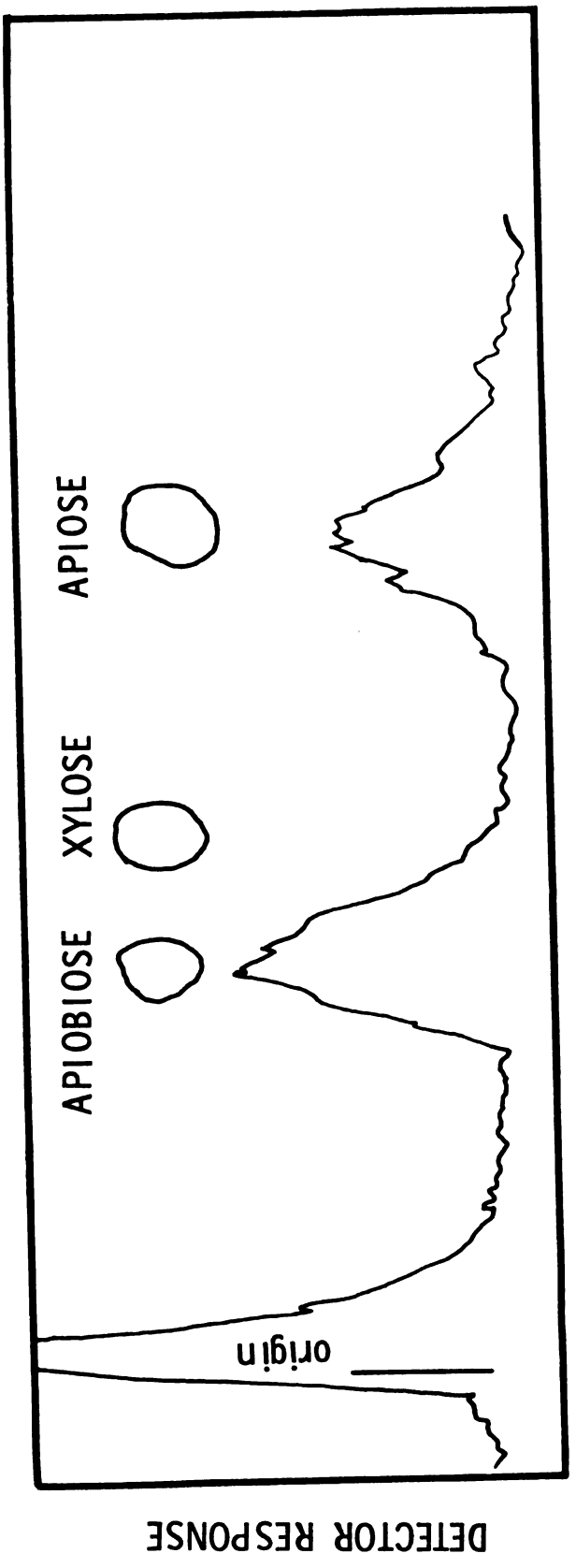

APIOBIOSE
XYLOSE
APIOSE
origin
DETECTOR RESPONSE
DISTANCE

Figure 4

TABLE 5. Hydrolysis at pH 4 of D-[U-^{14}C]Apiose Solubilized Product Synthesized in the Absence and Presence of Nonradioactive Sugar Nucleotides[a]

Sugar Nucleotide Added To Reaction Mixture	D-[U-^{14}C]Apiose (%)	[U-^{14}C]Apiobiose (%)
None (2)	11.6 ± 0.4	13.0 ± 0.4
UDPGalUA (2)	20.9 ± 2.8	25.4 ± 0.3
UDPGlcUA (1)	15.4	28.6
UDPXyl (1)	26.3	12.0

[a]The dialyzed D-[U-^{14}C]apiose solubilized product and the dialyzed D-[U-^{14}C]apiose solubilized products synthesized in the presence of UDPGalUA, UDPGlcUA, and UDPXyl described in Table 2 were hydrolyzed at pH 4 as described in the Experimental Procedures. The hydrolysates were analyzed by paper chromatography. The values represent the percent of D-[U-^{14}C]apiose contained in the solubilized products which was released as D-[U-^{14}C]apiose or [U-^{14}C]apiobiose. Therefore in this table the radioactivity in D-[U-^{14}C]apiose is equal to 100%. The values in parenthesis represent the number of times each experiment was performed.

Release of D-[U-^{14}C]Apiose and [U-^{14}C]Apiobiose as a Function of Hydrolysis Time

Samples of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA were subjected to hydrolysis at pH 4 for varying periods of time (Figure 5). These results showed that release of radioactivity from the D-[U-^{14}C]apiose solubilized product was essentially complete after a 3 hr hydrolysis period although 50% of the D-[U-^{14}C]apiose originally contained in the sample had not been released. We also investigated the hydrolysis at pH 4 of D-[U-^{14}C]apiose solubilized product and found that its hydrolysis at pH 4 was also complete after 3 hr.

Hydrolysis of [U-^{14}C]Apiobiitol

I determined whether both moieties of the [U-^{14}C]-apiobiose side chains of D-[U-^{14}C]apiose solubilized product were synthesized *in vitro*. [U-^{14}C]Apiobiose was isolated from D-[U-^{14}C]apiose solubilized product synthesized in the presence of 4 nmoles of UDPGalUA by hydrolysis at pH 4 and was purified by paper chromatography in Solvent A. A solution of [U-^{14}C]apiobiose (6,500 dpm) was titrated to pH 10 with 0.5 M NaOH and was then made 80 mM in $NaBH_4$. The solution was allowed to stand 16 hr at 22°C after which it was acidified to pH 3 with acetic acid. Addition of methanol and repeated concentration of the solution under reduced

Figure 5. Time-course of the hydrolysis at pH 4 of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA. D-[U-^{14}C]Apiose solubilized product, synthesized in the presence of UDPGalUA, was prepared and dialyzed as described in the Experimental Procedures and the legend of Figure 4. Samples of the dialyzed D-[U-^{14}C]apiose solubilized product, synthesized in the presence of UDPGalUA, (200 µl, 10,000 dpm) were hydrolyzed at pH 4 as described in the Experimental Procedures except that hydrolysis times of 1, 2, 3, 4, 6.25, 8, and 10 hr were used. The recovery of radioactivity on the chromatograms for hydrolysis times of 1, 2, 3, 4, 6.25, 8, and 10 hr were 89, 83, 79, 82, 87, 83, and 83%, respectively. In order to determine the D-[U-^{14}C]apiose content of the D-[U-^{14}C]apiose product synthesized in the presence of UDPGalUA, samples were hydrolyzed at pH 1 as described in the Experimental Procedures and the hydrolysates were chromatographed on paper in Solvent A. For each hydrolysis time at pH 4 the percent of D-[U-^{14}C]apiose remaining in solubilized product at the origin and released as D-[U-^{14}C]apiose and [U-^{14}C]-apiobiose was determined.

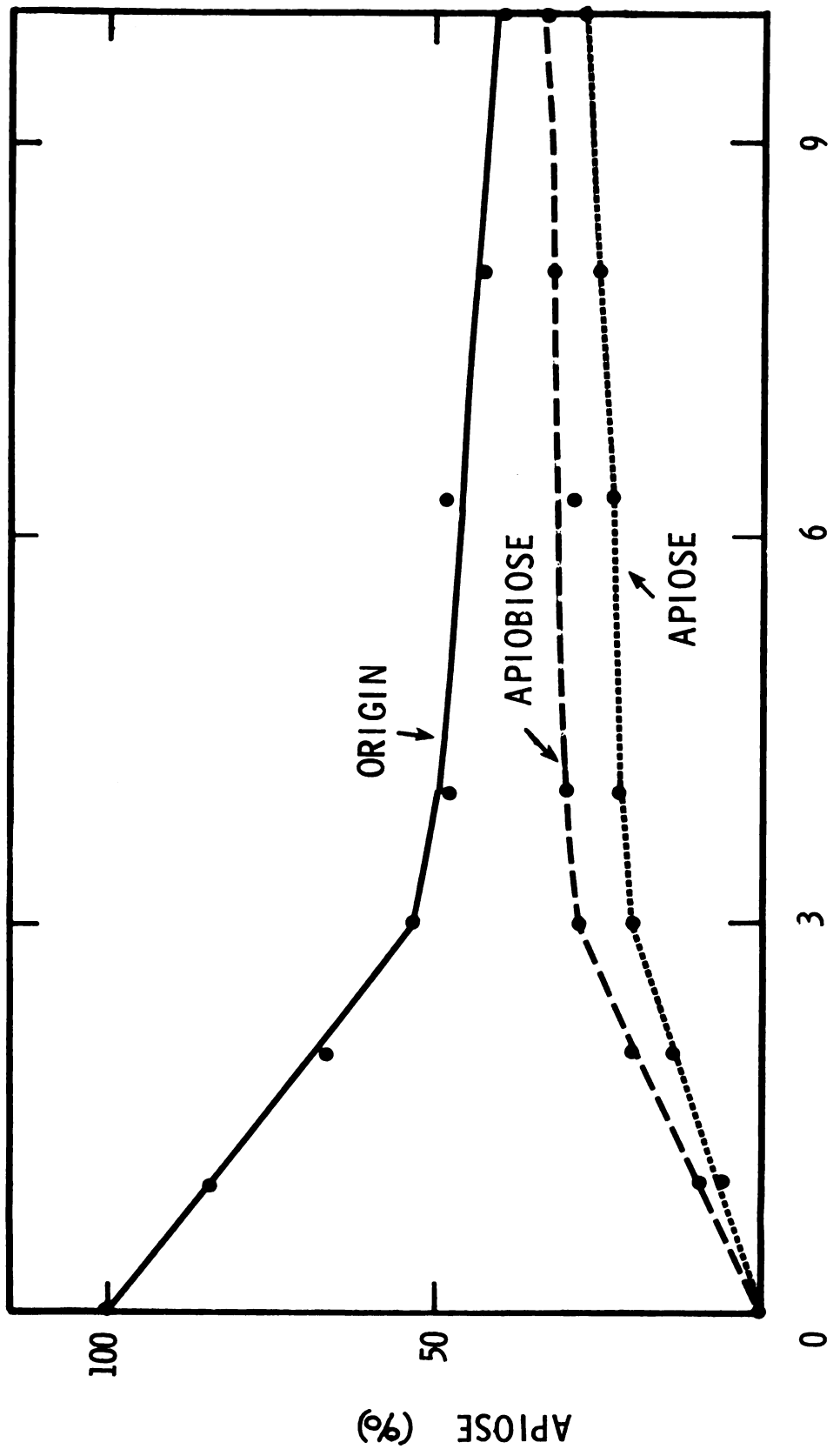

APIOSE (%)
100
50
0
3
6
9
HYDROLYSIS TIME (HOURS)
ORIGIN
APIOBIOSE
APIOSE

Figure 5

pressure removed the borate as methyl borate. The reduced disaccharide was purified by paper chromatography in Solvent A and hydrolyzed in 0.04 M trifluoroacetic acid for 30 min at 121°C. Conversion of [U-^{14}C]apiobiose to [U-^{14}C]apiobiitol after $NaBH_4$ treatment was greater than 90%. The hydrolysate was analyzed by paper chromatography in Solvent E (Figure 6). Two radioactive compounds were obtained which had the same R_f values as D-Apiitol and D-apiose. The amount of recovered radioactivity in D-[U-^{14}C]apiitol and D-[U-^{14}C]apiose was 50.2 and 49.8%, respectively. The amount of radioactivity from [U-^{14}C]apiobiitol that was recovered in D-[U-^{14}C]-apiitol and D-[U-^{14}C]apiose was 92.6%. This experiment established that synthesis of both moieties of the [U-^{14}C]apiobiose side chains occurred <u>in vitro</u>.

<u>Sodium Chloride Fractionation of D-[U-^{14}C]-Apiose Solubilized Product</u>

Hart and Kindel had shown that after 0.5% ammonium oxalate extraction authentic apiogalacturonans were fractionated into two components when treated with 1 M NaCl (102). Apiogalacturonans of high D-apiose content were soluble in 1 M NaCl while apiogalacturonans of low D-apiose content were insoluble in 1 M NaCl (102). I have investigated the solubility of D-[U-^{14}C]apiose solubilized product in 1 M NaCl. D-[U-^{14}C]Apiose solubilized product was dialyzed in water at 4° for

Figure 6. Radiochromatogram scan of the products obtained from the acid hydrolysis of [U-^{14}C]apiobiitol. [U-^{14}C]Apiobiitol was prepared from [U-^{14}C]apiobiose and was hydrolyzed and chromatographed as described in Results.

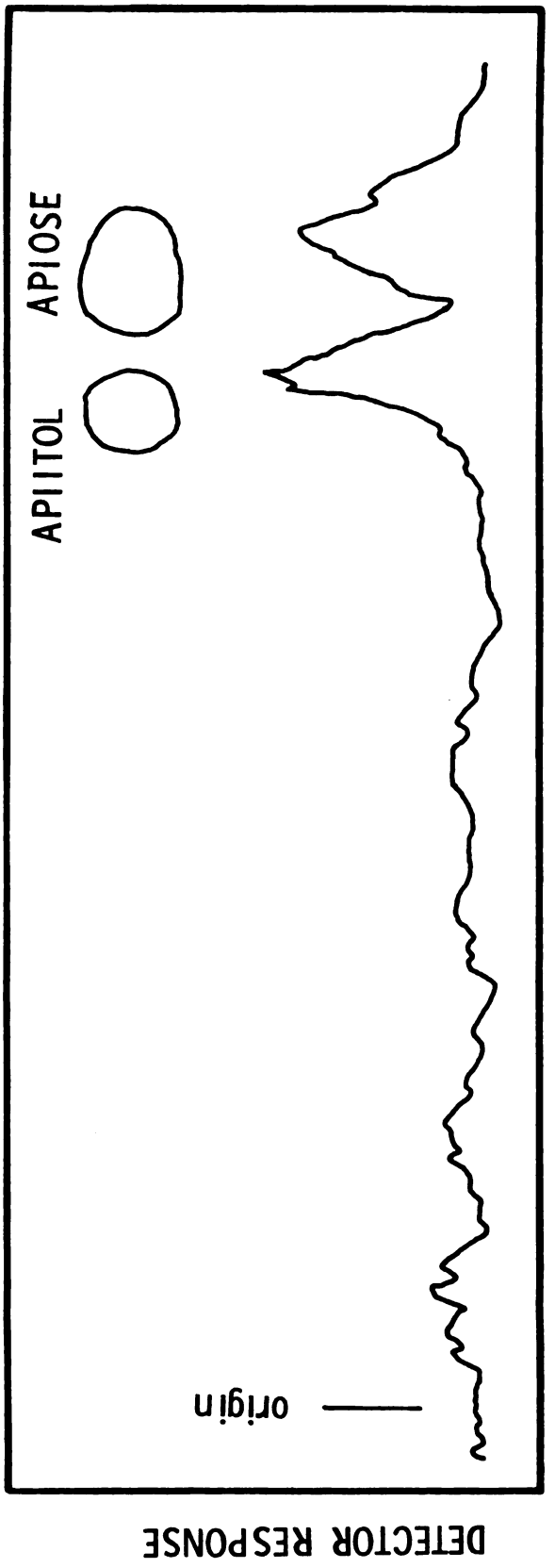


Figure 6

2 hr with changes of dialysis water after 30 and 60 min. The dialysate was diluted 1 to 10 with water. One mg of authentic 60° sodium chloride insoluble apiogalacturonan fraction was dissolved in 500 µl of the diluted dialysate. The solution was warmed to 22°C and stirred while 500 µl of 2 M NaCl was added dropwise. A precipitate formed during the addition of the NaCl. The suspension was centrifuged and the supernatant was removed. The precipitate was washed once each with 500 µl portions of 2 M and 1 M NaCl and then was dissolved in 1 ml of water. The radioactivity in the NaCl soluble and insoluble fractions was assayed with scintillation solution B. The NaCl soluble fraction contained 1958 dpm and the NaCl insoluble fraction contained 71 dpm. The results indicate that D-[U-^{14}C]apiose solubilized product has a high D-apiose content.

DEAE-Sephadex Column Chromatography

Chromatography of D-[U-^{14}C]Apiose Solubilized Product

Column chromatography with DEAE-Sephadex separated the D-[U-^{14}C]apiose solubilized product into 5 fractions (Figure 7). Recovery of solubilized products from the column was usually between 60 and 90% and in the experiment depicted in Figure 7, 81% of the D-[U-^{14}C]apiose solubilized product was recovered. One radioactive fraction was eluted with 0.1 M NaCl,

Figure 7. DEAE-Sephadex column chromatogram of D-[U-^{14}C]apiose solubilized product. D-[U-^{14}C]Apiose solubilized product was prepared and chromatographed on a DEAE-Sephadex column (0.8 cm internal diam x 5 cm) as described in the Experimental Procedures. The column flow rate was 20 ml/hr and 1.0 ml fractions were collected. Column fractions 1 and 2 were collected as the 1.8 ml sample (40,000 dpm) was placed on the column. The column was treated with phosphate buffer while column fractions 3 to 8 were collected and then with the step gradient of NaCl in phosphate buffer indicated in the figure. Aliquots were removed from the column fractions and were assayed for radioactivity with scintillation solution B. The indicated column fractions were then combined to form fractions A, B, C, D, and E.

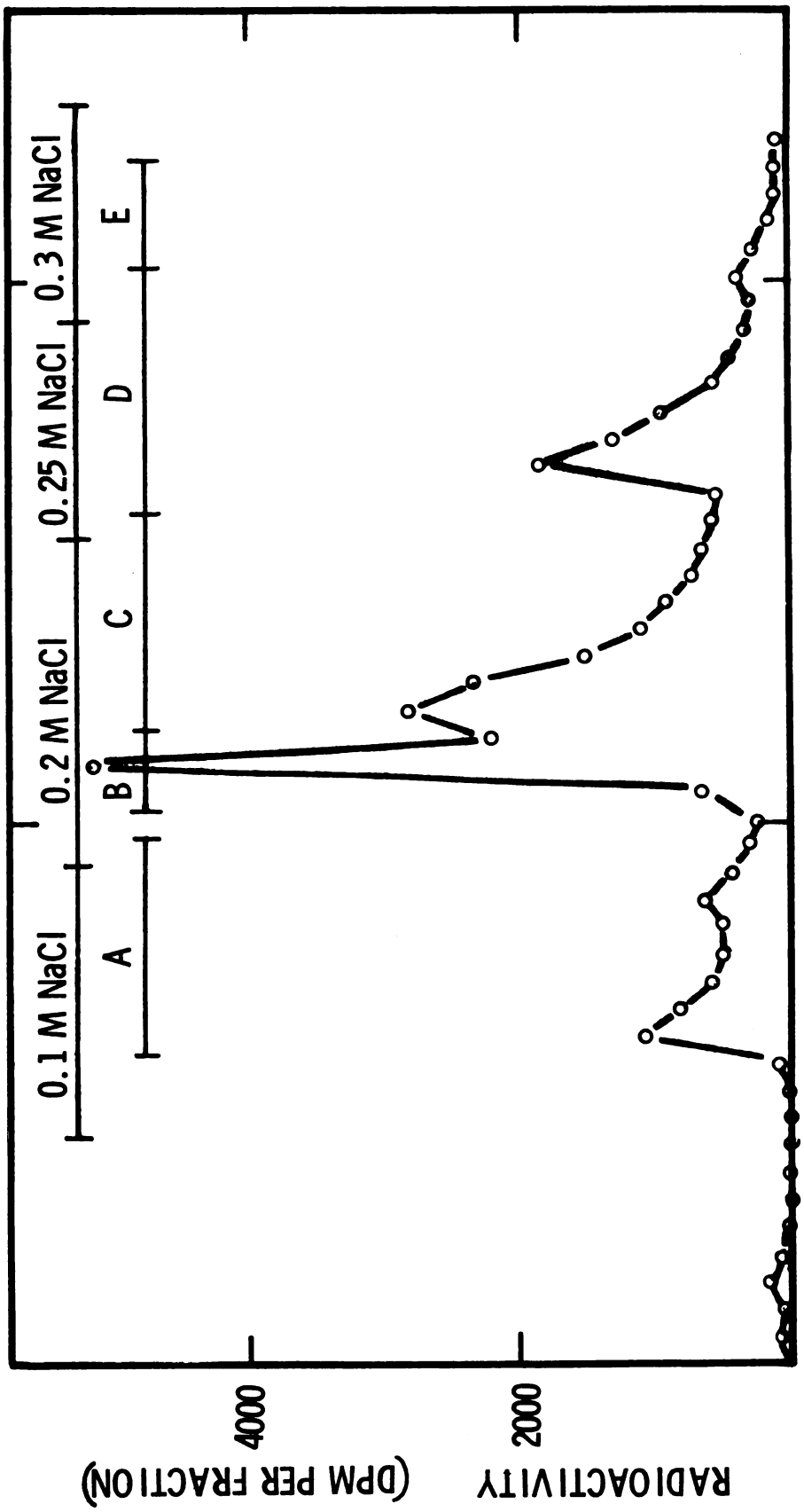

0.1 M NaCl
0.2 M NaCl
0.25 M NaCl
0.3 M NaCl
A
B
C
D
E
RADIOACTIVITY (DPM PER FRACTION)
4000
2000
0
20
40
FRACTION NUMBER

Figure 7

two with 0.2 M NaCl, one with 0.25 M NaCl, and a small fraction with 0.3 M NaCl. The indicated column fractions were combined to give 5 large fractions which were labelled, in order of their elution, A through E, with fraction A being eluted first and fraction E last. The percentage of recovered radioactivity contained in fractions A, B, C, D, and E was 15, 25, 32, 21, and 2%, respectively.

