CHARACTERIZATION OF A PARTICULATE UDP-GALACTURONATE: ACCEPTOR D-GALACTURONOSYLTRANSFERASE FROM LEMNA MINOR AND STUDIES ON THE PHYSICAL PROPERTIES OF THE PRODUCT

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ABSTRACT

CHARACTERIZATION OF A PARTICULATE UDP-GALACTURONATE:ACCEPTOR D-GALACTURONOSYLTRANSFERASE FROM *LEMNA MINOR* AND STUDIES ON THE PHYSICAL PROPERTIES OF THE PRODUCT

By

Edwin DeTurk Leinbach

Apiogalacturonans are pectic polysaccharides which constitute about 14% of the cell wall of *Lemna minor*. As a part of the study of their biosynthesis, a particulate UDP-galacturonate:acceptor D-galacturonosyltransferase, capable of incorporating D-galacturonic acid from UDP-galacturonic acid into polysaccharide, was isolated from *L. minor*.

The D-galacturonosyltransferase activity was associated with material sedimenting between 480 and 34,800*g*. Incorporation of D-[U-^{14}C]galacturonic acid into polysaccharide was constant with time for 1.5 to 2.0 min at 25°C. D-Galacturonosyltransferase activity was directly proportional to the amount of the particulate transferase preparation used, and activity was optimal in citric acid-sodium phosphate buffer at pH 6.0-6.2. The particulate D-galacturonosyltransferase had an apparent K_m for UDP-galacturonic acid of 8 μM. The presence of 10 mM $MnCl_2$, 0.4 M sucrose, and 1% bovine serum albumin in the particulate D-galacturonosyltransferase

preparation was required for both optimum transferase activity and for maximum stability.

The D-galacturonosyltransferase was stable for 30 min at 0°C. The transferase was stable for 2.0 to 2.5 min at 25°C, but only 10% of the initial activity remained after 10 min. In some experiments, greater than 50% of the initial activity could be retained for at least 60 h by storage of the transferase preparation at -20°C. None of the attempts to stabilize the D-galacturonosyltransferase activity by the addition of various stabilizing agents were successful.

D-Galacturonosyltransferase activity was slightly inhibited by the addition of 3 μM UDP-apiose. In contrast, the D-apiosyltransferase of *L. minor* is stimulated by the addition of UDP-galacturonic acid.

In an attempt to solubilize the particulate D-galacturonosyltransferase activity, the effects of various detergents, both ionic and non-ionic, were tested. All but Tween-20 caused almost complete loss of transferase activity at a concentration of 1.0%. Triton X-100, Tween-20, sodium cholate, and Emulgen-911 all failed to cause more than 20% solubilization with an accompanying 50% loss of total transferase activity. These results suggest the importance of an intact membrane structure for D-galacturonosyltransferase activity.

About 80% of the D-galacturonosyltransferase product was solubilized by extraction with 1% ammonium oxalate or 2% sodium hexametaphosphate at 50°C. However, upon chromatography of the two products on Bio-Gel P-300, the 2% sodium hexametaphosphate-solubilized product appeared to be considerably larger than the 1% ammonium

oxalate-solubilized product, with 30% of the former product exceeding an apparent molecular weight of 1×10^6.

The apparent size of the 1% ammonium oxalate-solubilized product decreased on standing or upon dialysis in water or 1% ammonium oxalate. The apparent size of the 2% sodium hexametaphosphate-solubilized product was unaffected by these treatments. The apparent size of the 1% ammonium oxalate-solubilized product was also increased by dialysis in 3% sodium hexametaphosphate and in 3% ammonium oxalate. The apparent size of both solubilized products was increased by elution with or by dialysis in 1.0 M NaCl.

These results suggest that the D-galacturonosyltransferase products solubilized with sodium hexametaphosphate or with 1% ammonium oxalate probably do not represent the true size of the polysaccharide as it is synthesized in the cell-free system. The apparent size of the solubilized product is sensitive to the ionic strength of the medium in which the product is placed, with aggregation occurring as the ionic strength is increased. In addition, various ions appear to differ in their ability to cause aggregation. Thus the 2% sodium hexametaphosphate-solubilized product is likely to represent an extensively and tightly aggregated form of the D-galacturonosyl-transferase product, whereas the 1% ammonium oxalate-solubilized product represents a less-extensively and less tightly aggregated form. Whether or not 1% ammonium oxalate also causes partial degradation of the D-galacturonosyltransferase product is still in question. The decrease in size of the 1% ammonium oxalate-solubilized product on standing or upon dialysis in 1% ammonium oxalate could be interpreted as either degradation or disaggregation.

The amount of the D-galacturonosyltransferase product solubilized decreased with decreasing concentrations of both ammonium oxalate and sodium hexametaphosphate, although the latter salt is a better solubilizing agent than the former at low concentrations. Also, the apparent size of the sodium hexametaphosphate-solubilized product decreased as the total amount of product solubilized decreased. Whether this size decrease represents a decreased aggregation at lower sodium hexametaphosphate concentrations or whether it represents solubilization of a different species of polysaccharide is unclear as yet. Preliminary results indicated that this product was not so sensitive to aggregation as was the 2% sodium hexametaphosphate-solubilized product.

The results presented demonstrate the ability of a cell-free system from *L. minor* to incorporate D-[U-^{14}C]galacturonic acid from UDP-galacturonic acid into a polysaccharide which behaves like a pectic substance. The techniques developed here can be applied to further study of the pectic polysaccharides of the *L. minor* cell wall.

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Edwin DeTurk Leinbach

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The road runs ever on and on,
   Down from the door where it began.
Now far away the road has run,
   And I must follow if I can;
Pursuing it with eager feet
   Until it joins some wider way
Where many paths and errands meet.
   And whither then, I cannot say.

from *The Hobbit*
by J. R. R. Tolkien

To my parents, John and Dorothy, who first set me on that road, and to Susan, my wife and colleague, who keeps me pursuing that road when my feet are far from eager.

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VITA

Edwin DeTurk Leinbach was born in Reading, Pennsylvania, on September 24, 1948, the only child of John A. and Dorothy D. Leinbach. He grew up in the suburban Reading area and graduated from Exeter Township High School in June, 1966, as class valedictorian.

In September of that same year he entered Ursinus College in Collegeville, Pennsylvania, where, under the guidance of several excellent and dedicated professors, he acquired a love for not only the science of chemistry and biology, but for their history and philosophy as well. His four years at Ursinus culminated in his receipt of the Bachelor of Science in Chemistry in June, 1970.

In September, 1970, Ed began his graduate studies in the Department of Biochemistry at Michigan State University, and in April of 1971 he entered the laboratory of Dr. Paul Kindel. In September of that year, he met Susan Singley, a new graduate student in that department, and they were married in June, 1972.

After having received his Ph.D. degree in September, 1975, Ed is now entering a year of postdoctoral work as an NIH fellow with Dr. Robert Barker while Sue finishes her Ph.D. studies under Dr. John Boezi.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetate, disodium salt
GDP	guanosine 5'-diphosphate
K_m	the Michaelis constant
$\underline{P}$	statistical probability
$\overline{P}_w$	weight-average degree of polymerization
SD	standard deviation
dTDP	thymidine 5'-diphosphate
Tris	tris(hydroxymethyl)aminomethane
U	uniformly labeled with radioactivity
UDP	uridine 5'-diphosphate
UDPGalA	uridine 5'-(α-D-galactopyranosyluronic acid pyrophosphate)
UTP	uridine 5'-triphosphate
V_o	void volume
V_t	total volume or column volume

STATEMENT OF THE PROBLEM

Despite the theoretical importance of the pectic polysaccharides in determining the physical properties of primary plant cell walls, little is known about their biosynthesis. No cell-free system has been described in which the incorporation of both the neutral and the acidic sugars of pectins has been studied.

The cell wall of *Lemna minor* contains large amounts of pectic heteropolysaccharides termed apiogalacturonans, the structure and physical properties of which have been described in part. Recently, a particulate enzyme preparation was isolated from *L. minor* which incorporates D-apiose from UDP-apiose into polysaccharides resembling these apiogalacturonans (64-66). If the enzyme activity responsible for the incorporation of D-galacturonic acid into these same polysaccharides could also be isolated and characterized, *L. minor* would become the first system in which the incorporation of both the acidic and neutral sugars of a pectin has been demonstrated.

In many of the cell-free polysaccharide synthesizing systems already described, the physiological significance of the reactions studied *in vitro* is uncertain since the presence in the cell wall of polysaccharides of the type synthesized was not determined. By comparing the properties of the polysaccharides synthesized from UDPGalA and from UDP-apiose *in vitro* with those of authentic apiogalacturonans it should be possible to remove some of this uncertainty.

The research described in this dissertation was undertaken to isolate and characterize the D-galacturonosyltransferase from *L. minor*, to relate its properties to those of D-apiosyltransferase, and to develop procedures for its solubilization so that the nature of its ultimate acceptor might be defined. To compare the D-galacturonosyltransferase product with the D-apiosyltransferase product and with authentic apiogalacturonans, the size and some of the physical properties of the D-galacturonosyltransferase product were investigated, and procedures were developed which can be used to study further the physical properties of the cell wall pectic substances.

LITERATURE REVIEW

Structure of the Plant Cell Wall

The Primary and Secondary Walls

The periphery of most cell types is surrounded by a coat of glycosubstances, sometimes referred to as a glycocalyx. In plants this glycocalyx has reached perhaps an extreme of development. The plant cell wall is almost entirely carbohydrate with protein constituting less than 10% of the total mass.

The basic plant cell wall structure is a microfibrillar array embedded in a complex heterogeneous polysaccharide matrix. In the mature cell this structure also contains a high molecular weight polyphenol, lignin.

Ultrastructurally, the plant cell wall is divided into several layers based upon the content and orientation of the microfibrils. Proceeding from the middle lamella toward the cell membrane, one finds first the primary cell wall, a loose, random net of microfibrils in a pectin-hemicellulose matrix. This wall is laid down during cell division and elongation. It is the only cell wall layer found in soft plant tissues (1).

The secondary wall is divided into three sublayers based upon the orientation of the numerous large and highly-oriented microfibrils. This layer, which is formed during thickening and

differentiation, provides greater strength and resistance than the more elastic primary wall (1).

The various components of the cell wall are modified in composition and ultrastructure in response to the changing conditions of growth and development. However, the basic arrangement of a fibrillar array in a complex matrix is maintained (2). This modification of the cell wall components may occur *in situ* or may result from deposition of different polysaccharides at different stages of growth (3).

The plant cell wall participates in many aspects of plant physiology: ultrastructure, growth, development, and host-pathogen interactions. For a long time, however, no clear idea of the molecular organization of this structure existed. With the development of gas-liquid chromatography, methylation analysis, and mass spectrometry, the various components are being resolved and defined to the point that a model for the interaction of various components can be proposed (4-6).

The microfibrillar structural material of higher plants is almost invariably cellulose. The molecular weight of primary wall cellulose is rather low and the degree of polymerization is more random than that of the higher molecular weight secondary wall cellulose (1). The microfibrillar array is largely responsible for giving shape and strength to the cell wall.

In lower plants such as yeast and some algae the microfibrils are composed of mannans. In other algae the microfibrils are formed from helical β-1,3-linked xylans (1).

In higher plants the variability in fibril composition is replaced by a variability in the structure and composition of the

hemicelluloses and pectins among plant species as well as between the two cell wall layers (1). Some of these polysaccharides are linear while others are highly branched. Because they are not primary gene products they are often polydisperse with respect to both size and carbohydrate content. Also, some are present only at certain stages of growth. All these factors combine to make studies of the structure of the matrix very complex (2,3).

The matrix formerly was considered to be merely an "amorphous" filler for the microfibrils. Recent studies, however, have indicated that its structure may be considerably more ordered (4-7).

In the final stages of secondary wall formation, lignification occurs. Lignin is a high molecular weight polyphenol containing oxidized *p*-hydroxycinnamyl alcohols. Its polymerization and deposition are purely chemical reactions with no enzymatic involvement (1,2). Several thorough reviews of the chemistry of lignin are available (8,9), and its structure and synthesis will not be discussed further in this dissertation.

Extraction of the Various Classes of Polysaccharides

The various classes of cell wall polysaccharides are defined more by the methods used to extract them from the cell wall than by their composition. A summary of these methods is presented in Figure 1. The delignification steps are eliminated for tissues containing only primary cell walls (11).

Pectins are removed from the primary wall by a combination of water extraction and treatment with chelating agents such as EDTA,

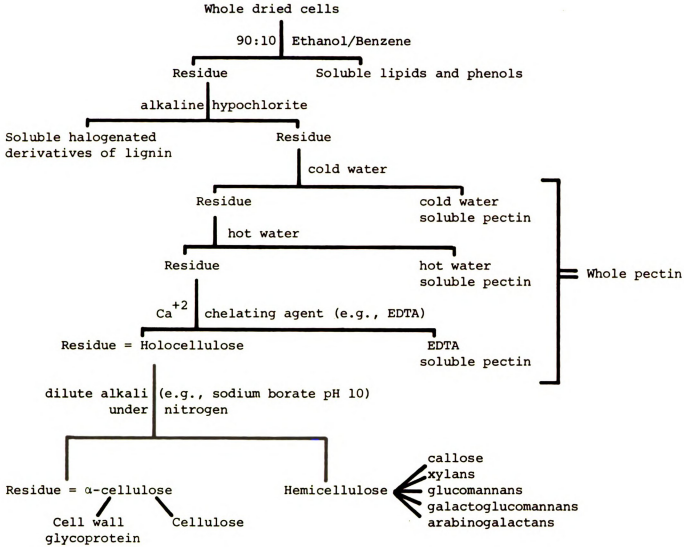

Figure 1. Procedures for extraction of plant cell walls.

ammonium oxalate, and sodium hexametaphosphate (11). The latter has been reported to cause minimal degradation of pectins (12).

The most important point to keep in mind is that each tissue studied requires its own modifications of the listed procedures in order to minimize degradation and other artifacts of isolation.

Cellulose

Cellulose is operationally defined to be the alkali-insoluble polysaccharide fraction from delignified cell walls. As isolated, it is seldom a pure β-1,4-glucan, but contains small amounts of other neutral sugars (3) which may result from the presence of enzymes capable of converting glucose to other sugars *in situ* (1,13). Alternatively, these sugars could belong to polymers which are merely adsorbed to the surface of the cellulose microfibril, or they could serve as a keying material for fibril-matrix interactions (2).

From viscosity measurements, $\overline{P}_w$ of total cell wall cellulose has been found to be 8,500 to 9,500. $\overline{P}_w$ for primary wall cellulose is approximately 1,500 while that for secondary wall cellulose is 11,500 in cotton, flax and spruce (14).

In the microfibril, the cellulose chains have long been assumed to have an antiparallel arrangement as in chitin (2). Recent evidence suggests, however, that this may be incorrect (15).

X-ray diffraction has shown that the microfibrils possess both crystalline and amorphous regions. This finding has been interpreted in various ways, and numerous models of fibrillar structure have been proposed (1-3,11,15). The precise ultrastructure of the microfibril is still in dispute.

Hemicelluloses

Among the matrix polysaccharides, the hemicelluloses are a group of neutral polysaccharides classified together on the basis of their method of extraction, illustrating a point made earlier. They are structurally heterogeneous, sharing none of the common properties of the pectins and cellulose (11). Several complete reviews of hemicelluloses are available (1,3,16-19), so only a brief listing will be presented here.

The xylans contain β-1,4-linked main chains of D-xylose with short, single-residue side chains of L-arabinose or D-glucuronic acid and its esters. Xylans comprise the bulk of the hemicelluloses in angiosperms (3) in which they are up to 50% acetylated at the 2- or 3-position of the D-xylose residue (20).

The galacto- and glucomannans are random copolymers of D-galactose or D-glucose in β-1,4-linkage with D-mannose. As in the xylans, the D-mannose residues are partially acetylated. These polysaccharides of $\overline{P}_w$ of approximately 100 to 200 are the major hemicelluloses of the gymnosperms (3).

Callose is a β-1,3-glucan elaborated in the development of phloem and in wound tissue (3). Because of its solubility properties, it is grouped with the hemicelluloses.

Two types of polysaccharide fit into neither the hemicellulose nor the pectin group, although they are often elaborated with the latter (21). The arabinogalactans, which are most abundant in conifers, consist of β-1,3- and β-1,6-linked galactan main chains with L-arabinose mono- or disaccharide side chains. The xyloglucans have

a β-D-glucan core with single D-xylose side chains which may be further substituted with L-fucose or D-galactose (16).

The Hydroxyproline-Rich Glycoproteins

The first report of a plant cell wall glycoprotein rich in hydroxyproline (hyPro) appeared in 1960 (22). At first it was suspected that this protein might merely be adsorbed to the cell wall polysaccharides during isolation. Now, however, the hyPro glycoprotein, termed "extensin" by Lamport, has been shown to be a definite wall component (10,23). It contains galactose in *O*-glycosyl linkage to serine as well as L-arabinose linked to hydroxyproline. In tomato, the glycosylated portion of the protein contains repeating units of the pentapeptide ser(hyp)$_4$ (24,25). Extensin is viewed as being a cross-linker in the cell wall with a role in cell extension (10).

Pectins

Pectins are matrix polysaccharides found mainly in primary cell walls where they help determine the physical properties of the wall which are important in elongation. Characteristically they are acidic polysaccharides, although a group of water-soluble arabino-galactans is generally classified as pectins (3). With the exception of the Characeae and the Zosteraceae, both seaweeds, and one or two other sources, pectins are found exclusively in terrestrial plants (11,26).

The basic structure of the main chain of acidic pectins is an α-1,4-linked galacturonan interspersed with L-rhamnose. Side chains

may contain L-arabinose, D-galactose, D-xylose, L-fucose, or D-apiose depending upon the plant species (3).

Pectins may be divided into two subgroups: those containing large "neutral blocks" of L-arabinose and D-galactose along the main chain, and those containing the neutral sugars in short, randomly-distributed side chains. These subgroups can be separated by electrophoresis, the latter subgroup being the more acidic because of its higher proportion of D-galacturonic acid residues (2,3). The pectins which contain the large neutral blocks might be characteristic of more mature primary cell walls (27,28).

D-Galacturonic acid in pectins is often methyl esterified and acetylated. The degree of esterification and acetylation can be used to modulate the physical properties of pectins. Other changes in structure and composition may occur in response to differing demands of growth (12). Some evidence suggests that these changes may occur by transglycosylation within the matrix (29).

The presence of rhamnosyl residues within the main chain causes kinks in an otherwise extended ribbon-like polysaccharide. Kinking was predicted on the basis of mathematical models (30) and was confirmed in an analysis of the rhamnogalacturonan of sycamore cells (5,7). The linear portions of the chain may form bundles while the kinked regions form a network which holds water molecules (31).

The ionic nature of the galacturonans also allows salt formation with calcium envisioned as the most important contributor to *in vivo* pectin structure. The physical properties of pectins in the presence of monovalent cations may be expected to differ from those observed in the presence of divalent cations (2).

Crystal structure analysis of calcium galacturonate has shown that the 9-coordinate calcium ion can interact with the *O*-6 carboxyl group and the *O*-5 ring oxygen of one galacturonic acid residue, the hydroxyl groups of adjacent residues, and waters of hydration (32). By regulating its degree of hydration, a group of pectin molecules can transform itself reversibly from a fairly rigid gel to a highly viscous solution (2).

Because they contain a variety of glycosidic linkages, pectins are especially susceptible to chemical modification during isolation. Acid precipitation removes neutral sugars and oligosaccharides (11, 33), while alkaline pH causes saponification of methyl esters and cleavage of the polyuronide chain (11). *Trans*- or β-elimination will occur even at neutral pH when polyuronides containing methyl esterified residues are heated (34). All these factors must be considered in devising extraction procedures.

At pH values of 4 to 6.5, EDTA is ineffective in extracting pectins, whereas ammonium oxalate may cause decarboxylation of uronic acids (35). Sodium hexametaphosphate chelates best at pH 4 and has been shown to be a suitable extractant for apple fruit pectin (28, 36). In contrast, in lemon peel it caused deesterification and some β-elimination (16).

Barrett and Northcote separated apple fruit pectin into a low molecular weight component which was an almost-pure galacturonan, and a high molecular weight polysaccharide (27). The latter component contained about 30% D-galacturonic acid and large amounts of neutral sugar, particularly L-arabinose and D-galactose. After β-elimination of this component, L-rhamnose was isolated linked to L-arabinose and

D-galactose oligosaccharides. Since L-rhamnose is a component of the galacturonan chain, it seemed to be the point of attachment for the neutral sugars. This was the first demonstration that pectins contain blocks of neutral sugar attached to the main chain at specific sites.

Conversely, the pectin of sycamore callus and other rapidly growing tissues is mainly a rhamnogalacturonan with short D-xylose side chains and no neutral blocks (12). This supports the hypothesis that neutral blocks are characteristic of the pectins of mature cells while galacturonans with short, randomly-distributed side chains are more typical of the pectins of rapidly growing and dividing cells (11).

Methylation analysis has confirmed the frequent substitution of L-rhamnose by neutral sugars at the *C*-4 position (4,37). L-Rhamnose residues, therefore, might be recognition sites for attachment of the neutral blocks (11).

The structure of the cell wall pectins of suspension cultured sycamore cells has been analyzed recently by Talmadge *et al.* (4). Difficulty in derivitizing D-galacturonic acid has delayed such analyses, but the development of an improved gas-liquid chromatographic technique (38) and the application of methylation analysis (39) and mass spectrometry (40) now permit thorough structural studies of pectins.

Talmadge *et al.* showed that the presence of 1,2-linked L-rhamnose units kinks the galacturonan chain as predicted by the mathematical models (4,31). In addition, attachment of neutral sugars to *C*-4 of about 50% of the L-rhamnose residues creates "Y-" shaped branches.

The distribution of L-rhamnose within the chain is not random; rather, it occurs as rhamnosyl-galacturonosyl-rhamnose trisaccharides which are separated by eight D-galacturonic acid residues. Hopefully, this is only the first of many studies on the structure of a variety of pectins which will clarify the concepts of pectin structure discussed in this review.

Galacturonans containing the neutral branched-chain aldopentose D-apiose have been isolated only from the aquatic angiosperm *Lemna minor* and three species of Zosteraceae (33,41). The apiogalacturonans of *L. minor* contain a galacturonan main chain with side chains of the disaccharide apiobiose and small amounts of D-apiose (41). No other neutral sugars were detected in these polysaccharides, but Beck has found D-xylose and D-galactose in apiogalacturonans (43).

The *L. minor* apiogalacturonans can be subfractionated on the basis of solubility in NaCl. The various subfractions differ only in their D-apiose content which ranges from 7.9% to 38.1% (42). This differential solubility is probably based on the ability of D-apiose to interfere with the formation of ionic bonds between the carboxyl groups of D-galacturonic acid residues and Na^+ and Cl^- which would tend to aggregate or precipitate the galacturonan chains.

