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Structural studies of some viscous, acidic bacterial exopolysaccharides

Costa, Jill Bonham, Ph.D.
The Ohio State University, 1991
STRUCTURAL STUDIES OF SOME VISCOS, ACIDIC
BACTERIAL EXOPOLYSACCHARIDES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in the Graduate School
of The Ohio State University

By
Jill Bonham Costa, B.A.

********

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1991

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To Greg and Shady
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Thanks to the Boss for being a really great advisor.
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LIST OF THREE-LETTER ABBREVIATIONS FOR MONOSACCHARIDES

Abbreviation — Monosaccharide

Gal — Galactose
Glc — Glucose
GlcA — Glucuronic Acid
Man — Mannose
ManA — Mannuronic Acid
Rha — Rhamnose
CHAPTER I

INTRODUCTION

1. WATER-SOLUBLE POLYMERS

Water-soluble polymers are of vital importance to many industries. Though comprising only a small fraction of consumer and industrial products and systems, they improve almost every system in which they are used in ways ranging from an ingredient to provide easier and faster processing in a manufacturing step, to an additive in order to obtain enhanced properties in a finished product. Nearly every conceivable industry, ranging from metal working to mining to detergents, explosives, and foods utilizes water-soluble polymers in some capacity.

Two broad categories of water-soluble polymers include the synthetic polymers, such as poly(acrylamide) and poly(ethylene glycol), and the polysaccharide and polysaccharide-derived polymers, such as gum arabic, starch, and O-(carboxymethyl)cellulose. The focus here will be on underivatized polysaccharide polymers of potential or actual industrial importance.
2. ACTIVITY

2.A. Mode of Action

The use of polysaccharides in industry is based on their capacity to alter the fundamental rheological properties of water while being only a minor component (<10%) of the system. Their function is primarily the thickening and gelling of aqueous solutions. Useful secondary functions include stabilization of two-phase systems, encapsulation, flocculation, film-forming, binding, and coating.1

Stabilizers are needed for three types of two-phase systems. In a solid–aqueous suspension, a polysaccharide increases the viscosity of the aqueous phase so that the rate of settling of the solids is slower. Some polysaccharides, such as xanthan gum, may also impart a shear force (defined as solution yield value), in which particles that do not have enough mass will be unable to move against the force and thereby remain suspended.1 In immiscible liquid–aqueous emulsions, the polysaccharide serves to increase the viscosity of the aqueous layer to greater than or equal to that of the oil-based layer. In addition, some polysaccharides, such as gum tragacanth, help achieve stable emulsions by being surface-active.2 In gas–aqueous dispersions (foams), a polysaccharide affects the surface properties and interfacial tension of the system, or may stabilize the system by reaction with with another component of the system, such as protein, to form a stable foam.1

Though the primary function of polysaccharides is to increase the viscosity of an aqueous solution, the various desirable secondary properties may actually dictate which polysaccharide is used for a specific application,
since different polysaccharides have differing secondary properties. Aspects such as solubility in cold water or over various pH ranges, stability in the presence of metal ions, and interactions with other system components also help dictate the choice of a specific polysaccharide. Often, combinations of polysaccharides are used to achieve all desired properties.

2.B. Mechanism of Action

Some polysaccharides control the rheology of the aqueous phase merely as simple thickeners. Other polysaccharides confer novel rheological properties to the aqueous phase, such as rigid gel formation, thermoreversible gel formation, or weak gel properties. These three types of gels are formed by a specific molecular association of the polysaccharide chains, either by the aggregation of ordered ribbon conformations or by the association of multiple helices in a gel-network formation.