Chromatography of D-[U-^{14}C]Galacturonic Acid Solubilized Product

When D-[U-^{14}C]galacturonic acid solubilized product was chromatographed on a DEAE-Sephadex column 2 major fractions and 3 minor fractions were recovered (Figure 8). Recovery of D-[U-^{14}C]galacturonic acid solubilized product from the column in this experiment was 76%. Radioactive material eluted from the column in the same positions in the elution gradient as was seen for the chromatography of D-[U-^{14}C]apiose solubilized product depicted in Figure 7 although the amount of radioactivity in the fractions was different. In Figure 8 the percentage of recovered radioactivity found in fractions A, B, C, D, and E was 3, 7, 41, 40, and 6%, respectively.

Figure 8. DEAE-Sephadex column chromatogram of D-[U-^{14}C]galacturonic acid solubilized product. D-[U-^{14}C]Galacturonic acid solubilized product was prepared as described in the Experimental Procedures and was chromatographed on a DEAE-Sephadex column (0.8 cm internal diam x 5 cm) as described in the legend of Figure 7. The sample (16,000 dpm) was placed on the column in a volume of 2 ml. The indicated column fractions were combined to form fractions A, B, C, D, and E.

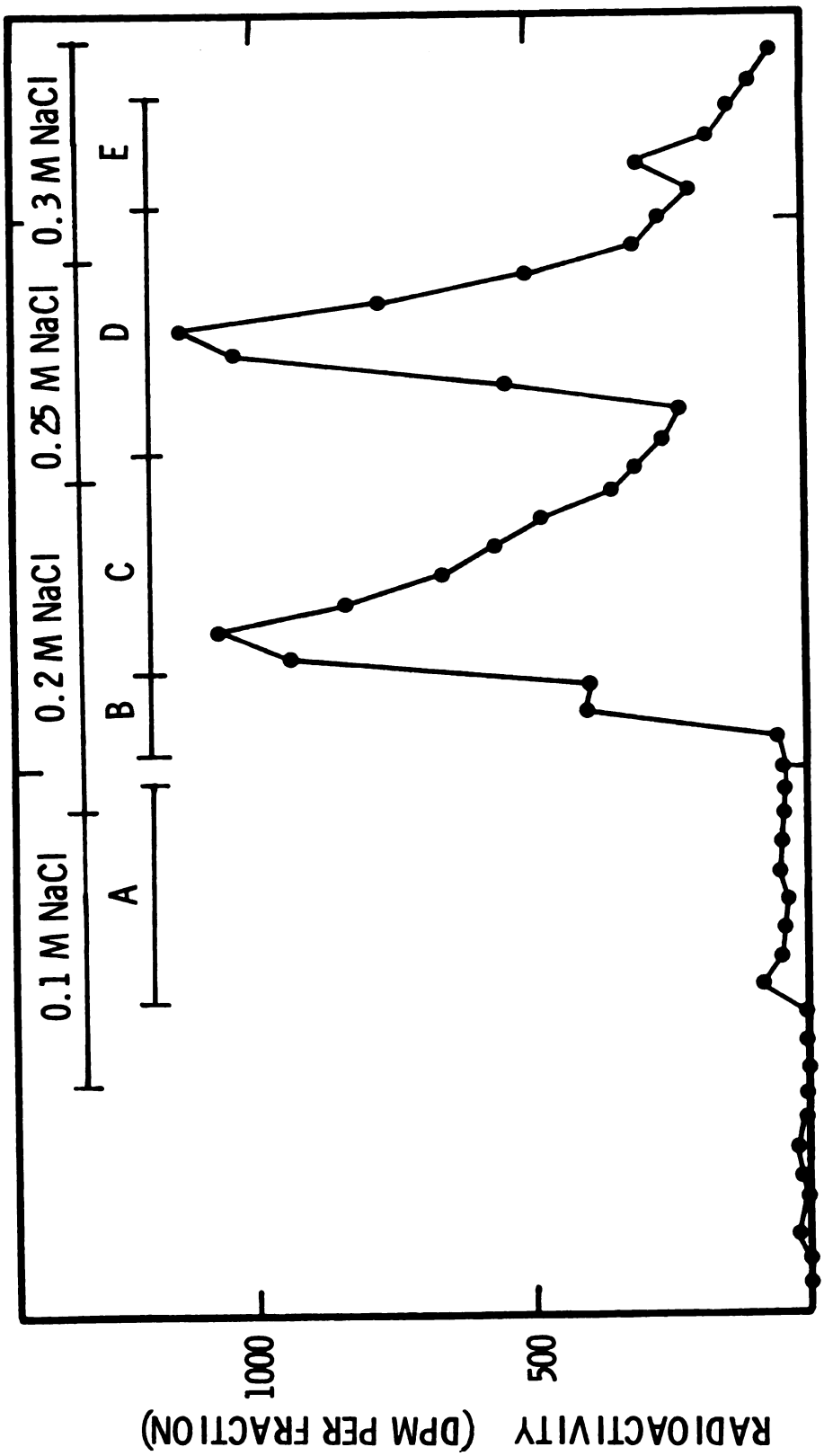

0.1 M NaCl
0.2 M NaCl
0.25 M NaCl
0.3 M NaCl
A
B
C
D
E
1000
500
0
20
40
RADIOACTIVITY (DPM PER FRACTION)
FRACTION NUMBER

Figure 8

Chromatography of D-[U-^{14}C]Apiose Solubilized Product Synthesized in the Presence of UDPGalUA

Comparison of the two elution profiles shown in Figures 7 and 8 indicated that a larger percentage of radioactivity present in D-[U-^{14}C]galacturonic acid solubilized product had was recovered in fractions C, D, and E than was recovered in the same fractions when D-[U-^{14}C]apiose solubilized product was chromatographed on DEAE-Sephadex. If D-[U-^{14}C]apiose and D-galacturonic acid are incorporated from their respective sugar nucleotides into the same polysaccharides then the acidity of the polysaccharide should increase because of the increased ratio of D-galacturonic acid to D-[U-^{14}C]apiose. D-[U-^{14}C]Apiose solubilized product synthesized in the presence of UDPGalUA would therefore, when chromatographed on the DEAE-Sephadex column, have a higher percentage of radioactivity recovered in the more acidic fractions, which elute with the higher salt concentrations, than would D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA. When D-[U-^{14}C]apiose solubilized product, synthesized in the presence of UDPGalUA, was subjected to DEAE-Sephadex column chromatography 5 fractions were obtained (Figure 9). In the experiment reported recovery of radioactivity from the column was 78%. Radioactive material eluted from the column in the same positions in the elution gradient as

Figure 9. DEAE-Sephadex column chromatogram of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA. D-[U-^{14}C]Apiose solubilized product, synthesized in the presence of UDPGalUA, was prepared as described in Table 2 and the Experimental Procedures and was chromatographed on a DEAE-Sephadex column (0.8 cm internal diam x 5 cm) as described in the legend of Figure 7. The sample (56,000 dpm) was placed on the column in a volume of 1.8 ml. The indicated column fractions were combined to form fractions A, B, C, D, and E.

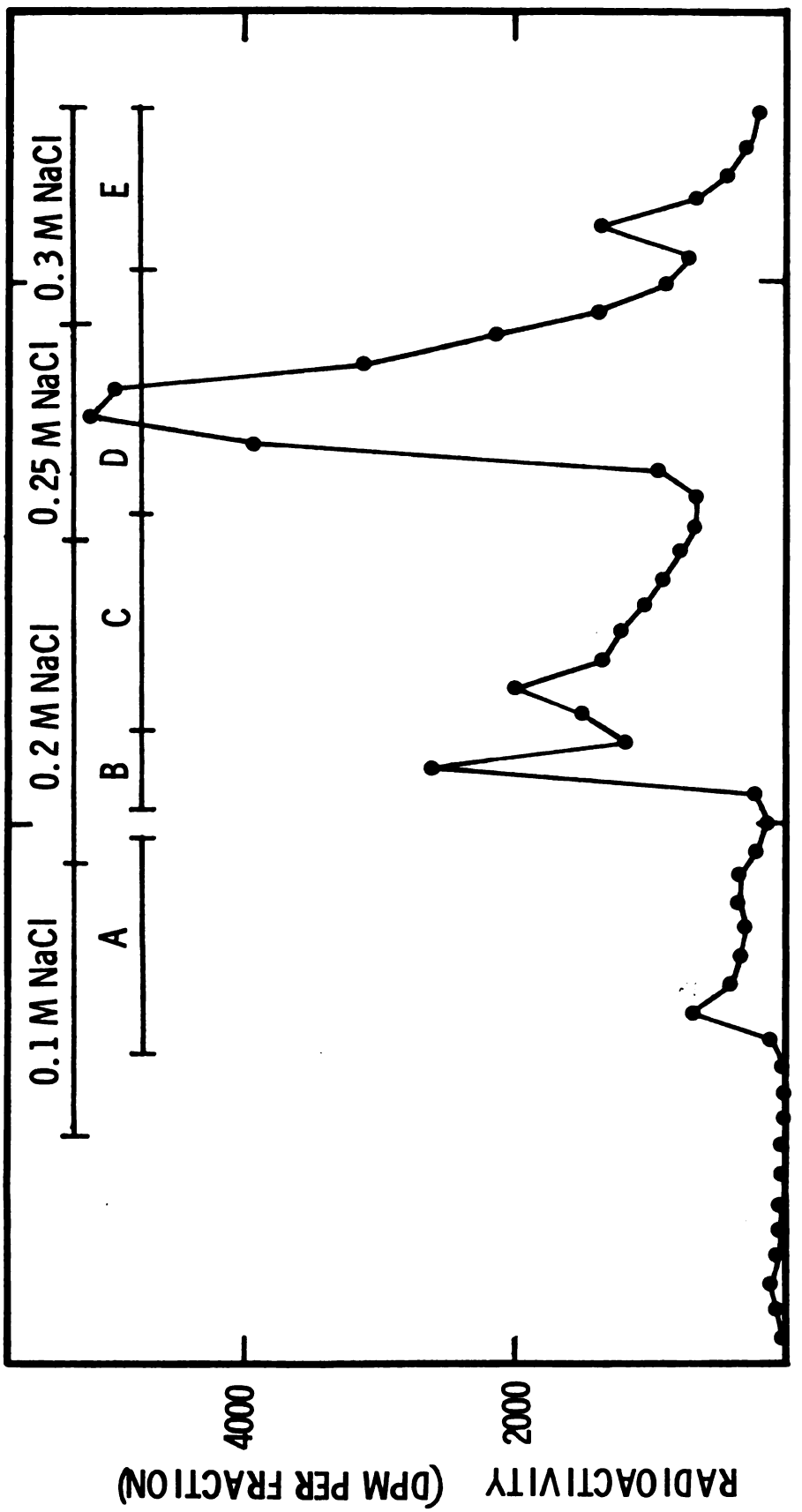

0.1 M NaCl
0.2 M NaCl
0.25 M NaCl
0.3 M NaCl
A
B
C
D
E
4000
2000
0
20
40
RADIOACTIVITY (DPM PER FRACTION)
FRACTION NUMBER

Figure 9

was seen in Figures 7 and 8. In Figure 9 the percentage of recovered radioactivity contained in fractions A, B, C, D, and E was 6, 9, 22, 53, and 7%, respectively. These data show that the D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA contained an increased amount of radioactive material that eluted as fractions D and E with a concurrent decreased amount of radioactive material that eluted as fractions A, B, and C compared to the results obtained when the D-[U-^{14}C]apiose solubilized product was synthesized in the absence of UDPGalUA. The increase in the amount of material which eluted in fractions D and E when UDPGalUA was present during synthesis confirms the theory that both D-[U-^{14}C]-apiose and D-galacturonic acid were incorporated into the same polysaccharide.

The optimum concentration of UDPGalUA needed to cause maximum formation of fractions D and E and minimum formation of fractions A through C was determined (Table 6). These results showed that the maximum shift of radioactivity into fractions D and E occurred when 4.0 nmoles of UDPGalUA was present. The presence of 16.0 nmoles of UDPGalUA yielded results that were basically the same as those obtained when 4.0 nmoles of UDPGalUA was present. However, D-[U-^{14}C]apiose solubilized product synthesized in the presence of 0.5 nmoles of UDPGalUA showed an elution pattern intermediate

TABLE 6. Distribution of Radioactivity in Fractions Obtained After DEAE-Sephadex Chromatography of D-[U-^{14}C]Apiose Soluble Product Synthesized in the Presence of Varying Quantities of UDPGalUA[a]

UDPGalUA Added to Reaction Mixture (nmoles)	Amount of Radioactivity in D-[U-^{14}C]Apiose Solubilized Product Present in D-[U-^{14}C]-Apiose[b] (%)	Amount of Radioactivity Recovered in Each Fraction				
		A (%)	B (%)	C (%)	D (%)	E (%)
none	75	21	22	29	20	4
none	80	15	25	32	21	2
0.5	80	14	9	25	38	8
4.0	88	6	9	22	53	7
16.0	90	8	9	22	54	6

[a]D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures. The reaction mixtures were as described in the Experimental Procedures except that 0.5, 4.0, or 16.0 nmoles of UDPGalUA was added to the appropriate reaction mixtures and the final volumes of the reaction mixtures were 75, 74, and 86 µl, respectively. The solubilized products were chromatographed on a DEAE-Sephadex column (0.8 cm internal diam x 5 cm) as described in the legend of Figure 7. The samples of D-[U-^{14}C]apiose solubilized product placed on the columns were each in 1.5 ml of buffer. The 2 samples of D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA each contained 40,000 dpm and the samples synthesized in the presence of 0.5, 4.0, and 16.0 nmoles of UDPGalUA contained 47,000, 56,000, and 44,000 dpm, respectively. Recovery of the radioactivity placed on the columns was 99 and 81% for the two experiments with D-[U-^{14}C]apiose solubilized product synthesized without exogenous UDPGalUA present and 91, 77, and 88% for the experiments with solubilized product synthesized with 0.5, 4.0, and 16.0 nmoles of exogenous UDPGalUA present, respectively. Fractions A through E were obtained by combining the same column fractions as indicated in Figure 7. The values reported for the amount of radioactivity recovered in fractions A through E were calculated from the total amount of radioactivity recovered after column chromatography.

[b]The remainder of the radioactivity in the D-[U-^{14}C]apiose solubilized product was present in D-[U-^{14}C]xylose. These values were determined by hydrolysis at pH 1.

between that synthesized in the absence of UDPGalUA and that synthesized in the presence of 4.0 nmoles of UDPGalUA. The results from the chromatography of two samples of D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA demonstrate that the ion-exchange chromatography of D-[U-^{14}C]apiose solubilized product was very reproducible (Table 6).

I have determined that the increased recovery of radioactivity in fractions D and E, seen when UDPGalUA was added to the reaction mixture, did not occur if UDP[U-^{14}C]Api and UDPGalUA were incubated separately with the particulate enzyme preparation. The experiment was performed by incubating 4 nmoles UDPGalUA with 50 µl of particulate enzyme preparation in a reaction volume of 54 µl for 15 min at 25°C. A control incubation was prepared by incubating 4 µl of H_2O with 50 µl of particulate enzyme preparation for 15 min at 25°C. After incubation the two reaction mixtures were extracted as described for the preparation of product and solubilized product in the Experimental Procedures. The solubilized extracts (0.5 ml) from the incubations with UDPGalUA and water were each combined with 0.5 ml samples of D-[U-^{14}C]apiose solubilized product (30,000 dpm) and were then dialyzed in phosphate buffer and chromatographed on columns of DEAE-Sephadex as described in the legend of Figure 7. Recovery of radioactivity from the

columns was 84% for the control experiment and 76% for the UDPGalUA experiment. A comparison of the elution profiles obtained from the two chromatographies showed that they were identical to each other and to Figure 7. Therefore, addition of solubilized material synthesized from UDPGalUA alone did not affect the migration of D-[U-^{14}C]apiose product through the DEAE-Sephadex column.

Chromatography of D-[U-^{14}C]Glucuronic Acid and D-[U-^{14}C]Xylose Solubilized Products

D-[U-^{14}C]Glucuronic acid and D-[U-^{14}C]xylose solubilized products were chromatographed on DEAE-Sephadex columns (Figure 10). The recovery of D-[U-^{14}C]-glucuronic acid and D-[U-^{14}C]xylose solubilized product from the ion-exchange columns in the experiments depicted in Figure 10 were 65 and 45%, respectively. Since the volume of NaCl solution in each of the steps in the gradient described in Figure 10 is different than in the gradients used in the previously discussed ion-exchange experiments, it is not possible to compare the percentage of radioactivity contained in Fractions A, B, C, D, and E for the D-[U-^{14}C]glucuronic acid and D-[U-^{14}C]xylose solubilized products. However, the elution profile for the D-[U-^{14}C]glucuronic acid solubilized product is similar to that seen in Figure 8 for the D-[U-^{14}C]galacturonic acid solubilized product.

Figure 10. DEAE-Sephadex column chromatograms of D-[U-^{14}C]glucuronic acid solubilized product (a) and D-[U-^{14}C]xylose solubilized product (b). D-[U-^{14}C]-glucuronic acid and D-[U-^{14}C]xylose solubilized products were prepared from reaction volumes of 55 and 70 µl, respectively, as described in the Experimental Procedures. The solubilized products were chromatographed on DEAE-Sephadex columns (0.8 cm internal diam x 10 cm) as described in the Experimental Procedures. The columns were developed identically. The column flow rate was 25 ml/hr and 2 ml fractions were collected. D-[U-^{14}C]-glucuronic acid and D-[U-^{14}C]xylose solubilized products containing 24,000 dpm and 16,000 dpm, respectively, in a 4 ml volume were placed on the column as column fractions 1 and 2 were collected. The columns were treated with phosphate buffer while column fractions 3 to 8 were collected and then in order with 16 ml each of 0.1 M, 0.2 M, and 0.25 M NaCl in phosphate buffer and 20 ml of 0.3 M NaCl in phosphate buffer. The fractions where the various NaCl solutions eluted were determined by conductivity measurements in an identical experiment except that no solubilized product was present and are indicated on the figure. Aliquots were removed from the column fractions and were assayed for radioactivity with scintillation solution B.