Overall Organization of the Cell Wall

The data which have been gathered by analysis of the cell wall of suspension-cultured cells (4,5) have led Albersheim to propose the model for cell wall organization pictured in Figure 2 (6,7,15).

Each cellulose microfibril in the primary wall is thought to be coated by a sheath of xyloglucan one molecule thick. The D-glucose

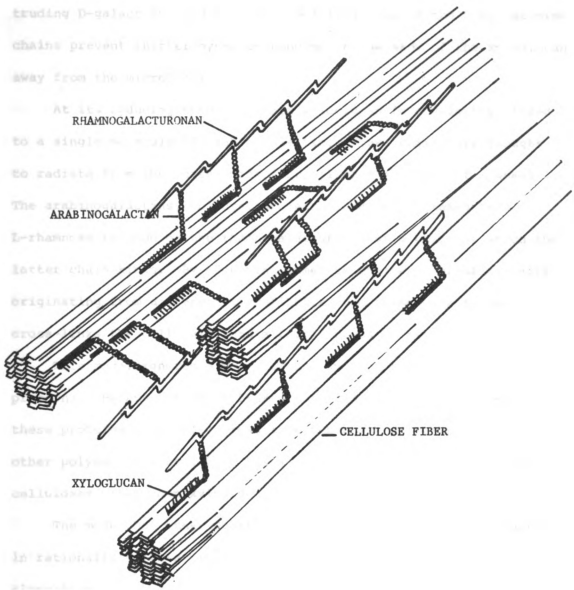

Figure 2. Model for the organization of the polysaccharides of the primary cell wall of suspension-cultured sycamore cells (15).

molecules of the xyloglucan are hydrogen-bonded to the D-glucose molecules of the cellulose on the surface of the microfibrils. Protruding D-galactosyl and L-fucosyl modifications of the D-xylose side chains prevent further hydrogen bonding on the side of the xyloglucan away from the microfibril.

At its reducing terminus, each xyloglucan is covalently linked to a single molecule of arabinogalactan. These chains are thought to radiate from the coated microfibril like the spokes of a wheel. The arabinogalactans, in turn, are each covalently linked to a L-rhamnose residue of the rhamnogalacturonan at a point at which the latter chain kinks. By receiving numerous chains of arabinogalactan originating from different microfibrils, the rhamnogalacturonan cross-links the cell wall into a fairly rigid matrix (15).

This model ignores a role for the hydroxyproline-rich glycoproteins. Because of their L-arabinose and D-galactose content, these proteins might also cross-link the rhamnogalacturonans with other polysaccharides (10). Likewise, no role is assigned to hemicelluloses other than xyloglucan.

The model has other shortcomings. Foremost is the difficulty in rationalizing this rigid structure with a mechanism for cell elongation. Such a process would require the breaking and reforming of many covalent bonds among the cross-links. While this may seem an unnecessarily cumbersome process, the search for enzymes that could mediate such changes is in progress (15).

Alternatively, while this model provides a good description of the mature primary wall, the newly-formed and elongating walls may be more plastic structures. In rapidly growing cells the physical

properties of the matrix polysaccharides might be more important. The most important stabilizing forces in such cases would arise from the ability of these polysaccharides to form hydrogen bonds, to form salts with ions such as calcium, and to alter the water structure of the wall by undergoing reversible gel-sol transitions. Then as the wall matured, the covalent cross-linking by proteins and polysaccharides would assume increasing importance until the rigid structure of Albersheim's model was achieved.

Of course, all of this is speculation, but it reemphasizes the need to investigate carefully the structure and properties of cell walls from many different sources, from different cell types, and from cells in various stages of growth. Only then can a general model be devised which provides for both maintenance of structure and cell elongation.

Polysaccharide Biosynthesis

Sugar Nucleotides

Sugar nucleotides appear to serve as the immediate glycosyl donors for the synthesis of oligosaccharides and polysaccharides (44). These donors, in turn, arise from the phosphorylated monosaccharides of the photosynthetic carbon reduction cycle by the action of pyrophosphorylases and enzymes of sugar interconversion. Sugar nucleotides are favored thermodynamically as donors for polysaccharide synthesis because their free energy of hydrolysis is higher than that of other glycosyl compounds.

Glycosyl derivatives of all the nucleosides have been found (11,44); however, not all sugars are linked to all nucleoside

diphosphates. Polysaccharide synthesizing enzymes are often specific for the nucleoside diphosphate portion as well as for the sugar portion of the sugar nucleotide. This latter point is particularly well illustrated in glucan synthesis. In cotton boll and in mung bean, synthesis of cellulose, a β-1,4-glucan is specific for GDP-glucose whereas UDP-glucose is used for synthesis of callose, a β-1,3-glucan (48). ADP-Glucose is generally the precursor to synthesis of starch, an α-1,4-glucan (11), while TDP-glucose has been shown to be involved in sucrose metabolism in sugar beet (48).

The sugar nucleotides involved in pectin and hemicellulose synthesis are formed from glucose 6-phosphate by two basic pathways. Loewus has named these the glucose oxidation pathway and the *myo*-inositol oxidation pathway (49).

In the glucose oxidation pathway, glucose 6-phosphate is converted first to glucose-1-phosphate and then to UDP-glucose which can be converted to UDP-galactose by an epimerase. The most important enzyme of the pathway is UDP-glucose dehydrogenase which, in the presence of NAD^+, converts UDP-glucose to UDP-glucuronic acid (50). An epimerase converts UDP-glucuronic acid to UDP-galacturonic acid for pectin synthesis. Oxidative decarboxylation by a carboxy-lyase converts UDP-glucuronic acid to UDP-xylose which is then epimerized to UDP-arabinose (11,51).

Loewus has described an alternate pathway from glucose 6-phosphate to UDP-glucuronic acid which bypasses the dehydrogenase (49). All four key enzymes of this pathway have been found in various plants. These enzymes are:

(1) a cycloaldolase for converting glucose 6-phosphate to the cyclitol 1L-*myo*-inositol 1-phosphate,

(2) an oxygenase for conversion of *myo*-inositol 1-phosphate to glucuronic acid,

(3) a kinase for phosphorylating glucuronic acid, and

(4) a pyrophosphorylase for synthesizing UDP-glucuronic acid. From UDP-glucuronic acid on, the same enzymes are used as were described for the glucose oxidation pathway.

The presence of the *myo*-inositol oxidation pathway has been reported in a number of plant species (52). The cell wall D-apiose of *Lemna* is supposedly formed by this route (53). Recent studies (54), however, have questioned the significance of this pathway. In *Fraxinus pennsylvanica* only 0.5% of the cell wall galacturonic acid was found to be derived from *myo*-inositol, the remainder coming from UDP-glucose (54). It was speculated that the *myo*-inositol oxidation pathway is involved mainly in the degradation of *myo*-inositol which is a ubiquitous constituent of plant cells (55).

UDP-Apiose is synthesized by concomitant cyclization and decarboxylation of UDP-glucuronic acid (56-58). The properties of UDP-apiose have been described by Kindel and Watson (58). Two carboxy-lyases (I and II) which convert UDP-glucuronic acid to UDP-xylose are present in *L. minor* together with the cyclase that synthesizes UDP-apiose. The purification and characterization of these enzymes together with the separation of carboxy-lyase II from the other two enzymes have recently been described (59).

Synthesis of Pectins

Despite the importance of pectic polysaccharides in determining the properties of primary cell walls, little is known of their synthesis. *In vitro* synthesis of the main galacturonan chain has been studied only in mung bean (60,61) and in green tomato and turnip (62).

Villimez *et al.* (61) found UDPGalA to be the preferential donor for galacturonan synthesis in mung bean. Incorporation of D-galacturonic acid from all other sugar nucleotide donors including UDP-methyl-D-galacturonate was negligible. In tomato, some incorporation was found with dTDP-galacturonic acid (62).

The basic characteristics of the mung bean D-galacturonsyl-transferase have been described (60,61). The enzyme is optimally active at pH 6.7 and 30°C, with an apparent K_m for UDPGalA of 1.7 μM. The activity was increased 50% by the addition of 1.7 mM $MnCl_2$.

One of the most serious problems in studying *in vitro* pectin synthesis is the instability of the particulate glycosyltransferase preparations. For example, the mung bean D-galacturonosyltransferase is spontaneously and totally inactivated in 5 min at 37°C, in 20 min at 30°C, and in 24 h at 0°C. Even at -18°C, total inactivation occurs in slightly greater than three weeks (61).

Almost no evidence is available on the incorporation of L-rhamnose residues or on the synthesis of the side chains or neutral blocks, because of the difficulty of analysis of components present in very small amounts. This is true in the study of the incorporation of L-arabinose (63). In these experiments it was impossible to distinguish incorporation of L-[^{14}C]arabinose into pectin from

incorporation into hemicellulose because the main chain sugars were unlabeled (55).

The apiogalacturonans of *Lemna minor* represent one of the few systems in which synthesis of both the main chain and the side chains of the polysaccharide are being studied. Pan (64) has isolated and characterized a D-apiosyltransferase which, according to Mascaro (65, 66) incorporates D-apiose into acidic polysaccharides which appear to be apiogalacturonans of the type isolated from *L. minor* cell walls.

Apart from these studies, the only reports of incorporation of any unit other than D-galacturonic acid into pectin have concerned the methyl esterification of polygalacturonate. Using a particulate preparation from mung bean, Kauss showed incorporation of the methyl group from S-adenosylmethionine into pectin (55). Only endogenous pectin or a galacturonan prepared *in situ* could be esterified. Exogenous acceptor had no effect on esterification, apparently because it lacked access to the methyltransferase.

The newly esterified pectin was also protected from the action of both exogenous and endogenous pectin methylesterase (67). Agents that disrupt lipid membranes, such as Triton X-100, sodium dodecyl-sulfate and phospholipase A, removed this protection. Thus, it appears that all the enzymes needed for polymerization and esterification are contained in the same membrane-bound particle (55).

Site of Synthesis

The close proximity of the cell wall and cell membrane suggests that the biosynthesis and development of each should be closely coordinated. This has been found to be true.

Studies in *Lolium longiflorum* have shown that the polysaccharides found in Golgi vesicles are similar in composition to the polyuronides (pectins) of the cell wall matrix (68,69). These findings agree with previous reports of a vesicular origin for the matrix polysaccharides (70-72). In addition, a wide range of ultrastructural, histochemical and biochemical studies point to participation of the Golgi apparatus in hemicellulose and pectin synthesis. The membranes of the Golgi vesicles fuse with and become a part of the plasma membrane while the contents of the vesicles are deposited in the cell wall as precursors to the matrix phase (69).

Despite the evidence presented by Ray *et al.* (73) that Golgi vesicles are able to synthesize a β-1,4-glucan, the higher degree of order of the cellulose microfibrils makes it more likely that the enzymes responsible for cellulose synthesis are present in or on the surface of the plasma membrane or even within the wall itself (3,72). Vesicle fusion with the plasma membrane is almost never observed during late primary and secondary wall formation (69), further supporting this argument. Also, cell elongation in pollen tubes can be inhibited by $CaCl_2$. When this is done, the number of vesicles is reduced as is their sugar content and the labeling of the matrix polysaccharides. Cellulose synthesis, however, is unaffected, strongly suggesting that it proceeds independently of the Golgi vesicles (69).

Reexamining Ray's results (73), vander Woude *et al.* (74) found that both Golgi fractions and plasma membrane fractions synthesized β-1,3- and β-1,4-glucans. However, which synthesis predominated depended on the concentration of UDP-glucose. At low concentrations

(1.5 μM), glucan synthesis in the Golgi fractions predominated, but at high UDP-glucose concentrations (1 mM), the plasma membrane fractions were most active in glucan synthesis. Thus, they attributed Ray's findings to the low concentrations of UDP-glucose which his group used. *In vivo* UDP-glucose concentrations in excess of 30 μM have been reported (75). This concentration is intermediate between the concentrations used by vander Woude (74).

The significance of the Golgi glucan synthetase is unclear. One possible explanation is that it represents the cellulose synthesizing enzyme which has been synthesized on the endoplasmic reticulum and within the smooth membrane system for transport in an inactivated form to the plasma membrane where it is activated.

Recent brief reports suggest that the Golgi glucan synthetase activity may be involved in xyloglucan synthesis (76) or that it may be a precursor to cell surface glucan synthetase (77).

There is also evidence that the plasma membrane glucan synthetase may be a mobile enzyme complex, the movements of which are directed by cellulose microfilaments (78). Hopefully, the morphology of cellulose synthesis may be clarified in the near future.

Solubilization of Particulate Enzyme Activities

One reasonable approach to the problem of the instability of particulate polysaccharide synthesizing systems is to try to solubilize the enzymes. This involves treating the particulate preparation with some agent which disrupts the lipid membrane of the particle. In theory, the solubilized preparation will either be more stable than the particulate form or, should solubilization

cause loss of activity, reconstitution of the enzyme in an artificial membrane may cause reactivation and stabilization. Numerous bacterial or animal systems have been treated in this way, and the techniques involved have been reviewed thoroughly (79-81). Unfortunately, little success has been reported in plant systems.

The greatest success has been achieved with the glucan synthetases. Two studies indicated that digitonin treatment of particulate mung bean preparations "solubilized" both β-1,3- and β-1,4-synthesizing activities (47,82). These preparations could be precipitated by centrifugation at 30,000*g*. Such preparations have been shown to contain particulate matter. A truly "soluble" activity remains in the supernatant after centrifugation at 100,000*g* (83). Nonetheless, these digitonin-treated preparations were more stable than their particulate counterparts (82). Sonication and treatment with trypsin and lipase or phospholipase did not cause solubilization.

Subsequently, Tsai and Hassid (84) showed that following treatment of the particulate glucan synthetase from *Avena* coleoptiles with 8% digitonin, 40 to 50% of the initial activity remained soluble after centrifugation at 100,000*g*. This soluble preparation contained both β-1,3- and β-1,4-glucan synthetase activities which could be separated by column chromatography on hydroxyapatite.

The only report of the successful solubilization of a heteropolysaccharide synthesizing system concerns the glucomannan of mung bean (85). Treatment of the particulate mannosyltransferase with 0.5% Triton X-100 produced an enzyme preparation which remained soluble after centrifugation at 300,000*g*. Unfortunately, this preparation, which also could utilize GDP-glucose, was still

relatively unstable, losing two-thirds of its activity in 5 h at 0°C. Thus, solubilization does not automatically guarantee stabilization.

This last study is an important advance in attacking one of the biggest problems of studying polysaccharide synthesis *in vitro*. Particulate enzyme preparations contain endogenous polysaccharides which are presumably used as acceptors by the transferase under study. In order to relate *in vitro* observations of polysaccharide synthesis to *in vivo* mechanisms, systems must be devised in which the nature of the acceptor can be completely defined. Solubilization of the particulate glycosyltransferases represents a critical step in this direction.

Lipid Intermediates

Related to the acceptor problem is the possibility that other intermediates are synthesized that function between the sugar nucleotides and the completed glycan chain. A critical advance in understanding the synthesis of bacterial cell wall glycans was the discovery of sugars bound to polyisoprenoids. These polyisoprenoid lipids are likely intermediates in polysaccharide and glycoprotein synthesis in plant and animal systems as well. These studies have been reviewed recently (86) and only the highlights of the plant studies will be discussed.

Preliminary studies on a cell-free system from mung bean showed that a lipid-like material served as a precursor to mannan synthesis from GDP-mannose (87). The intermediate had the properties of a complex glycolipid. Significantly, it could not be isolated by common lipid solvents until the particulate system was treated with

detergent which also destroyed mannosyltransferase activity. Thus, the intermediate seems to be held in the same compartment as the transferase. The sugar moiety of the intermediate was identified as a monosaccharide (88). An oligosaccharide derivative of a low molecular weight membrane protein was suggested as a second intermediate. The protein might act as a primer for polysaccharide synthesis or serve to anchor the completed polysaccharide in the cell wall (88). Unfortunately, no further reports on these studies have appeared.

The reactions by which the bacterial lipid intermediates are formed from sugar nucleotides are freely reversible, indicating that the intermediates have almost the same group transfer potential as sugar nucleotides (86). Kauss (89) confirmed this reversibility for mannose transfer in the mung bean system. However, he cautioned that the kinetics of transfer are such that the labeled sugar could be transferred to polysaccharide via back-donation from glycosyl-lipid to nucleotide. This argument is supported by further studies which failed to show transfer of label from partially purified ^{14}C-mannosyl-lipid to polysaccharide (90). These same studies confirmed the exchange between endogenous ^{14}C-mannosyl-lipid and GDP and showed that in the absence of GDP or GDP-mannose, mannose is hydrolyzed from the lipid rather than transferred to polysaccharide (90).

Clark and Villemez (91) have shown that phytanol, a saturated commercial isoprenol, can substitute for endogenous mannosyl acceptor in mung bean. The authors hope that the availability of this commercial substrate will speed the solubilization and purification of the transferases involved.

The only other plant system in which glycosyl-polyisoprenol synthesis has been described is cotton fibers (92). In this system both D-mannose from GDP-mannose and D-glucose from GDP-glucose are incorporated into acidic lipids of the type described in bacteria. Extraction of cotton bolls with chloroform-methanol has demonstrated the presence of significant amounts of L-arabinose and D-galactose in the lipid phase (92).

By analogy with the bacterial systems, the structure and transfer potential of the lipids described in these studies suggests that they may participate in the synthesis of at least the terminal branches of polysaccharides or in glycoprotein synthesis. Until transfer of the sugar moieties from these glycosyl-lipids to such macromolecular acceptors has been conclusively documented, however, the lipids cannot be regarded as obligatory intermediates in *in vivo* systems in plants.

EXPERIMENTAL PROCEDURES

Materials

D-Glucuronic acid, D-galactose 1-phosphate, D-galacturonic acid 1-phosphate, UDPGalA, inorganic pyrophosphatase (pyrophosphate phosphohydrolase, E.C. 3.6.1.1) and Sepharose 2B, 4B, and 6B were purchased from Sigma Chemical Co. UTP was supplied by P-L Biochemicals, Inc. Platinum oxide (Adams' catalyst) was obtained from MC/B Manufacturing Chemists. Bio-Gel P-30 (100-200 mesh), Bio-Gel P-100 (100-200 mesh), Bio-Gel P-200 (100-200 mesh) and Bio-Gel P-300 (50-100 mesh) were purchased from Bio-Rad Laboratories. Ultrogel AcA-22 and Ultrogel AcA-34 were supplied by LKB Instruments, Inc. *p*-Hydroxydiphenyl was purchased from ICN/K&K Laboratories, Inc. Triton X-100 for solubilization of membranes was supplied by Research Products International Corp., Elk Grove Village, Illinois.

α-D-[U-^{14}C]Galactose 1-phosphate (214 mCi/mmol) and UDP-[U-^{14}C]glucuronic acid (190 mCi/mmol) were purchased from New England Nuclear Corp. UDP-[U-^{14}C]apiose (2.8 mCi/mmol) was enzymatically synthesized from UDP-[U-^{14}C]glucuronic acid (58, and Pan and Kindel, unpublished experiments).

Butylated hydroxytoluene, Tween-20, Emulgen-911, Emulgen-913, sodium cholate, and sodium deoxycholate were gifts from Dr. S. D. Aust. Phenylmethylsulfonyl fluoride was a gift from Dr. W. C. Deal. Nupercaine hydrochloride was generously provided by Dr. L. L. Bieber.

NP-40 and Sarkosyl-97 were gifts from Drs. J. A. Boezi and L. F. Lee.

General Methods

Paper chromatograms were scanned for radioactivity with a Packard radiochromatogram scanner, Model 7201 (Packard Instrument Co., Inc.). All other radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer, Model 3310.

The following solutions were used for liquid scintillation counting:

(A) Bray's solution (93),

(B) 5.5 g of 2,5-diphenyloxazole and 0.1 g of 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 of 2,5-bis-2-(5-*t*-butylbenzoxazolyl)-thiophene in 1.0 l of reagent grade toluene.

Aqueous radioactive samples (1.0-1.3 ml) were counted in 10 ml of solutions A or B. Radioactive samples on paper were counted by complete immersion of the paper in solution C. The counting efficiencies of solutions A, B and C were 77, 81, and 63%, respectively.

Gel chromatography columns were prepared according to the manufacturers' recommendations. All columns were eluted at 24-27°C by downward flow. V_o and V_t for each column were determined by measuring the elution volumes of solutions of blue dextran (V_o) and phenol red (V_t). The dimensions of all columns were 1.0 cm i.d. x 40 cm unless otherwise noted.

The total uronic acid content of solubilized products was determined colorimetrically using *p*-hydroxydiphenyl according to the method of Blumenkranz and Abou-Hansen (94).

The mean, the standard deviation, and the standard error were calculated for results based on two or more observations. The Student's *t* test was used for comparing sample means. The levels of probability upon which decisions of significance were based are given at the appropriate place in the text.

Electrophoresis and Paper Chromatography

Electrophoresis of D-galactose 1-phosphate, D-galacturonic acid 1-phosphate, and UDPGalA was performed using a Pherograph Original Frankfurt, Type 64 electrophoresis apparatus (distributed by Brinkmann Instruments, Inc.). Samples were applied to Schleicher and Schuell 589c paper and were electrophesed in 0.2 M ammonium formate (pH 3.6) at 4°C at constant current.

Descending paper chromatography was performed with Schleicher and Schuell 589c paper at 4°C or at 24-27°C. The solvents were: (A) 95% ethanol-1.0 M ammonium acetate (pH 3.8) (7:3, v/v), and (B) 95% ethanol-1.0 M ammonium acetate (pH 7.5) (7:3, v/v).

Non-radioactive sugar 1-phosphates were detected by the procedure of Bandurski and Axelrod (95). Non-radioactive sugar nucleotides were visualized in the dark using a Mineralight Model UVS-11 ultraviolet lamp (λ_{max} = 2735 Å).

Samples were eluted from paper chromatograms with cold water by centrifugal extraction in plastic spin thimbles (Reeve-Angel, Clifton, N.J.).

Preparation of UDP[U-^{14}C]GalA

D-[U-^{14}C]Galacturonic acid 1-phosphate was prepared by catalytic oxidation of D-[U-^{14}C]galactose 1-phosphate by modification of the method of Marsh (97). PtO_2 (Adams' catalyst - 50 mg) was activated by bubbling H_2 gas through an aqueous suspension of the catalyst at 1 atm for 2 h. Activated catalyst was resuspended in 0.1 N $NaHCO_3$ and combined with about 4.5 µCi of D-[U-^{14}C]galactose 1-phosphate in 7% aqueous ethanol. O_2 gas was bubbled through this suspension at 25°C and 1 atm for 7-8 h. The catalyst was then removed by centrifugation and the clear supernatant was chromatographed in solvent A or electrophoresed. Yields of D-[U-^{14}C]galacturonic acid 1-phosphate averaged 50-55%.