2.B.1. Simple Thickeners

The simple thickeners include sodium alginate, O-(carboxymethyl)amylose, and λ-carrageenan. (See Figure 1.) These molecules exist in aqueous solution as random coils (disordered chains). The viscosity (η) under shear stress is defined as the applied shear stress (τ) over the resulting rate of shear (dγ/dt),

\[ η = \tau \left( \frac{dγ}{dt} \right)^{-1} \]  

For a Newtonian liquid, the viscosity is constant at any shear rate, or, the shear rate increases linearly with the applied shear stress. Polymer solutions usually show non-Newtonian behavior, and, as a result, exhibit "shear thinning" (thixotropy). The shear viscosity of a polymer solution
Partial Structure of Sodium Alginate (Alternating Region)
\[ (\beta-D-mannuronate - (1 \rightarrow 4) - \alpha-L-guluronate - (1 \rightarrow 4) - \beta-D-mannuronate - (1 \rightarrow n)\]

Partial Structure of Sodium O-(Carboxymethyl)amylose
(Degree of Substitution = 1.5)
\[ (\rightarrow 4) - \alpha-D-glucopyranose - (1 \rightarrow n) + \text{Sodium O-Carboxymethyl Ethers}\]

Partial Structure of \(\lambda\)-Carrageenan
30\% of the \(\beta\)-linked units carry a 2-sulfate residue.
\[ (\rightarrow 3) - \beta-D-galactopyranose - 2\text{-sulfate} - (1 \rightarrow 4) - \alpha-D-galactopyranose - 2,6\text{-di-sulfate} - (1 \rightarrow n)\]

**Figure 1.** The Structures of the Simple Thickeners Sodium Alginate, O-(Carboxymethyl)amylose, and \(\lambda\)-Carrageenan
will decrease when more shear stress is applied, or, the rate of shear increases faster than the applied shear stress. Therefore, the viscosities of polymer solutions are extrapolated to a zero-shear, or maximum rate, known as the zero-shear viscosity ($\eta_0$), in order to have a standard by which to compare different polymers.

For polymers in a random-coil conformation, the intrinsic viscosity ([\eta]) varies with the coil dimensions according to the Flory–Fox relationship:\(^3\)

$$[\eta] = AR^3/M_r$$  \hspace{1cm} (2)

where $A$ is a constant, $R$ is the radius of gyration, and $M_r$ is the molecular weight of the polymer. The coil can rotate in three dimensions, so that each coil can be treated as a sphere with radius $R$. Therefore, the hydrodynamic volume will be proportional to $[\eta]M_r$. The total number of coils present is proportional to $c/M_r$, where $c$ is the concentration of the solution. The degree of occupancy of space in a solution may be characterized by the "coil overlap parameter", which is defined as $c[\eta]$.

When the specific viscosity ($\eta_{sp}$) at zero shear is plotted against the coil overlap parameter on a log-log plot, a general curve is observed for the simple thickeners dextran, $O$-(carboxymethyl)amylose, alginate, and $\lambda$-carrageenan.\(^4\) (See Figure 2. Specific viscosity takes into account the fractional enhancement of viscosity due to the solvent, and is defined as

$$\eta_{sp} = (\eta_s[\eta]) / \eta_s$$  \hspace{1cm} (3)

where $\eta_s$ is the viscosity of the solvent and $\eta$ is the solution viscosity.) Polysaccharides which are thought to thicken aqueous solutions by another mechanism (see Sections 2.B.2. and 2.B.3.) do not fit this curve.
Figure 2. Generalized Concentration Dependence of Specific Viscosity for a Variety of Polysaccharide Simple Thickeners
The lower portion of the curve in Figure 2 represents the viscosity behavior of simple thickeners in dilute solution, as when the individual polymers are present as isolated coils. The equation describing this behavior, reduced from the empirically derived graph, is

\[ \eta_0 = \eta_s \{1.5(c[\eta])^{1.4} + 1\} \quad (4) \]

where the symbols have the meanings previously described. The upper portion of the curve in Figure 2 represents the behavior of simple thickeners in concentrated solutions, or when the total hydrodynamic volume of the individual chains exceeds the volume of the solution and the polymer coils interpenetrate and are tangled. The solvent viscosity is negligible, the slope is steeper, and the empirical equation becomes

\[ \eta_0 = \eta_s (c[\eta])^{3.3}/10. \quad (5) \]