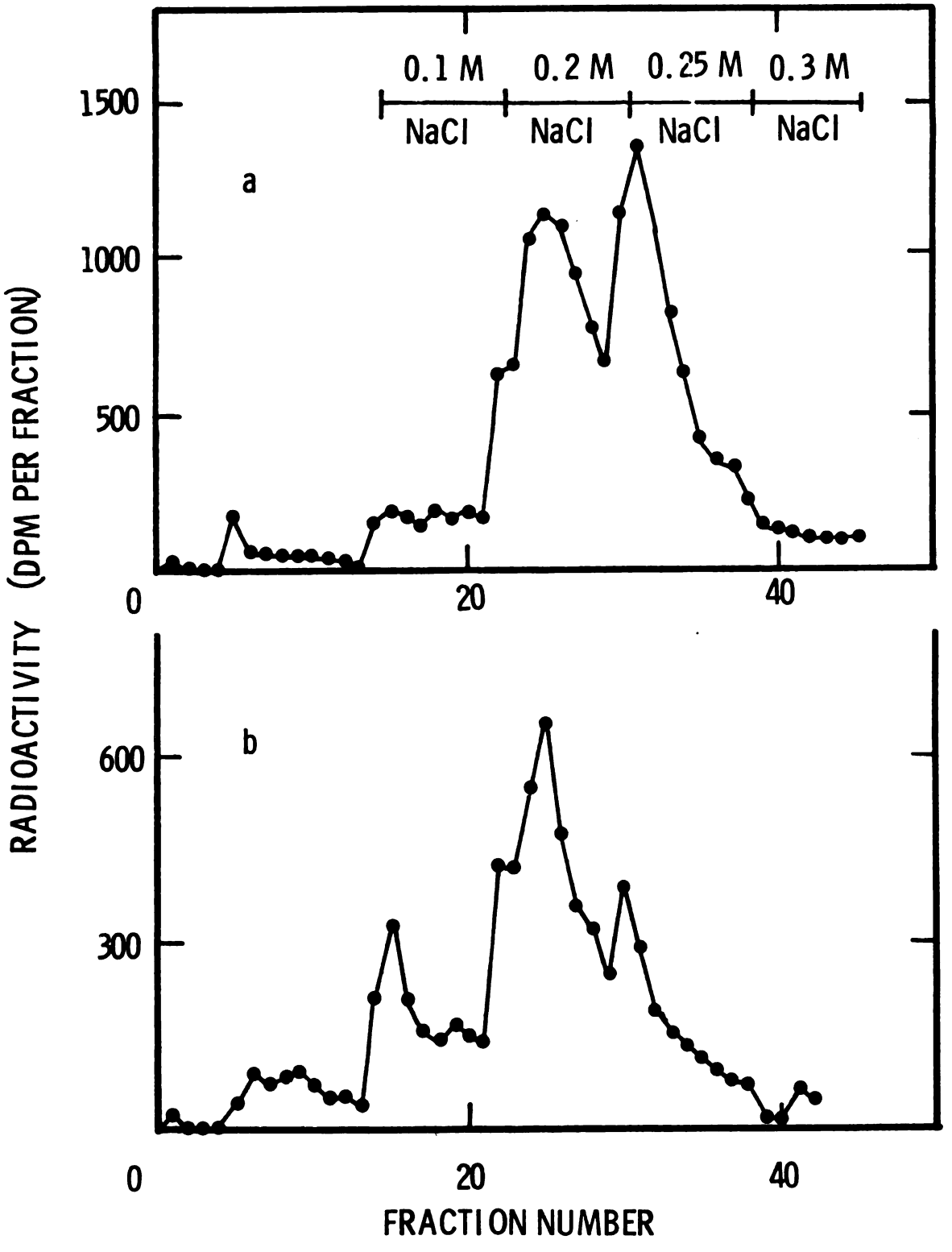

0.1 M
NaCl
0.2 M
NaCl
0.25 M
NaCl
0.3 M
NaCl
a
b
1500
1000
500
0
600
300
0
20
40
20
40
RADIOACTIVITY (DPM PER FRACTION)
FRACTION NUMBER

Figure 10

This is unexpected since a previous hydrolysis experiment disclosed that the majority of the radioactivity in the D-[U-^{14}C]glucuronic acid product was contained in D-[U-^{14}C]xylose. The elution profile for the D-[U-^{14}C]xylose solubilized product is similar to that seen in Figure 7 for the D-[U-^{14}C]apiose solubilized product. This shows that D-[U-^{14}C]xylose was incorporated into an acidic polysaccharide.

Chromatography of D-[U-^{14}C]Apiose Solubilized Products Synthesized in the Presence of UDPGlcUA and UDPXyl

The elution of D-[U-^{14}C]apiose solubilized products synthesized in the presence of UDPGlcUA and UDPXyl through the DEAE-Sephadex column was investigated. These experiments were performed to show whether the addition of UDPGlcUA and UDPXyl to the reaction mixture would cause the same changes in ion-exchange properties that were seen when UDPGalUA was present during synthesis. The dialyzed D-[U-^{14}C]apiose solubilized products synthesized in the presence of UDPGlcUA and UDPXyl described in Table 2 were used in this experiment. Three ml samples of the two solubilized products, each containing 69,000 dpm, were chromatographed on columns of DEAE-Sephadex (0.8 cm internal diam x 10 cm) as described in the legend of Figure 7 except that 2 ml fractions were collected at a rate

of 25 ml/hr. The same fractions as described in Figure 7 were combined to form fractions A through E. Recovery of D-[U-^{14}C]apiose solubilized products synthesized in the presence of UDPGlcUA and UDPXyl from the DEAE-Sephadex column in these experiments was 80 and 75%, respectively. For D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGlcUA the percentage of recovered radioactivity contained in fractions A, B, C, D, and E was 12, 10, 19, 46, and 10%. For D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPXyl the percentage of recovered radioactivity contained in fractions A, B, C, D, and E was 14, 15, 34, 29, and 3%. Comparison of these values with the ones in Table 6 shows that addition of UDPGlcUA to the reaction mixture caused nearly the same change in distribution of radioactivity in the fractions that addition of UDPGlcUA caused. Addition of UDPXyl caused a slight increase in the amount of radioactivity recovered in fraction D at the expense of fraction C.

Characterization of Fractions Obtained from the DEAE-Sephadex Chromatography of D-[U-^{14}C]Apiose Solubilized Product Synthesized in the Absence and Presence of Nonradioactive Sugar Nucleotides

The results described in the preceding sections established that D-[U-^{14}C]apiose solubilized product

synthesized in the presence and absence of nonradioactive sugar nucleotides could be separated into 5 fractions by DEAE-Sephadex column chromatography. The fractions were characterized on the basis of their content of D-[U-^{14}C]apiose and D-[U-^{14}C]xylose and on their ability to release D-[U-^{14}C]apiose and [U-^{14}C]apiobiose after pH 4 hydrolysis.

Table 7 shows the results of the characterization of the individual fractions obtained from DEAE-Sephadex chromatography of D-[U-^{14}C]apiose solubilized product synthesized in the absence of nonradioactive sugar nucleotide and D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA. These data show that the fractions differed in their D-[U-^{14}C]apiose and D-[U-^{14}C]xylose content as well as in their ability to release D-[U-^{14}C]apiose and [U-^{14}C]apiobiose when hydrolyzed at pH 4. The results also indicate that regardless of whether D-[U-^{14}C]apiose solubilized product was synthesized in the presence or absence of UDPGalUA, the fractions were eluted from the column in the order of their increasing D-[U-^{14}C]-apiose content. I examined fractions A through D in order and found that each fraction contained a greater percentage of its radioactivity in D-[U-^{14}C]apiose than did the preceding fraction, as determined by hydrolysis at pH 1. Examination of the fractions by hydrolysis at pH 4 in the same order also showed that each

TABLE 7. Release of D-[U-^{14}C]Apiose and [U-^{14}C]Apiobiose from Fractions Obtained from DEAE-Sephadex Column Chromatography of D-[U-^{14}C]Apiose Solubilized Product Synthesized in the Absence of Nonradioactive Sugar Nucleotide and in the Presence of UDPGalUA[a]

Fraction from DEAE-Sephadex Column	Amount of Radioactivity Present in D-[U-^{14}C]-Apiose[b]	Hydrolysis at pH 4 Yield of D-[U-^{14}C]-Apiose	Hydrolysis at pH 4 Yield of [U-^{14}C]-Apiobiose[c]	$\frac{[U\text{-}^{14}C]\text{Apiobiose}}{D\text{-}[U\text{-}^{14}C]\text{Apiose}}$
	(%)	(%)	(%)	
Solubilized Product Synthesized in the Absence of Exogenous Nonradioactive Sugar Nucleotides				
A	66.3 ± 2.8	8.1 ± 0.4	3.3 ± 1.5	0.4
B	72.3 ± 0.2	12.4 ± 2.5	9.4 ± 1.7	0.8
C	86.7 ± 2.5	12.1 ± 6.2	25.1 ± 6.1	2.1
D	87.9 ± 2.2	16.0 ± 1.4	46.5 ± 1.5	2.9
Solubilized Product Synthesized in the Presence of Exogenous UDPGalUA				
A[d]	76.0	-	-	-
B[d]	80.9	-	-	-
C	90.7 ± 1.2	38.6 ± 2.6	31.4 ± 2.6	0.8
D	95.2 ± 0.3	33.2 ± 0.7	47.4 ± 0.2	1.4

[a]D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures and chromatographed on a DEAE-Sephadex column (0.8 cm internal diam x 15 cm) as described in the Experimental Procedures and the legend of Figure 7. Each experiment was performed twice. The reaction mixtures used to synthesize solubilized product in the presence of UDPGalUA contained 4 nmoles of UDPGalUA and the final volume of the reaction mixtures was 75 µl. For chromatography the samples of

TABLE 7. Continued

D-[U-^{14}C]apiose solubilized product synthesized in the absence of nonradioactive sugar nucleotide were each in the 6 ml of buffer and contained 158,000 and 326,000 dpm and the samples of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA were each in 3.4 ml of buffer and contained 148,000 and 160,000 dpm. The column flow rate was 30 ml/hr and 3.0 ml fractions were collected. Fractions A through D were obtained by combining the same numbered column fractions that were combined in Figure 7. Recovery of radioactivity from the column was 81 and 42% for the two experiments with D-[U-^{14}C]solubilized product synthesized in the absence of nonradioactive sugar nucleotide and 85 and 77% for the two experiments with D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA. The fractions were dialyzed in water at 4°C for 12 hr, concentrated at 30°C, and hydrolyzed. Hydrolysates were chromatographed in Solvent A. Means and mean deviation are reported for the duplicate experiments.

[b]The fractions were hydrolyzed at pH 1 as described in the Experimental Procedures. The values represent the percent of radioactivity recovered from the chromatogram which was present in D-[U-^{14}C]apiose. The remainder of the radioactivity was present in D-[U-^{14}C]xylose.

[c]The fractions were hydrolyzed at pH 4 as described in the Experimental Procedures. The values represent the percent of D-[U-^{14}C]apiose contained in the fractions which was released as D-[U-^{14}C]apiose or [U-^{14}C]apiobiose.

[d]A single hydrolysis at pH 1 was performed. Hydrolysis at pH 4 was omitted.

fraction was capable of releasing a greater percentage of its D-[U-^{14}C]apiose as [U-^{14}C]apiobiose and D-[U-^{14}C]apiose than the preceding fraction. I also found, when the fractions were subjected to hydrolysis at pH 4 in the same order, that they released an increasing percentage of their D-[U-^{14}C]apiose as [U-^{14}C]apiobiose rather than as D-[U-^{14}C]apiose as shown by the increase in the [U-^{14}C]apiobiose to D-[U-^{14}C]apiose ratio (Table 7).

Once again, there were differences between identical fractions obtained from D-[U-^{14}C]apiose solubilized products synthesized in the absence and presence of UDPGalUA (Table 7). The fractions obtained after chromatography from D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA contained a greater percentage of their radioactivity in D-[U-^{14}C]apiose than did the corresponding fractions obtained from solubilized product synthesized in the absence of UDPGalUA (Table 7). After hydrolysis of fractions C and D at pH 4 a higher yield of D-[U-^{14}C]apiose and [U-^{14}C]apiobiose was obtained from D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA than from D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA. This is seen in fraction D, for example, where fraction D from D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA contained 88% of its radioactivity in D-[U-^{14}C]apiose

of which 62% of the D-[U-^{14}C]apiose was released after hydrolysis at pH 4 as D-[U-^{14}C]apiose and [U-^{14}C]apiobiose. Fraction D obtained from D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA contained 95% of its radioactivity in D-[U-^{14}C]apiose of which 81% of the D-[U-^{14}C]apiose was released after hydrolysis at pH 4. However, the ratio of [U-^{14}C]-apiobiose to D-[U-^{14}C]apiose was lower in fractions obtained from solubilized product synthesized in the presence of UDPGalUA than in the fractions obtained from D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA (Table 7).

Fraction D obtained from the DEAE-Sephadex chromatography of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGlcUA was also characterized. The fraction D material was obtained from the DEAE-Sephadex chromatography of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGlcUA which was described in the previous section. Fraction D was dialyzed 12 hr in H_2O at 4°C and concentrated to 1.1 ml. Two samples, each containing 4800 dpm in 500 µl of solution, were hydrolyzed; one at pH 1 and the other at pH 4 as described in the Experimental Procedures and the hydrolysates were chromatographed on paper in Solvent A. Hydrolysis at pH 1 showed that 95% of the recovered radioactivity was contained in D-[U-^{14}C]apiose. The remainder of the radioactivity

was contained in D-[U-^{14}C]xylose. Hydrolysis at pH 4 resulted in the release of 25.2% of the radioactivity in D-[U-^{14}C]apiose as D-[U-^{14}C]apiose and 58.8% as [U-^{14}C]apiobiose. The ratio of [U-^{14}C]apiobiose to D-[U-^{14}C]apiose was 2.3. These results showed that fraction D from D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGlcUA and fraction D from D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA were similar in D-[U-^{14}C]apiose and D-[U-^{14}C]xylose content and in their ability to release D-[U-^{14}C]apiose and [U-^{14}C]apiobiose after hydrolysis at pH 4.

Rechromatography of Fractions Obtained from DEAE-Sephadex Column Chromatography of D-[U-^{14}C]Apiose Solubilized Product

The homogeneity of the fractions collected from the DEAE-Sephadex column was investigated by chromatographing D-[U-^{14}C]apiose solubilized product on a column of DEAE-Sephadex, isolating fractions A, B, C, and D, rechromatographing the fractions on a second column of DEAE-Sephadex, and determining the D-[U-^{14}C]-apiose content of the different fractions before and after rechromatography (Table 8). Fraction B rechromatographed as a single component in its original position in the gradient as evidenced by the single peak that was obtained. Fractions A, C, and D each yielded two

TABLE 8. Rechromatography of Fractions Obtained from DEAE-Sephadex Column Chromatography of D-[U-^{14}C]Apiose Solubilized Product[a]

Fraction from First DEAE-Sephadex Column	D-[U-^{14}C]Apiose Content After First Chromatography[b] (%)	Fraction from Second DEAE-Sephadex Column	Amount of Recovered Radioactivity Contained in Major Components After Second Chromatography (%)	D-[U-^{14}C]Apiose Content After Second Chromatography[b] (%)
A	66.4	A	15.1	-[c]
		B	73.2	66.1
B	68.9	B	69.5	69.0
C	87.6	BC	35.4	78.9
		D	54.3	93.3
D	88.4	D	64.5	89.7
		E	20.7	91.6

[a]D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures and chromatographed on a DEAE-Sephadex column (0.8 cm internal diam x 15 cm, 7.5 ml vol) as described in Figure 7. The sample (300,000 dpm) added to the column was in 4 ml of buffer. The column flow rate was 30 ml/hr and 3 ml fractions were collected. The recovery of radioactivity from the column was 74%. The same column fractions were combined to form fractions A through D as indicated in Figure 7. Fractions A, B, C, and D contained 26, 18, 29, and 15%, respectively, of the radioactivity that was recovered from the column. The four fractions were dialyzed in water at 4°C for 13 hr and concentrated to 1.1 ml at 30°C. Aliquots of the

TABLE 8. Continued

concentrated fractions (250 µl) were hydrolyzed at pH 1. The remainder of each concentrated fraction was rechromatographed, separately, through a second DEAE-Sephadex column (0.5 cm internal diam x 5 cm, 1 ml vol). A NaCl step-gradient, proportional in size to the one used on the column of 7.5 ml volume, was used to develop the column of 1 ml volume. Recovery of radioactivity from the column was 83, 82, 100, and 79% for fractions A, B, C, and D, respectively. Appropriate column fractions from the second DEAE-Sephadex column were combined and the combined fractions were dialyzed for 10 hr in water at 4°C, concentrated at 30°C, and hydrolyzed at pH 1. The amount of D-[U-^{14}C]apiose and D-[U-^{14}C]xylose in the hydrolysates was determined by paper chromatography in Solvent A.

[b]The fractions were hydrolyzed at pH 1 as described in the Experimental Procedures. The values represent the percent of radioactivity recovered from the paper chromatogram which was present in D-[U-^{14}C]apiose. The remainder of the radioactivity was present in D-[U-^{14}C]xylose.

[c]Material was not hydrolyzed because insufficient radioactivity was present.

peaks on rechromatography. The 2 components of fraction A chromatographed in the positions expected of fractions A and B. The 2 components of fraction C chromatographed in the position expected of fractions B and C combined and in the position expected of fraction D. The 2 components of fraction D chromatographed in the positions expected of fractions D and E. The fourth column of data in Table 8 shows what percentage of the radioactivity recovered after rechromatography was contained in each of the fractions. For the rechromatography of fractions A, C, and D greater than 85% of the radioactivity recovered from the column was present in the 2 fractions reported in each Table 8. The rest of the recovered radioactivity was spread over the remainder of the column. For the rechromatography of fraction B, 30% of the radioactivity recovered did not elute with the single fraction reported in Table 8. Most of the radioactivity not contained in the reported fraction was present in the positions expected of fractions C and D as a trailing shoulder of the single large fraction.

The amount of radioactivity that was present in each of the fractions after rechromatography was not invariant. An identical rechromatography experiment was performed, except that analyses for D-[U-^{14}C]apiose and D-[U-^{14}C]xylose were not made. In this experiment the fractions rechromatographed in the same positions in

the elution gradient as in the experiment described in Table 8. The percentages of radioactivity which were recovered from the column in the individual fractions after rechromatography were as follows: for fraction A, 52.5% of the recovered radioactivity was found in the position of fraction A and 37.9% in the position of fraction B, for fraction B, 81.5% of the recovered radioactivity was found in the position of fraction B, for fraction C, 58.2% of the recovered radioactivity was found in the combined positions of fractions B and C and 32.8% in the position of fraction D, and for fraction D, 72.5% of the recovered radioactivity was found in the position of fraction D and 13.9% in the position of fraction E.

A comparison of the D-[U-^{14}C]apiose content of fractions before and after rechromatography was made (Figure 8). In the case of fractions A, B, and D there were no changes in the D-[U-^{14}C]apiose content even though rechromatography had resulted in fractions A and D each being eluted as two separate fractions. However, the two components that were recovered from rechromatography of fraction C had different D-[U-^{14}C]apiose content from each other and from the original fraction C.

Gel Chromatography of D-[U-^{14}C]Apiose Solubilized Product and D-[U-^{14}C]Galacturonic Acid Solubilized Product

Column chromatography of D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products on Bio-Gel P-300 showed that both solubilized products consisted of molecules of different sizes (Figure 11). Dextrans with a weight average molecular weight in the range 5,000 to 125,000 are fractionated by this gel (114). The chromatograms in Figure 11 show that D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products both eluted from the column over the entire fractionation range. Based on radioactivity measurements the recoveries of D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products from the column were 79 and 71%, respectively. Whether the peak of radioactive material that was eluted in the void volume of the column is also of variable size has not been investigated. A peak of radioactive material always eluted at V_t when D-[U-^{14}C]galacturonic acid solubilized product was chromatographed on the Bio-Gel P-300 column. However, the peak of radioactive material depicted in Figure 11 which eluted at V_t when D-[U-^{14}C]-apiose solubilized product was chromatographed on Bio-Gel P-300 was not always seen (Figure 12a). The appearance of this peak with D-[U-^{14}C]apiose solubilized product was random between experiments; the conditions

Figure 11. Bio-Gel P-300 column chromatograms of D-[U-^{14}C]apiose solubilized product and D-[U-^{14}C]galacturonic acid solubilized product. D-[U-^{14}C]Apiose and D-[U-^{14}C]galacturonic acid solubilized products were prepared as described in the Experimental Procedures and chromatographed on a Bio-Gel P-300 column (1 cm internal diam x 40 cm) which was equilibrated and developed with 0.05 M sodium phosphate buffer, pH 6.8. D-[U-^{14}C]Apiose and D-[U-^{14}C]-galacturonic acid solubilized products, containing 21,000 and 13,000 dpm, respectively, in a volume of 0.5 ml, were placed on the column. One ml fractions were collected at a flow rate of 3 ml/hr. Aliquots were removed from the column fractions and were assayed for radioactivity with scintillation solution B. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each fraction.