UDP-[U-^{14}C]GalA was prepared from D-[U-^{14}C]galacturonic acid and UTP by the method of Feingold *et al.* (97) using a nonspecific uronic acid pyrophosphorylase (uronic acid 1-phosphate uridyltransferase) isolated from mung beans. The standard reaction mixture contained: 2 mM UTP, 40 µM $MgCl_2$, 5 µg inorganic pyrophosphatase, 0.2 µCi D-[U-^{14}C]galacturonic acid 1-phosphate and 0.5 mg of crude pyrophosphorylase preparation in 0.1 M Tris-HCl (pH 7.3) containing 50 mM 2-mercaptoethanol. Total assay volume was 100 µl.

Incubations were performed at 30°C for 1 h. Samples were immediately chromatographed in solvents A or B or were electrophoresed. Yields averaged 60-70% from D-galacturonic acid 1-phosphate.

In the synthesis of both D-[U-^{14}C]galacturonic acid 1-phosphate and UDP[U-^{14}C]GalA, the radioactive products were identified by their co-migration with non-radioactive standards or with radioactive

compounds previously identified by their co-migration with these non-radioactive standards upon chromatography or electrophoresis.

Culture of *Lemna minor*

Lemna minor was cultured according to previously described methods (58). For these studies, plants were grown on 4 l of inorganic culture medium in uncovered white plastic pans (29 x 32 cm). Fronds were harvested from 1-2 d after their growth had just covered the surface of the pan, usually 18-22 d after inoculation of the culture.

Isolation of the Particulate D-Galacturonosyltransferase

Preparation

Lemna minor was harvested on a double layer of cheesecloth, washed thoroughly with distilled water, blotted to remove excess water, and weighed. All remaining steps were performed at 4°C.

The fronds were chilled for 5 min, then ground gently with a mortar and pestle for 1 min. The standard grinding medium was 50 mM sodium phosphate buffer (pH 7.3) containing 1% BSA (w/v). Two milliliters (2.0 ml) of this buffer were used for each gram of wet plant material.

The crude homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 480*g* for 10 min. The precipitate was discarded, and the supernatant was recentrifuged at 34,800*g* for 10 min. The supernatant was discarded.

The 34,800*g* precipitate was resuspended by gentle trituration in 0.1 M citric acid-50 mM sodium phosphate buffer (pH 5.7) containing 10 mM $MnCl_2$, 0.4 M sucrose, and 1% BSA (w/v). Five-tenths milliliter

(0.5 ml) of buffer was used for each gram of plant material. Any modifications of this buffer or of the grinding medium will be described where appropriate in the text.

This resuspended 34,800*g* precipitate is termed the particulate D-galacturonosyltransferase preparation.

Standard Assay for UDPGalA:Acceptor D-Galacturonosyltransferase

For characterization of the D-galacturonosyltransferase, the standard reaction mixture contained 20 to 40 pmol of UDP[U-^{14}C]GalA (10,000-20,000 dpm), 1 or 5 nmol of UDPGalA, and 50 µl of the particulate D-galacturonosyltransferase preparation. The mixture was incubated for 1.0 min at 25°C.

For characterization of the D-galacturonosyltransferase product, the standard reaction mixture contained 0.10 to 0.20 nmol of UDP[U-^{14}C]GalA (50,000-100,000 dpm), 1.0 nmol of UDPGalA and 100 µl of particulate D-galacturonosyltransferase preparation. These assay mixtures were incubated at 25°C for 20 min.

Variations in standard assay procedures are noted at the appropriate places in the text. Each reaction mixture was extracted with 1 ml of 1% KCl in 75% (v/v) aqueous methanol. Following brief centrifugation, the supernatant was discarded, and each precipitate was then extracted in turn with two 1 ml portions of absolute methanol, with one more 1 ml portion of KCl-methanol, and with two 1 ml portions of water. All extractions were performed at 24-27°C.

The material which remained insoluble after these extractions was termed the D-galacturonosyltransferase product. The radioactivity

in this material was assayed in scintillation solution C as a measure of D-galacturonosyltransferase activity.

Solubilization of the D-Galacturonosyltransferase Product with Ammonium Oxalate and with Sodium Hexametaphosphate

In product characterization studies, the D-galacturonosyltransferase product was extracted at 50°C with freshly prepared solutions of sodium hexametaphosphate or ammonium oxalate. Each sample of D-galacturonosyltransferase product was extracted in turn with one 0.3 ml portion and three 0.1 ml portions of the appropriate extractant. Each extraction was performed for 15 min, after which the samples were centrifuged and the supernatants were removed. The supernatants from the four extractions were pooled and portions were withdrawn and assayed for radioactivity in scintillation solution B.

The residue remaining after the four extractions was resuspended by boiling for 10 min in 2% NaOH. This material was then streaked on Whatman 3MM paper and counted in scintillation solution C.

The material solubilized by these extractions was named for the particular concentration of extractant used. Thus, the material solubilized by extraction with 1% ammonium oxalate was termed the 1% ammonium oxalate-solubilized product.

RESULTS

Partial Characterization of UDPGalA:Acceptor D-Galacturonosyltransferase

Linearity of the D-Galacturonosyltransferase Assay

The amount of D-[U-^{14}C]galacturonic acid incorporated into water- and methanol-insoluble product is a linear function of the amount of particulate D-galacturonosyltransferase preparation used between 0 and 100 µl (Figure 3).

At 25°C, the amount of D-[U-^{14}C]galacturonic acid incorporation is a linear function of time for about 1.5 to 2 min, as shown in Figure 4. Incorporation is almost complete by 10 min.

At 0°C, the rate of incorporation is much lower, but it remains linear for at least 15 min.

Addition of more UDPGalA at 1.5 min failed to increase the rate of incorporation, suggesting that the decreased rate of D-[U-^{14}C]-galacturonic acid incorporation after 1.5 min is not due to depletion of substrate.

Effect of pH on D-Galacturonosyltransferase Activity

As shown in Figure 5, the D-galacturonosyltransferase is optimally active between pH 6.0 and 6.2. Activities were approximately equal at the same pH in citric acid-sodium phosphate and Tris-maleate

Figure 3. The effect of enzyme concentration on the rate of incorporation of D-[U-^{14}C]galacturonic acid into D-galacturonosyltransferase product. Assays were performed as described in the Experimental Procedures, but the amount of D-galacturonosyltransferase preparation was varied as indicated. Assays were performed for 5 min.

Figure 4. The effect of time and temperature on the incorporation of D-[U-^{14}C]galacturonic acid into D-galacturonosyltransferase product. Assays were performed as described in the Experimental Procedures, but the length of the incubation period and the temperature of the incubation were varied as indicated. Incubations were performed at 25°C (●) or at 0°C (O). The concentration of UDPGalA in the assay was 10 μM.

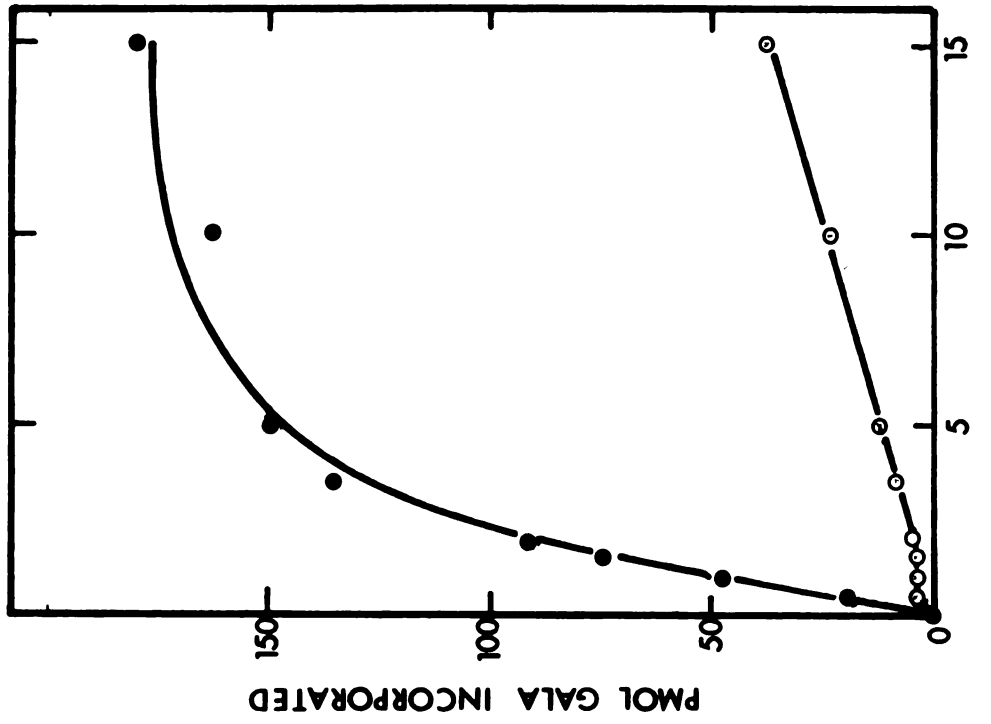

Figure 4

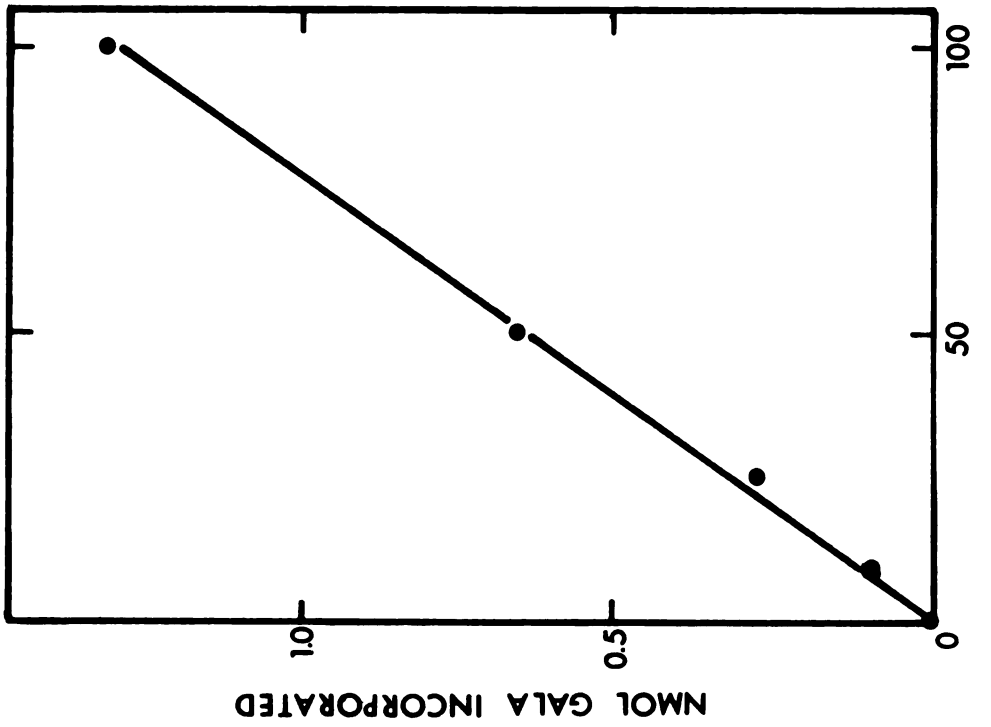

Figure 3

Figure 5. The effect of pH on the rate of incorporation of D-[U-^{14}C]galacturonic acid into D-galacturonosyltransferase product. The values depicted represent data from four separate experiments. In each experiment the rate of D-[U-^{14}C]galacturonic acid incorporation at the pH measured which was closest to 6.0 was set equal to 100. All other rates measured within that experiment were reported relative to this rate. Rates of incorporation were measured in 0.1 M citric acid-50 mM sodium phosphate buffer (●), in 50 mM Tris-maleate buffer (O), or in 50 mM Tris-HCl buffer (▲). All assays were performed as described in the Experimental Procedures, but the composition and pH of the resuspension buffer were varied. The pH values reported in Figure 5 represent the final pH of the reaction mixture. The concentration of UDPGalA in the assay was 10-12 μM.

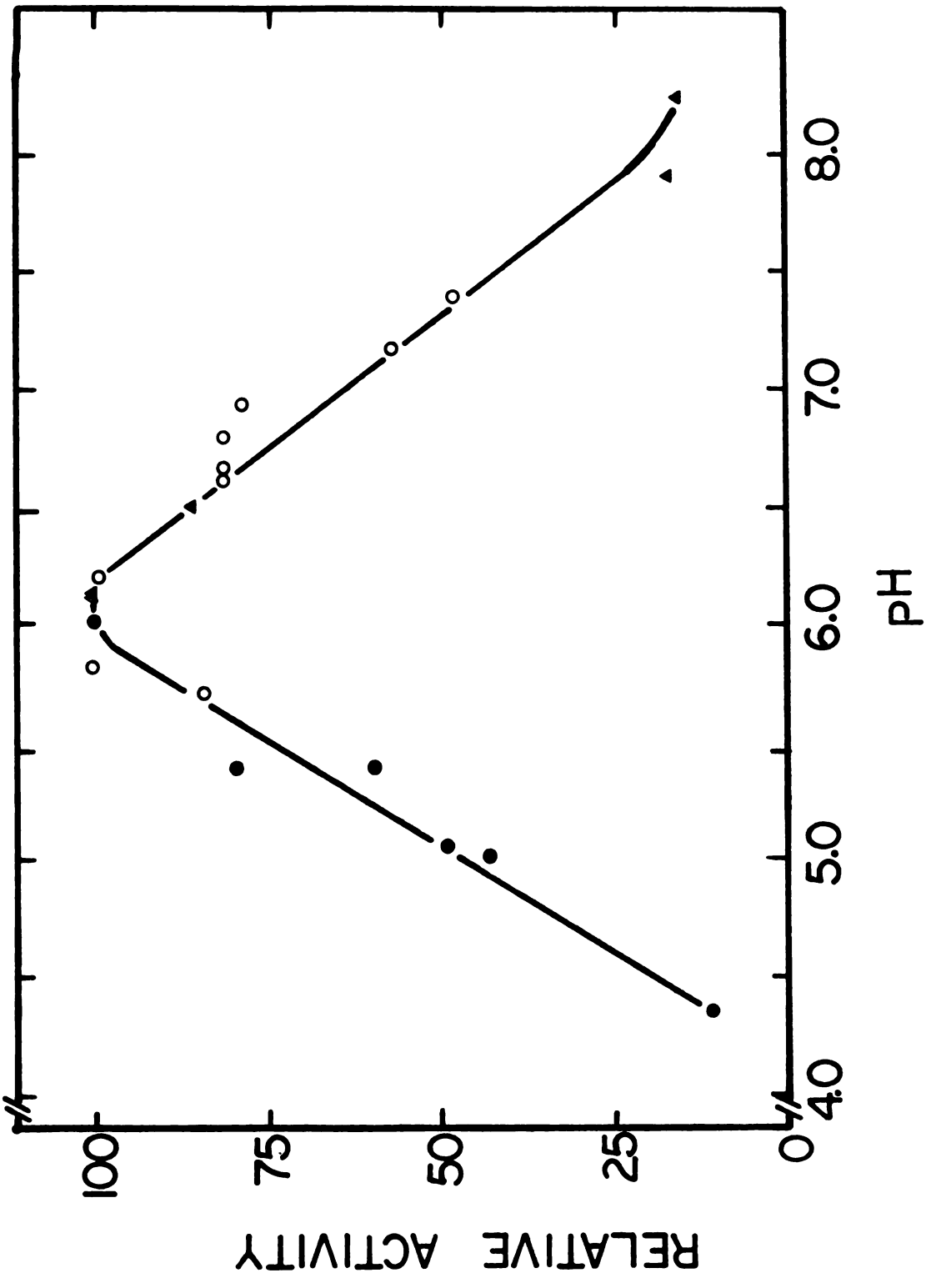

Figure 5

buffers; however, at pH values greater than 6.5, the D-galacturonosyltransferase activity in Tris-HCl buffer was slightly higher than in citric-acid sodium phosphate buffer at the same pH values.

The high buffering capacity of the transferase preparation required use of a resuspension buffer of pH 5.7 to achieve the optimal pH in the assay.

Effect of Various Ions on D-Galacturonosyltransferase Activity

Of all the cations tested, only Mn^{+2} caused significant stimulation of D-galacturonosyltransferase activity (Table 1). The apparent slight stimulations caused by Co^{+2}, Zn^{+2}, and Fe^{+2} are not significant at the $P=0.05$ level. The inhibitions caused by $NiCl_2$ and by all the salts listed below it in Table 1 are significant. Mg^{+2} had no significant effect. The chlorides seem to be the most effective anions. Although all the results presented in Table 1 were obtained at salt concentrations of 5 mM, the same trends were observed at 10 mM.

$MnCl_2$ caused maximum stimulation of the transferase at a concentration of 10 mM whether the $MnCl_2$ was added to the resuspension buffer or to the assay (Figure 6). The sharp decrease in activity at $MnCl_2$ concentrations greater than 10 mM may be a general ionic strength effect since similar decreases were caused by $MgCl_2$ and NaCl.

Although only $MnCl_2$ caused significant stimulation, the presence of 2.5 mM EDTA in the reaction mixture decreased D-galacturonosyltransferase activity by about 40%. Addition of equal concentrations of $MnCl_2$ or $MgCl_2$ with the EDTA did not reverse EDTA inhibition.

Table 1. Effect of various ions on the incorporation of D-[U-^{14}C]-galacturonic acid into D-galacturonosyltransferase product

Addition	nmol D-galacturonic acid incorporated per min ± SD
None	0.161 ± 0.036
$MnCl_2$	0.186 ± 0.037
$MnSO_4$	0.164 ± 0.025
$MgCl_2$	0.155 ± 0.027
$MgSO_4$	0.159 ± 0.041
$Mg(C_2H_3O_2)_2$	0.165 ± 0.055
$CoCl_2$	0.178 ± 0.028
$FeCl_2$	0.173 ± 0.022
$ZnCl_2$	0.171 ± 0.058
$NiCl_2$	0.136 ± 0.018
NH_4Cl	0.133 ± 0.022
NaCl	0.130 ± 0.013
$CaCl_2$	0.114 ± 0.018
$CuCl_2$	0.100 ± 0.012
$HgCl_2$	0.016 ± 0.006

The D-galacturonosyltransferase preparation was isolated as described in the Experimental Procedures except that the resuspension buffer contained no metal ions. The standard D-galacturonosyltransferase assay was modified to contain the ion being tested at a final concentration of 5 mM. The concentration of UDPGalA was 50 μM in all assays.

The effects of adding higher metal ion concentrations were not studied.

Effect of Sucrose on D-Galacturonosyltransferase Activity

The addition of sucrose to the resuspension buffer caused a marked increase in D-galacturonosyltransferase activity. As shown in Figure 7, optimal stimulation occurred at 0.4 to 0.5 M sucrose. However, sucrose decreased D-[U-^{14}C]galacturonic acid incorporation when it was added to the grinding buffer.

Determination of the Apparent K_m for UDPGalA

The effect of UDPGalA concentration on D-galacturonosyltransferase activity is shown in Figure 8, which is a typical Lineweaver-Burk plot of the results. From these plots, an apparent K_m for UDPGalA of 8 μM was determined. The highest rate of incorporation shown in Figure 8 is 0.108 nmol D-galacturonic acid per min, but this value varied considerably depending upon the particular D-galacturonosyltransferase preparation. This must be accepted in a crude particulate enzyme system. In contrast, the apparent K_m value varied little.

Stability of D-Galacturonosyltransferase

Inactivation of the D-galacturonosyltransferase is both spontaneous and temperature-dependent. The enzyme is stable for 2.0 to 2.5 min at 25°C, but less than 10% of the initial activity remains after 10 min. At 0°C, 10% of the initial transferase activity is lost in 30 min. About 60% remains after 3 h, and less than 45% after 5 h. The presence of 10 mM $MnCl_2$ and 0.4 M sucrose provided only slight stabilization at 0°C.

Figure 6. The effect of $MnCl_2$ on the rate of incorporation of D-[U-^{14}C]galacturonic acid into D-galacturonosyltransferase product. Assays were performed as described in the Experimental Procedures. $MnCl_2$ was added to the resuspension buffer at the concentration indicated in Figure 6 ($MnCl_2$ was also added to the reaction mixture so that the concentrations of $MnCl_2$ in the resuspension buffer and in the reaction mixture were the same). The concentration of UDPGalA was 10 µM.

Figure 7. The effect of sucrose on the rate of incorporation of D-[U-^{14}C]galacturonic acid into D-galacturonosyltransferase product. Assays were performed as described in the Experimental Procedures, but the concentration of sucrose in the resuspension buffer was varied as indicated. (The final concentration of sucrose in the reaction mixture was one-half that shown in Figure 7). The grinding medium and the resuspension buffer both contained 5 mM $MnCl_2$. The final concentration of UDPGalA was 50 µM.