Examining the factors which influence the value of \([\eta]\), and hence \(\eta_0\), is informative when comparing the thickening ability of polysaccharides to that of synthetic polymers. Two principal factors define the magnitude of \([\eta]\). These factors include the chain length (molecular weight) of the polymer, and the chain stiffness. Molecular weights are not unusually high in the polysaccharides compared to synthetic polymers, but polysaccharide chains are in general far less flexible\(^5\)\(^6\) than synthetic polymers and therefore adopt a more extended coil conformation, which in turn increases the radius of gyration \(R\). Increasing \(R\) only a small amount will, as seen in equation (2), increase \([\eta]\) dramatically. Therefore, according to equations (4) and (5), the maximum viscosity will be much greater for a solution of polysaccharide than for a synthetic polymer at the same concentration and molecular weight.
As their viscosity behavior is non-specific, and all simple thickeners show a similar concentration dependence vs. specific viscosity plot, the cheapest simple thickener is typically used if possible, since there is very little difference in the properties of these polymers.4

2.6.2. Association of Ribbonlike Conformations

Calcium alginates and pectins form rigid gels in aqueous solutions. Alginates are linear polymers of 4-linked β-D-mannuronic and α-L-guluronic acids. (See Figure 1.) Alginate contains blocks of homopolymeric sequences of both mannnuronic and guluronic acids, as well as regions containing alternating sugars. The quantity of these types of regions depends upon the biological source of the alginate. It has been established that the cross-linking to form a gel in alginate involves the cooperative binding of calcium ion between aligned guluronan blocks.7 This is known as the "egg-box" model of gel formation. (See Figure 3.) Pectins contain α-D-galacturonan regions which, when in a hydrated state, associate in the same manner as the guluronan regions of alginate, and form a similar type of gel.8

Xanthan gum, a polysaccharide with a cellulose (4-linked β-D-glucan) backbone and a charged side-chain every second residue (see Figure 4), forms a weak gel at rest in aqueous solution, but this weak gel flows easily under stress.9 Spectroscopic evidence shows that xanthan gum undergoes an order–disorder transition at the temperature which parallels its weak gel formation and melting. X-ray diffraction studies show that the ordered conformation of xanthan is single-stranded,10 and it is generally accepted that the weak gel properties of xanthan are due to the alignment,
Figure 3.
Representations of the "Egg-box" Model of Gel Formation
Associations of Guluronan Ribbons with Calcium Ions
Figure 4. Structure of Xanthan Gum
aggregation and association of the rigid rods, or ribbons, in solution to give a 3-dimensional structure. The average length of the ordered segments has been shown to be much smaller than the entire length of the xanthan molecule, such that the association of the ordered chain sequences is easily broken under stress, allowing the solution to flow.9 (See Figure 5.)

2.B.3. Association of Helices

Both τ- and κ-carrageenan from the red seaweeds form rigid thermoreversible gels. These carrageenans are alternating copolymers containing 3-linked β-D-galactose and 4-linked 3,6-anhydro-α-D-galactose residues. τ-Carrageenan contains two sulfate groups per disaccharide repeating-unit, and κ-carrageenan contains one sulfate group per repeating-unit. (See Figure 6.)

As in the case of xanthan, the temperature of gel setting and melting is paralleled by an order–disorder transition of the carrageenan molecules. In contrast to xanthan, however, which is single-stranded in solution, both τ- and κ-carrageenan exist as 3-fold right-handed double-helices in solution. Gel formation can occur only when helix aggregation can occur. Therefore, gelation is thought to be caused by aggregation of the helical segments. (See Figure 7.) The extent of the aggregation, and therefore the rigidity of the gel, depends on factors such as sulfate content of the carrageenan, ionic strength of the solution, and type of cation present.11 κ-Carrageenan, which has a lower sulfate content than τ-carrageenan, and therefore is less charged, forms a more rigid and opaque gel than τ-carrageenan.