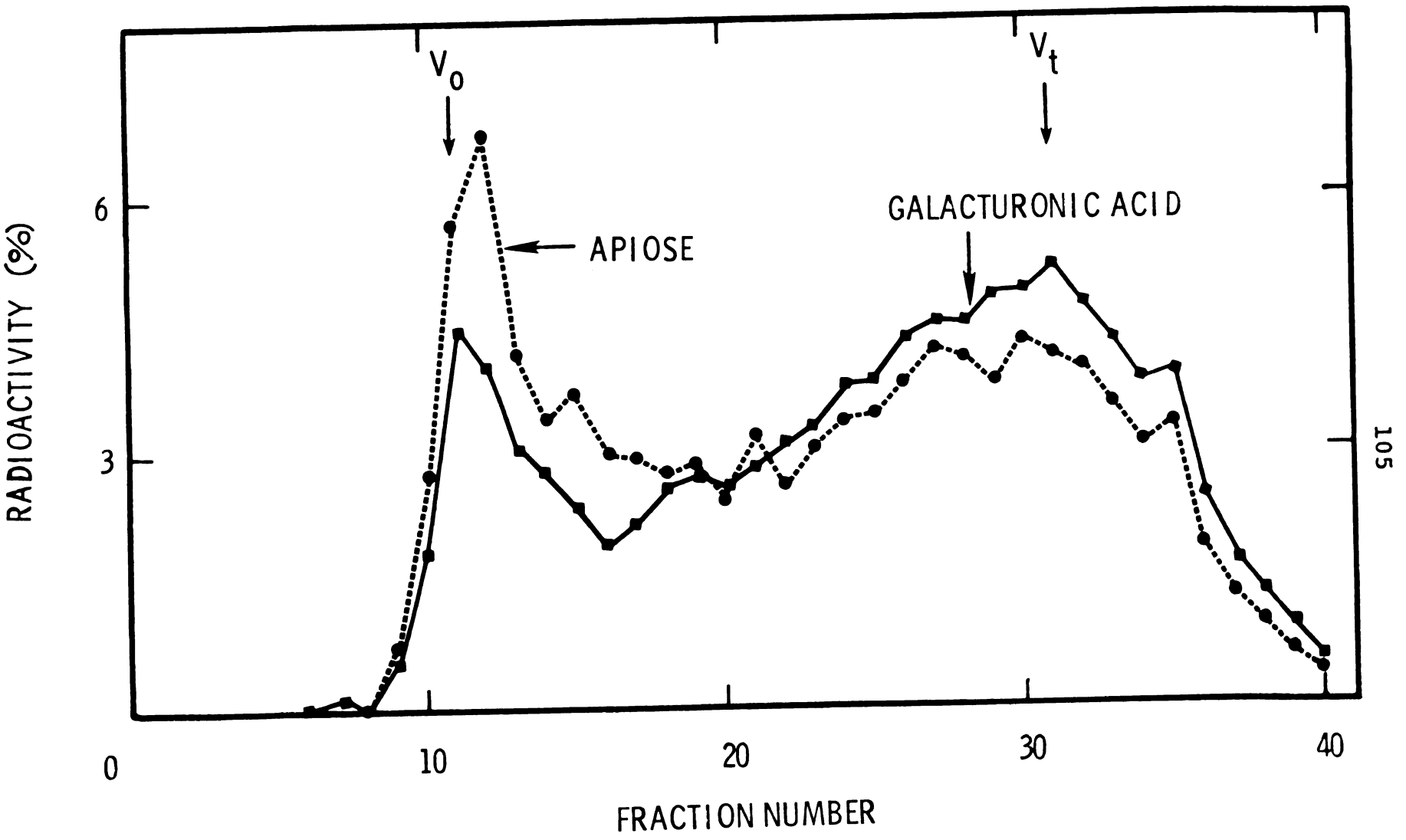

V_o
V_t
APIOSE
GALACTURONIC ACID
RADIOACTIVITY (%)
6
3
0
10
20
30
40
FRACTION NUMBER
105

Figure 11

Figure 12. Bio-Gel P-300 column chromatograms of D-[U-^{14}C]apiose solubilized product (a) and the rechromatography of selected fractions (b). D-[U-^{14}C]Apiose solubilized product (166,000 dpm, in 4 ml) prepared as described in the Experimental Procedures was dialyzed against 0.05 M sodium phosphate buffer, pH 6.8, for 2 hr at 4°C with a buffer change after 1 hr. A 3.5 ml sample of the dialysate (137,000 dpm) was concentrated to 0.6 ml at 27°C and chromatographed on a Bio-Gel P-300 column as described in the legend of Figure 11. Aliquots were removed from the column fractions and were assayed for radioactivity with scintillation solution B. The recovery of radioactivity from the column was 78%. The indicated column fractions in (a) were combined to form fraction I, II, and III, each of which was concentrated to 0.7 ml at 22°C. Portions (200 µl) of each fraction were removed and hydrolyzed at pH 1 as described in the Experimental Procedures. The remainder of each concentrated fraction was then rechromatographed separately on the Bio-Gel P-300 column as described in the legend of Figure 11. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each fraction.

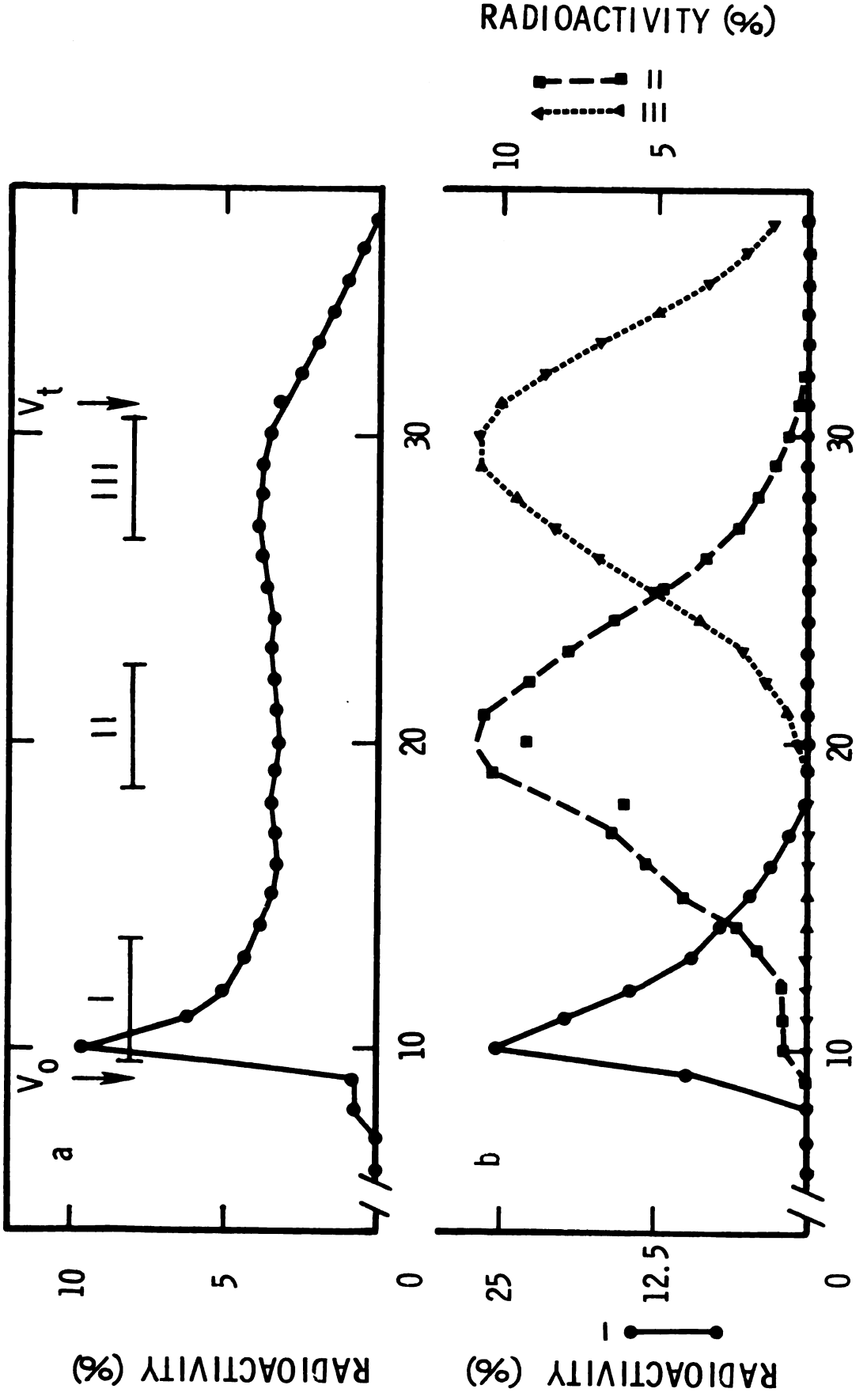

RADIOACTIVITY (%)
I
II
III
V_o
V_t
a
b
RADIOACTIVITY (%)
RADIOACTIVITY (%)
FRACTION NUMBER
10
5
0
25
12.5
20
30

Figure 12

for its appearance were not investigated. Comparison of the two chromatograms in Figure 11 showed that the D-[U-^{14}C]apiose solubilized product contained more molecules of large size which eluted in the void volume than did the D-[U-^{14}C]galacturonic acid solubilized product.

The homogeneity of the fractions obtained from the chromatography of D-[U-^{14}C]apiose solubilized product on the Bio-Gel P-300 column was investigated (Figure 12). D-[U-^{14}C]Apiose solubilized product was chromatographed on the Bio-Gel P-300 column and the fractions indicated in Figure 12a representing large, medium, and small molecular weight components of the D-[U-^{14}C]apiose solubilized product were labelled fractions I, II, and III, respectively, and were rechromatographed on the Bio-Gel P-300 column. The results showed that fractions I, II, and III had the same elution volumes on rechromatography as they had in the initial chromatography on Bio-Gel P-300 although each distributed itself over a larger molecular weight range on rechromatography (Figure 12b). Samples of D-[U-^{14}C]apiose solubilized product prior to rechromatography and fractions I, II, and III were hydrolyzed at pH 1 and chromatographed on paper with Solvent A. Analysis of the chromatograms showed that the D-[U-^{14}C]apiose solubilized product before chromatography contained 79.5% of its radioactivity in D-[U-^{14}C]apiose.

Fractions I, II, and III contained 82.4, 76.8, and 80.4%, respectively, of their radioactivity in D-[U-^{14}C]apiose. The remainder of the radioactivity was contained in D-[U-^{14}C]xylose. The similar D-[U-^{14}C]apiose content of the three fractions and of D-[U-^{14}C]apiose solubilized product showed that D-[U-^{14}C]apiose and D-[U-^{14}C]xylose were not concentrated in molecules of a particular size.

The chromatograms obtained from the chromatography of D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products on a Bio-Gel P-100 column were dissimilar (Figure 13). D-[U-^{14}C]Apiose solubilized product eluted from the column predominantly at the V_o (Figure 13a). There was no elution of radioactive material at V_t. On the other hand, D-[U-^{14}C]galacturonic acid solubilized product eluted from the column over the entire fractionation range of the Bio-Gel P-100 column. These results indicate that the smallest molecules of D-[U-^{14}C]galacturonic acid solubilized product are smaller than the smallest molecules of D-[U-^{14}C]apiose solubilized product.

"Degradation" of D-[U-^{14}C]Apiose Solubilized Product and D-[U-^{14}C]Galacturonic Acid Solubilized Product

If D-[U-^{14}C]apiose solubilized product was dialyzed in water prior to column chromatography on Bio-Gel P-300 there was a significant change in elution profile when compared with the chromatogram obtained from nondialyzed D-[U-^{14}C]apiose solubilized product

Figure 13. Bio-Gel P-100 column chromatograms of D-[U-^{14}C]apiose solubilized product (a) and D-[U-^{14}C]-galacturonic acid solubilized product (b). D-[U-^{14}C]-Apiose and D-[U-^{14}C]galacturonic acid solubilized products were prepared as described in the Experimental Procedures and chromatographed on a Bio-Gel P-100 column (1 cm internal diam x 40 cm) which was equilibrated and developed with 0.05 M sodium phosphate buffer, pH 6.8. D-[U-^{14}C]Apiose and D-[U-^{14}C]galacturonic acid solubilized products, containing 24,000 and 12,000 dpm, respectively, in a volume of 0.6 ml were placed on the column. One ml fractions were collected at a flow rate of 20 ml/hr. Aliquots were removed from the column fractions and were assayed for radioactivity with scintillation solution B. Based on radioactivity measurements the recoveries of D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products were 95 and 79%, respectively. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each fraction.

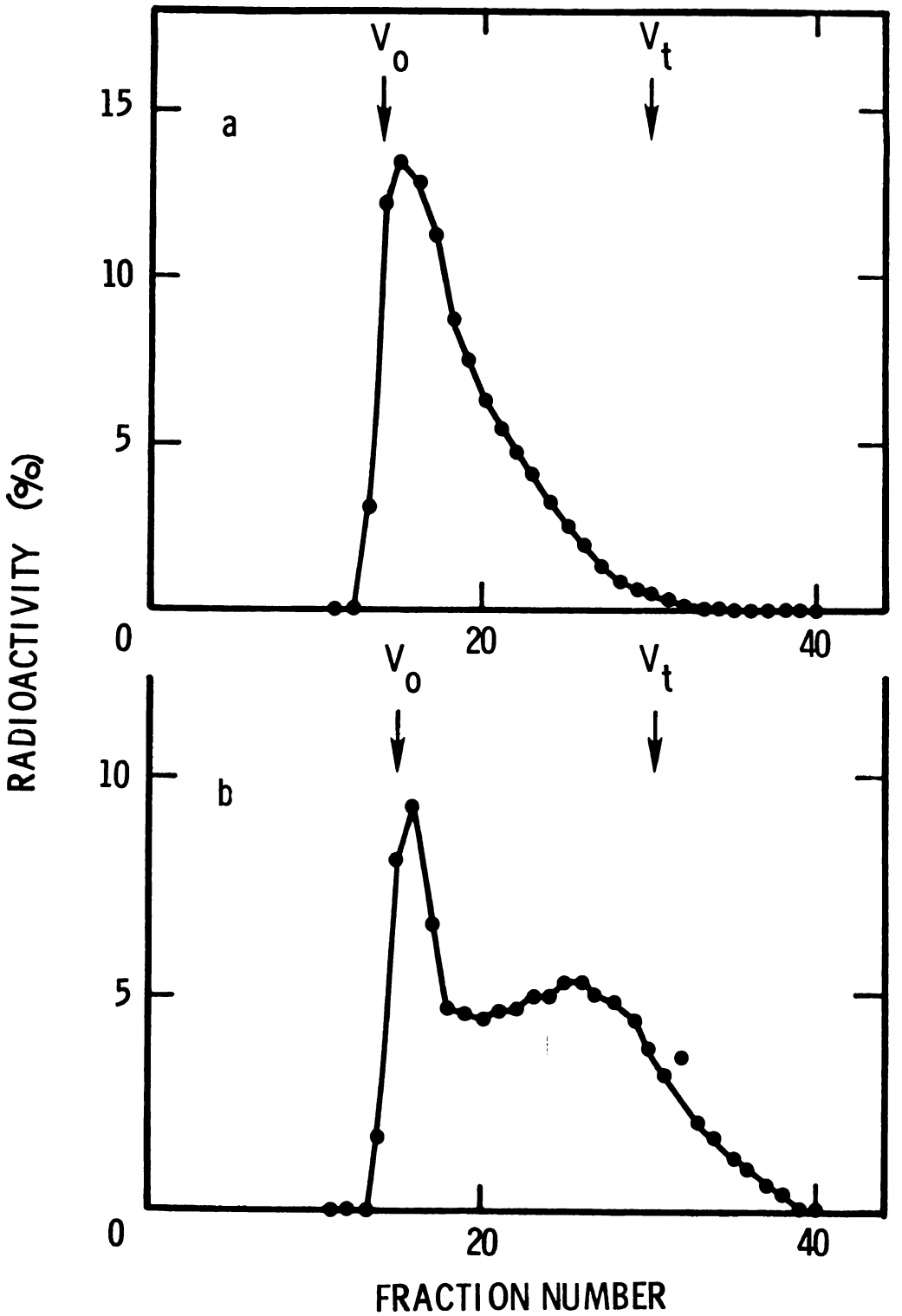

V_o
V_t
15
a
10
5
0
20
40
V_o
V_t
10
b
5
0
20
40
RADIOACTIVITY (%)
FRACTION NUMBER

Figure 13

(Figure 14). Dialysis in water resulted in a decrease in the amount of radioactive material which eluted at V_o and a concurrent increase in the amount of radioactive material which eluted at V_t. However, dialysis of D-[U-^{14}C]apiose solubilized product in sodium phosphate buffer prior to chromatography resulted in no change in elution from the Bio-Gel P-300 column (Figure 14). This process which resulted in a decrease in the amount of large molecular weight components of the solubilized product as demonstrated by gel-chromatography was named "degradation" by us.

I have attempted to reverse the "degradation" of D-[U-^{14}C]apiose solubilized product caused by dialysis in water by performing a second dialysis in sodium phosphate buffer. D-[U-^{14}C]Apiose solubilized product was prepared and divided into 2 samples. One sample, containing 27,000 dpm in a volume of 0.5 ml, was chromatographed on the Bio-Gel P-300 column. The other sample containing 60,000 dpm was dialyzed in water for 16 hr at 4°C with changes in water after 1 and 4.5 hr. The recovery of radioactive material after dialysis in water was 65%. A sample of the water dialysate, containing 17,000 dpm in a volume of 0.5 ml, was chromatographed on the Bio-Gel P-300 column. The remainder of the water dialysate was then dialyzed in 0.1 M sodium phosphate buffer, pH 6.8, at 4°C for 24 hr

Figure 14. Bio-Gel P-300 column chromatograms of nondialyzed, water dialyzed, and sodium phosphate dialyzed D-[U-^{14}C]apiose solubilized product. D-[U-^{14}C]-Apiose solubilized product was prepared as described in the Experimental Procedures and was divided into 3 samples (24,000 dpm, in 500 µl). Two of the samples were dialyzed for 2.5 hr at 4°C with one change of buffer after 1 hr; one sample in water and the other sample in 0.05 M sodium phosphate buffer, pH 7.7. Recoveries of radioactive material after dialysis in water and phosphate was 78 and 98%, respectively. The three samples were then chromatographed on a Bio-Gel P-300 column (1 cm internal diam x 38 cm) as described in the legend of Figure 11. Based on radioactivity measurements the recoveries from the column were 100, 85, and 78% for the nondialyzed, water dialyzed, and phosphate dialyzed samples, respectively. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each fraction.