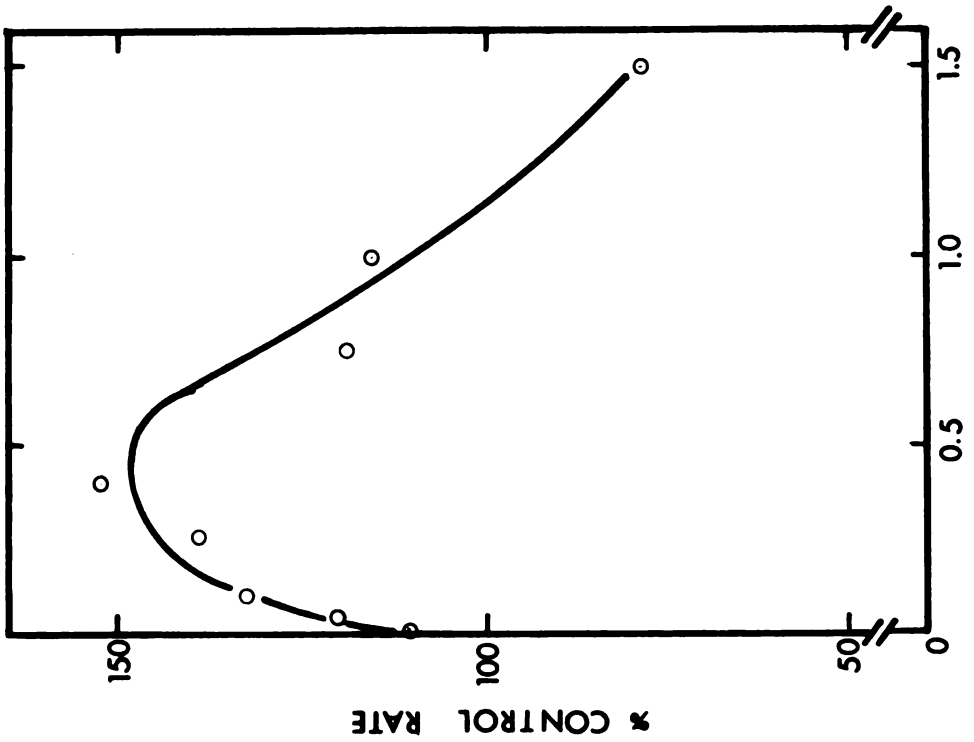

Figure 7

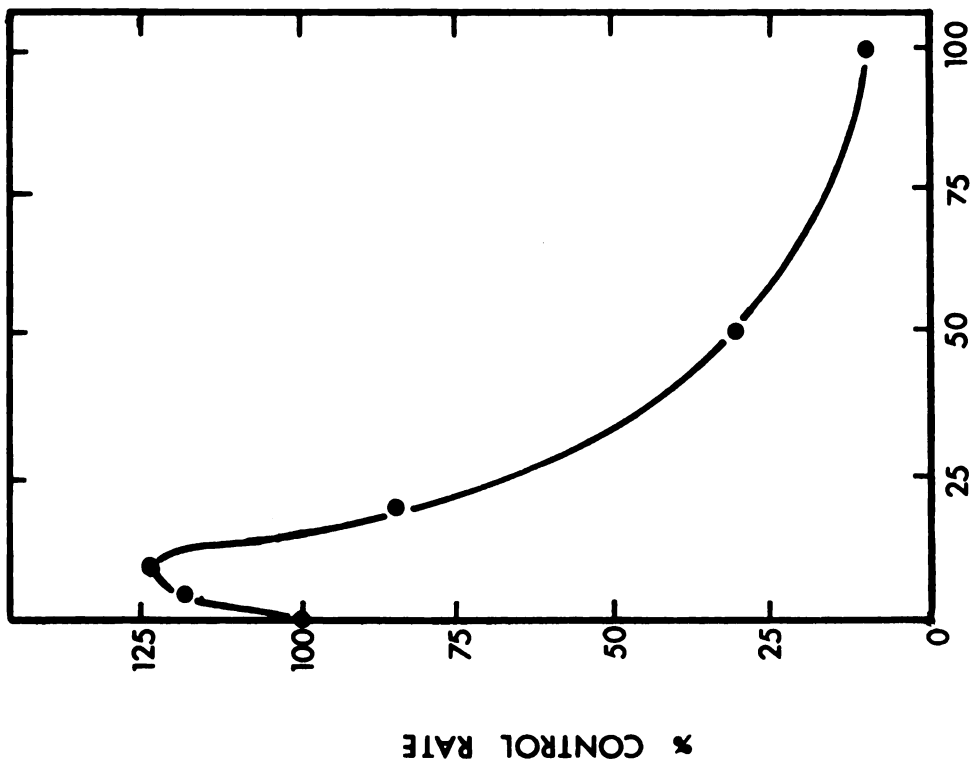

Figure 6

Figure 8. The effect of UDPGalA concentration on the rate of incorporation of D-[U-^{14}C]galacturonic acid into D-galacturonosyltransferase product. Assays were performed as described in the Experimental Procedures, except that the concentration of UDPGalA was varied as indicated.

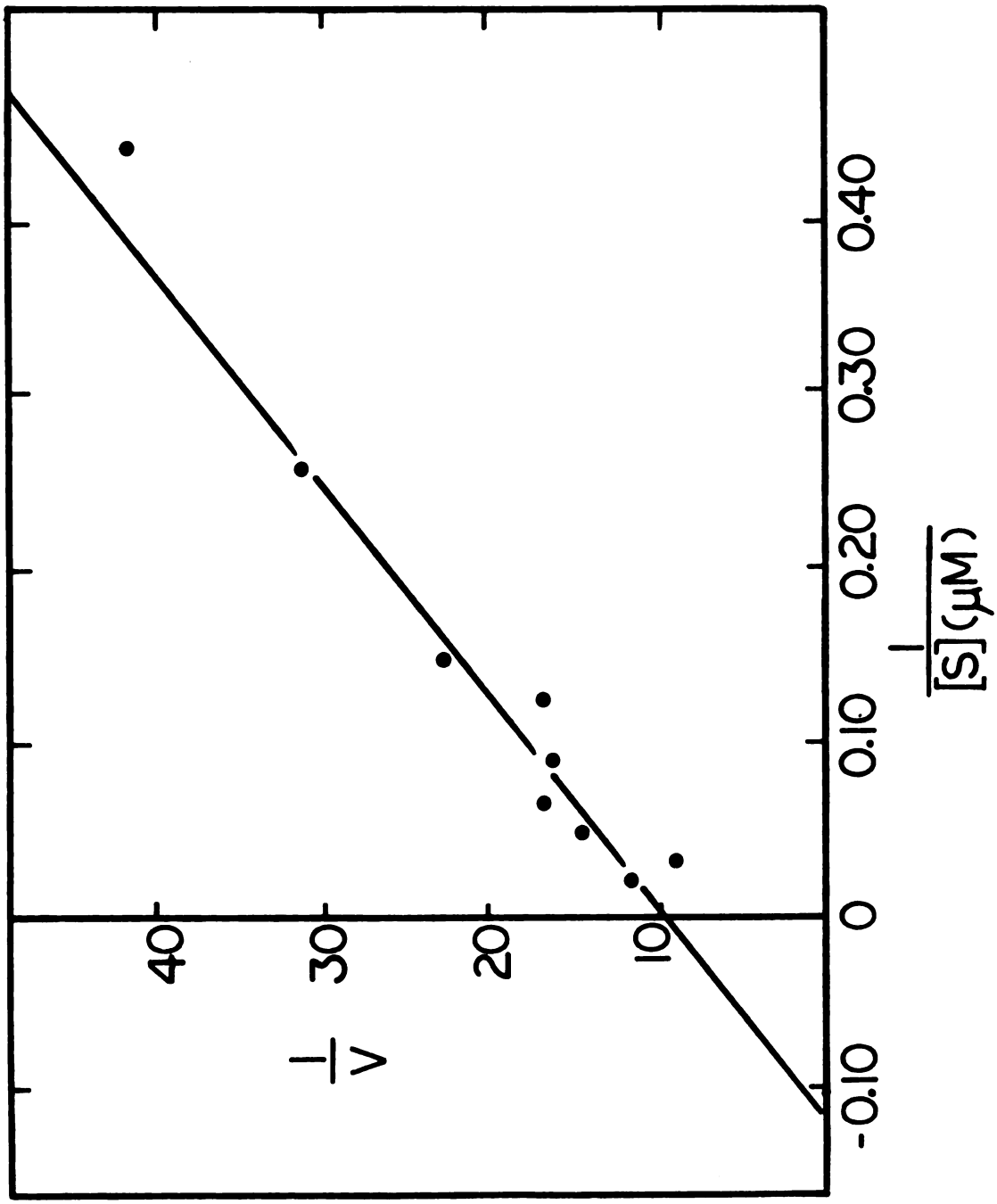

Figure 8

The stability at -20°C is variable. In three experiments, 50% of the initial transferase activity was lost in 24-36 h, whereas in two other experiments, greater than 50% of the initial activity remained after 60 h. The activity seemed to be very sensitive to the method of thawing the frozen transferase preparation. Quick-thawing at 25°C produced greater recovery of activity than slow-thawing in ice water. This may explain some of the variability.

Regardless of the variability in the rate of inactivation, storage in 50% glycerol always caused a 10% to 20% increase in the recovery of transferase activity up to 60 h at -20°C.

A survey of a number of reagents which stabilize other enzymes failed to find any that would significantly stabilize the D-galacturonosyltransferase at 0°C. These reagents included butylated hydroxytoluene, dithiothreitol, 3-mercapto-1,2-propanediol, phenylmethylsulfonyl fluoride and Nupercaine hydrochloride.

Storage of the enzyme in high ionic strength solutions of NaCl and $MgCl_2$ also failed to provide any significant stabilization. As shown previously, high ionic strength also decreased initial D-galacturonosyltransferase activity.

Effect of UDP-Apiose on D-Galacturonosyltransferase Activity

The major known galacturonan of *L. minor* cell walls is a heteropolysaccharide containing D-apiose. Therefore, the effect of UDP-apiose on D-[U-^{14}C]galacturonic acid incorporation was studied. The incorporation of D-[U-^{14}C]galacturonic acid into polysaccharide with time in the absence of UDP-apiose and in the presence of 3.0 μM UDP-apiose is shown in Figure 9. The presence of UDP-apiose did not

Figure 9. The effect of UDP-apiose on the incorporation of D-[U-^{14}C]galacturonic acid into D-galacturonosyltransferase product. Assays were performed as described in the Experimental Procedures, except that 50 mM Tris-maleate buffer (pH 5.7) was used for the resuspension buffer. Assays contained 10 µM UDPGalA (●) or 10 µM UDPGalA + 3 µM UDP-apiose (O).

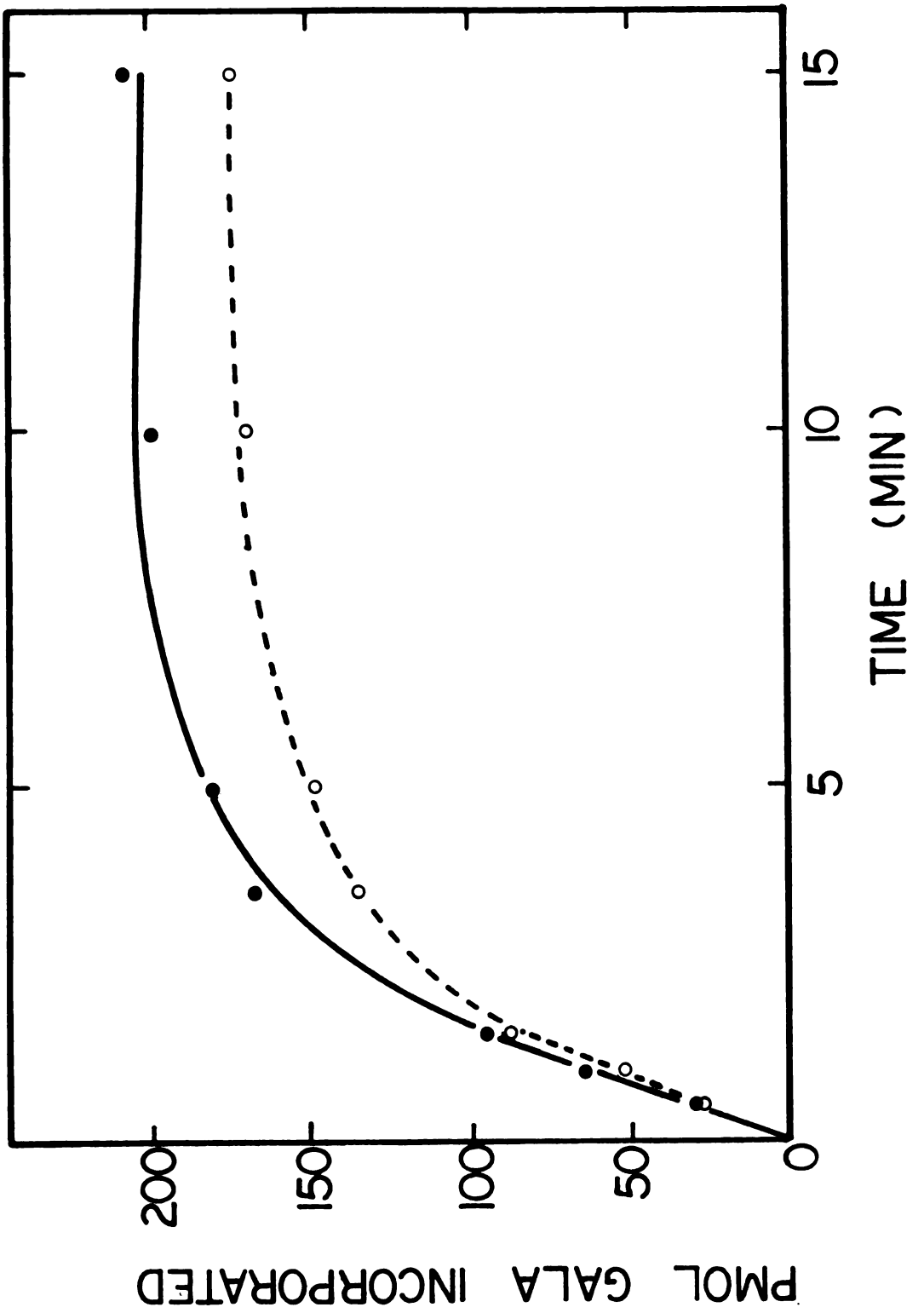

Figure 9

increase D-[U-^{14}C]galacturonic acid incorporation. In fact, UDP-apiose slightly decreased both the rate and the extent of D-[U-^{14}C]-galacturonic acid incorporation. These results are in marked contrast to the increase in D-[U-^{14}C]apiose incorporation caused by UDPGalA (64).

Effects of Detergents on Activity and Solubilization

As a prelude to attempting to solubilize the particulate D-galacturonosyltransferase, the effects of a number of ionic and non-ionic detergents on incorporation of D-[U-^{14}C]galacturonic acid were studied (Table 2). At a concentration of 0.1% most detergents decreased incorporation by no more than 20% when they were added to the D-galacturonosyltransferase preparation just before assay. At this concentration sodium cholate, Emulgen-911 and Tween-20 had no significant effect at the P=0.1 level. However, all but Tween-20 caused almost complete loss of activity by the time their concentration was increased to 1.0%. Mixing Tween-20 with any other detergent caused significant inhibition at all concentrations (P=0.1).

Tween-20, Triton X-100, Emulgen-911, and sodium cholate were tested for their ability to solubilize the particulate D-galacturonosyltransferase. The transferase was prepared by the usual procedure (see Experimental Procedures) except that the 34,800*g* precipitate was resuspended in buffer containing the appropriate detergent at concentrations from 0.1 to 1.0%. The resuspended preparation was then centrifuged for 10 min and the supernatant and pellet were assayed for D-galacturonosyltransferase.

In none of these studies was more than 20% solubilization achieved. The presence of detergent caused at least 50% loss of

Table 2. Effect of various detergents on D-galacturonosyltransferase activity

Addition	Concentration in % (v/v)	Amount of original D-galacturonosyltransferase activity remaining (%) ± SD
None	--	100 ± 6
Triton X-100	0.01	103 ± 16
	0.10	91 ± 15
	1.0	24 ± 14
NP-40	0.01	74 ± 3
	0.10	76 ± 6
	1.0	7 ± 0
Emulgen-911	0.10	102 ± 5
	1.0	18 ± 3
Emulgen-913	0.10	80 ± 0
	1.0	31 ± 1
Tween-20	0.10	97 ± 6
	1.0	92 ± 6
Sarkosyl-97	0.01	80 ± 3
	0.10	75 ± 0
	1.0	12 ± 4
Sodium cholate	0.10	108 ± 1
	1.0	6 ± 1
Sodium deoxycholate	0.10	84 ± 1
	1.0	11 ± 0

The D-galacturonosyltransferase preparation was isolated as described in the Experimental Procedures except that the detergent being tested was added to the resuspension buffer at the appropriate concentration. All samples were assayed within five minutes of resuspension. The standard D-galacturonosyltransferase assay contained 10 mM UDPGalA.

initial D-galacturonosyltransferase activity during this second 34,800*g* centrifugation. Addition of various amounts of glycerol up to a final concentration of 75% had varying effects on the recovery of transferase activity. As a further complication, the presence of glycerol made separation of the supernatant and pellet fractions more difficult after the second 34,800*g* centrifugation, so that determination of "solubilized" activity was much more uncertain.

Attempts to restore activity by adding boiled transferase preparation to the second 34,800*g* supernatant or by recombining supernatant and pellet fractions were also unsuccessful. Apparently, any treatment that is capable of severely disrupting the membrane system which encloses the transferase will cause significant loss of activity. Further attempts at solubilization will require procedures more elaborate than those described here.

Characterization of the Product of the D-Galacturonosyltransferase Reaction

Efficiency of Solubilization

The efficiency of solubilization of the D-galacturonosyltransferase product by ammonium oxalate and by sodium hexametaphosphate was determined at 50°C (Table 3).

Sodium hexametaphosphate is the more effective solubilizing agent (extractant). The amount of product solubilized remains unchanged at hexametaphosphate concentrations as low as 0.10%. The efficiency of solubilization decreases rapidly at ammonium oxalate concentrations below 0.5%.

Fifty percent extraction requires at least a five-fold higher concentration of ammonium oxalate than of sodium hexametaphosphate.

Table 3. Efficiency of solubilization of D-galacturonosyltransferase product with ammonium oxalate and sodium hexametaphosphate

Concentration of extractant (%) (w/v)	Amount of product solubilized by: Ammonium oxalate (%)	Sodium hexametaphosphate (%)
2.0	ND[a]	78
1.0	86	83
0.50	84	80
0.25	55	78
0.10	33	82
0.050	ND	68
0.025	ND	23
0.010	ND	11

[a]ND: value not determined.

D-Galacturonosyltransferase product was prepared and was extracted with ammonium oxalate or with sodium hexametaphosphate as described in the Experimental Procedures, except that the concentration of extractant was varied as indicated.

Size of the Solubilized Product

The sizes of the D-galacturonosyltransferase products solubilized with the two extractants were compared by chromatography on Bio-Gel P-300 with 50 mM sodium phosphate buffer (Figure 10). Both the 1% ammonium oxalate-solubilized product and the 2% sodium hexametaphosphate-solubilized product eluted throughout the fractionation range of Bio-Gel P-300. This gel reportedly separates dextrans with weight-average molecular weights between 5,000 and 125,000 (99). About 40% of the 2% sodium hexametaphosphate-solubilized product eluted from the P-300 column in V_o (fractions 8-11) versus less than 10% of the 1% ammonium oxalate-solubilized product. Thus, the 2% sodium hexametaphosphate-solubilized product apparently contained more material of apparent high molecular weight than did the 1% ammonium oxalate-solubilized product. This was surprising since these two extractants solubilize approximately equal amounts of D-galacturono-syltransferase product.

The size of the 2% sodium hexametaphosphate-solubilized product was studied further by chromatography on agarose gels with higher exclusion limits. A molecular weight of 1×10^6 is the upper exclusion limit of Sepharose 6B for polysaccharides (99). When the 2% sodium hexametaphosphate-solubilized product was chromatographed on this gel with 50 mM sodium phosphate buffer, about 30% of the radioactive product eluted in V_o (fractions 10-13) (Figure 11A). This product is larger than any previously reported galacturonan.

To study the size of this product further, material eluting from Bio-Gel P-300 in V_o was rechromatographed on Sepharose 4B (Figure 12B) with 50 mM sodium phosphate buffer. The

Figure 10. Chromatography of 1% ammonium oxalate-solubilized product and 2% sodium hexametaphosphate-solubilized product on Bio-Gel P-300.

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate (●) or 2% sodium hexametaphosphate (O) as described in the Experimental Procedures. The two products were chromatographed on a Bio-Gel P-300 column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 3-4 ml per h. The two products contained 25,000 and 19,500 dpm, respectively. Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity which was collected in each fraction. Total recoveries of the two products from the columns were 85% and 90%, respectively.

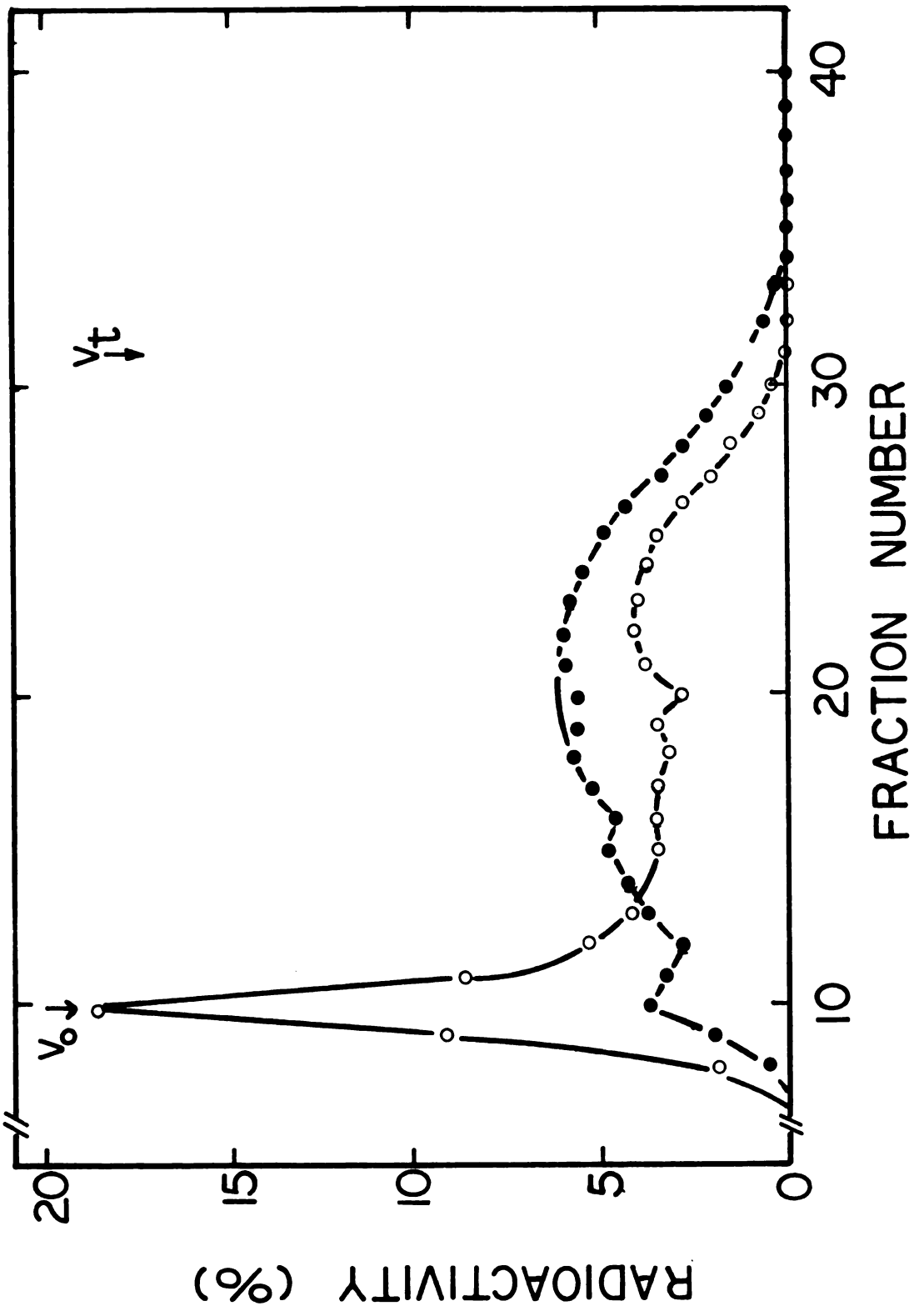

Figure 10

Figure 11. Chromatography of 2% sodium hexametaphosphate-solubilized product on Sepharose 6B (A) and rechromatography of Fraction 11 on Sepharose 2B (B).