Agarose, which contains 3-linked β-D-galactose and 4-linked 3,6-anhydro-α-L-galactose residues, has a similar structure to the carrageenans.
Weak Gel Formation in Xanthan Gum

A) Random Conformation

B) Local Order

C) Weak Gel Network

Figure 5. Schematic Representation of the Formation of a Weak Gel Network in Xanthan Gum by a Disorder—Order Transition
Figure 6. The Structures of i- and κ- Carrageenans and Agarose
Figure 7. The Association of Double-Helix \( \eta \)-Carrageenan Structures to Form a Rigid Gel in Solution.

Light triangles represent the top view of a helix structure, while dark triangles represent the bottom view of the helix. Side chains represent sulfate residues. The circles represent a divalent cation such as calcium.
In contrast to the carrageenans, which form right-handed helices, agarose forms 3-fold left-handed double-helices in solution. (See Figures 8 and 9.) Agarose is a neutral polysaccharide and, in keeping with the trend in the carrageenans, forms a very rigid thermoreversible gel even at low concentrations.\(^{12}\)

Association of helices is thought to be the gelation mechanism of in a number of polysaccharides.\(^{13}\) Double-helix formation is implicated in the gelation of amylose, the linear 4-linked \(\alpha\)-D-glucan portion of starch. Aggregation of triple-helix formations is implicated in the gelling of 3-linked \(\beta\)-D-glucan (curdlan), a branched 3-linked \(\beta\)-D-glucan,\(^{14}\) and gellan gum (acidic linear tetrasaccharide\(^{15}\) repeating-unit).\(^{16}\)

3. USES AND APPLICATONS OF POLYSACCHARIDES

The two major markets for natural gums can be loosely divided into the food and non-food industries. The major difference between these industries is the grade of gum which is used, as the highly purified gums used in the food industry serve many of the same purposes as those used in the non-food industries.

3.A. Non-Food Industries

Major non-food applications of natural gums include adhesives, agricultural chemicals, detergents, explosives, paint, and the paper, textile, and petroleum industries. The polysaccharide properties utilized include the ability to suspend and stabilize, thicken, disperse, form films, and gel. Polysaccharides are used as suspending agents in solid–liquid systems such
Figure 8. Schematic of the Three-fold Double Helix Structure of the Gel-Forming Polysaccharides Agarose (see ref. a) and t-Carrageenan (see ref. b)


Figure 9. Schematic View down the Axis of the Helix formed by Agarose
as inks, abrasives, drilling muds, and polishes. They are also used as flocculents to aid in separations in boiler compounds and the mining industry. Many polysaccharides are used for their film-forming abilities in the paper and textile industries, both to aid in the manufacture and to enhance the finished product. Table 1 outlines uses of polysaccharides in various non-food industries.