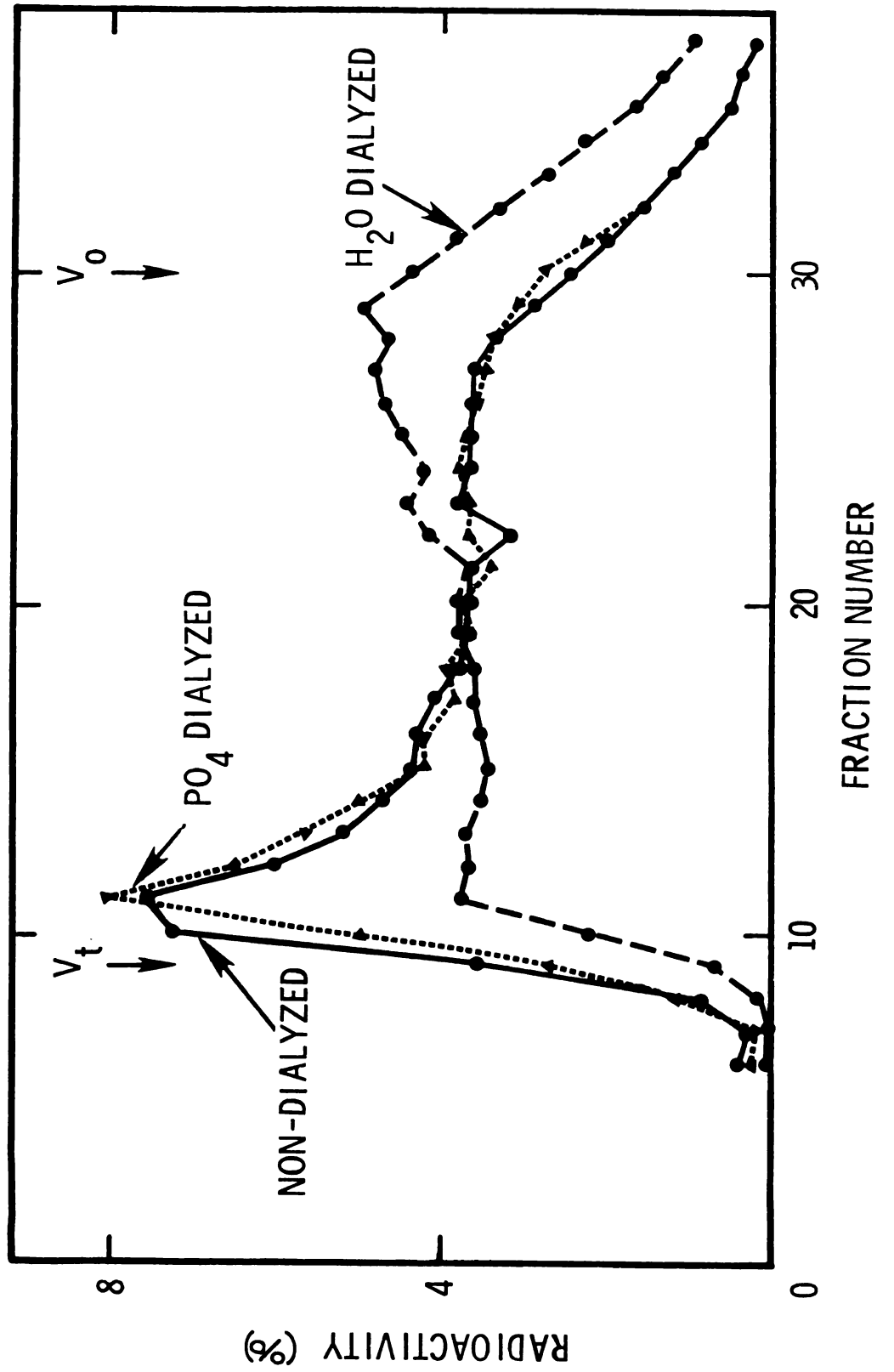

RADIOACTIVITY (%)
8
4
0
10
20
30
FRACTION NUMBER
V_t
V_o
PO_4 DIALYZED
H_2O DIALYZED
NON-DIALYZED

Figure 14

with changes of buffer after 2 and 5 hr. Recovery of radioactive material after dialysis in phosphate buffer was 69%. A sample of the phosphate dialysate, containing 13,000 dpm in a volume of 0.5 ml, was chromatographed on the Bio-Gel P-300 column. In all cases chromatography on the Bio-Gel P-300 column was performed as described in the legend of Figure 11. The recoveries of radioactive material from the columns for the nondialyzed sample, water dialyzed material, and water dialyzed then phosphate dialyzed material 88, 88, and 92%, respectively. Comparison of the three Bio-Gel P-300 chromatograms showed that dialysis in sodium phosphate buffer was unable to reverse the "degradation" caused by dialysis in water.

Dialysis in water also "degraded" D-[U-^{14}C]-galacturonic acid solubilized product. Although a chromatogram from this experiment is not presented D-[U-^{14}C]galacturonic acid solubilized product was dialyzed for 3 hr in water at 4°C and chromatographed on a Bio-Gel P-100 column as described in Figure 13 except that 2 ml fractions were collected. A sample of nondialyzed D-[U-^{14}C]galacturonic acid solubilized product was similarly chromatographed on the column. Recovery of radioactivity after dialysis in water was 78% and recoveries of radioactivity from the Bio-Gel P-100 column after chromatography of nondialyzed solubilized product and solubilized product

dialyzed in water were 100 and 84%, respectively. The water dialysate had a decreased amount of radioactive material that eluted in the column V_o and an increased amount of radioactive material eluted at V_t when compared with the sample that was not dialyzed. D-[U-^{14}C]-Galacturonic acid solubilized product was also dialyzed in 0.05 M sodium phosphate buffer (pH 6.8) for 3 hr at 4°C and was chromatographed on the Bio-Gel P-100 column as described above. The recovery of radioactivity after dialysis was 98% and recovery after column chromatography was 74%. "Degradation" did not occur during dialysis in phosphate buffer as there was essentially no difference in the elution profiles of D-[U-^{14}C]galacturonic acid solubilized product dialyzed in sodium phosphate buffer and of nondialyzed D-[U-^{14}C]galacturonic acid solubilized product.

When D-[U-^{14}C]apiose solubilized product was chromatographed on a Bio-Gel P-300 column and then dialyzed in water, it was not "degraded" (Figure 15). After water dialysis fraction I still eluted at V_o, thus indicating that under these conditions "degradation" did not occur. Although the data are not presented here, degradation was also not obtained when the V_o fraction obtained from the Bio-Gel P-100 column chromatography of D-[U-^{14}C]galacturonic acid

Figure 15. Bio-Gel P-300 column chromatograms of D-[U-^{14}C]apiose solubilized product (a) and the rechromatography of a selected fraction after dialysis in water (b). D-[U-^{14}C]Apiose solubilized product (200,000 dpm) was dialyzed in 0.05 M sodium phosphate buffer, pH 6.8 concentrated, and chromatographed on a Bio-Gel P-300 column as described in the legends of Figures 11 and 12. The concentrated sample (160,000 dpm) was placed on the column in a volume of 0.5 ml. Recovery of radioactivity after column chromatography was 89%. The indicated column fractions in (a) were combined to form fraction I. A sample of fraction I (20,000 dpm) was dialyzed in water for 6 hr at 4°C with changes of water after 2 and 4 hr. Recovery of radioactive material after dialysis was 89%. The dialysate was concentrated to 0.5 ml and was rechromatographed on the Bio-Gel P-300 column as described in Figure 11. Recovery of radioactivity from the column was 66%.

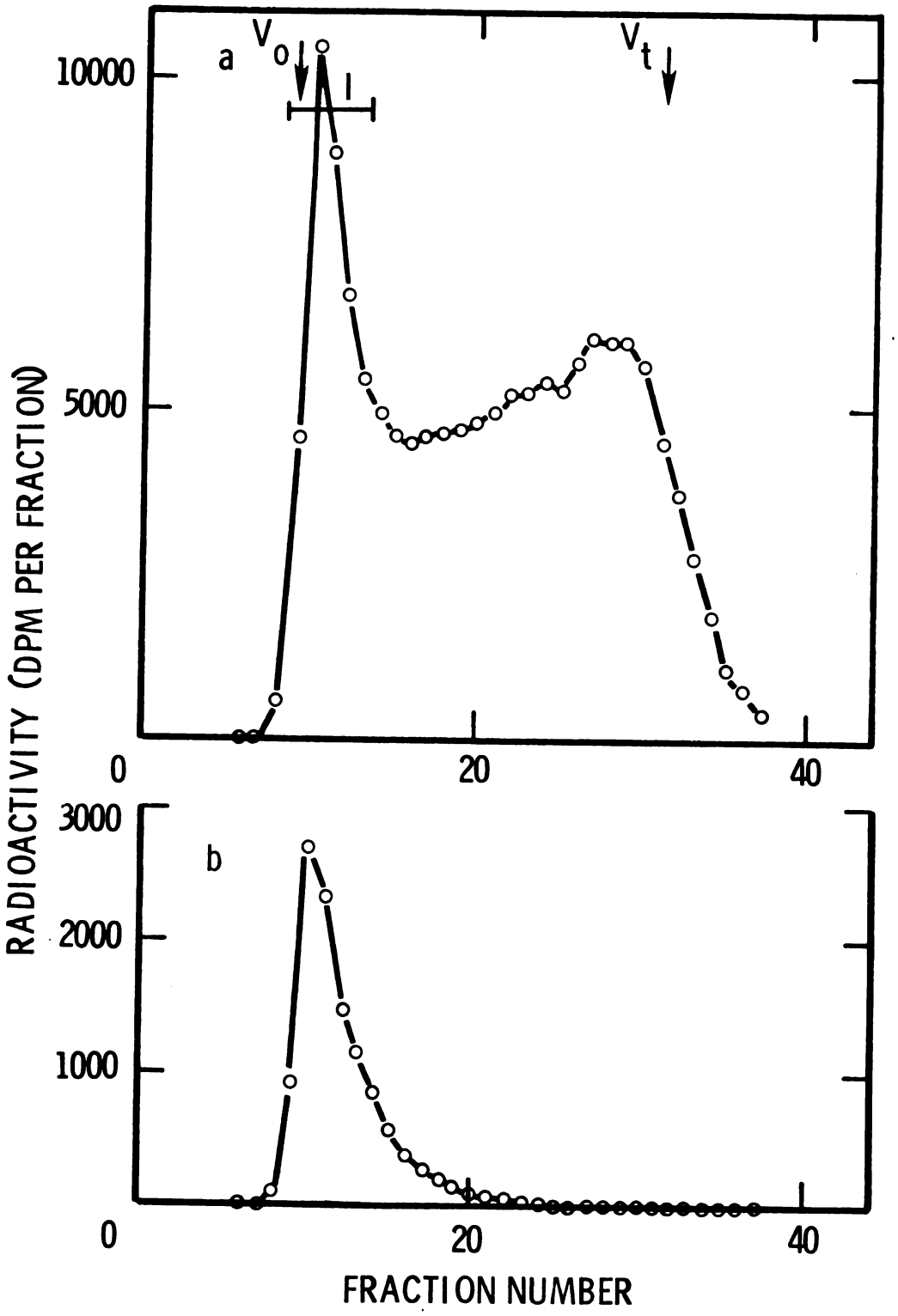

a
V_o
V_t
I
10000
5000
0
20
40
b
3000
2000
1000
0
20
40
RADIOACTIVITY (DPM PER FRACTION)
FRACTION NUMBER

Figure 15

solubilized product was dialyzed in water at 4°C and rechromatographed on the same column.

D-[U-^{14}C]Apiose solubilized product was also "degraded" when chromatographed on DEAE-Sephadex even though the material was never dialyzed in water. D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures and was dialyzed in 0.05 M sodium phosphate buffer, pH 7.7, containing 0.002% Hibitane (1, 1'-hexamethyl lenebis [5-(p-chlorophenyl)biguanide]) for 2 hr at 4°C. This material is referred to as the PO_4 dialysate. A sample of dialysate was chromatographed on the Bio-Gel P-300 column. A second sample of the PO_4 dialysate (23,000 dpm) in a volume of 2.5 ml was placed on a DEAE-Sephadex column (0.8 cm internal diam x 5 cm) prepared as described in the Experimental Procedures. The DEAE-Sephadex column was washed with 7 ml of 0.05 M sodium phosphate buffer, pH 7.7, containing 0.002% Hibitane. The column was then developed with 10 ml of the above phosphate buffer containing 0.3 M NaCl containing 0.002% Hibitane as 1 ml fractions were collected at a flow rate of 20 ml/hr. Aliquots of each column fraction were assayed for radioactivity with scintillation solution B. Total recovery of radioactive material from the column was 68%. Column fractions 3 to 8, containing 53% of the radioactivity originally placed on the column, were

combined and a 4 ml sample was concentrated to 1.2 ml at 22°C. This material is referred to as the DEAE-Sephadex fraction. The DEAE-Sephadex fraction was also chromatographed on the Bio-Gel P-300 column (Figure 16). Comparison of the two chromatograms showed that "degradation" had occurred. In this particular experiment the Bio-Gel P-300 column was equilibrated and developed with 1 M NaCl in order to minimize association between molecules of D-[U-^{14}C]apiose solubilized product and because of the high salt concentration of the DEAE-Sephadex fraction after concentration. Hibitane was added to the solutions to preclude the growth of bacteria capable of metabolizing D-[U-^{14}C]apiose solubilized product.

In the experiment depicted in Figure 16 a sample of D-[U-^{14}C]apiose solubilized product (10,000 dpm) was dialyzed at 4°C for 2 hr in water followed by 2 hr in sodium phosphate buffer, pH 7.7. Solid NaCl was dissolved in the dialysate to give a NaCl concentration of 1 M and the dialysate was then chromatographed on the Bio-Gel P-300 column as described in the legend of Figure 16. Although the column chromatogram obtained from the water dialysate is not shown in Figure 16, it was nearly identical to the one obtained from the DEAE-Sephadex fraction. These results show that the amount of degradation caused by water dialysis and DEAE-Sephadex chromatography was identical.

Figure 16. Bio-Gel P-300 column chromatography of D-[U-^{14}C]apiose solubilized product after dialysis in sodium phosphate buffer and after elution from a DEAE-Sephadex column. D-[U-^{14}C]Apiose solubilized product was dialyzed in sodium phosphate buffer (PO_4 dialysate fraction) and eluted from a DEAE-Sephadex column (DEAE-Sephadex fraction) as described in the text. Samples of PO_4 dialysate fraction (7,000 dpm) and DEAE-Sephadex fraction (3,000 dpm) in a volume of 0.5 ml were chromatographed on a Bio-Gel P-300 column (1 cm internal diam x 21 cm) that was equilibrated and developed with 1 M NaCl containing 0.002% hibitane. Prior to gel chromatography, solid NaCl was dissolved in the PO_4 dialysate fraction to give a NaCl concentration of 1 M. One ml fractions were collected at a rate of 3 ml/hr. Based on radioactivity measurements the recovery of the PO_4 dialyzed fraction and the DEAE-Sephadex fraction from the column was 85 and 87%, respectively. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each fraction.

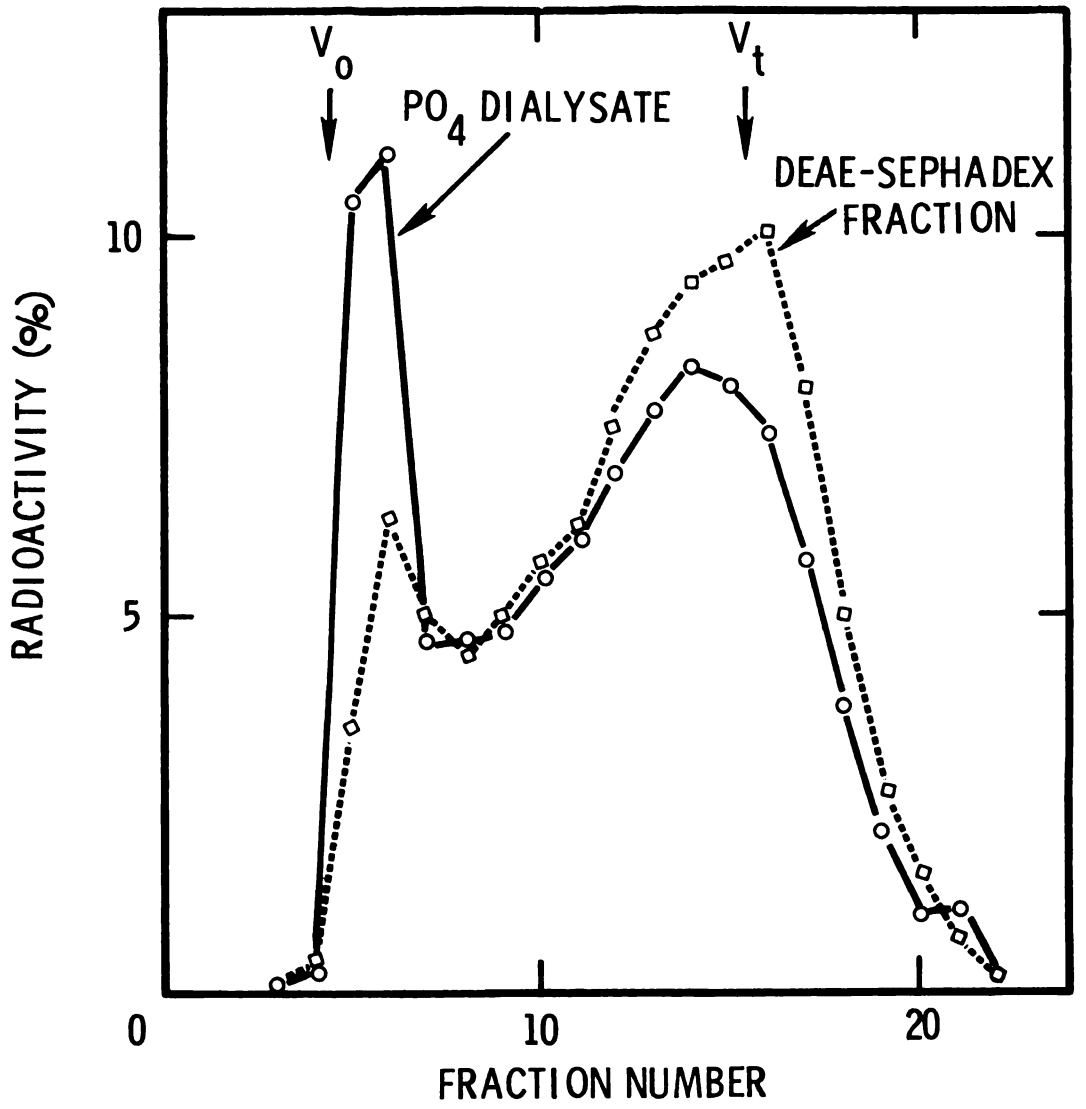

V_o
V_t
PO_4 DIALYSATE
DEAE-SEPHADEX FRACTION
RADIOACTIVITY (%)
10
5
0
10
20
FRACTION NUMBER

Figure 16

Effect of UDPGalUA on the Size of D-[U-^{14}C]-Apiose Solubilized Product

D-[U-^{14}C]Apiose solubilized products synthesized in the absence and presence of UDPGalUA were chromatographed on the same Bio-Gel P-300 column (Figure 17). The recovery of radioactive material from the column for D-[U-^{14}C]apiose solubilized product synthesized in the absence and presence of UDPGalUA was 72.3 and 89.6%, respectively. Comparison of the two elution profiles showed there was little difference in the amount of radioactive material which eluted in the void volume of the column. However, there was a shift in the position of the broad peak which represents the elution of the smaller molecules. These results show that the presence of UDPGalUA during synthesis caused a relative increase in the size of the smaller radioactive molecules compared to those synthesized in the absence of UDPGalUA.

Effect of Incubation Time on the Size of D-[U-^{14}C]-Apiose Solubilized Product and D-[U-^{14}C]Galacturonic Acid Solubilized Product

The length of the incubation period used in synthesis of D-[U-^{14}C]galacturonic acid solubilized product affected its size as determined by chromatography with Bio-Gel P-100 (Figure 18). For incubation times of 0.5, 2, and 15 min, 9.1, 32.1, and 37.9%, respectively, of the starting radioactivity was incorporated into

Figure 17. Bio-Gel P-300 column chromatograms of D-[U-^{14}C]apiose solubilized product synthesized in the presence and absence of UDPGalUA. D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures. The reaction mixture used to synthesize D-[U-^{14}C]apiose solubilized product in the presence of UDPGalUA contained 4 nmoles of UDPGalUA in a final volume of 75 µl. D-[U-^{14}C]Apiose solubilized products synthesized with (29,000 dpm) and without (23,000 dpm) UDPGalUA were applied in a volume of 0.5 ml to the Bio-Gel P-300 column and eluted as described in the legend of Figure 11. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each individual fraction.