D-Galacturonosyltransferase product was prepared and was extracted with 2% sodium hexametaphosphate as described in the Experimental Procedures. The solubilized product, containing 28,000 dpm, was chromatographed on a Sepharose 6B column that was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 5 ml per h (A). Fractions (1.0 ml) were collected and 100 µl aliquots were assayed for radioactivity in scintillation solution B. The material collected in Fraction 11 (2,900 dpm) was rechromatographed on a Sepharose 2B column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 5 ml per h (B). Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Radioactivity values (%) represent the percent of total eluted radioactivity collected in each fraction. Total recovery from the columns was 85% for the product and 97% for Fraction 11.

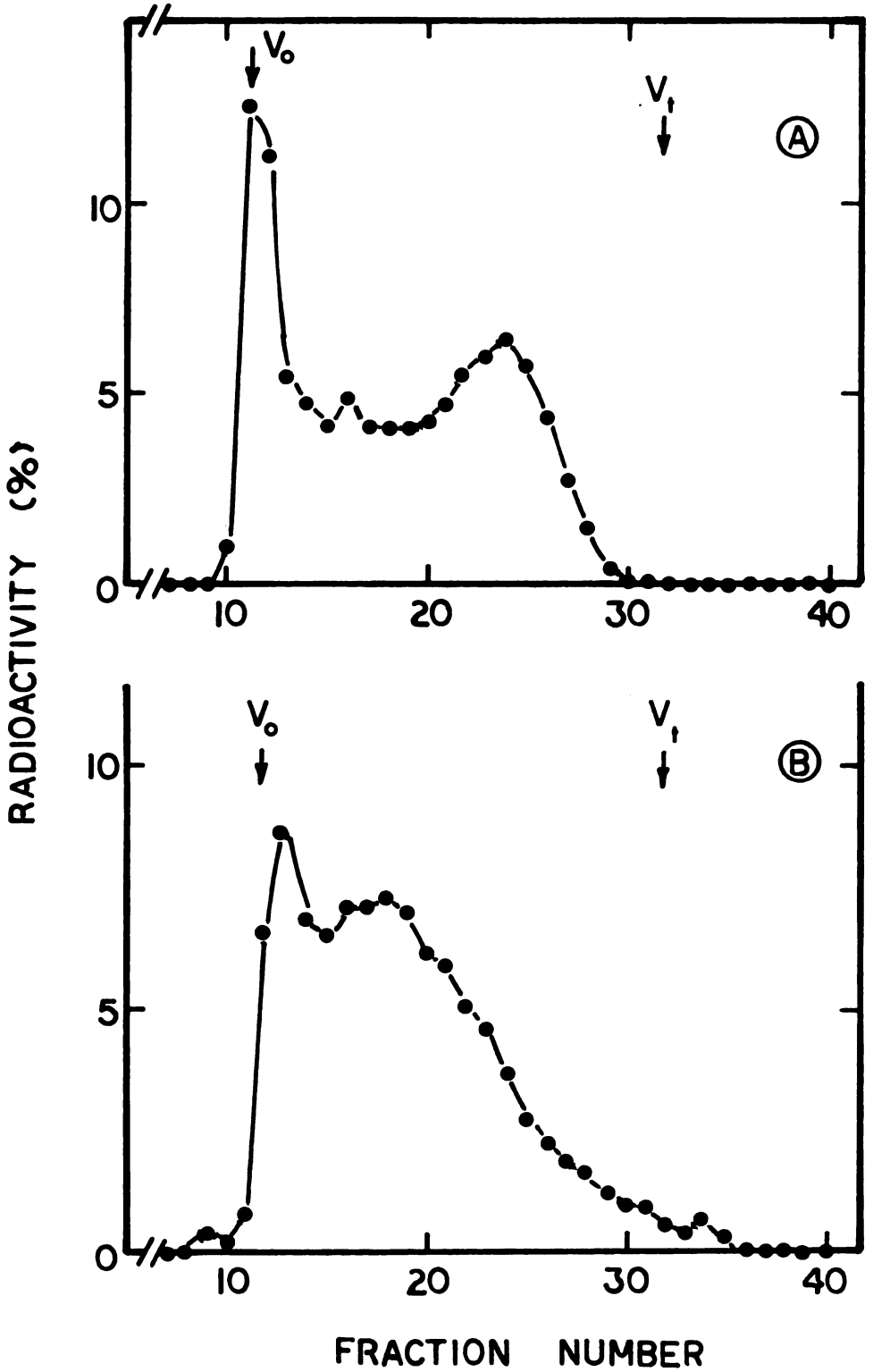

Figure 11

Figure 12. Chromatography of 2% sodium hexametaphosphate-solubilized product on Bio-Gel P-300 (A) and rechromatography of Fraction 10 on Sepharose 4B (B).

D-Galacturonosyltransferase product was prepared and was extracted with 2% sodium hexametaphosphate as described in the Experimental Procedures. The solubilized product, containing 35,500 dpm, was chromatographed on a Bio-Gel P-300 column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 4 ml per h (A). Fractions (1.0 ml) were collected and 100 µl portions were assayed for radioactivity in scintillation solution B. The material collected in Fraction 10 (4,400 dpm) was rechromatographed on a Sepharose 4B column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 5 ml per h (B). Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Radioactivity (%) values represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from the columns were 80% for both the 2% sodium hexametaphosphate-solubilized product and for Fraction 10.

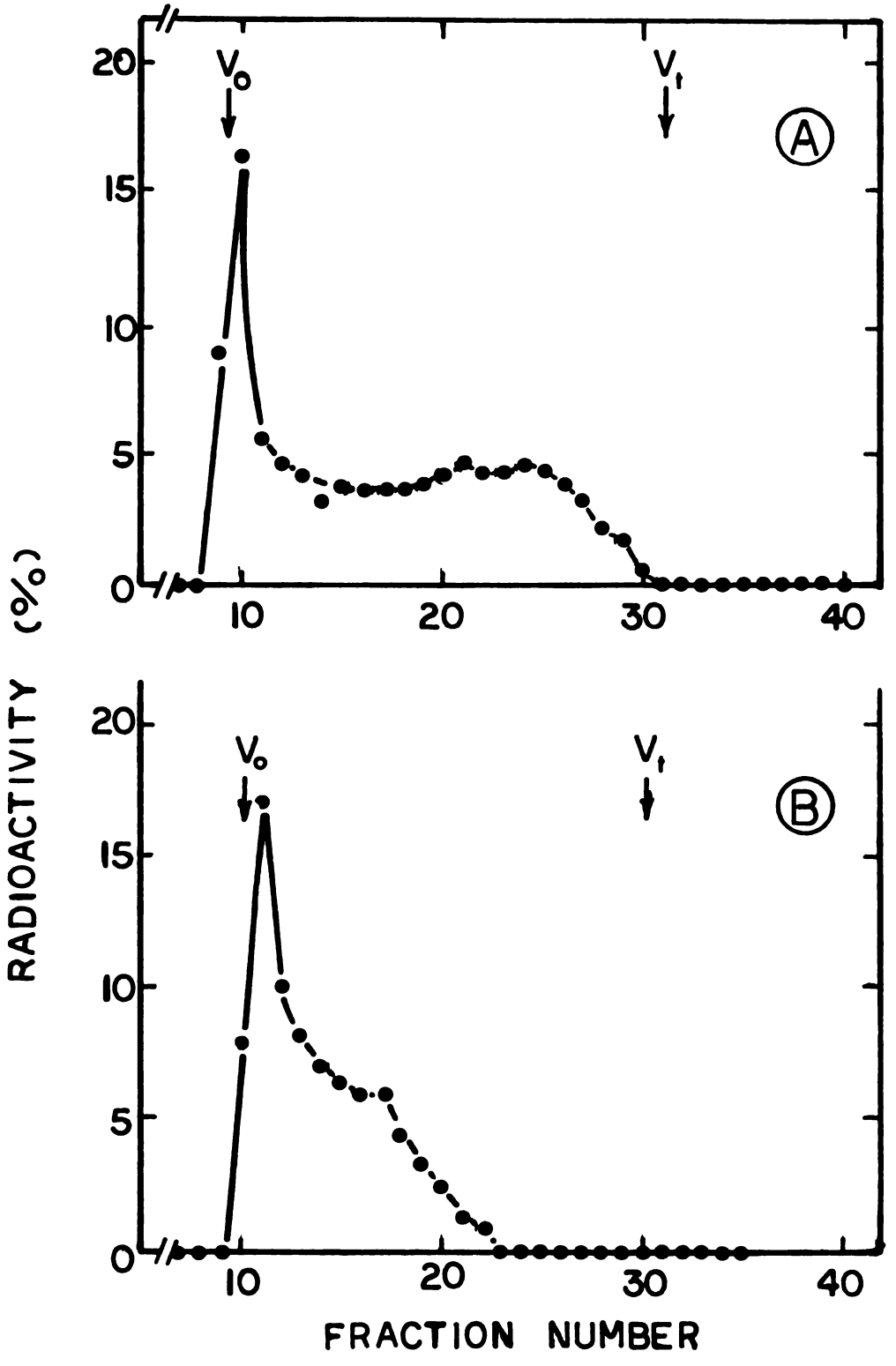
RADIOACTIVITY (%)
20
15
10
5
0
V_o
V_t
A
10
20
30
40
20
15
10
5
0
V_o
V_t
B
10
20
30
40
FRACTION NUMBER

Figure 12

rechromatographed material contained a wide range of sizes of polysaccharides. However, 40% of this material still eluted in V_o (fractions 9-12), indicating that its apparent molecular weight exceeded 4×10^6 (99).

Sepharose 2B, with an upper exclusion limit of molecular weight 2×10^7 (100) was used to determine the upper size limit of the 2% sodium hexametaphosphate-solubilized product. The product was first chromatographed on Sepharose 6B, and the material eluting in V_o was rechromatographed on Sepharose 2B (Figure 11). Although the bulk of the material eluted within the fractionation range of the column, about 20% still eluted in V_o (fractions 11-14).

Based on these results, two possibilities were considered: either sodium hexametaphosphate and ammonium oxalate solubilize different populations of radioactively labeled polysaccharides, or extraction with one or both of these solubilizing agents alters the actual size of the polysaccharides. The former seems unlikely, since both extractants solubilize about 80% of the total radioactively labeled product. Experiments were designed to test the latter possibility.

Stability of the Solubilized Products

The 2% sodium hexametaphosphate-solubilized product was unaffected by dialysis in 50 mM sodium phosphate buffer (pH 6.8) for 3 h at 4°C, nor was its size decreased by dialysis in water for up to 43 h at 4°C based on chromatography on Bio-Gel P-300 with 50 mM sodium phosphate buffer. Recoveries after dialysis varied from 65% to 89% for dialysis in water and from 67% to 76% for dialysis in sodium

phosphate buffer. The cause of this loss and its variability is unknown; however, radioactivity did not seem to be lost from any particular portion of the elution profile since the shape of the profile was unchanged by dialysis.

In contrast, dialysis in water for 3 h converted the 1% ammonium oxalate-solubilized product to a product that eluted as a single broad peak throughout the fractionation range of a Bio-Gel P-100 column. Before dialysis, 40% of the 1% ammonium oxalate-solubilized product eluted from P-100 in V_o, but after dialysis, only 25% eluted in V_o.

These results suggest that the 2% sodium hexametaphosphate-solubilized product may represent an extensively and tightly aggregated form of the D-galacturonosyltransferase product. The 1% ammonium oxalate-solubilized product seems to be more loosely and less extensively aggregated.

When the product solubilized with 1% ammonium oxalate was allowed to stand on ice at 4°C for 12-24 h, its size decreased dramatically (Figure 13A). Although dialysis in water decreased the size of the 1% ammonium oxalate-solubilized product, the dialyzed product was almost completely stable for at least 24 h (Figure 13B). Prolonged storage of the solubilized product in ammonium oxalate may cause some degradation such as has been reported previously (35). No such decrease in size on standing was seen in the case of the 2% sodium hexametaphosphate-solubilized product.

When 1% ammonium oxalate-solubilized product was dialyzed in 1% ammonium oxalate, the size of the product decreased such that

Figure 13. Decrease in size of the 1% ammonium oxalate-solubilized product on standing at 4°C (A) and the effect of dialysis in water on this decrease (B).

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures.

(A) One sample of the solubilized product, containing 45,000 dpm, was divided, and one-half was chromatographed immediately on a Bio-Gel P-200 column that was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 3-4 ml per h (●). The other half was stored at 4°C for 18 h and then was chromatographed in the same manner as the first half (O).

(B) Another sample of the solubilized product, containing 48,000 dpm, was dialyzed in water at 4°C for 3 h. Recovery from dialysis was 69%. The dialyzed product was divided, and one-half was chromatographed immediately on Bio-Gel P-200 as described above (●). The other half was stored at 4°C for 18 h and then was chromatographed on Bio-Gel P-200 as described above (O).

For all samples, 1.0 ml fractions were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from chromatography for the samples shown in (A) were 98% (●) and 78% (O). For the samples in (B), total recoveries from chromatography were 88% (●) and 100% (O).

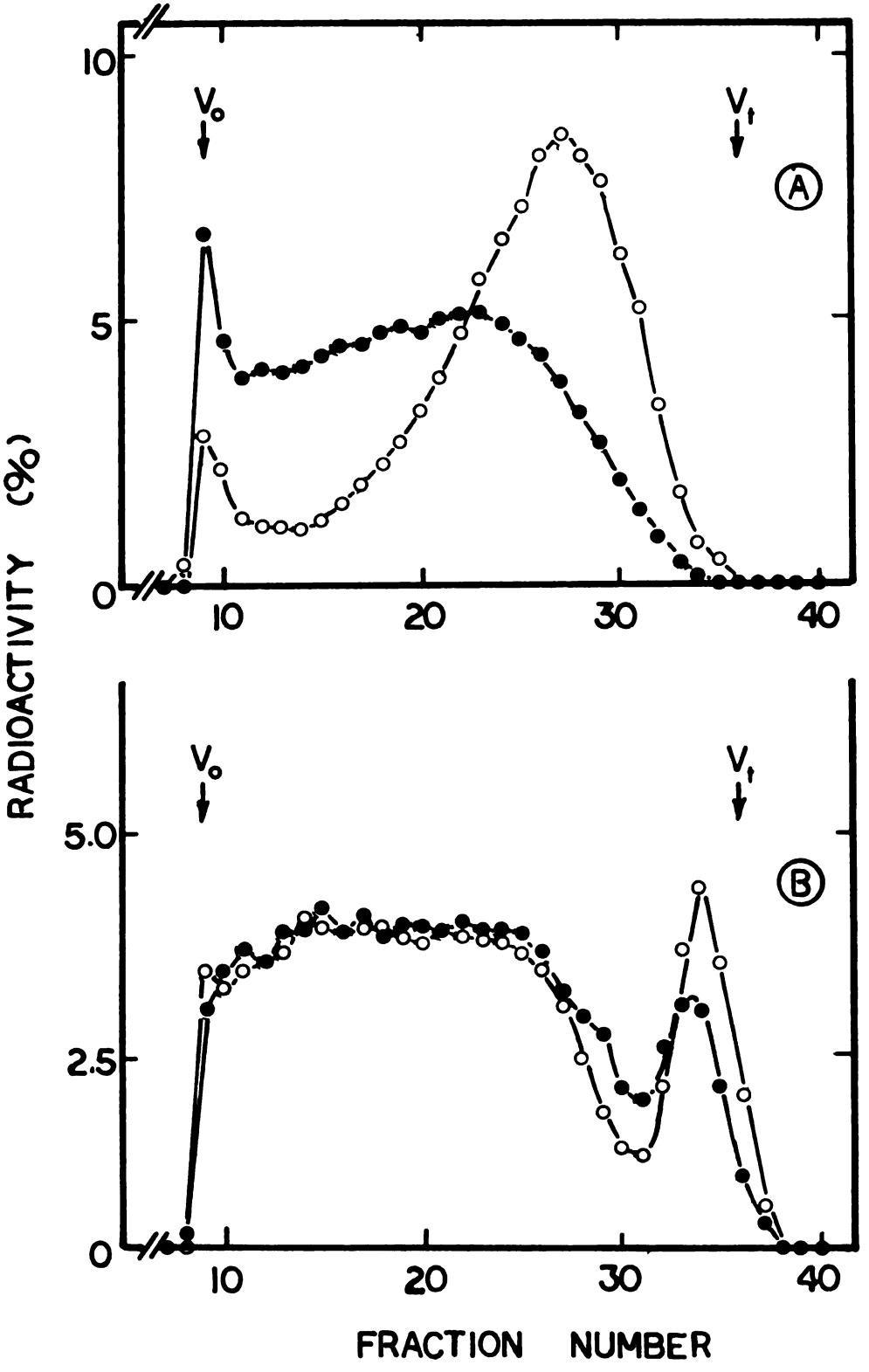
RADIOACTIVITY (%)
10
5
0
V_o
V_t
A
10
20
30
40
5.0
2.5
0
B
FRACTION NUMBER

Figure 13

most of the radioactivity was associated with material eluting from Bio-Gel P-300 near V_t (Figure 14). In contrast, the size of the 2% sodium hexametaphosphate-solubilized product was not altered by dialysis in 1% ammonium oxalate (data not presented).

As a further test of the apparent degradative effect of 1% ammonium oxalate, 1% ammonium oxalate-solubilized product was first dialyzed in water for 3 h and then in 1% ammonium oxalate for 3 h, both at 4°C (Figure 15). Although dialysis in water decreased the size of the solubilized product somewhat, dialysis in 1% ammonium oxalate decreased the size of the water-dialyzed product even more. These results provide the strongest evidence that I have obtained that 1% ammonium oxalate degrades or disaggregates the solubilized D-galacturonosyltransferase product. They also suggest that extraction with 1% ammonium oxalate may partially degrade the galacturonans synthesized *in vitro*. Similar experiments with material synthesized *in vivo* have not been performed yet.

Induction of Aggregation

The suggestion that the 2% sodium hexametaphosphate-solubilized product might represent an aggregated form of the D-galacturonosyltransferase product was tested by dialyzing 1% ammonium oxalate-solubilized product in 2% sodium hexametaphosphate for 3 h. When the dialyzed and undialyzed products were chromatographed on Bio-Gel P-300 with 50 mM sodium phosphate buffer, the elution profiles of the two products were almost identical. However, when the concentration of sodium hexametaphosphate used for dialysis was increased to 3%, the amount of the 1% ammonium oxalate-solubilized product eluting

Figure 14. Effect of dialysis in 1% ammonium oxalate on the size of the 1% ammonium oxalate-solubilized product.

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures. One sample (A), containing 25,000 dpm, was chromatographed directly on a Bio-Gel P-300 column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 3-4 ml per h. A second sample (B) containing 25,000 dpm was dialyzed in 1% ammonium oxalate for 3 h at 4°C. Recovery from dialysis was 71%. The dialyzed sample was then chromatographed on a Bio-Gel P-300 column in the same manner as sample (A). For both samples, 1.0 ml fractions were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from chromatography for samples (A) and (B) were 85 and 87%, respectively.

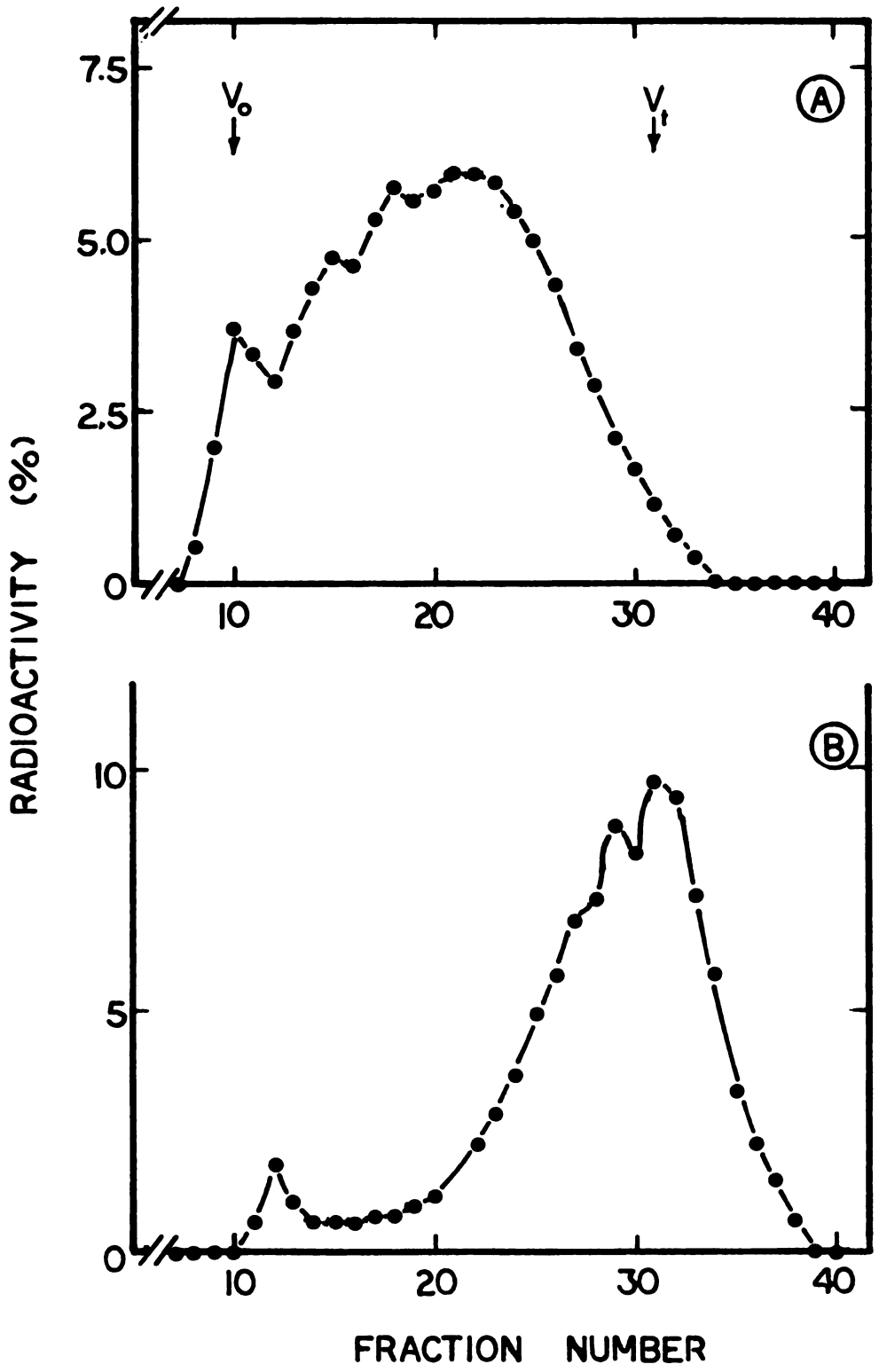
7.5
5.0
2.5
0
V_o
V_t
A
10
20
30
40
RADIOACTIVITY (%)
10
5
0
B
10
20
30
40
FRACTION NUMBER

Figure 14

Figure 15. Effect of dialysis in 1% ammonium oxalate on the size of 1% ammonium oxalate-solubilized product that was dialyzed in water.