3.B. Food Industries

In the food industry, polysaccharides are also used for their water-holding, gelling, emulsifying, and stabilizing properties. A polysaccharide can provide a pleasant texture or "mouth feel" for various baked and processed foods. Many manufacturers of processed foods also take advantage of a polysaccharide's ability to hold water to prevent syneresis, or "weeping". Emulsifying properties are used in products such as salad dressings and butter-containing syrups. A polysaccharide's ability to suspend solids is used in maintaining fruit juices containing pulp, while polysaccharides also stabilize whipped toppings and foam on beer. Table 2 summarizes the major uses of polysaccharides in the food industry.
<table>
<thead>
<tr>
<th>Application</th>
<th>Property</th>
<th>Poly-saccharide</th>
<th>Specific Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abrasives</td>
<td>Stabilizing</td>
<td>Carrageenan</td>
<td>Suspending agent</td>
</tr>
<tr>
<td>Adhesives</td>
<td>Water-holding</td>
<td>Alginate, Gum Arabic, Tamarind Gum</td>
<td>Control penetration to improve adhesion and application</td>
</tr>
<tr>
<td>Agricultural Chemicals</td>
<td>Suspension, Film-forming</td>
<td>Xanthan Gum, Carrageenan, Gum Tragacanth</td>
<td>Suspending agent for solids, impart cling of chemical to substrate</td>
</tr>
<tr>
<td>Air Fresheners</td>
<td>Gelling</td>
<td>Alginate, Carrageenan</td>
<td>Form firm stable gel from cold water</td>
</tr>
<tr>
<td>Boiler Compounds</td>
<td>Flocculent</td>
<td>Alginate</td>
<td>Form soft flocs easily separated from treated water</td>
</tr>
<tr>
<td>Ceramics, Ceramic Glazes</td>
<td>Stabilizing</td>
<td>Alginate, Carrageenan, Gum Tragacanth, Xanthan Gum</td>
<td>Impart plasticity, suspend solids</td>
</tr>
<tr>
<td>Explosives</td>
<td>Gelling, Thickening</td>
<td>Alginate, Guar Gum, Xanthan Gum</td>
<td>Form rubbery, elastic gels with borates, thicken nitrate salt solutions</td>
</tr>
<tr>
<td>Inks</td>
<td>Stabilizer</td>
<td>Gum Arabic</td>
<td>Suspending agent</td>
</tr>
<tr>
<td>Latex Paints</td>
<td>Emulsifying, Thickening</td>
<td>Alginate, Carrageenan</td>
<td>Stabilize emulsions and provide viscosity</td>
</tr>
<tr>
<td>Mining</td>
<td>Flocculent</td>
<td>Guar Gum</td>
<td>Aid in liquid-solid separations, adsorbed onto hydrated mineral surfaces</td>
</tr>
<tr>
<td>Paper Sizing</td>
<td>Film-forming, Water-holding, Thickening</td>
<td>Agar, Alginate, Gum Arabic, Starches, Tamarind Gum</td>
<td>Coating for specialty papers, improve rheology of coating, improve surface characteristics</td>
</tr>
<tr>
<td>Petroleum Industry</td>
<td>Thickener, Emulsifier</td>
<td>Guar Gum, Gum Ghatti, Gum Karaya</td>
<td>Thicken drilling muds and water at low concentration to fracture wells</td>
</tr>
<tr>
<td>Polishes</td>
<td>Emulsifying</td>
<td>Agar, Alginate, Gum Ghatti, Xanthan Gum</td>
<td>Emulsify oils, suspend solids</td>
</tr>
<tr>
<td>Textile Dyeing</td>
<td>Thickening, water holding</td>
<td>Alginate, Guar Gum, Gum Karaya, Gum Tragacanth, Xanthan Gum</td>
<td>Thicken dye baths for sharp, bright colors, prevent migration of dyestuffs, modify flow characteristics</td>
</tr>
<tr>
<td>Textile Sizing</td>
<td>Film-forming, binding</td>
<td>Gum Arabic, Gum Tragacanth</td>
<td>Sizing agent for cloth, add body to certain fabrics</td>
</tr>
</tbody>
</table>
Table 2. Selected Applications of Some Natural Gums in the Food Industry