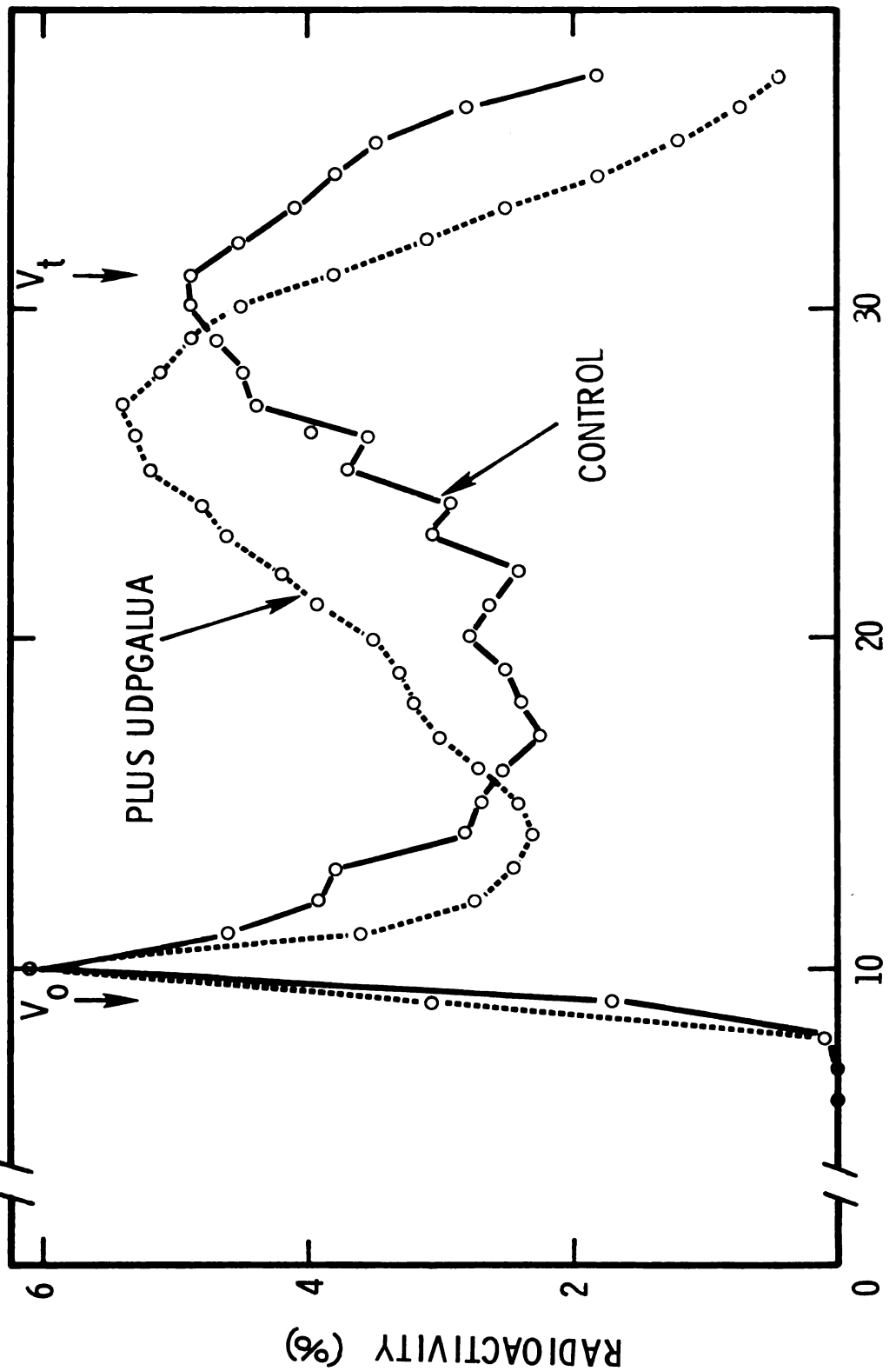

RADIOACTIVITY (%)
0
2
4
6
10
20
30
FRACTION NUMBER
V_o
V_t
PLUS UDPGALUA
CONTROL

Figure 17

Figure 18. Bio-Gel P-100 column chromatograms of D-[U-^{14}C]galacturonic acid solubilized product synthesized in 0.5, 2, and 15 min. Three identical reaction mixtures for the synthesis of D-[U-^{14}C]galacturonic acid solubilized product were prepared as described in the Experimental Procedures. All three reaction mixtures were incubated at 25°C; one for 0.5 min, one for 2 min, and the other for 15 min. Solubilized product was prepared from these assays as described in the Experimental Procedures. After isolation, the D-[U-^{14}C]galacturonic acid solubilized products in a volume of 0.5 ml were chromatographed on the Bio-Gel P-100 column as described in the legend of Figure 13. The amount of radioactivity isolated as solubilized product was 3,200, 10,900, and 12,200 dpm for incubations of 0.5, 2, and 15 min, respectively. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each individual fraction.

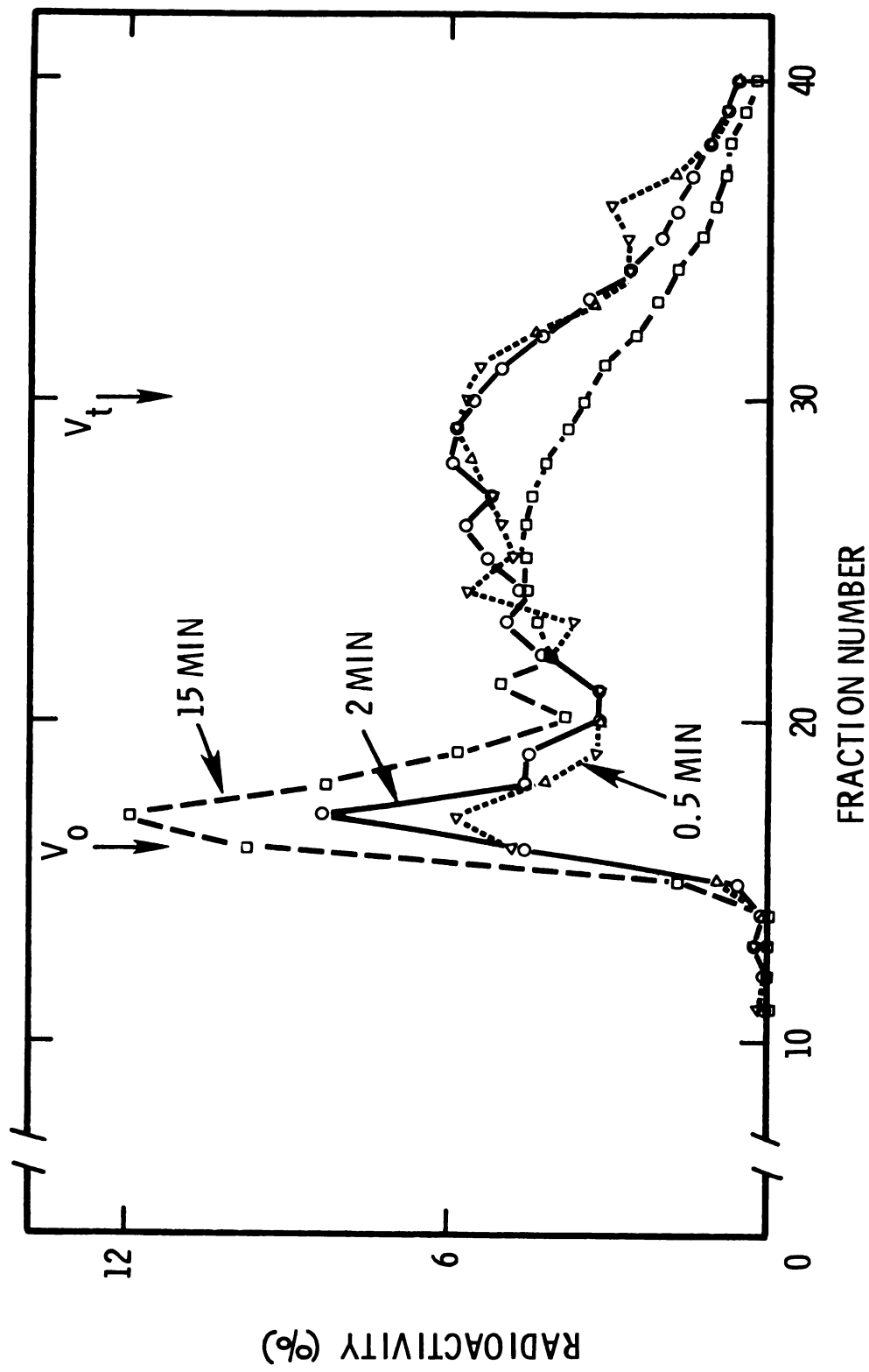

RADIOACTIVITY (%)
12
6
0
10
20
30
40
FRACTION NUMBER
V_o
V_t
15 MIN
2 MIN
0.5 MIN

Figure 18

D-[U-^{14}C]galacturonic acid product and 96, 94, and 89%, respectively, of the D-[U-^{14}C]galacturonic acid product was solubilized by ammonium oxalate extraction. Recovery of radioactivity from the column was 84, 76, and 83% for material synthesized with incubation times of 0.5, 2, and 15 min, respectively. Comparison of the elution profiles showed that as the reaction mixtures were incubated for longer periods of time the percent of radioactive material which eluted in the V_o increased. These results indicated that the size of the molecules of D-[U-^{14}C]galacturonic acid solubilized product increased with increasing incubation time.

The size of the D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA, as determined by chromatography with Bio-Gel P-300, was also affected by the length of the incubation period used in synthesis (Figure 19). For the 0.5 and 15 min incubations 6.4 and 44.1%, respectively, of the starting radioactivity was incorporated into D-[U-^{14}C]apiose product of which 91.4 and 90.2%, respectively, was solubilized by ammonium oxalate extraction. Recovery of radioactivity from the column for D-[U-^{14}C]apiose solubilized product synthesized with incubation times of 0.5 and 15 min was 83 and 77%, respectively. Comparison of the two elution profiles showed that D-[U-^{14}C]apiose solubilized product synthesized in

Figure 19. Bio-Gel P-300 column chromatograms of D-[U-^{14}C]apiose solubilized product synthesized in 0.5 min and 15 min. Two identical reaction mixtures for the synthesis of D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA were prepared as described in the Experimental Procedures. Each assay mixture contained 4 nmoles of UDPGalUA and had a final volume of 75 µl. Both assays were incubated at 25°C, one for 0.5 min and the other for 15 min. Solubilized product was prepared from these assays as described in the Experimental Procedures. The amount of radioactivity isolated as D-[U-^{14}C]apiose solubilized product was 3,400 dpm from the 0.5 min incubation and 23,000 dpm from the 15 min incubation. The D-[U-^{14}C]apiose solubilized products, in a volume of 0.5 ml, were chromatographed on a Bio-Gel P-300 column (1 cm internal diam x 40 cm) as described in the legend of Figure 11. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each individual fraction.

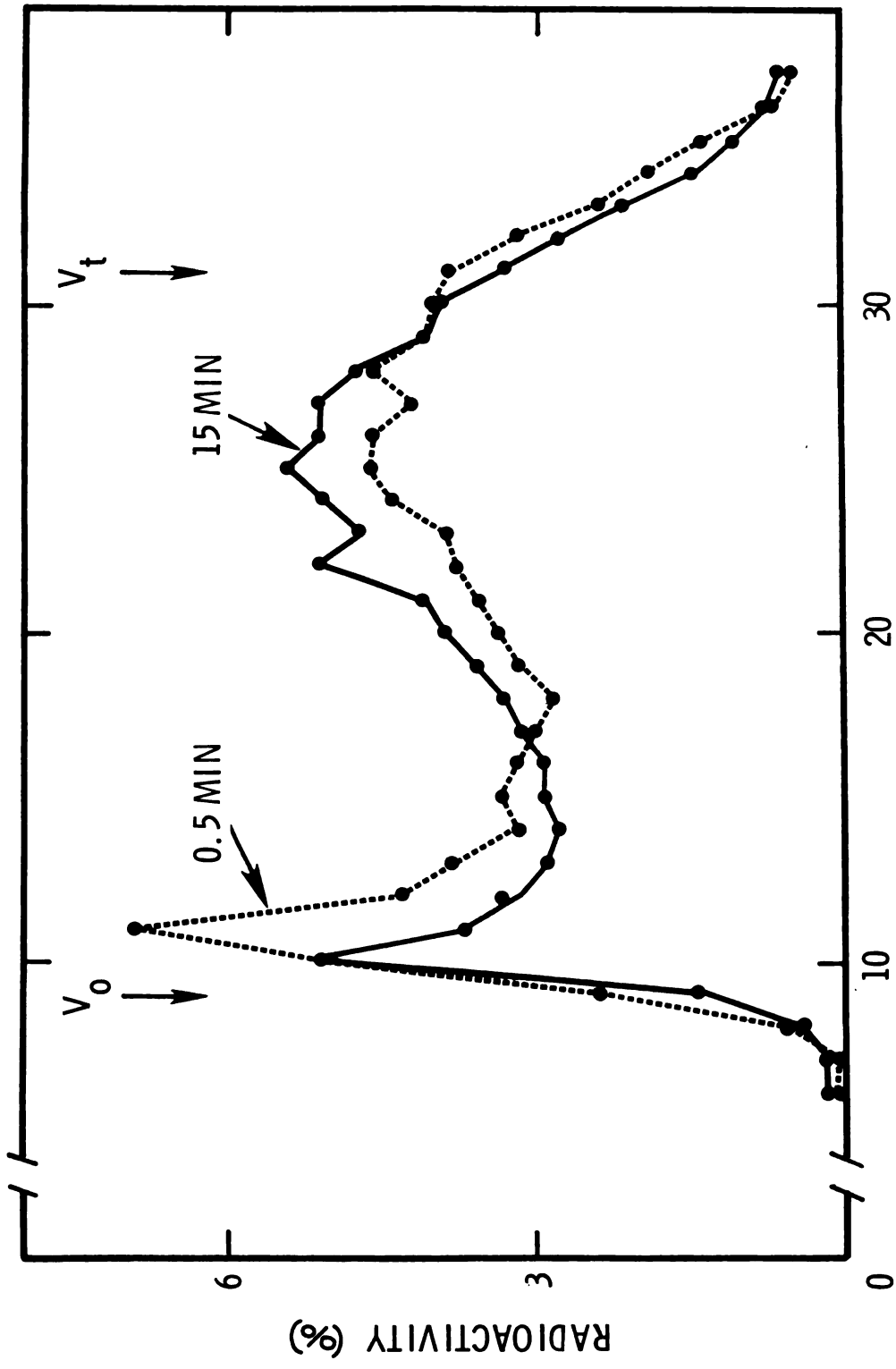

RADIOACTIVITY (%)
0
3
6
FRACTION NUMBER
10
20
30
V_o
V_t
0.5 MIN
15 MIN

Figure 19

0.5 min contained a greater percentage of radioactivity in molecules which eluted in the column V_o than did D-[U-^{14}C]apiose solubilized product which was synthesized in 15 min. These data suggest that acceptor molecules of larger size were preferentially used in the synthesis of the polysaccharide.

Pectinase Hydrolysis of Solubilized Products

Hydrolysis of D-[U-^{14}C]Apiose Solubilized Product

Untreated D-[U-^{14}C]apiose solubilized product and D-[U-^{14}C]apiose solubilized product treated with pectinase were chromatographed on a Bio-Gel P-100 column (Figure 20). Most of the untreated D-[U-^{14}C]-apiose solubilized product eluted from the column in the V_o. The treated material, on the other hand, eluted over the entire fractionation range of the column indicating that enzyme catalyzed hydrolysis of the D-[U-^{14}C]apiose solubilized product had occurred.

The amount of D-[U-^{14}C]apiose and D-[U-^{14}C]-xylose contained in the fragments resulting from pectinase hydrolysis was investigated. D-[U-^{14}C]Apiose solubilized product was treated with pectinase and then chromatographed on a column of Bio-Gel P-30 (Figure 21). The fractions indicated in Figure 21 and labelled as 1, 2, and 3 were each concentrated to approximately 200 µl at 30°C. The three samples were hydrolyzed at

Figure 20. Bio-Gel P-100 column chromatograms of D-[U-^{14}C]apiose solubilized product that was untreated and was treated with pectinase. D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures and was dialyzed in water at 4°C for 2 hr with changes of water after 0.5 and 1 hr. Two equal portions (200 µl) of the solution of dialyzed, D-[U-^{14}C]-apiose solubilized product, each containing 6,000 dpm, were placed in separate tubes. One portion of solubilized product was treated with pectinase as described in the Experimental Procedures. To the other portion (untreated) was added 20 µl of 0.5 M sodium acetate buffer, pH 4.5. The two samples were incubated for 25 hr at 37°C. After incubation the samples were chromatographed on a Bio-Gel P-100 column (0.7 cm internal diam x 10 cm) which had been equilibrated with 0.01 M sodium phosphate buffer; the untreated sample was chromatographed first. The flow rate of the column was 1.3 ml/hr and 0.25 ml fractions were collected. Radioactivity was assayed by using scintillation solution B. Recoveries from the P-100 column were 67% for untreated D-[U-^{14}C]-apiose solubilized product and 80% for pectinase treated D-[U-^{14}C]apiose solubilized product.

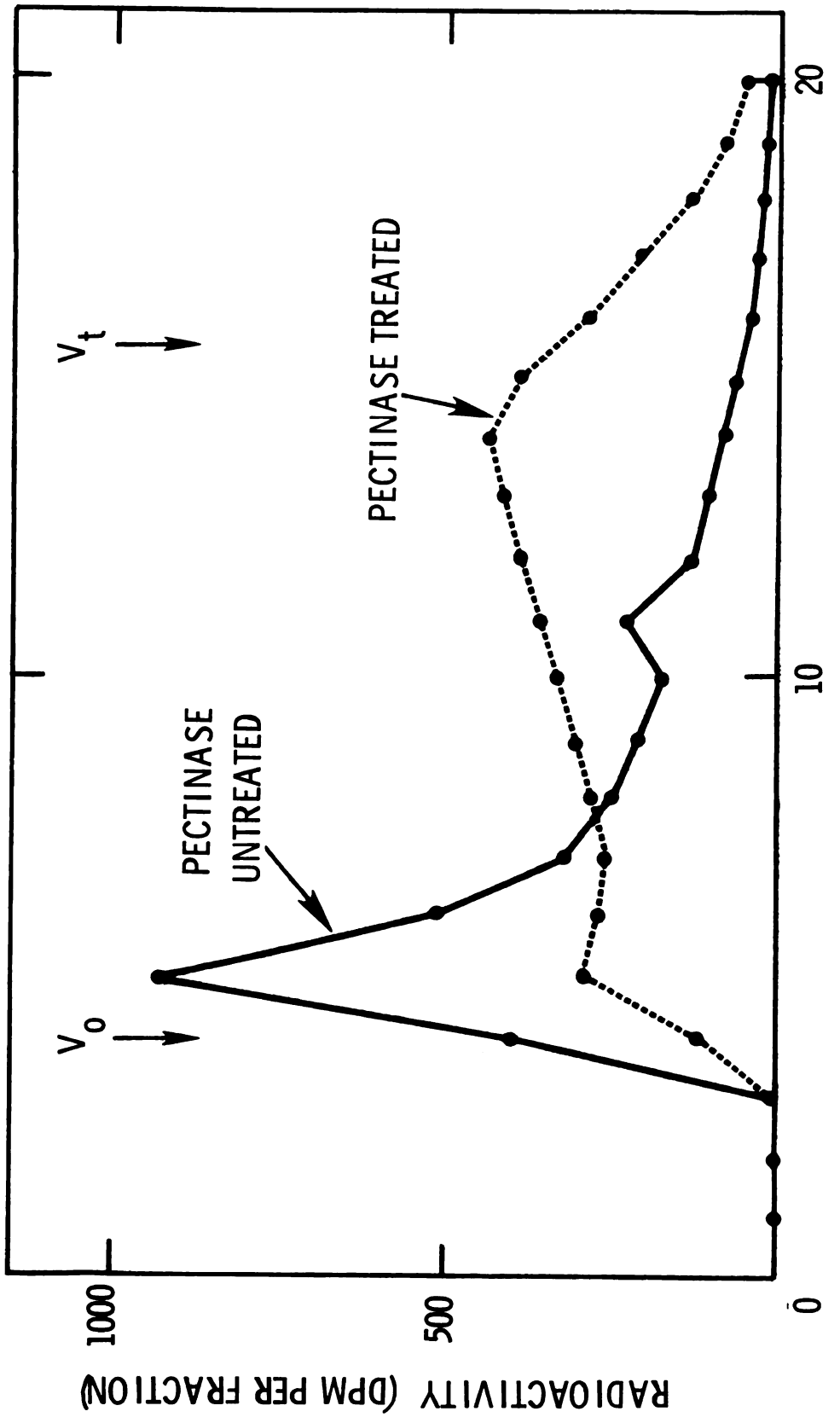

RADIOACTIVITY (DPM PER FRACTION)
1000
500
0
10
20
FRACTION NUMBER
V_o
V_t
PECTINASE UNTREATED
PECTINASE TREATED

Figure 20

Figure 21. Bio-Gel P-30 column chromatogram of D-[U-^{14}C]apiose solubilized product that was treated with pectinase. D-[U-^{14}C]Apiose solubilized product was prepared as described in the Experimental Procedures except that the reaction mixture contained 100 µl of particulate enzyme preparation and 50 µl of UDP[U-^{14}C]Api solution. The D-[U-^{14}C]apiose solubilized product was dialyzed in water for 14 hr with one water change after 4 hr. The dialysate (40,000 dpm, in 750 µl) was then treated with pectinase for 2 days as described in the Experimental Procedures. After pectinase treatment the D-[U-^{14}C]apiose solubilized product (220 µl) was chromatographed on a Bio-Gel P-30 column (0.8 cm internal diam x 47 cm) that was equilibrated and developed with 0.05 M ammonium formate buffer, pH 6.2. The column flow rate was 20 ml/hr and 1.0 ml fractions were collected. Aliquots of the column fractions were assayed for radioactivity in scintillation solution B. Recovery of radioactive material from the column was 84%. The indicated column fractions were then combined to form fractions 1, 2, and 3.