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures. One sample of the solubilized product (O) was dialyzed in water at 4°C for 3 h and then for 3 h in 1% ammonium oxalate at 4°C. Another sample of solubilized product (●) was dialyzed in water at 4°C for 6 h. Recoveries from dialysis were 60 and 71%, respectively. Each sample was then chromatographed on a Bio-Gel P-100 column that was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 4 ml per h. For both samples, 1.0 ml fractions were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from chromatography were 90% for the sample dialyzed in both water and ammonium oxalate and 91% for the sample dialyzed in water alone.

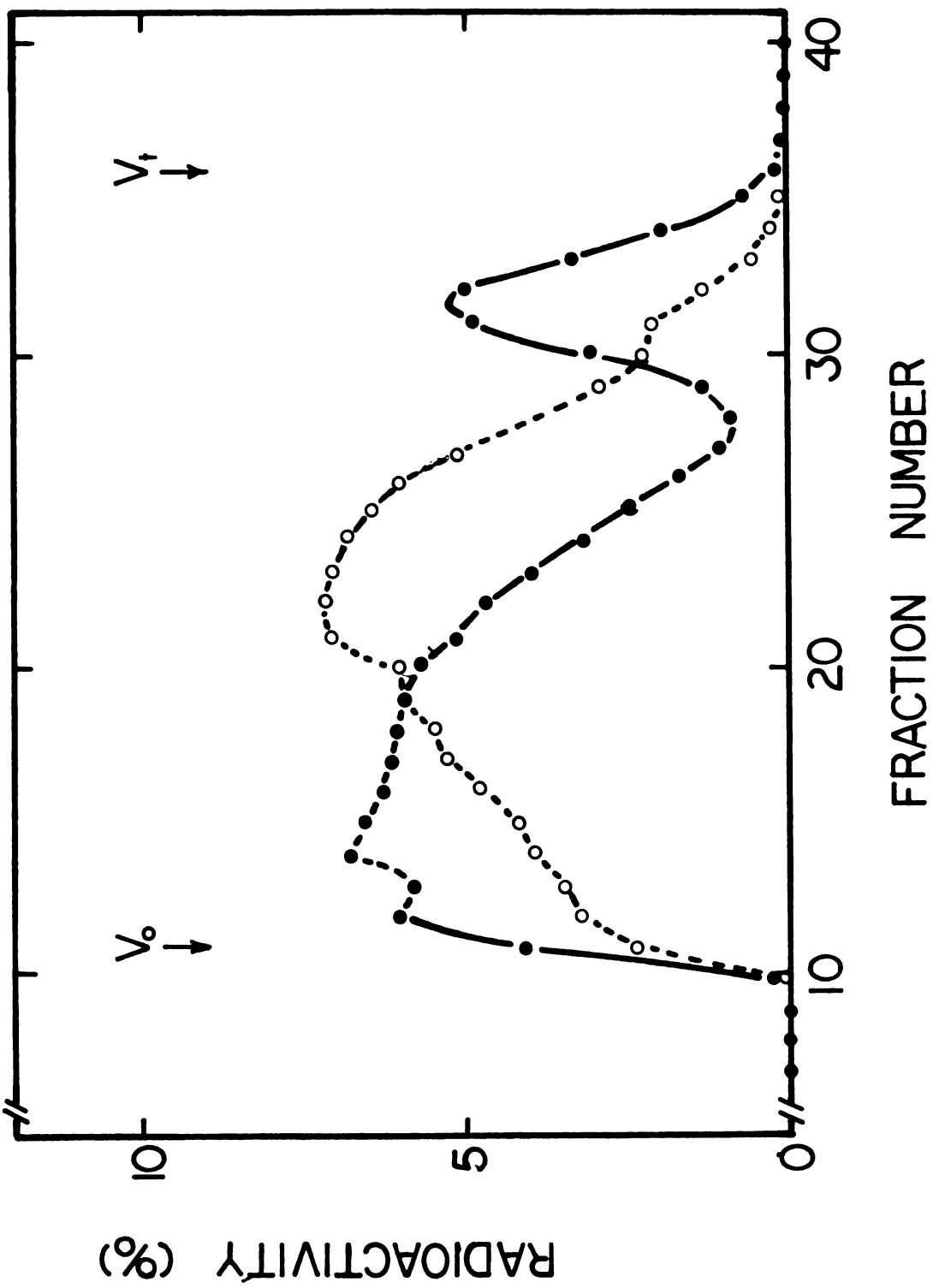

Figure 15

from P-300 in V_o (fractions 8-11) tripled (Figure 16). Therefore, since sodium hexametaphosphate aggregated ammonium oxalate-solubilized product, the 2% sodium hexametaphosphate-solubilized product may well have containted polysaccharides aggregated to various extents.

When 1% ammonium oxalate-solubilized product was dialyzed for 3 h in 3% ammonium oxalate, the Bio-Gel P-300 elution profile of the 1% ammonium oxalate-solubilized product was not altered significantly in contrast to the apparent degradation caused by dialysis in 1% ammonium oxalate as shown in Figure 14. When 1% ammonium oxalate-solubilized product was first dialyzed in 1% ammonium oxalate and then was dialyzed for 3 h in 3% ammonium oxalate, the size of the product increased substantially (Figure 17). Thus it appears that the ability to aggregate the solubilized D-galacturonosyltransferase product is not a property of sodium hexametaphosphate alone, but rather that the aggregation may be caused, at least in part, by increasing the ionic strength of the medium in which the solubilized product is placed.

If aggregation is ionic strength-dependent, then dialysis in solutions of salts other than ammonium oxalate and sodium hexametaphosphate should also cause aggregation. When 1% ammonium oxalate-solubilized product was dialyzed in 1.0 M NaCl for 3 h and then was chromatographed on Ultrogel AcA 22, 22% of the radioactive material eluted in V_o (fractions 9-12) as opposed to less than 1.0% for undialyzed product (Figure 18). Dialysis in 0.33 M $CaCl_2$, which has an ionic strength equal to that of 1.0 M NaCl, led to formation of a thick white precipitate which could not be chromatographed. Therefore,

Figure 16. Effect of dialysis in 3% sodium hexametaphosphate on the size of 1% ammonium oxalate-solubilized product.

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures. One sample of solubilized product, containing 25,000 dpm, was chromatographed directly on a Bio-Gel P-300 column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 3-4 ml per h (●). A second sample, containing 46,000 dpm, was dialyzed for 3 h in 3% sodium hexametaphosphate at 4°C. Recovery from dialysis was 69%. The dialyzed sample was then chromatographed on a Bio-Gel P-300 column (O) in the same manner as was the undialyzed sample. For both samples, 1.0 ml samples were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from chromatography were 85% for the undialyzed sample and 100% for the dialyzed sample.

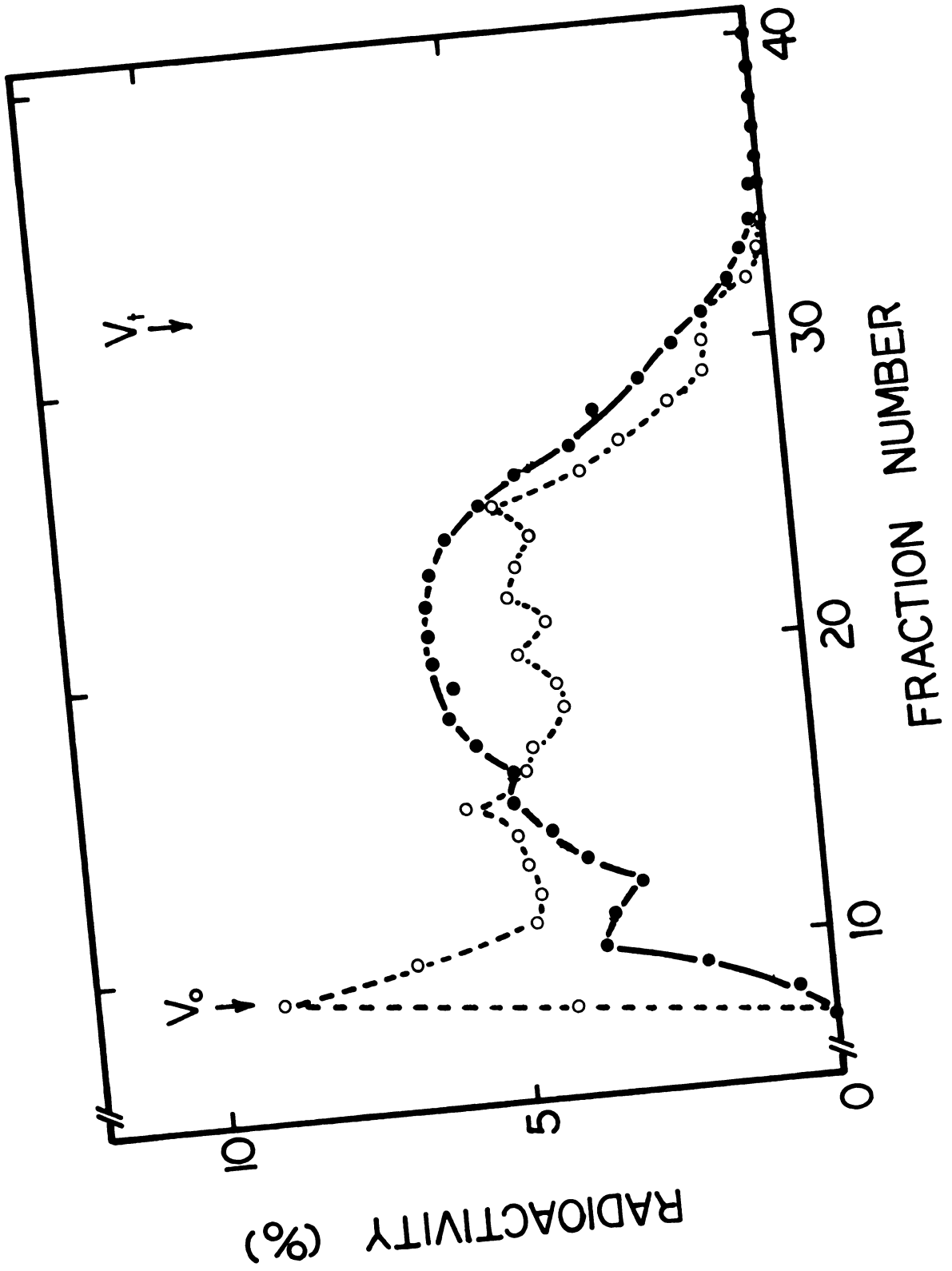

Figure 16

Figure 17. Effect of dialysis in 3% ammonium oxalate on the size of the 1% ammonium oxalate-solubilized product which was dialyzed in 1% ammonium oxalate.

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures. One sample (●), containing 25,000 dpm, was dialyzed in 1% ammonium oxalate at 4°C for 3 h. A second sample (○), containing 52,000 dpm, was dialyzed in 1% ammonium oxalate for 3 h and then in 3% ammonium oxalate for 3 h at 4°C. Recoveries from dialysis were 71% (●) and 54% (○). Each sample was then chromatographed on a Bio-Gel P-300 column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 3-4 ml per h. For both samples, 1.0 ml fractions were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from chromatography were 87% for the sample dialyzed in 1% ammonium oxalate alone and 92% for the sample dialyzed in both 1% and 3% ammonium oxalate.

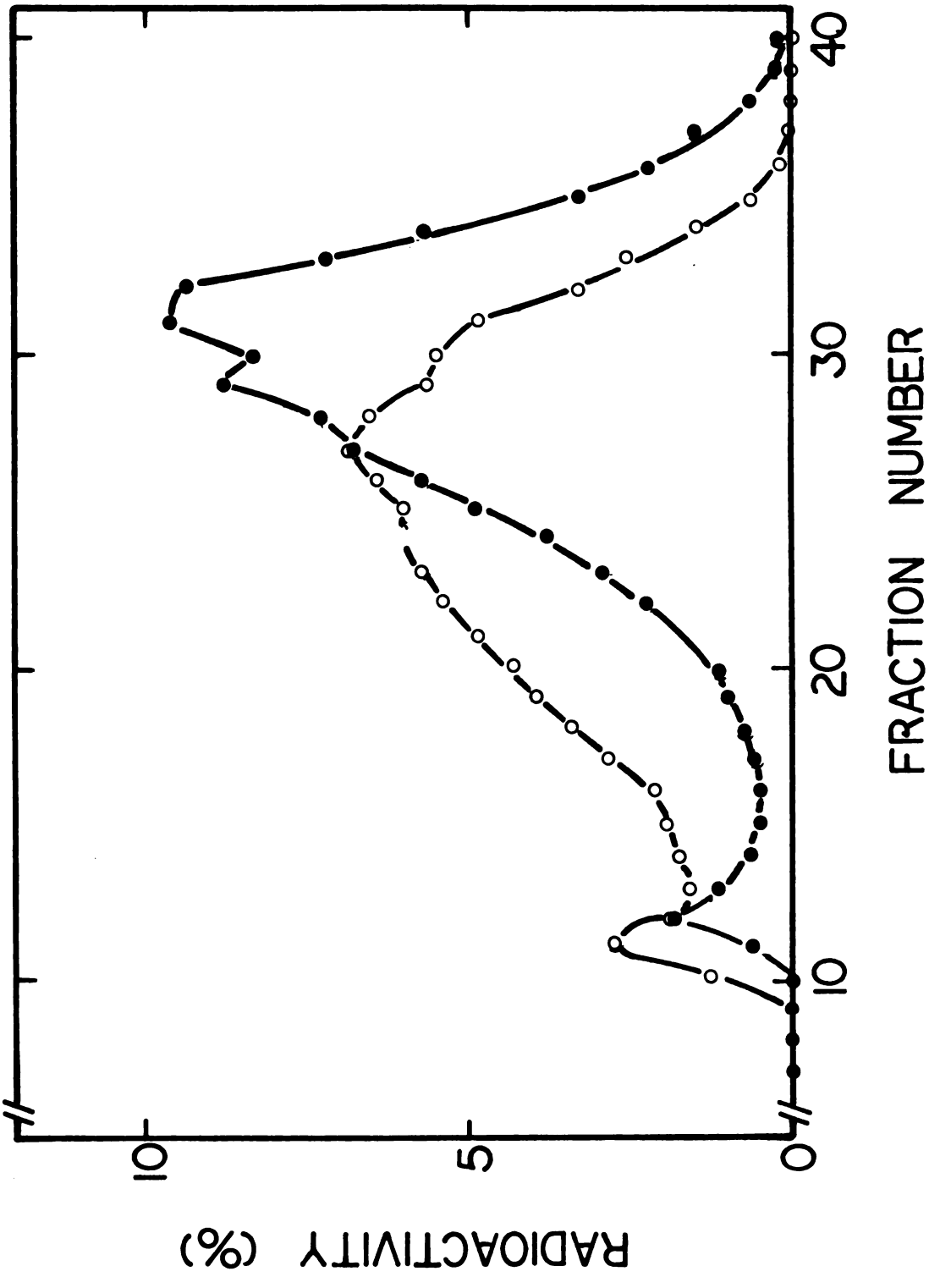

Figure 17

Figure 18. Effect of dialysis in 1.0 M NaCl on the size of the 1% ammonium oxalate-solubilized product.

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures. One sample of the solubilized product, containing 40,000 dpm, was chromatographed directly on an Ultrogel AcA 22 column that was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 5 ml per h (●). A second sample, containing 32,000 dpm, was dialyzed in 3 l of 1.0 M NaCl for 3 h at 4°C. Recovery from dialysis was 67%. The dialyzed sample was chromatographed in the same manner as the non-dialyzed sample (O). Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recovery for the non-dialyzed sample was 98%; for the dialyzed sample, total recovery was 90%.

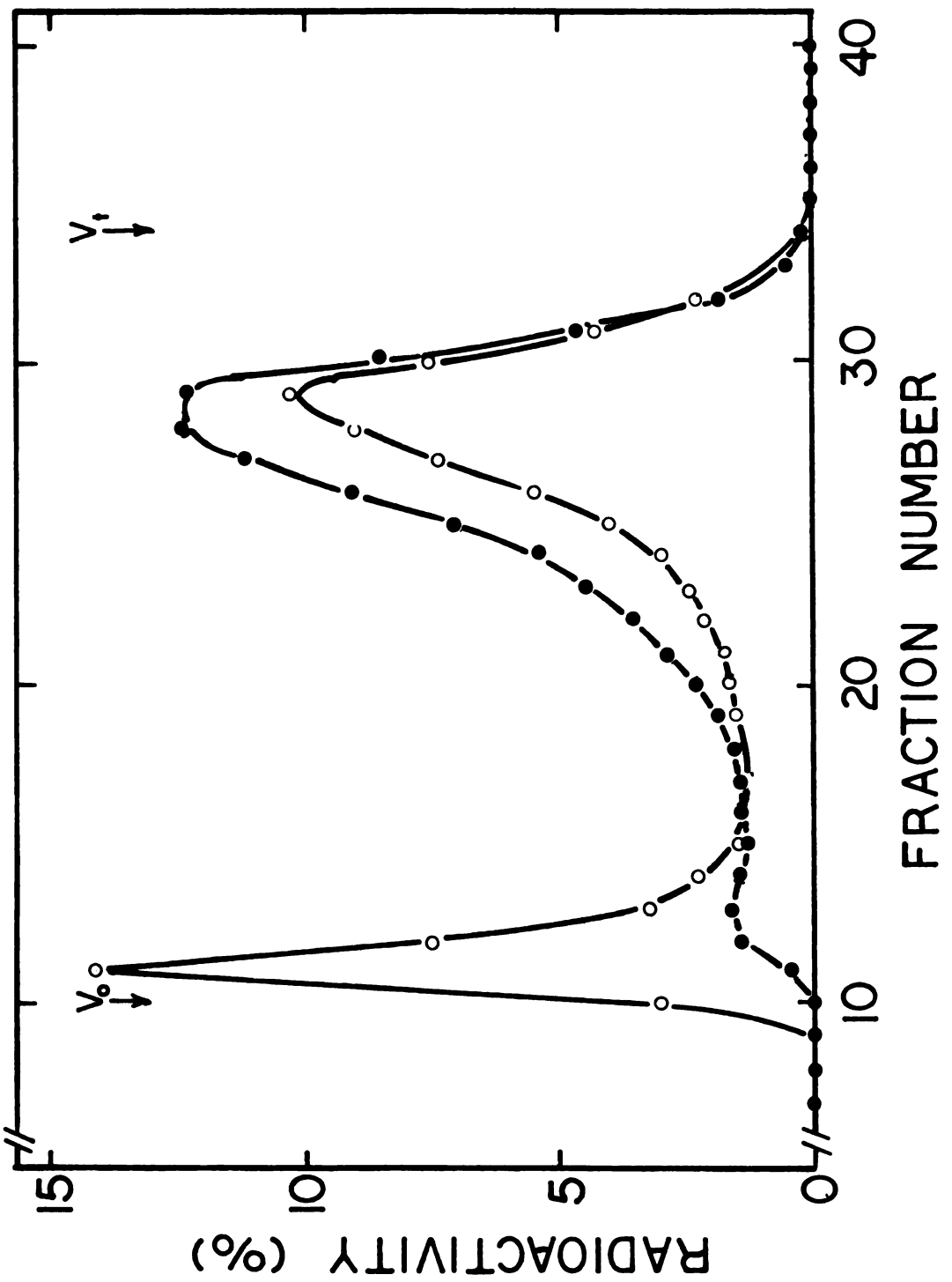

Figure 18

although ionic strength is a contributing factor in determining the degree of aggregation, there is some ionic specificity as well.

Aggregation Induced by 1.0 M NaCl

Both 2% sodium hexametaphosphate- and 1% ammonium oxalate-solubilized products were aggregated by chromatography on Sepharose 4B with 1.0 M NaCl. When 2% sodium hexametaphosphate-solubilized product was chromatographed on Sepharose 4B with 50 mM sodium phosphate buffer, 15% of the applied radioactivity eluted in V_o. Elution of this same product with 1.0 M NaCl caused 49% of the applied radioactive material to elute in V_o, and total recovery from the column dropped from 87 to 60% (Figure 19).

Only 4.5% of the 1% ammonium oxalate-solubilized product eluted in V_o when chromatographed on Sepharose 4B with 50 mM sodium phosphate buffer, but elution with 1.0 M NaCl increased this to 50% as shown in Figure 20. Again, the total recovery from the column dropped sharply from 94 to 58%.

Therefore, although the 2% sodium hexametaphosphate-solubilized product contained more large-sized material than did the 1% ammonium oxalate product, 1.0 M NaCl aggregated both to almost the same extent.

The aggregation induced by NaCl did not require that the chromatography be performed in NaCl, but merely that the sample be exposed to NaCl before chromatography. When NaCl was added to 1% ammonium oxalate-solubilized material to a concentration of 1.0 M immediately before chromatography on Sepharose 4B with 50 mM sodium phosphate buffer, the elution profile was almost identical to that obtained upon elution with 1.0 M NaCl, although recovery was 83%. When 1%

Figure 19. Chromatography of 2% sodium hexametaphosphate-solubilized product on Sepharose 4B with 50 mM sodium phosphate (A) buffer and with 1.0 M NaCl (B).

D-Galacturonosyltransferase product was prepared and was extracted with 2% sodium hexametaphosphate as described in the Experimental Procedures. One sample of solubilized product, containing 30,500 dpm, was chromatographed on a Sepharose 4B column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) (A) at a rate of 5 ml per h. A second sample, containing 43,000 dpm, was chromatographed on a Sepharose 4B column which was equilibrated and eluted with 1.0 M NaCl (B) at a rate of 5 ml per h. Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from the columns for the two samples were 87 and 60%, respectively.