<table>
<thead>
<tr>
<th>Application</th>
<th>Property</th>
<th>Polysaccharide</th>
<th>Specific Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakery Goods</td>
<td>Adhesive, Thickener</td>
<td>Gum Arabic, Pectin, Xanthan Gum</td>
<td>Stabilize glazes and toppings, add desirable &quot;mouth-feel&quot;</td>
</tr>
<tr>
<td>Beer</td>
<td>Stabilizing</td>
<td>Gum Arabic</td>
<td>Stabilize foam</td>
</tr>
<tr>
<td>Dry Mixes</td>
<td>Water-holding</td>
<td>Alginate, Xanthan Gum</td>
<td>Absorb liquid rapidly in reconstitution</td>
</tr>
<tr>
<td>Fabricated Foods</td>
<td>Gelling, Water-holding</td>
<td>Alginate, Gum Karaya, Gum Tragacanth</td>
<td>Binding system, prevent syneresis</td>
</tr>
<tr>
<td>Frozen Foods</td>
<td>Water-holding</td>
<td>Alginate, Carrageenan, Gum Arabic, Gum Karaya, Gum Tragacanth, Tamarind Gum</td>
<td>Maintain texture throughout freeze-thaw cycle</td>
</tr>
<tr>
<td>Fruit Juice</td>
<td>Stabilizing</td>
<td>Alginate, Gum Tragacanth, Pectin, Xanthan Gum</td>
<td>Stabilize pulp in concentrated and finished products</td>
</tr>
<tr>
<td>Ice Cream</td>
<td>Stabilizing</td>
<td>Carrageenan, Locust Bean Gum</td>
<td>Retard crystal formation, impart smooth, chewy texture</td>
</tr>
<tr>
<td>Instant Pudding</td>
<td>Gelling</td>
<td>Alginate</td>
<td>Form firm pudding</td>
</tr>
<tr>
<td>Jelly</td>
<td>Gelling</td>
<td>Pectin, Tamarind Gum</td>
<td>Form stable gel</td>
</tr>
<tr>
<td>Pet Foods</td>
<td>Stabilizing, Thickening</td>
<td>Carrageenan</td>
<td>Stabilize fats, suspend solids</td>
</tr>
<tr>
<td>Relishes</td>
<td>Thickening</td>
<td>Alginate, Xanthan Gum</td>
<td>Improve clinging to substrate</td>
</tr>
<tr>
<td>Salad Dressings</td>
<td>Emulsifying</td>
<td>Alginate, Gum Tragacanth, Xanthan Gum</td>
<td>Emulsify and stabilize two-phase systems</td>
</tr>
<tr>
<td>Syrups</td>
<td>Water-holding, Emulsifying</td>
<td>Alginate, Xanthan Gum, Gum Tragacanth</td>
<td>Suspend solids, emulsify and stabilize butter-containing syrups</td>
</tr>
<tr>
<td>Whipped Toppings</td>
<td>Stabilizing</td>
<td>Alginate, Carrageenan</td>
<td>Stabilize fat dispersion</td>
</tr>
</tbody>
</table>
4. SOURCES OF INDUSTRIAL POLYSACCHARIDES

Industrial polysaccharides can be divided into four broad categories with respect to their sources.

4.A. Exudates

Plant exudates have been used for centuries as a source of water-soluble polymers. Traditionally called "gums", these sticky masses occur in the plant kingdom as a plant's response to physical injury. Most plant exudates come from trees native to East Asia and West Africa, and are harvested by unskilled workers in small villages. Simple harvesting procedures such as tapping a tree or scarring its bark and tearing off the resulting gum at the scar have developed over time.

Exudates are typically acidic polysaccharides containing various sugars other than glucose. They most often occur as the calcium, magnesium, or potassium salts of their carboxylic acids. Examples of common plant exudates include gum arabic, from the acacia tree, and gum tragacanth, from a species of shrubs from the genus Astragalus. These gums have continued to be irreplaceable in both their cost-effectiveness and range of properties.17

4.B. Extractives

Plant extractives are obtained from both land and marine plants and seeds. These polysaccharides are produced as a result of the plant's normal metabolic processes, or may be the plant's reserve carbohydrate source. The harvesting procedure for plant extractives typically are more varied and complex than those for the exudates. When the carbohydrate is to be
extracted from a plant's seed, great care is often given to the seed selection, collection, and processing. The gum is extracted by a series of crushing, sifting, and grinding steps to purify the seeds, followed by an aqueous extraction. In order to extract polysaccharides from trees and seaweed, the entire plant is harvested to facilitate handling. The polysaccharides are obtained by water-leaching of the plant. Unlike plant exudates, many of the processing procedures for extractives include a fractionation step to improve gum quality.17

Plant extractives, like exudates, are typically salts of acidic polysaccharides. Common plant extractives include the alginates, from brown seaweed, starch, from various sources such as corn and potatoes, and locust bean gum, from the carob tree.