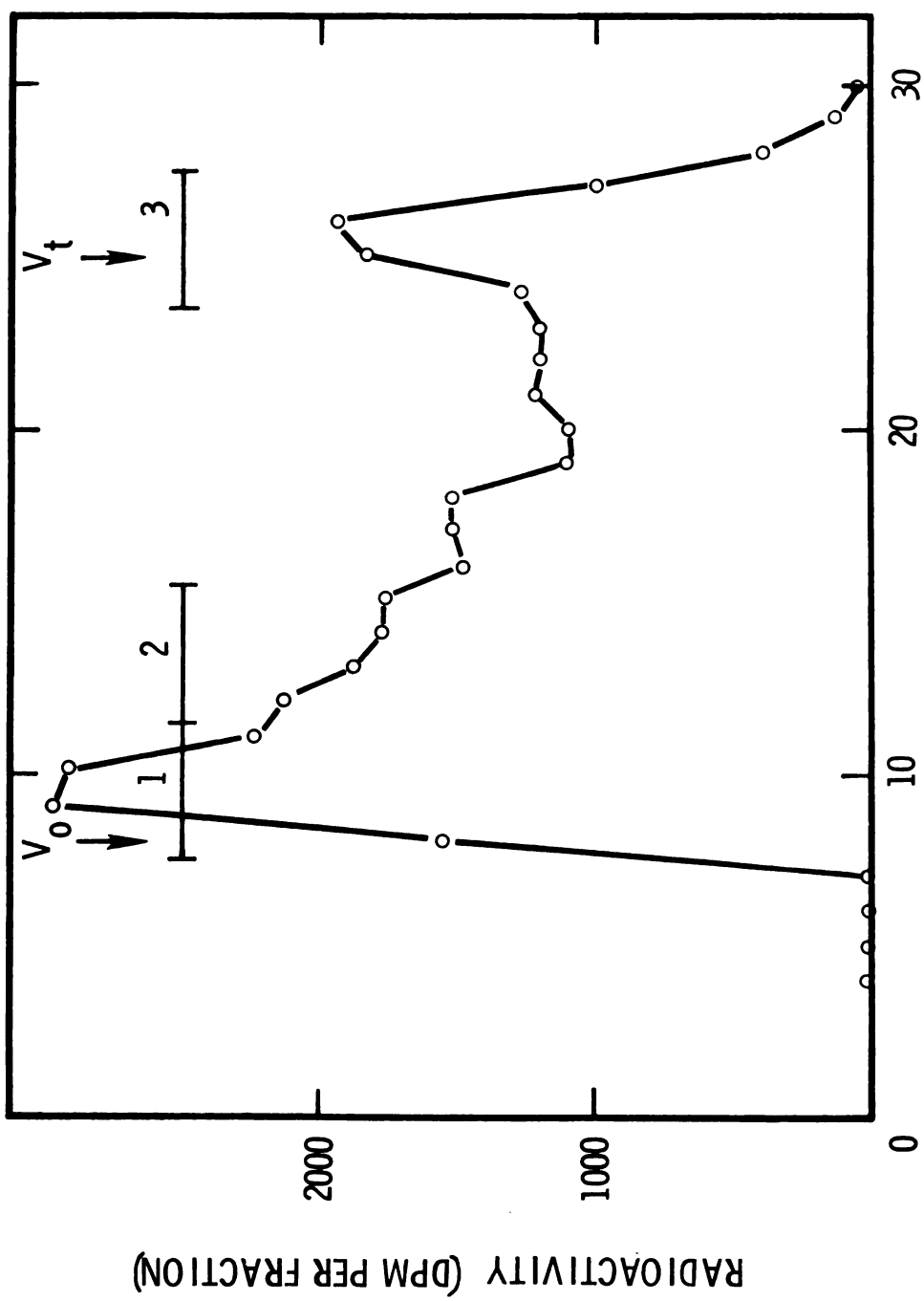

RADIOACTIVITY (DPM PER FRACTION)
2000
1000
0
10
20
30
FRACTION NUMBER
V_o
V_t
1
2
3

Figure 21

pH 1 as described in the Experimental Procedures and the hydrolysates were analyzed by paper chromatography with Solvent A. All of the radioactivity in the samples was present in D-[U-^{14}C]apiose and D-[U-^{14}C]xylose, however, the relative amounts of the two sugars varied. The amount of radioactivity present as D-[U-^{14}C]xylose in fractions 1, 2, and 3 was 13, 8, and 64%, respectively. In a second, similar experiment the amount of radioactivity present as D-[U-^{14}C]xylose in fractions 1, 2, and 3 was 15, 10, and 28%, respectively. These results show that the small fragments of D-[U-^{14}C]apiose solubilized product isolated after pectinase treatment were enriched in D-[U-^{14}C]xylose.

Hydrolysis of D-[U-^{14}C]Galacturonic Acid Solubilized Product

In order to investigate the pectinase susceptibility of D-[U-^{14}C]galacturonic acid solubilized product, a sample of this material was chromatographed on a Bio-Gel P-100 column and the radioactive material which eluted at V_o was isolated and concentrated. This material was divided into two portions; one of which was treated with pectinase while the other was untreated. The two samples were then chromatographed again on the Bio-Gel P-100 column (Figure 22). Recovery of radioactive sample from the column was 87% for treated material and 54% for untreated material. Most of the

Figure 22. Bio-Gel P-100 column chromatograms of D-[U-^{14}C]galacturonic acid solubilized product treated and untreated with fungal pectinase. D-[U-^{14}C]Galacturonic acid solubilized product was prepared as described in the Experimental Procedures. The solubilized product (8,700 dpm, in 0.5 ml) was chromatographed on a column of Bio-Gel P-100 as described in the legend of Figure 13 except that 2 ml fractions were collected. The material which eluted in elution volumes of 14 to 20 ml, corresponding to the V_o, were combined and dialyzed in H_2O for 2 hr at 4°C. After dialysis the material was concentrated to 0.8 ml at 22°C and divided into 2 equal 400 µl portions (1800 dpm). One portion (treated) had 160 µl of 0.5 M sodium acetate buffer, pH 4.5, containing pectinase at a concentration of 5 mg/ml added to it. To the other portion (untreated) was added 160 µl of 0.5 M sodium acetate buffer, pH 4.5. Both tubes were incubated at 37°C for 18 hr. After incubation the samples were chromatographed on Bio-Gel P-100 as described in the legend of Figure 13 and 1 ml fractions were collected. Values of radioactivity (%) represent the percent of total recovered radioactivity which was collected in each fraction.

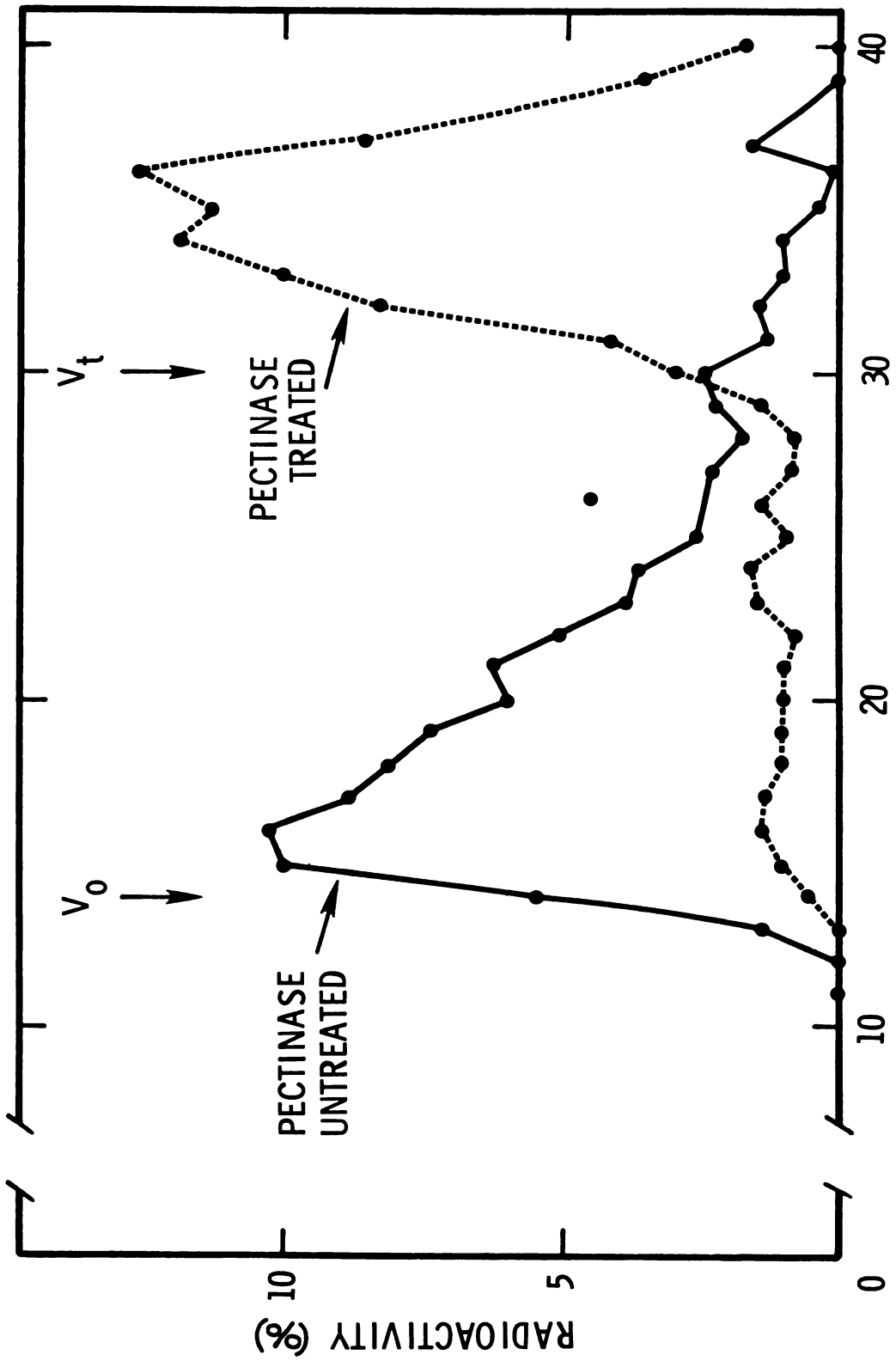

RADIOACTIVITY (%)
0
5
10
10
20
30
40
FRACTION NUMBER
V_o
V_t
PECTINASE UNTREATED
PECTINASE TREATED

Figure 22

untreated material eluted in the V_o of the column while the pectinase treated material eluted at the V_t of the column. These results indicate that D-[U-^{14}C]galacturonic acid solubilized product was also capable of being degraded by pectinase.

DISCUSSION

Characterization of Solubilized Products

Procedures have been developed for the characterization of radioactive polysaccharides synthesized with a particulate enzyme preparation. Radioactive products were synthesized from "UDP[U-^{14}C]Api," UDP-[U-^{14}C]GalUA, UDP[U-^{14}C]GlcUA, and UDP[U-^{14}C]Xyl in large enough yields that the products could be partially characterized (Table 1). However, not all of the radioactivity in the reaction mixtures was incorporated into product. I have not attempted to isolate or characterize the radioactive compounds in the reaction mixtures that were not incorporated into product. The inability of the particulate enzyme preparation to incorporate all of the radioactive sugar into product under these reaction conditions is presumably caused by rapid inactivation of enzymatic activity, metabolism of the sugar nucleotides by other enzymatic pathways, or an insufficient quantity of acceptor molecules for the transferase reactions or all or a combination of these. Glycosyl transferase activities in the particulate enzyme preparation from

L. minor are indeed unstable. Studies of the enzymes responsible for the synthesis of D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid products by Pan, Leinbach and Kindel have shown that the particulate enzyme preparation lost 50% of both activities when stored for 6 min at 25°C (unpublished results). Despite the incomplete incorporation of radioactive sugars into product the yields were sufficient to partially characterize the products.

I have not characterized the insoluble radioactive material remaining after ammonium oxalate extraction of the products. In the case of D-[U-^{14}C]-glucuronic acid and D-[U-^{14}C]xylose products the insoluble radioactive material may be hemicelluloses since D-glucuronic acid and D-xylose are important constituents of hemicellulose polysaccharides. It is also possible that the nonsolubilized materials have structures identical to the solubilized products but that ammonium oxalate extraction did not solubilize them for unknown reasons.

The presence of D-[U-^{14}C]xylose and D-[U-^{14}C]-galacturonic acid in the D-[U-^{14}C]glucuronic acid indicated that the particulate enzyme preparation contained UDPGlcUA carboxy-lyase and UDPGlcUA 4-epimerase activities. The absence of D-[U-^{14}C]apiose in the D-[U-^{14}C]-glucuronic acid solubilized product does not preclude

the existence of UDPGlcUA cyclase activity in the particulate enzyme preparation, however. The pH optima for UDPGlcUA cyclase activity is 7.8 in potasium phosphate buffer and the incorporation assays were performed at pH 6.1 (115).

The absence of incorporation of D-[U-^{14}C]glucuronic acid into solubilized product may explain why the amount of radioactivity in the D-[U-^{14}C]glucuronic acid product was less than for the other 3 products (Table 1) since before radioactivity could be incorporated into solubilized product D-[U-^{14}C]GlcUA in the reaction mixture had to be converted to UDP[U-^{14}C]Xyl and UDP U-^{14}C]GalUA.

I have not investigated whether the UDPGlcUA 4-epimerase and carboxy-lyase are membrane bound or in organelles or both. L. minor seems to be an excellent system for investigating Northcote's theory that the synthesis of polysaccharides is controlled in the golgi apparatus by control of the synthesis of the different sugar nucleotides (33). Plant cell wall synthesis has the potential of being an important tool in studying the development of cell surfaces because of the change in the types of polysaccharides synthesized when primary cell wall synthesis is replaced by secondary cell wall synthesis.

The identity of the radioactive sugars contained in the insoluble material remaining after ammonium oxalate extraction was not determined. Other radioactive sugars such as D-[U-^{14}C]glucuronic acid and L-[U-^{14}C]arabinose may have been contained in this material.

DEAE-Sephadex chromatography of the 4 solubilized products showed that they were all negatively charged since they were initially bound to the column (Figures 7, 8, and 10). This result was expected for the D-[U-^{14}C]galacturonic acid and D-[U-^{14}C]glucuronic acid solubilized products since they both contained uronic acid sugars. However, since the D-[U-^{14}C]apiose and D-[U-^{14}C]xylose solubilized products were also bound by the DEAE-Sephadex column this suggests that they also contained uronic acid residues.

Presumably the solubilized products were fractionated on the DEAE-Sephadex column according to their ratio of acidic to neutral sugars. The higher this ratio the higher the concentration of NaCl necessary to disassociate the solubilized product from the ion-exchange column. The column chromatograms indicate that the D-[U-^{14}C]apiose and D-[U-^{14}C]xylose solubilized products contain less of the highly acidic material than does the solubilized products synthesized from the uronic acids (Figures 7, 8, and 10). Hart and

Kindel have reported that the polygalacturonic acid backbone of apiogalacturonan, obtained by mild acid hydrolysis of apiogalacturonan, was recovered after DEAE-Sephadex chromatography in a fraction which eluted from the column in 0.3 M NaCl (102). When the D-[U-^{14}C]-galacturonic acid solubilized product was chromatographed on the DEAE-Sephadex column most of the radioactive material was recovered in fractions which eluted with NaCl concentrations of 0.25 M or less (Figure 8). This result suggests that the D-[U-^{14}C]galacturonic acid solubilized product contains some neutral sugars.

The fractions obtained from the DEAE-Sephadex chromatography of D-[U-^{14}C]apiose solubilized product were not homogeneous as determined by rechromatography on DEAE-Sephadex (Table 8). The lack of homogeneity may have been caused by "degradation" as shown in Figure 16. Rechromatography did not, however, change the D-[U-^{14}C]apiose content of the fractions recovered from fractions A, B, and D. The significance of the change in D-[U-^{14}C]apiose content of the fractions recovered from the rechromatography of fraction C is unknown.

The polysaccharide nature of the solubilized products is evidenced by their high molecular weight as shown by their impermeability to dialysis membranes and the results obtained by gel chromatography of the

D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products. From the elution of the D-[U-^{14}C]-apiose and D-[U-^{14}C]galacturonic acid solubilized products through a column of Bio-Gel P-300 the molecules had a disperse molecular weight range from at least 125,000 to 5,000 (Figure 11). The D-[U-^{14}C]galacturonic acid solubilized product contained lower molecular weight molecules than were contained in the D-[U-^{14}C]-apiose solubilized product (Figure 13). These in vitro synthesized pectic materials are especially suited for studying molecular size differences by gel chromatography because they are solubilized by relatively gentle techniques. However, the ammonium oxalate extraction procedure may result in some breakdown (116). Villemez has described a procedure for the gel chromatography of the acetate derivatives of in vitro synthesized [^{14}C]glucomannans (117). He resolved the [^{14}C]glucomannan into two fractions; one with a minimum molecular weight of 200,000 and the other with a minimum molecular weight of 60,000.

Identification of D-[U-^{14}C]Apiose Solubilized Product as an Apiogalacturonan

Although direct evidence for the identification of the D-[U-^{14}C]apiose product as an apiogalacturonan was not obtained, there is an abundance of indirect evidence which confirms this conclusion.

D-[U-^{14}C]Apiose solubilized product has many of the same properties as authentic apiogalacturonan isolated from intact plants.

Like authentic apiogalacturonan, the D-[U-^{14}C]-apiose product could be extracted by ammonium oxalate treatment (Table 1). I have shown that the solubilization is dependent on the presence of ammonium oxalate which is an indication of the pectic nature of the D-[U-^{14}C]apiose product.

The release of [U-^{14}C]apiobiose from D-[U-^{14}C]-apiose solubilized product after hydrolysis at pH 4 (Figure 4) also shows that the solubilized product has a structure similar to apiogalacturonan (104). Hart and Kindel postulated that the cleavage of the glycosidic bond between the apiobiose side chains and the polygalacturonic acid backbone is the result of the transannular participation of the free carboxylic group of the D-galacturonic acid residue (104). Similar release of [U-^{14}C]apiobiose from the solubilized products suggests a similar structure of a polygalacturonic acid backbone with apiobiose side chains.

The D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products probably contain a backbone of α-1,4-galacturonan since they were both hydrolyzed by treatment with pectinase (Figures 20 and 22). It is not surprising that the D-[U-^{14}C]apiose solubilized

product was not hydrolyzed completely since side chains of D-apiose are known to confer pectinase resistance on the polygalacturonic acid backbone of apiogalacturonans (102, 120). The small fragments of D-[U-^{14}C]apiose solubilized product resulting from treatment with pectinase were found to be enriched in D-[U-^{14}C]xylose content (Figure 21). This result may be explained by assuming that the D-[U-^{14}C]xylose residues are bound as side chains to the polygalacturonic acid backbone but do not confer resistance to pectinase hydrolysis. Therefore treatment with pectinase will result in the cleavage of the backbone near the point of D-[U-^{14}C]-xylose attachment thus releasing small fragments of polysaccharide containing D-[U-^{14}C]xylose. A similar release of small oligosaccharides containing D-xylose and D-galacturonic acid was reported when a pectic polysaccharide isolated from Zosteraceae and containing D-galacturonic acid, D-xylose, D-apiose, and other neutral sugars was hydrolyzed with pectinase (120).

As was the case with authentic apiogalacturonans from intact plants, D-[U-^{14}C]apiose solubilized product was fractionated by chromatography on a DEAE-Sephadex column (Figure 7).