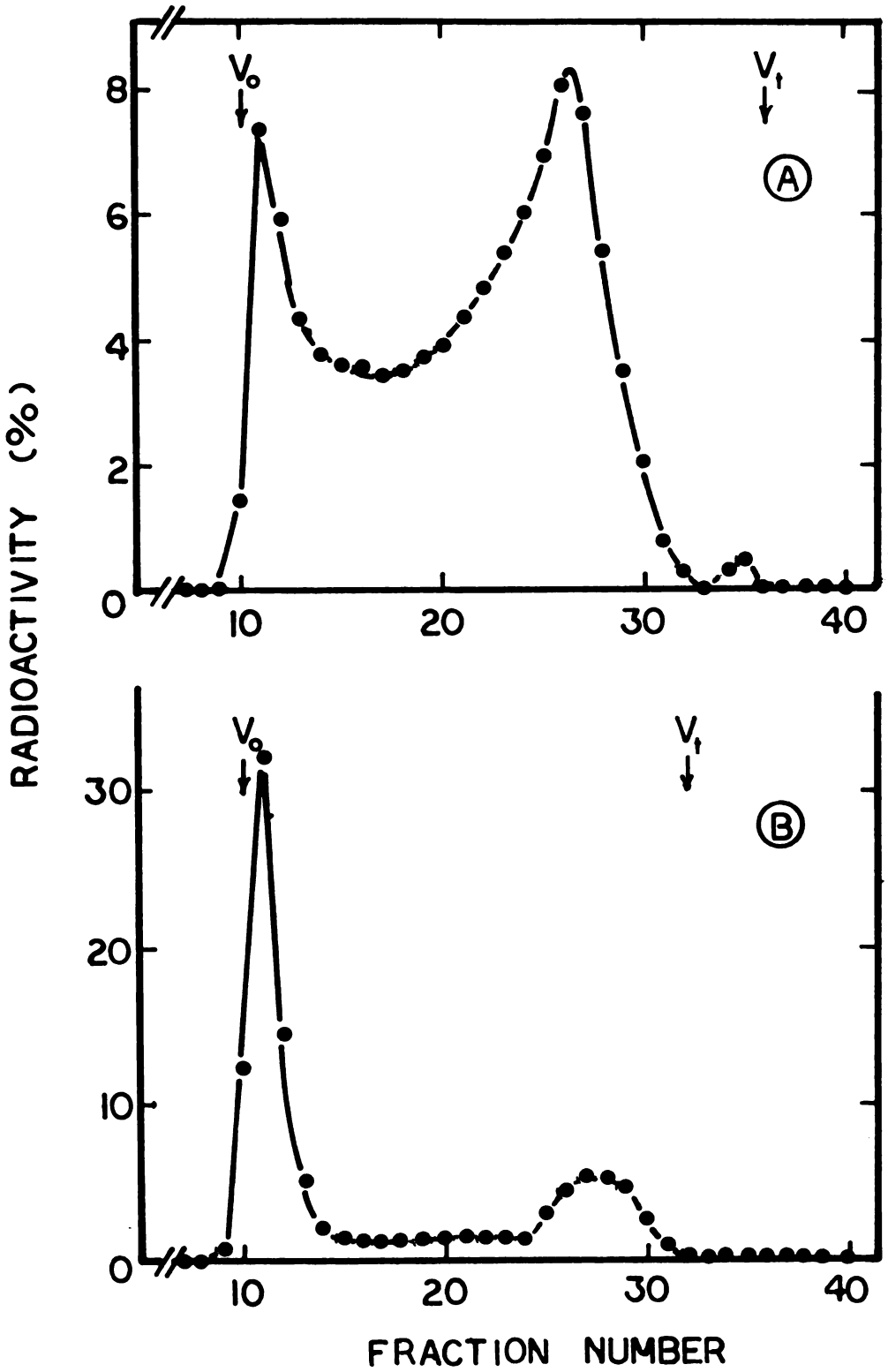

Figure 19

Figure 20. Chromatography of 1% ammonium oxalate-solubilized product on Sepharose 4B with 50 mM sodium phosphate buffer (A) and with 1.0 M NaCl (B).

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures. One sample of the solubilized product, containing 42,000 dpm, was chromatographed on a Sepharose 4B column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) (A) at a rate of 5 ml per h. A second sample, containing 46,500 dpm, was chromatographed on a Sepharose 4B column which was equilibrated and eluted with 1.0 M NaCl (B) at a rate of 5 ml per h. Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from the columns for the two samples were 94 and 58%, respectively.

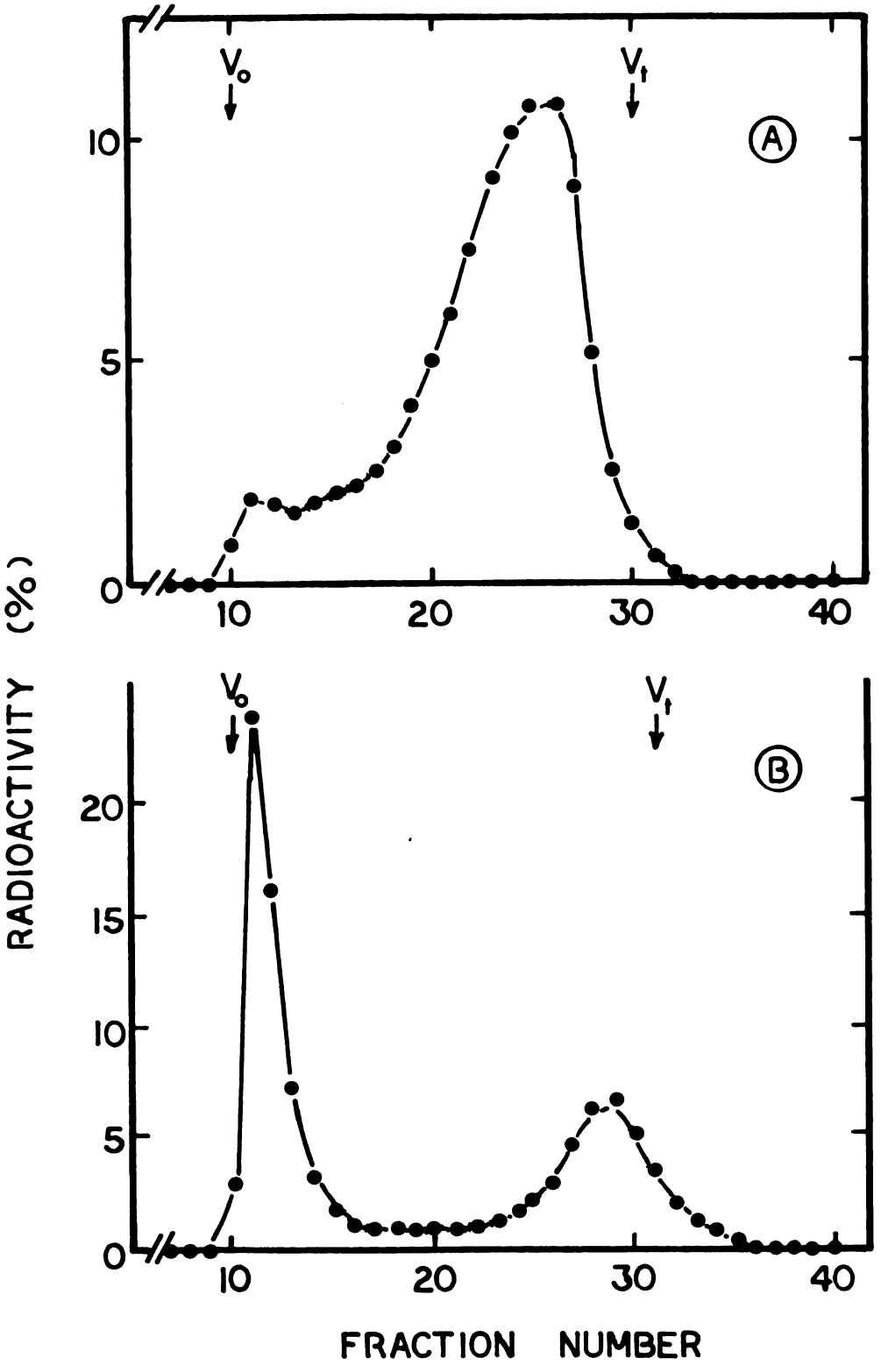
V0
Vt
A
10
5
0
10
20
30
40
V0
Vt
B
20
15
10
5
0
10
20
30
40
RADIOACTIVITY (%)
FRACTION NUMBER

Figure 20

ammonium oxalate-solubilized product was dialyzed in 1.0 M NaCl before chromatography in 50 mM sodium phosphate buffer, 50% eluted in V_o and recovery was 100%. The differences in recovery from the columns might be due to a further NaCl-induced aggregation during elution with 1.0 M NaCl which caused the product to stick to the column.

The aggregated species was stable toward rechromatography in buffers of lower ionic strength. When 1% ammonium oxalate-solubilized product which had been aggregated with 1.0 M NaCl was rechromatographed on Sepharose 4B with 50 mM sodium phosphate buffer, the radioactive material remained in V_o (Figure 21). However, if the aggregated product which eluted from Sepharose 4B in V_o was dialyzed in water before rechromatography on 4B, the size of the V_o peak was halved, and greater than 50% of the previously aggregated material eluted as a fairly symmetrical peak within the fractionation range of the column as shown in Figure 21.

Solubilization of D-Galacturonosyltransferase Product with Various Concentrations of Sodium Hexametaphosphate and Ammonium Oxalate

Dialyzing 1% ammonium oxalate-solubilized product in various concentrations of both ammonium oxalate and sodium hexametaphosphate altered the size of the product. Therefore, products which are solubilized with various concentrations of ammonium oxalate and sodium hexametaphosphate might also vary in size.

Various concentrations of sodium hexametaphosphate were used to solubilize the D-galacturonosyltransferase product, and the solubilized products were chromatographed on Ultrogel AcA 34 with 50 mM

Figure 21. Effect of dialysis in water for 3 h on the size of 1% ammonium oxalate-solubilized product which was aggregated by dialysis in 1.0 M NaCl.

D-Galacturonosyltransferase product was prepared and was extracted with 1% ammonium oxalate as described in the Experimental Procedures. Two samples of solubilized product, one (a) containing 69,000 dpm and the other (b) containing 72,500 dpm, were dialyzed for 3 h in 3 l of 1.0 M NaCl at 4°C. Recoveries from dialysis were about 60% in both cases. Each sample was chromatographed immediately on a Sepharose 4B column which was equilibrated and eluted with 50 mM sodium phosphate buffer at a rate of 5 ml per h (data not shown). Fractions (1.0 ml) were collected and 100 µl aliquots of each fraction were assayed for radioactivity in scintillation solution B. The portion of sample (a) which eluted in Fraction 11 (V_O) and which contained 18% of the total eluted radioactivity was rechromatographed on a Sepharose 4B column in the manner described above (●). The portion of sample (b) which eluted in Fraction 11 (V_O) and which contained 24% of the total eluted radioactivity was dialyzed in water for 3 h at 4°C. Recovery from dialysis was 71%. Dialyzed Fraction 11 was then immediately rechromatographed on a Sepharose 4B column as described above (○). For both rechromatographed samples, 1.0 ml fractions were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. The total recovery after chromatography for Fraction 11 from sample (a) was 93%, and the total recovery after chromatography for dialyzed Fraction 11 from sample (b) was 100%.

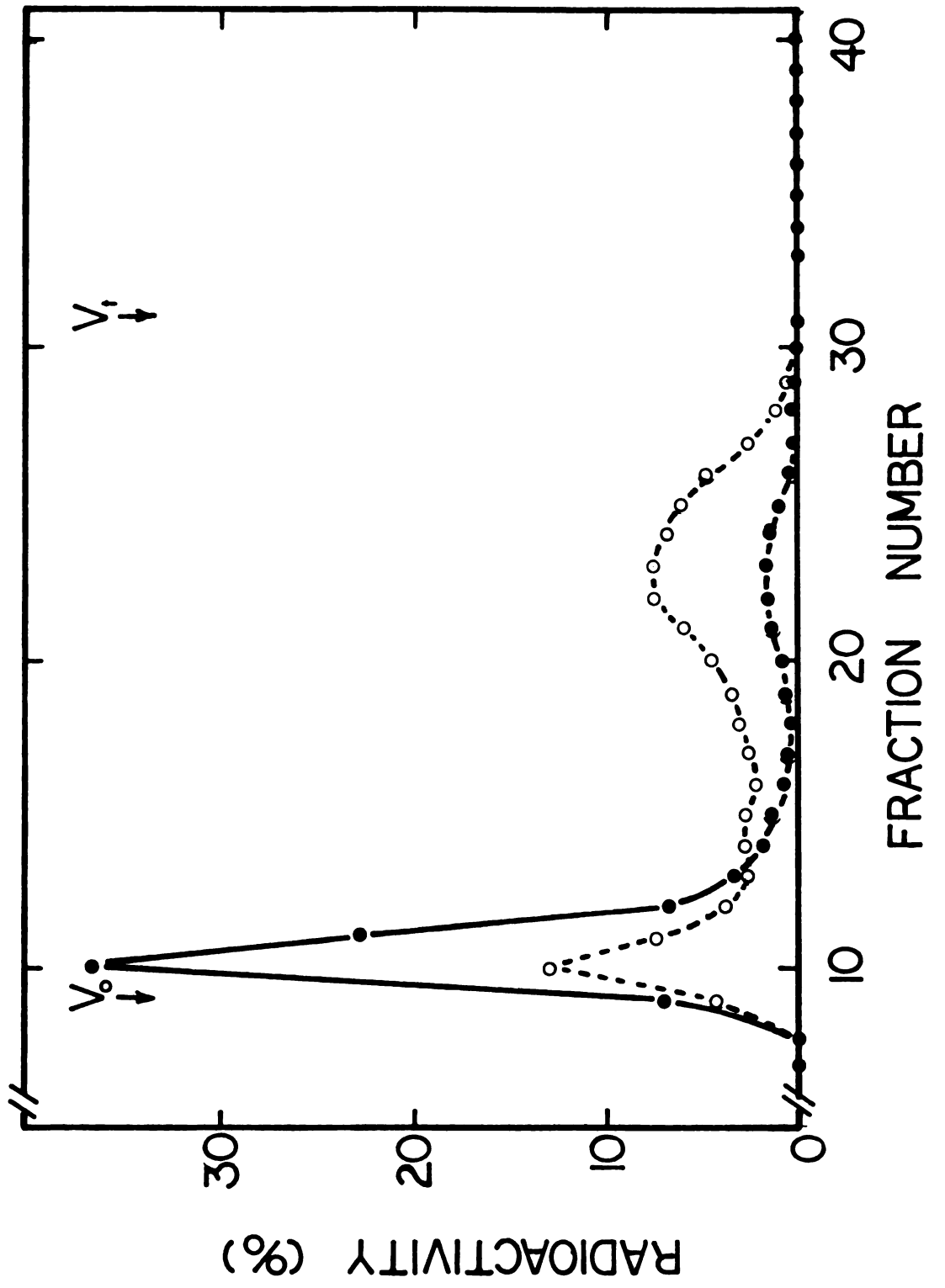

Figure 21

sodium phosphate buffer. The results are summarized in Table 4 and in Figure 22.

The size of the product did not decrease significantly until the total amount solubilized began to decrease. This could mean either that concentrations of sodium hexametaphosphate below 0.05% failed to extract larger molecular weight products, or that the product solubilized simply appeared to be of lower molecular weight because the lower sodium hexametaphosphate concentration caused less aggregation.

In contrast, preliminary studies indicated that lowering the concentration of ammonium oxalate used for extraction to 0.1% did not affect the Ultrogel AcA 34 elution profile substantially (Figure 23).

Both ammonium oxalate and sodium hexametaphosphate solubilized a species of polysaccharide which could not be aggregated by NaCl. This material eluted as a symmetrical peak near V_t on Sepharose 4B with 1.0 M NaCl. Depending on the experiment, it contained up to one-third of the total radioactive polysaccharide eluted from the column. When 2% sodium hexametaphosphate-solubilized product was chromatographed on Sepharose 4B with 50 mM sodium phosphate buffer and the material eluting near V_t was rechromatographed on Sepharose 4B with 1.0 M NaCl, no significant aggregation occurred (Figure 24). Rechromatography in 1.0 M NaCl of a fraction of NaCl-aggregated, 1% ammonium oxalate-solubilized product which eluted near V_t also failed to cause aggregation.

Product which was solubilized with 0.025% sodium hexametaphosphate and which represented only 20-25% of the total incorporated

Table 4. Effect of sodium hexametaphosphate concentration on the size of the solubilized product

Exp. no.	Concentration of sodium hexametaphosphate (%) (w/v)	Amount solubilized (%)	Amount eluted in V_o (%)
A	2.0	77	34
	0.50	79	28
	0.10	81	27
B	2.0	75	40
	0.050	68	35
	0.025	23	14
	0.010	11	9

D-Galacturonosyltransferase product was prepared and was solubilized with sodium hexametaphosphate as described in the Experimental Procedures except that the concentration of sodium hexametaphosphate was varied. Fifty microliter (50 µl) portions of solubilized products were assayed for radioactivity in scintillation solution B for determining the percent of the total D-galacturonosyltransferase product solubilized. The remaining solubilized product was chromatographed on an Ultrogel AcA 34 column that was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8). Fractions (1.0 ml) were collected and were assayed for radioacti-ity in scintillation solution B. Values of "Amount eluted in V_o" represent the percent of total eluted radioactivity collected in the V_o fractions.

Figure 22. Effect of sodium hexametaphosphate concentration on the size of the sodium hexametaphosphate-solubilized product.

D-Galacturonosyltransferase product was prepared and was extracted with sodium hexametaphosphate as described in the Experimental Procedures except that the concentration of sodium hexametaphosphate was varied. Products solubilized with 0.01% (●), 0.025% (○), 0.025% (▲), or 2.0% (■) sodium hexametaphosphate contained 3,000, 4,500, 15,000 and 16,500 dpm, respectively. Each sample was chromatographed on an Ultrogel AcA 34 column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 5 ml per h. Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries for the four products were 58%, 90%, 91%, and 100%, respectively.

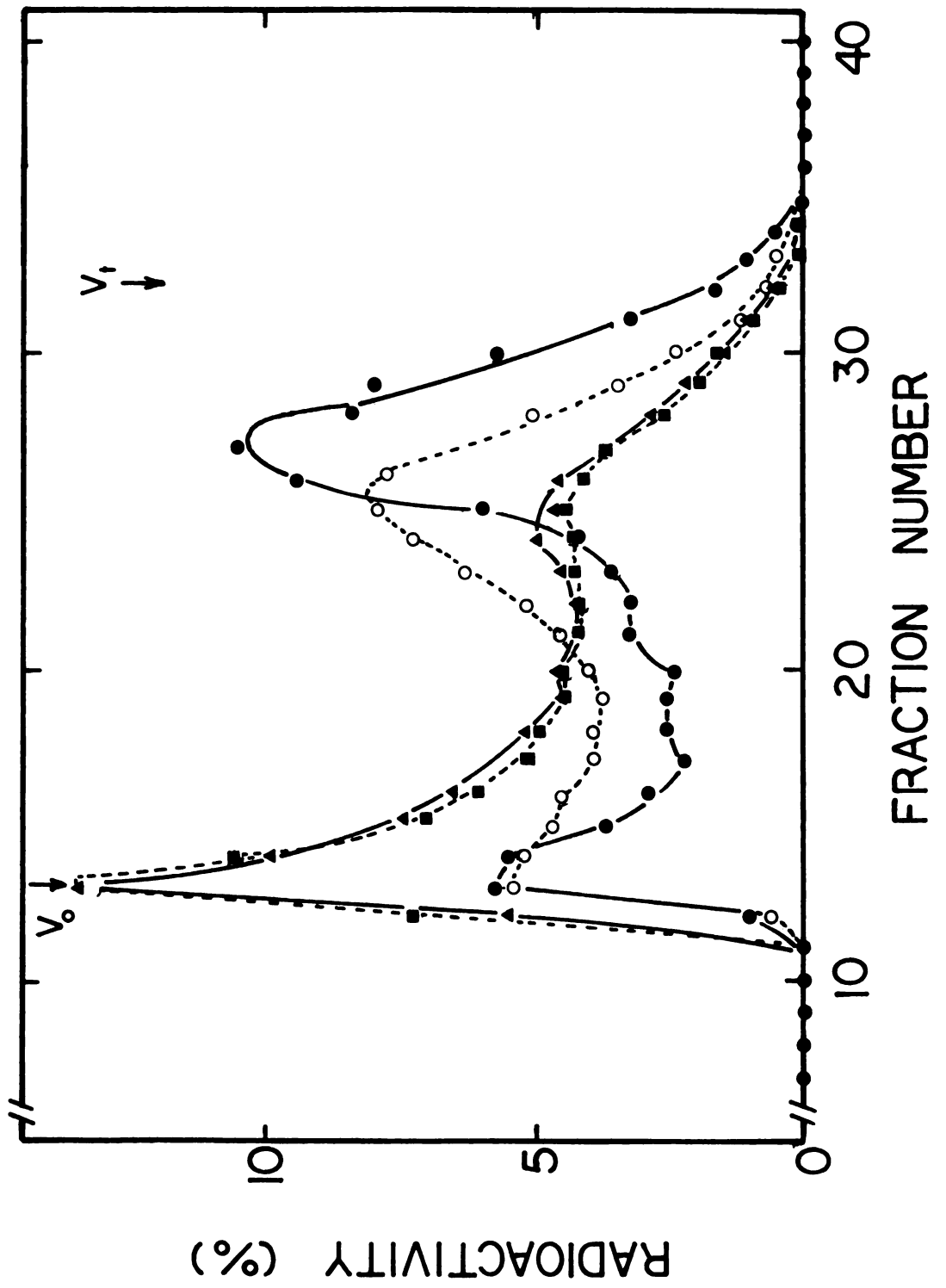

Figure 22

Figure 23. Effect of ammonium oxalate concentration on the size of the ammonium oxalate-solubilized product.

D-Galacturonosyltransferase product was prepared and was extracted with ammonium oxalate as described in the Experimental Procedures, except that the concentration of ammonium oxalate was varied. Products solubilized with 0.1% (O) or with 1.0% (●) ammonium oxalate contained 10,000 dpm and 22,000 dpm, respectively. Both products were chromatographed on an Ultrogel AcA 34 column which was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) at a rate of 5 ml per h. Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries for the two samples were 98 and 100%, respectively.