4.C. Naturally Derived Semi-Synthetic Polysaccharides

Semi-synthetic polysaccharides consist mainly of modified celluloses and starches, with the former holding the largest market. Cellulose modifications include the acidic cellulose ethers, of which O-(carboxymethyl)cellulose (CMC) is the most common, and neutral cellulose ethers, which include O-methylcellulose and O-(hydroxyethyl)cellulose. Derivatives of starch include both anionic and cationic group modifications.17 Derivatives of guar and locust bean gums are also used industrially, but these markets are much smaller than the cellulose and starch derivative market.

Semi-synthetic polysaccharides were developed many years before they came into popular use. CMC was developed shortly after World War-I, but its popularity increased greatly only in the early 1940's due to World
War-II, when fatty acids were diverted from soaps and detergents to the military explosives industry. It was found that CMC could increase the effectiveness of synthetic detergent systems, where it prevented soil redeposition. CMC was used primarily in its detergent role until shortly after 1945, when it began to be used in systems containing insoluble solids, such as latex paints. Purified (99.5+%) sodium (carboxymethyl)cellulose has now been approved for use as a food additive in the United States when it has a degree of substitution not exceeding 0.95 carboxymethyl groups per glucose residue in the repeating-unit.18

4.D. Microbial Polysaccharides

Polysaccharides are found associated with all classes of bacteria, yeasts, and molds. Only bacterial polysaccharides will be discussed here.

There may be considered to be three general types of polysaccharides according to their physical location in the bacterium. Intracellular polysaccharides are located inside or as a part of the cytoplasmic membrane. Cell-wall polysaccharides occur as a constituent of the outer membrane, and include the lipopolysaccharides (O-antigens). Extracellular or exopolysaccharides reside outside the cell wall. (See Figure 10.) Exopolysaccharides are loosely subdivided into the capsular polysaccharides (K-antigen, CPS), which may or may not be covalently attached to the cell, but in any case are intimately associated with the cell. These polysaccharides are not easily removed except by chemical or extreme physical procedures. In contrast, the unattached, or slime exopolysaccharides (EPS), are only loosely associated with the cell or are excreted into the growth medium, and are therefore more easily isolated.
Figure 10. Schematic Locations of Various Polysaccharides in Gram-Negative Organisms
(See Figure 11.) Some microbes produce both a capsular and slime exopolysaccharide.

Slime exopolysaccharides have enjoyed the most study and use, since the purification procedures can be much much simpler than for the CPS or internal polysaccharides. EPS can contain component residues of nearly any sugar type and many types of non-carbohydrate organic substituents, although the non-carbohydrate substituents are frequently present in non-stoichiometric proportions which can depend on the growth conditions and phase.19

Unlike plant exudates and extractives, microbes which produce polysaccharides can be grown almost anywhere under controlled conditions. Microbes utilize simple nutrients to produce polysaccharides characterized by highly regular repeating-sequences, and they are able to produce polysaccharides in great abundance. Therefore, there is no dependence upon a favorable growing season in order to maintain a constant supply of the polymer; neither do the problems of an unfavorable world situation affect availability. Furthermore, there is typically little purification involved in the isolation of EPS from bacteria. Microbial polysaccharides, then, have an advantage over traditional exudates and extractives in being stable with respect to availability and quality, and a predictable cost.