Additional evidence for the identification of D-[U-^{14}C]apiose solubilized product as an apiogalacturonan is seen by the effect of adding UDPGalUA to the reaction

mixture used to synthesize D-[U-^{14}C]apiose product. Addition of UDPGalUA to the "UDP-[U-^{14}C]Api" reaction mixture resulted in an increase in the incorporation of radioactive sugars into the product (Table 2), an increase in the ratio of D-[U-^{14}C]apiose to D-[U-^{14}C]-xylose (Table 4) an increase in susceptibility to hydrolysis at pH 4 (Table 5), a change in chromatography properties when chromatographed on DEAE-Sephadex (Figure 9), and an increase in the size of the molecules in the small molecular weight fraction (Figure 17). These changes indicate that addition of UDPGalUA to the reaction mixture resulted in the incorporation of D-[U-^{14}C]apiose and D-galacturonic acid into the same molecules of polysaccharide.

Additional galacturonans would be synthesized when UDPGalUA is present in the "UDP-[U-^{14}C]Api" reaction mixture. The increase in incorporation of radioactivity into D-[U-^{14}C]apiose product synthesized in the presence of UDPGalUA (Table 2) could be explained by the presence of additional galacturonan acceptors for the incorporation of radioactive side chains.

The increase in the ratio of D-[U-^{14}C]apiose to D-[U-^{14}C]xylose seen in the D-[U-^{14}C]apiose solubilized product could have resulted from the dilution of UDP-[U-^{14}C]Xyl by UDPXyl synthesized in the reaction mixture from UDPGalUA. This conversion of UDPGalUA to

UDPXyl is possible because of the presence of UDPGlcUA 4-epimerase and UDPGlcUA carboxy-lyase activities in the particulate enzyme preparation. However, the amount of conversion of UDPGalUA to UDPXyl cannot be large since the D-[U-^{14}C]apiose product synthesized in the presence of UDPGalUA did not exhibit the lower amount of solubilization with ammonium oxalate treatment that was seen when D-[U-^{14}C]apiose product was synthesized in the presence of UDPXyl. Also, there was little D-[U-^{14}C]-xylose found in D-[U-^{14}C]galacturonic acid solubilized product. Therefore, the decrease in content of D-[U-^{14}C]xylose with addition of UDPGalUA to the reaction mixture may be caused instead by UDPGalUA effecting the control mechanisms for D-apiosyl and D-xylosyl transferase activities. Pan and Kindel (unpublished results) have found that addition of UDPGalUA to the reaction mixture used to synthesize D-[U-^{14}C]apiose product resulted in an increased rate of D-[U-^{14}C]apiose incorporation and a decreased rate of D-[U-^{14}C]xylose incorporation.

The increase in the release of D-[U-^{14}C]apiose and [U-^{14}C]apiobiose after hydrolysis at pH 4 from D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA (Table 5) may be the result of increased incorporation of D-galacturonic acid into the D-[U-^{14}C]apiose containing polysaccharide thus

resulting in a more acidic polysaccharide which can better participate in the hydrolysis.

It has been reasoned that if D-apiose and D-galacturonic acid are incorporated into the same polysaccharide then D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA should have a higher ratio of D-galacturonic acid to D-[U-^{14}C]apiose then would D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA. The polysaccharide with the higher ratio would be more acidic than the other. As determined by DEAE-Sephadex chromatography, D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA was more acidic than the product synthesized without UDPGalUA (Figures 7 and 9). The more acidic polysaccharides elute from the DEAE-Sephadex column in fractions D and E. Twenty-three percent of the recovered radioactivity was contained in these 2 fractions combined when D-[U-^{14}C]apiose solubilized product synthesized in the absence of UDPGalUA was chromatographed. Sixty percent of the recovered radioactivity was contained in fractions D and E combined when D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA was chromatographed. The increase in the amount of highly acidic polysaccharide fraction was dependent on the amount of UDPGalUA added to the reaction mixture (Table 6).

When I first saw the change that addition of UDPGalUA to the reaction mixture caused in the chromatography of D-[U-^{14}C]apiose solubilized product on the DEAE-Sephadex column, I thought that a galacturonan may have been synthesized from UDPGalUA which then complexed with the D-[U-^{14}C]apiose solubilized product. The change in the elution profile would have then been caused by the galacturonan dragging the D-[U-^{14}C]apiose product with it rather than the D-galacturonic acid being incorporated into the D-[U-^{14}C]apiose solubilized product. This theory is incorrect, however, since mixing galacturonan synthesized from UDPGalUA with D-[U-^{14}C]-apiose solubilized product did not change the elution profile of D-[U-^{14}C]apiose solubilized product on the DEAE-Sephadex column.

Characterization and comparison of the DEAE-Sephadex fractions obtained from D-[U-^{14}C]apiose solubilized product synthesized in the absence and presence of UDPGalUA showed that UDPGalUA affected the D-[U-^{14}C]-apiose/D-[U-^{14}C]xylose ratio and susceptibility to hydrolysis at pH 4 of corresponding fractions (Table 7). These results show that the higher the D-[U-^{14}C]xylose content of a fraction the lower the acidity was as determined by elution order from the DEAE-Sephadex column. Fraction D eluted from the DEAE-Sephadex column in a similar position in the gradient as the

22° sodium chloride soluble apiogalacturonan IIa fraction characterized by Hart and Kindel (102). When hydrolyzed at pH 4 this authentic apiogalacturonan totally released its D-apiose (104). In the case of fraction D obtained from D-[U-^{14}C]apiose solubilized product synthesized in the presence of UDPGalUA 81% of the incorporated D-[U-^{14}C]-apiose was released after hydrolysis at pH 4 (Table 7). This shows that fraction D has similar hydrolysis properties at pH 4 to the authentic apiogalacturonan fraction.

Although Hart and Kindel did not find D-xylose in the apiogalacturonans that they isolated from the cell wall of L. minor (102), I was able to demonstrate by hydrolysis at pH 1 the existance of D-[U-^{14}C]xylose in the [^{14}C]60° sodium chloride soluble apiogalacturonan fraction isolated by them. D-Xylose seems to be a normal constituent of the apiogalacturonan fractions isolated at higher temperatures as evidenced by these results and those of Beck (103) albeit D-xylose is not contained in the fractions isolated by ammonium oxalate extraction at 22°C.

Beck was unable to establish whether D-apiose and D-xylose were contained in his 60°C extract as separate apiogalacturonan and xylogalacturonan polysaccharides or as an apioxylogalacturonan (103). He did suggest that both pentoses were contained in the

same polysaccharide because the D-xylose and D-apiose containing polysaccharides were not separated from one another by DEAE-Cellulose chromatography (103). The characterization experiments performed with D-[U-^{14}C]-apiose solubilized product also did not determine whether D-[U-^{14}C]xylose and D-[U-^{14}C]apiose were incorporated in vitro into the same polysaccharide. However, I was unable to completely separate incorporated D-[U-^{14}C]apiose from D-[U-^{14}C]xylose by DEAE-Sephadex chromatography (Tables 7 and 8) and this also suggests that both pentoses were incorporated into the same molecules. A possible experimental approach to answer this question would be to prepare an affinity column for D-apiose using an antibody specific for this sugar. If the D-[U-^{14}C]apiose and D-[U-^{14}C]xylose incorporated in the D-[U-^{14}C]apiose solubilized product were contained in different polysaccharide molecules than the D-apiose affinity column would bind the D-[U-^{14}C]apiose containing material but not the D-[U-^{14}C]xylose containing material.

Evidence that D-[U-^{14}C]xylose was incorporated into the apiogalacturonan molecules, however, is seen from the effect that the presence of UDPXyl in the reaction mixtures used to synthesize D-[U-^{14}C]apiose product has on the properties of the resultant product. The changes seen when UDPGlcUA was added are also

probably due in part by incorporation of D-xylose because of the presence of UDPGlcUA carboxy-lyase activity in the particulate enzyme preparation. There is less conversion of UDPGlcUA to UDPGalUA in the reaction mixture than there is conversion to UDPXyl as shown by the larger quantity of D-[U-^{14}C]xylose as compared to D-[U-^{14}C]galacturonic acid contained in the D-[U-^{14}C]glucuronic acid solubilized product.

Addition of UDPGlcUA and UDPXyl to the reaction mixtures used to synthesize D-[U-^{14}C]apiose product resulted in a decreased solubilization of radioactive material by ammonium oxalate extraction (Table 2). Addition of these nonradioactive sugar nucleotides may have promoted the synthesis of a D-apiose containing polysaccharide which was not a pectin. Approximately 80% of the D-apiose in the cell wall of L. minor was not extracted by ammonium oxalate and the structure of this D-apiose-containing material is unknown (102). The large amount of D-[U-^{14}C]apiose product synthesized in the presence of UDPXyl which was not solubilized by ammonium oxalate treatment may indicate that the cell wall of L. minor contains an apioxylan. However, the ammonium oxalate solubilized and nonsolubilized materials may also have the same structure except that the nonsolubilized material has more D-xylose in the side chains. The D-xylose side chains for unknown

reasons may cause the polysaccharide to be less susceptible to solubilization by ammonium oxalate. Addition of UDPXyl to the reaction mixture would increase the number of D-xylose side chains in the product and thus decrease the amount of material solubilized. This last possibility agrees with the observation that D-xylose containing polysaccharides were extracted from the cell wall of L. minor only at higher temperatures (103).

When hydrolyzed at pH 4 D-[U-^{14}C]apiose solubilized product, synthesized in the presence of UDPXyl, released a larger amount of D-[U-^{14}C]apiose as D-[U-^{14}C]apiose than did D-[U-^{14}C]apiose solubilized product synthesized in the absence of nonradioactive sugar nucleotide (Table 5). There was no increase in the amount of [U-^{14}C]apiobiose released. This suggests that D-xylose was incorporated into the polysaccharide and this affected the hydrolysis at pH 4 in a presently unknown manner.

The experimental results described above all show that an apiogalacturonan, containing some D-xylose side chains, can be synthesized with the particulate enzyme preparation from L. minor. However, the results obtained from hydrolyzing the D-[U-^{14}C]apiose solubilized product at pH 4 are not exactly the same as was obtained by Hart and Kindel (104) with authentic

apiogalacturonans. When they hydrolyzed an apiogalacturonan fraction, which had been purified by DEAE-Sephadex chromatography, at pH 4 all of D-apiose was released from the polysaccharide and recovered as apiobiose (104). On the other hand, I was unable to obtain total release of D-[U-^{14}C]apiose from any of the fractions of D-[U-^{14}C]apiose solubilized product by hydrolysis at pH 4 (Tables 5 and 7). I also found that the ratio of [U-^{14}C]apiobiose to D-[U-^{14}C]apiose released from the fractions of D-[U-^{14}C]apiose solubilized product was lower than was reported by Hart and Kindel (104). However, hydrolysis of the [^{14}C]-22°C and 60°C sodium chloride soluble apiogalacturonan fractions of Hart and Kindel at pH 4 also resulted in incomplete release of D-[^{14}C]apiose from the polysaccharides (Hart and Kindel, unpublished results). In the case of the [^{14}C]60°C sodium chloride soluble apiogalacturonan fraction only 41% of the D-[^{14}C]apiose was released from the polysaccharide after a 3 hr hydrolysis period and the ratio of [^{14}C]apiobiose to D-[^{14}C]apiose was 2.7 (Hart and Kindel, unpublished results). This may indicate that the completeness of hydrolysis at pH 4 may depend on small differences in structure or preparation of apiogalacturonan. Some of the D-[U-^{14}C]apiose may be contained in the solubilized product as monosaccharide side chains.

Possible Mechanisms of Synthesis

I do not know whether apiogalacturonans were synthesized de novo by the particulate enzyme preparation from L. minor. However, the amount of radioactivity contained in the largest molecules of D-[U-^{14}C]-galacturonic acid increased as the reaction mixture was incubated for longer periods of time (Figure 18). One interpretation of this is that more than one D-[U-^{14}C]-galacturonic acid residue was incorporated into each molecule of D-[U-^{14}C]galacturonic acid solubilized product. In order to determine whether de novo synthesis has occurred additional experiments will have to be performed to determine whether the reducing ends and interior residues of the D-[U-^{14}C]galacturonic acid solubilized product were synthesized in vitro.

There is a second interpretation for the results shown in Figure 18. It is still possible to incorporate a single D-[U-^{14}C]galacturonic acid residue into preformed chains and obtain an increase in the relative amount of radioactivity into the large molecular weight fraction with increasing synthesis times if the rate of incorporation into large chains is faster than into small chains.

There are at least 2 possible mechanisms for synthesis of apiogalacturonans. The first mechanism is similar to one seen in bacterial systems where an

oligosaccharide-repeating unit containing D-apiose and D-galacturonic acid is first synthesized as part of a lipid intermediate and then transferred to the growing polysaccharide (78). This mechanism allows for a highly uniform structure. A second mechanism of synthesis is that the polygalacturonic acid backbone be synthesized first after which the pentose sugar side chains are incorporated. This mechanism is similar to the one found for the methylation of pectins (74, 75).

Based on the gel chromatography experiments some proposals about the mechanism of apiogalacturonan synthesis can be made. Since D-[U-^{14}C]apiose is incorporated in the absence of exogenous UDPGalUA and the D-[U-^{14}C]galacturonic acid solubilized product is smaller than the D-[U-^{14}C]apiose solubilized product (Table 13), it is doubtful that synthesis occurs by means of an apiogalacturonasyl lipid intermediate. Therefore synthesis most likely occurs by the formation of the polygalacturonic acid backbone followed by incorporation of the D-apiose side chains. Such a synthesis mechanism can be used to explain why the percent of D-[U-^{14}C]apiose solubilized product of high molecular weight decreased when the incubation times were increased from 0.5 to 15 min (Figure 19) even though in the case of D-[U-^{14}C]galacturonic acid

solubilized product the percent of high molecular weight material increased as the length of incubation increased (Fraction 18). At the beginning of the in vitro incorporation of D-[U-^{14}C]apiose the largest molecules of polygalacturonic acid are probably used preferentially as acceptor molecules for D-[U-^{14}C]apiose transfer. As the incorporation reaction proceeds then the smaller molecules of polygalacturonic acid would act as acceptors and thus increase the fraction of lower molecular weight D-[U-^{14}C]apiose solubilized product.

An alternate explanation for the results shown in Figure 19 is that there is a degradative enzyme in the particulate enzyme preparation which hydrolyzes the backbone of the galacturonan acceptor. Therefore, initially the D-[U-^{14}C]apiose side chains were incorporated into large molecular weight polysaccharides, but as the synthesis reaction proceeded, the apiogalacturonans and the galacturonan acceptors would be cleaved into smaller pieces by the degradative enzyme. This theory is unlikely, however, since the D-[U-^{14}C]galacturonan did not show evidence of being cleared during synthesis (Figure 18).

As discussed previously the D-[U-^{14}C]galacturonic acid solubilized product probably contains neutral sugar side chains. Some of these neutral sugar side chains may be the result of the conversion

of UDP[U-^{14}C]GalUA to UDP[U-^{14}C]Xyl. A second possibility is that the particulate enzyme preparation contains acceptor molecules for D-galacturonic acid transfer which contain neutral sugars. This suggests that apiogalacturonan synthesis occurs by alternating periods of backbone elongation with periods of incorporation of the side chains. I do not know if the particulate enzyme preparation contains nonradioactive sugar nucleotides when isolated. If it did this would also explain why the D-[U-^{14}C]galacturonic acid solubilized product contains neutral sugars.

Since both residues in [U-^{14}C]apiobiose were incorporated <u>in vitro</u> this suggests that incorporation of the two D-[U-^{14}C]apiose residues of the disaccharide into the apiogalacturonan occurred simultaneously. Otherwise some of the [U-^{14}C]apiobiose molecules would have contained an <u>in vivo</u> synthesized D-apiose moiety at the reducing end of the disaccharide. A reaction mechanism involving incorporation of intact apiobiose residues through a lipid intermediate could account for these results. Pan and Kindel were unable to isolate a D-[U-^{14}C]apiose containing lipid intermediate from this system, however (unpublished results). A second mechanism can be imagined where the polygalacturonic acid acceptor was bound to an enzyme complex containing 2 apiosyl transferase enzymes. The first transferase

would transfer D-apiose to a D-galacturonic acid residue in the backbone. The second transferase would immediately transfer another D-apiose to the first apiosyl residue.

Degradation of D-[U-^{14}C]Apiose and D-[U-^{14}C]Galacturonic Acid Solubilized Products

The mechanism by which D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products were "degraded" by dialysis in water or by chromatography on DEAE-Sephadex is unknown (Figures 14 and 16). Any possible mechanism must be able to account for the absence of "degradation" when the solubilized products were dialyzed in sodium phosphate buffer (Figure 14). The mechanism would also have to explain why dialysis in water does not "degrade" fractions of solubilized products that have been chromatographed on a column of Bio-Gel equilibrated with sodium phosphate buffer.

The "degradative" process may indicate that the solubilized product consists of aggregates of non-covalently bound polysaccharide molecules which are disassociated by dialysis in water or by DEAE-Sephadex chromatography. However, this hypothesis seems questionable since the "degradation" was not reversed by dialysis in sodium phosphate buffer. Also this hypothesis does not explain why solubilized product

recovered after gel chromatography was not degraded by water dialysis (Figure 15).

If the solubilized products are in solution as single molecules then "degradation" may result from the enzymatic cleavage of glycosidic bonds. However, the inactivation of such enzymes by sodium phosphate buffer is hard to understand. Another possible explanation for the "degradation" mechanism is that during dialysis in water or chromatography on DEAE-Sephadex the larger molecules of solubilized product were selectively lost. The decrease in the amount of radioactive material contained in the V_o of the gel chromatography column after dialysis or DEAE-Sephadex chromatography would be caused by this selective loss of large molecular weight material. The amount of radioactive material not recovered after dialysis or DEAE-Sephadex chromatography is large enough to support this mechanism although it too is highly speculative.

An understanding of this "degradation" process may be important in the study of cell wall structure. This is especially true in the light of recent models of cell wall structure based on extensive cross linking of polysaccharides and proteins (21).

I do not know whether the D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid solubilized products used in the gel-chromatography experiments were degraded

during the solubilization procedure with ammonium oxalate. Such degradation of pectic polysaccharides by ammonium oxalate treatment has been reported (116). Initial experiments by Leinbach and Kindel suggest that extraction of D-[U-^{14}C]galacturonic acid product with sodium hexametaphosphate rather than ammonium oxalate resulted in a greater percentage of the radioactivity being isolated in the V_o when chromatographed on a Bio-Gel P-300 column (Leinbach and Kindel, unpublished results). Apiogalacturonans are not methyl esterifred (102) so that degradation of the solubilized products by β-elimination is not probable.

It is possible that as polysaccharides were synthesized in the particulate enzyme preparation they were also degraded enzymatically. This possibility was not investigated and finding proper control experiments for such an investigation is not practical. The obvious experiment of isolating solubilized product, determining its molecular weight distribution by chromatographing a portion on a gel chromatography column, incubating the rest of the solubilized product with particulate enzyme preparation, and then rechromatographing on the gel chromatography column to see if there is any change in the molecular weight distribution is not valid. This is true because the polysaccharide may be synthesized within a membrane-bound organelle (75).

Addition of isolated solubilized product to the particulate enzyme preparation will not result in the polysaccharide being in the same environment as it was synthesized in since it will not be able to penetrate the lipid membrane. This is important because the organelle may protect the newly synthesized polysaccharide from degradative enzymes in the cytoplasm or in other organelles. Addition of isolated solubilized product to the particulate enzyme preparation may result in the exposure of the product to degradative enzymes that it would normally be protected from before extraction from the reaction mixture or *vice versa*.

Summary

I have shown that the particulate enzyme preparation from *L. minor* is capable of synthesizing D-apiose and D-galacturonic acid containing polysaccharides with structures similar to the apiogalacturonan components of the cell wall. The *in vitro* synthesized apiogalacturonans seem to contain side chains of D-xylose also. Studies with *L. minor* on the cell-free incorporation of D-[U-^{14}C]apiose and D-[U-^{14}C]galacturonic acid, from their respective sugar nucleotides, into polysaccharides can be done with the knowledge that an actual component of the plant cell wall is synthesized. In addition I have shown that the synthesis of cell wall polysaccharides in the particulate enzyme preparation from *L. minor* is

an integrated system where the products formed are influenced by the species and relative amounts of sugar nucleotides present in the reaction mixture.

The results of several experiments have allowed for the proposal of a tentative mechanism of apiogalacturonan synthesis where the backbone of polygalacturonic acid is synthesized first from UDPGalUA after which the D-apiose containing side chains are added.

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