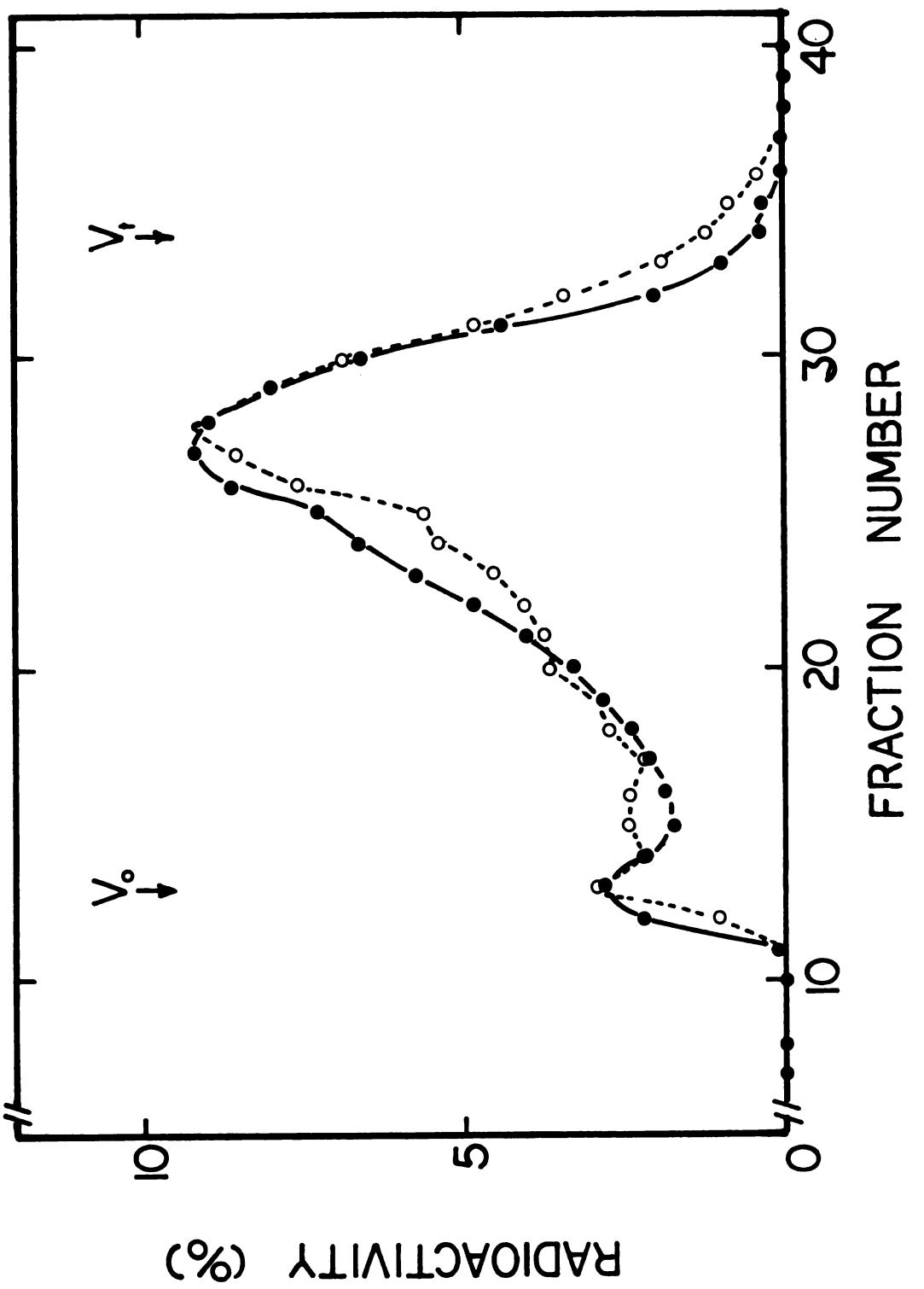

Figure 23

Figure 24. Effect of 1.0 M NaCl on 2% sodium hexametaphosphate-solubilized product that elutes near V_t from Sepharose 4B.

D-Galacturonosyltransferase product was prepared and was extracted with 2% sodium hexametaphosphate as described in the Experimental Procedures. Solubilized product, containing 23,000 dpm, was chromatographed on a Sepharose 4B column that was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 6.8) (A) at a rate of 5 ml per h. Fractions (1.0 ml) were collected, and a 100 µl portion of each fraction was assayed for radioactivity in scintillation solution B. Material eluting in Fraction 25, which contained 6% of the total eluted radioactivity, was rechromatographed on a Sepharose 4B column that was equilibrated and eluted with 1.0 M NaCl (B) at a rate of 5 ml per h. Fractions (1.0 ml) were collected and were assayed for radioactivity in scintillation solution B. Values of radioactivity (%) represent the percent of total eluted radioactivity collected in each fraction. Total recoveries from columns A and B were 95 and 98%, respectively.

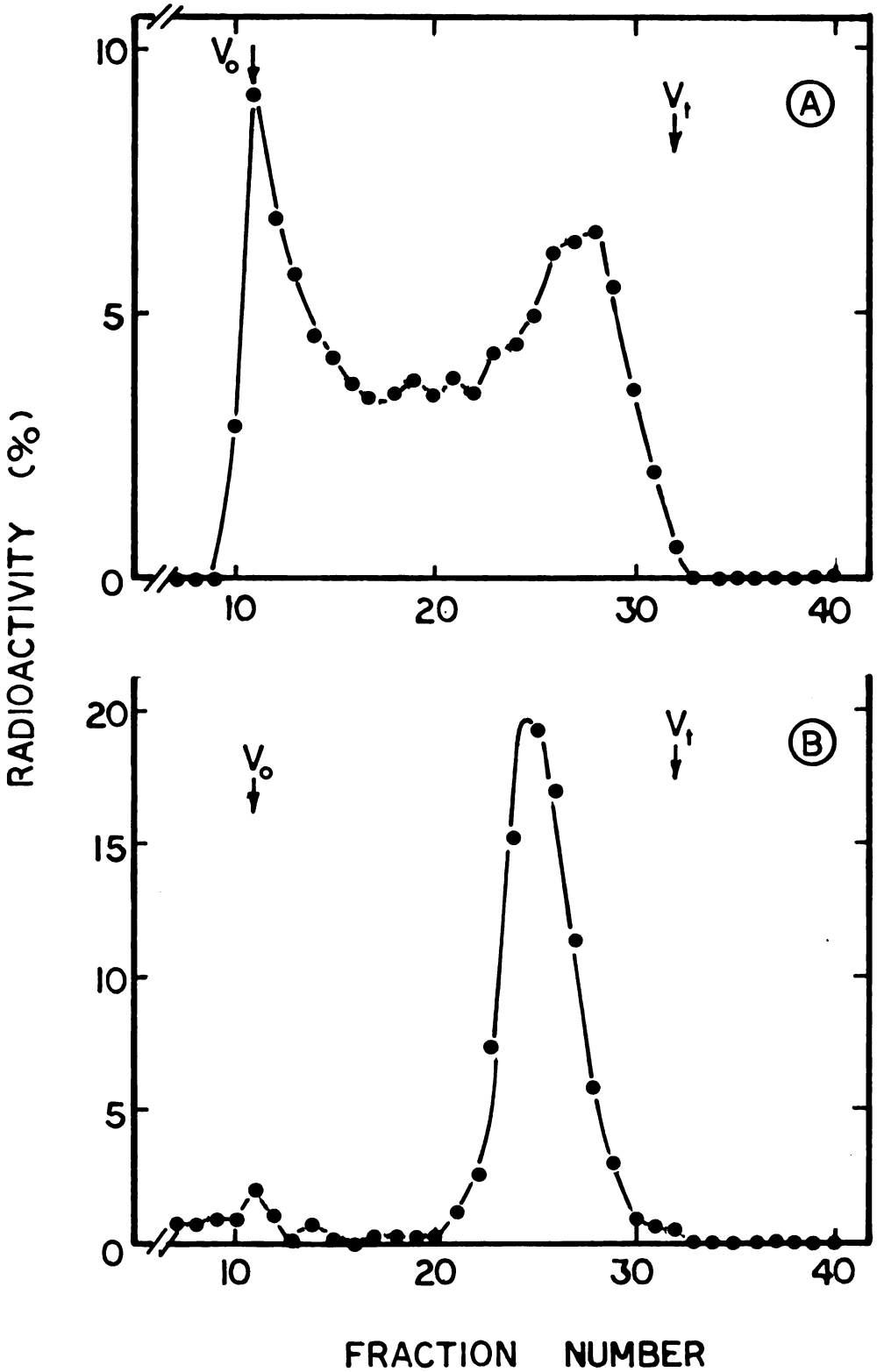
RADIOACTIVITY (%)
10
5
0
V_o
V_t
A
10
20
30
40
20
15
10
5
0
V_o
V_t
B
10
20
30
40
FRACTION NUMBER

Figure 24

radioactivity in the D-galacturonosyltransferase product eluted in this same region near V_t when it was eluted with 50 mM phosphate buffer. Preliminary results indicated that the material solubilized by 0.025% sodium hexametaphosphate could not be aggregated by elution with 1.0 M NaCl.

The structures of these products which elute near V_t were not analyzed, but their failure to aggregate suggests that their structures may differ from that of the bulk of the polysaccharide isolated with 1% ammonium oxalate or 2% sodium hexametaphosphate.

DISCUSSION

Characteristics of UDPGalA:Acceptor D-Galacturonosyltransferase

Lemna minor is the only system other than *Phaseolus aureus* (mung bean) in which an active particulate UDPGalA:acceptor D-galacturonosyltransferase has been described. Like the mung bean preparation (61), the D-galacturonosyltransferase preparation from *L. minor* appears to contain all the necessary elements for polysaccharide synthesis with the possible exception of substrate. Mn^{+2} is the only ion which causes significant stimulation of D-galacturonosyltransferase activity. Although this stimulation is not nearly as great as that achieved in mung bean, the *L. minor* enzyme was significantly inhibited by EDTA. This result suggests that the *L. minor* transferase has a metal ion requirement which is satisfied by endogenous levels in the absence of EDTA. The pH optimum of both transferases is 6.0 to 6.2; however, the apparent K_m for UDPGalA for the *L. minor* enzyme is four-fold higher than that for the mung bean transferase.

Because the properties of the *L. minor* D-galacturonosyl- and D-apiosyltransferases are quite similar (64, and Pan and Kindel, unpublished results), the two transferase activities responsible for the synthesis of the galacturonan main chain and the addition of the D-apiosyl- and apiobiosyl- side chains of cell wall

apiogalacturonans may be in the same particle. This would allow a close coordination of the main events of apiogalacturonan synthesis.

D-Apiosyltransferase activity was stimulated by the addition of UDPGalA to the assay. This observation can be explained by assuming that the polymerization of D-galacturonic acid by the D-galacturonosyltransferase which is isolated with the D-apiosyltransferase provides new acceptor sites for D-apiose transfer (64). While the failure of UDP-apiose to stimulate D-galacturonosyltransferase activity shows that galacturonan synthesis is not obligatorily coupled to the addition of D-apiose, it does not prove that the two processes are totally separate either spatially or temporally. The apparent K_m for UDP-apiose is about one-half that for UDPGalA (64), which could account for the unexpected slight inhibition of the D-galacturonosyltransferase by UDP-apiose. If the two transferase activities belonged to one relatively non-specific transferase system, the presence of UDP-apiose might tie up part of the system that would otherwise be used for galacturonan synthesis. However, this apparent inhibition must be studied more carefully before any inferences about the mechanism of synthesis are drawn from it.

The rapid departure from linearity of the time course for D-galacturonic acid incorporation is probably not caused by depletion of substrate because addition of UDPGalA shortly after the rate of incorporation began to decrease failed to stimulate incorporation. An inhibitor might be formed during the course of the reaction; this possibility was not tested. Alternatively, such a decrease in rate of incorporation with time could be explained by disruption of the particle with which the transferase is associated, by protein

denaturation, or by the exhaustion of endogenous acceptor polysaccharide. All these processes would be expected to be temperature-dependent. Therefore, any one or a combination of them could explain the prolonged linearity of the incorporation at 0°C.

Stability, Solubilization, and the Role of the Particle Which Contains UDPGalA:Acceptor D-Galacturonosyltransferase

The rapid and spontaneous inactivation of the D-galacturonosyltransferase could be explained by several of these same proceses. Although the failure of Mn^{+2}, sucrose, and BSA, and of dithiothreitol and mercaptopropanediol to improve the stability significantly weighs against protein denaturation, the kinetics of inactivation have not been studied enough to rule it out. The phenylmethylsulphonyl fluoride and Nupercaine hydrochloride studies, while not conclusive, suggest that proteases and phospholipases are not primary causes of inactivation. The inability of butylated hydroxytoluene to stabilize the transferase activity tends to eliminate the possibility of lipid peroxidation.

The effects of a number of detergents demonstrated the importance of intact membrane structure for maximum D-galacturonosyltransferase activity. These surface-active agents are known to disrupt biological membranes. All the detergents tested, both ionic and non-ionic, except Tween-20, caused a marked decrease in D-galacturonosyltransferase activity when used at concentrations of 1%. Also, solutions of high ionic strength which solubilize some membrane-bound enzymes decreased D-galacturonic acid incorporation. Brief sonication of the transferase preparation and addition of small amounts of ethanol

and 2-propanol had similar effects. The apparent destabilization of the transferase during attempts at solubilization further supports the need for an intact particle.

The membrane in which the transferases are apparently embedded might control transferase activity simply by providing a hydrophobic environment which is essential to its function. In addition, the particle might serve as a barrier to attack of the growing polysaccharide chain or the transferases by degradative enzymes. Kauss has shown that an intact particle prevents the attack of pectin methylesterase (67). Thus, transferase instability might be caused by several factors such as a disruption of the particle followed by the attack of degradative enzymes. Further attempts at stabilization should involve testing combinations of stabilizing agents which both maintain membrane integrity and inhibit the action of degradative enzymes.

If the main function of the particle is protection of the transferases and their products, the problems of stabilization and solubilization are tractable. The same is true if the membrane's purpose is to provide the proper hydrophobic environment for enzyme function. Numerous membrane-bound enzymes have been solubilized and their activity has been restored by reconstitution in an artificial lipid membrane (79-81). Experiments along this line, while complex, have a reasonable chance of success.

Besides stabilization, solubilization of the transferase is essential for another reason. All particulate transferase preparations probably contain many types of endogenous polysaccharides which can serve as acceptors for radioacstively-labeled sugar nucleotides.

Until these can be eliminated, the true nature of the ultimate acceptor cannot be defined, and the various transferase reactions that lead to heteropolysaccharide synthesis cannot be described with the precision that is common to biochemists who work with soluble enzymes. In the absence of a defined acceptor, the validity of these studies rests on comparisons of the products synthesized *in vitro* with polysaccharides known to be present in the cell wall.

Similarity of the Solubilized D-Galacturonosyltransferase Product to Cell Wall Apiogalacturonans

Mascaro (66) showed that the D-galacturonosyltransferase product is probably a galacturonan according to several criteria:

(1) About 80% of the product is solubilized by ammonium oxalate,

(2) The solubilized product binds to DEAE-Sephadex, and most of it elutes at a NaCl concentration of about 0.25 M, and

(3) The solubilized product is almost completely degraded by fungal pectinase.

Mascaro also presented several lines of evidence which suggest that the *L. minor* transferase preparation incorporates both D-apiose and D-galacturonic acid into a molecule similar to cell wall apiogalacturonans.

The studies described in this dissertation extend our knowledge of the size and peculiar properties of the D-galacturonosyltransferase product. They also suggest a further similarity of the *in vitro* synthesized product and authentic apiogalacturonans based on their behavior toward NaCl.

Size of the Product

The solubility of about 80% of the D-galacturonosyltransferase product in ammonium oxalate and in sodium hexametaphosphate classifies it as a pectin. The solubility also suggests that even as the product is synthesized in the particle before deposition in the cell wall matrix, it exists as a calcium salt or the salt of some other cation which can be chelated by ammonium oxalate or sodium hexametaphosphate. Although both chelating agents presumably solubilize pectins by the same mechanism, sodium hexametaphosphate is more effective at lower concentrations, presumably because of its polyvalency.

Gel chromatography showed that the 2% sodium hexametaphosphate-solubilized product appeared to be larger than the product solubilized by 1% ammonium oxalate. About 25% of the former product had an apparent molecular weight greater than 1×10^6, which is much greater than any previously reported molecular weight for a pectin.

Although dialysis in water reduced the size of the 1% ammonium oxalate-solubilized product, the elution profile of the 2% sodium hexametaphosphate-solubilized product was insensitive to dialysis. However, at least 30% of the total solubilized radioactivity was lost on dialysis in water in both cases. In the case of the sodium hexametaphosphate-solubilized product this loss appeared to come equally from all segments of the elution profile. It is unclear whether this loss represented disaggregation or degradation of the solubilized product.

The 1% ammonium oxalate-solubilized product decreases in size both upon standing and upon dialysis in water, the former decrease

being greater. However, when the solubilized product was dialyzed in water immediately the decrease in size due to standing was minimized. This suggests that ammonium oxalate may also cause degradation of the product. There is no evidence for degradation on standing in the case of the 2% sodium hexametaphosphate-solubilized product.

Degradation by ammonium oxalate was also suggested by experiments in which dialysis in 1% ammonium oxalate decreased the size of products which had been solubilized in 1% ammonium oxalate or which had been solubilized by 1% ammonium oxalate and then dialyzed for 3 h in water.

Alternatively, the decrease in size upon standing and upon dialysis in 1% ammonium oxalate might be explained by assuming that the product solubilized by 1% ammonium oxalate is still partially aggregated due to incomplete removal of Ca^{+2} or other cations. Standing or dialysis in 1% ammonium oxalate would then allow chelation of these ions by oxalate to proceed further, resulting in a further disaggregation of the solubilized product. The ability of 3% ammonium oxalate and 1 M NaCl to partially reaggregate the solubilized product that was dialyzed in 1% ammonium oxalate supports this latter alternative; however, the weight of evidence is not yet sufficient to permit a choice between these two possible explanations. Further studies on reaggregation and on the structure of the solubilized product after standing and after dialysis are needed.

Treatment of the 1% ammonium oxalate-solubilized product with 3% sodium hexametaphosphate increased the size of the former product, suggesting that sodium hexametaphosphate also causes aggregation of

the D-galacturonosyltransferase product. A number of experiments indicate that this aggregation is partly ionic strength-dependent, although there may also be some salt specificity. The valence and coordinating ability of the particular ions may affect the extent and strength of the aggregation. This would explain why the sodium hexametaphosphate-induced aggregation seems to be so much more irreversible than that caused by ammonium oxalate.

The size of the solubilized product also depends on the concentration of the solubilizing agent at concentrations of sodium hexametaphosphate below those which cause maximum solubilization. The trend is clear-cut: as the concentration of hexametaphosphate decreases, the amount of product solubilized decreases along with the size of the solubilized product at hexametaphosphate concentrations of 0.05% and below. The product solubilized with 0.025% sodium hexametaphosphate was not aggregated substantially by dialysis in 2% hexametaphosphate or by chromatography with 1.0 M NaCl. Apparently the structure and/or composition of the product solubilized at 0.025% sodium hexametaphosphate differs from that of the product solubilized at higher concentrations. Since even the 2% sodium hexametaphosphate-solubilized product seems to contain some material that is not aggregated, the 2%-solubilized product may contain a variety of types of galacturonans of differing solubilities and abilities to be aggregated.

The effect of the concentration of ammonium oxalate on the size distribution of the products solubilized requires further study, although preliminary results indicate that there is not much of an effect.

Relationship Between Aggregation and Galacturonan Structure

Most of our knowledge of the physical properties of polyuronides comes from studies of alginic acids and plant gums (30,32). What is known about the properties of the pectic polyuronides comes more from their use in the food industry than from a detailed analysis of their role in the cell wall. Reports of the size and state of aggregation of pectins isolated from plant cell walls are rare. The results presented in this dissertation represent the first attempt I know of to study the physical properties of an *in vitro*-synthesized pectin.

Barrett and Northcote (27) found that one of the products of the transelimination of apple fruit pectin appeared to be an almost pure galacturonan which was, however, highly esterified. This material did not seem to be aggregated in the presence of 0.1 M NaCl. Similarly, Anderson *et al.* (101) found no effect of 1.0 M NaCl on the size of an Acacia gum which contained about 16% uronic acid.

In contrast, Greenwood and Matheson (100) reported that a gum from *Khava gradifolia* containing 47% uronic acid could be aggregated to the point of gelation by 0.05 M Ca^{+2} and 0.15 M NaCl. They believed that cross-linking of the carboxyl groups by these ions caused aggregation.

Although they did not examine aggregation, Hart and Kindel (33) reported that the ammonium oxalate-solubilized apiogalacturonans of *L. minor* cell walls were differentially soluble in NaCl depending on their content of neutral sugar. The higher the proportion of D-apiose in the polysaccharide, the greater the solubility in NaCl. They also

postulated an interaction between polysaccharide chains mediated by Na^+ and Cl^- which could be interfered with by D-apiose.

Four factors emerge from these reports as possible determinants of the ability of polyuronides to aggregate: the content of uronic acid, the content of neutral sugar, the degree of esterification, and the presence of mono- or divalent ions. Significantly, these same four factors govern the formation and properties of gels formed from isolated and partially degraded pectins used commercially in food processing.

Most gel-forming pectins contain about 70% galacturonic acid, although other polysaccharides may aggregate without forming gels, especially in dilute solutions (30). Completely esterified pectins can gel in the absence of ions under proper conditions, indicating that gel formation involves a variety of types of interactions known collectively as "microcryatallite formation." Cations are required for gel formation when esterification is incomplete. Rather than forming only ionic bridges between polysaccharides, cations are thought to decrease the activity of water in the thermodynamic sense, meaning that the uronic acids become less highly hydrated and tend to form tangled microcrystalline regions (30).

In this context, the apparent aggregation of the solubilized products of D-galacturonosyltransferase becomes meaningful.

Hart and Kindel reported a very low degree of esterification for apiogalacturonans. While the ester content of the D-galacturonosyltransferase product was not examined, Mascaro has found the product to be acidic. Therefore, aggregation in the presence of cations such as Na^+ and Ca^{+2} and probably NH_4^+ would be expected,

and the degree of aggregation would depend to a large extent on the ionic strength of the medium.

Assuming that ammonium oxalate and sodium hexametaphosphate solubilize the D-galacturonosyltransferase product by chelating Ca^{+2}, the products which are solubilized at the lowest concentrations of the solubilizing agents might be expected to have the least Ca^{+2} associated with them in the particle which contains the transferase. This lower Ca^{+2} content might be due to a lower uronic acid content, a higher degree of esterification, or a higher content of neutral sugar. All these conditions would also reduce the ability of the polysaccharide to aggregate in the presence of cations, which is in line with the results obtained.

A more complete explanation of the aggregation phenomenon will require analysis of the structure and especially the composition of the D-galacturonosyltransferase product. The aggregation properties and structure of the cell wall apiogalacturonans should also be studied more closely so that the properties of the *in vivo*- and *in vitro*-synthesized products may be more closely compared. The aggregation results, when compared to the NaCl-solubility of the cell wall apiogalacturonans, strengthen the evidence that the D-galacturonosyltransferase product is similar to polysaccharides normally found in the *L. minor* cell wall.

Summary

A particulate UDPGalA:acceptor D-galacturonosyltransferase was isolated from *L. minor* and the activity responsible for the incorporation of D-galacturonic acid into polysaccharides was partially

characterized. The activity of the D-galacturonosyltransferase was not stimulated by the presence of UDP-apiose, indicating that galacturonan synthesis can proceed in the absence of the addition of neutral sugar. The D-galacturonosyltransferase was spontaneously inactivated and no treatment was found that could prevent this inactivation. The sensitivity of the transferase activity to the presence of detergents suggests that an intact membrane structure is necessary for maximum enzyme activity.

The D-galacturonosyltransferase product appears to be a galacturonan by several criteria. Most of the product is soluble in ammonium oxalate or sodium hexametaphosphate. The sodium hexametaphosphate-solubilized product seems to be aggregated and the extent of aggregation appears to be partly dependent on the ionic strength of the medium in which the product is placed. This aggregation is a type of behavior which would be expected of a highly acidic galacturonan. The properties of this D-galacturonosyltransferase product suggest that it is similar to apiogalacturonans isolated from cell walls of *L. minor*.

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