The most widely used microbial polysaccharide today is xanthan gum, the EPS excreted by the bacterium Xanthomonas campestris, an organism originally isolated from the rutabaga plant. Xanthan is produced in a carefully controlled fermentation culture, then isolated by precipitation using isopropyl alcohol for the purified grades, or simply spray-dried for a
Figure 11. Schematic Location of Exopolysaccharides in Gram-Negative Bacteria
technical-grade product. Xanthan gum of varying purities is used in applications ranging from tertiary oil recovery to a food additive.

Table 3 summarizes various widely used types of natural gums, and their sources, structures and utility.
Table 3. Structure, Source and Properties of Some Important Water-Soluble Industrial Gums

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Structure</th>
<th>Source</th>
<th>Most Desirable Properties or Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>Multiple components—Agarose (Gelling fraction)—Linear polymer: →3)-β-D-galactopyranose-(1→4)-α-L-3,6-anhydrogalactose-(1→</td>
<td>Extracted from red marine algae of class Rhodophyceae, species Gelidium and Gracilana</td>
<td>Very potent gelling agent, Not metabolized by microorganisms</td>
</tr>
<tr>
<td>Alginate</td>
<td>Linear polymer: →4)-β-D-mannuronic acid-(1→4)-α-L-guluronic acid-(1→</td>
<td>Extracted from brown seaweed (kelp)</td>
<td>Water-holding, Gelling, Stabilizing, Emulsifying</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>Linear polymer of alternating 3-linked galactose and 4-linked D-galactose, containing some 3,6-anhydro-α-D-galactopyranose, and various sulfate residues. Seven types: k, λ, i, τ, δ, ν, μ, of which k, i and λ are most important.</td>
<td>Extracted from red marine algae of class Rhodophyceae, such as Chondrus crispus (k, λ), Eucheuma spinosum (i) and Eucheuma cottonii (k) (North and South America, Korea, Europe, Pacific Islands)</td>
<td>λ: Thickening, i, k: Binding, Gelling</td>
</tr>
<tr>
<td>Guar Gum</td>
<td>Linear →4)-β-D-mannopyranose-(1→ backbone, with α-galactopyranose residues linked to 6-position of every second residue.</td>
<td>Seeds of leguminous plants of pea family Cyamopsis tetragonolobus and psoraleoides (India, Pakistan and SW USA)</td>
<td>Most highly efficient water-thickener known, Adsorbs to mineral and cellulosic surfaces</td>
</tr>
<tr>
<td>Gum Arabic</td>
<td>Ca, Mg and K salt of acidic polysaccharide consisting of L-arabinose, L-rhamnose, D-galactose, and D-glucuronic acid of varying proportion depending on source.</td>
<td>Exudate of acacia tree, primarily Acacia senegal (Africa)</td>
<td>Protective colloid, Emulsifier, Lowers surface tension of water, Adhesive</td>
</tr>
</tbody>
</table>

Table 3 Continued on Next Page
<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Structure</th>
<th>Source</th>
<th>Most Desirable Properties or Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gum Ghatti (Indian Gum)</td>
<td>Ca and Mg salt of an acidic polysaccharide containing a 6-linked β-galactopyranose backbone residues with side chains attached by L-arabinose. Also contains D-mannose, D-xylose and D-glucuronic acid</td>
<td>Exudate of <em>Anogeissus latifolia</em> (India, Sri Lanka)</td>
<td>Excellent emulsifier, Thickener, Adhesive</td>
</tr>
<tr>
<td>Gum Karaya (Sterculia Gum)</td>
<td>Partially acetylated polysaccharide containing D-galactose, L-rhamnose, and D-galacturonic acid</td>
<td>Exudate of <em>Sterculia urens</em> (India)</td>
<td>Strong adhesive, Thickener, Forms very smooth films</td>
</tr>
<tr>
<td>Gum Tragacanth</td>
<td>Two components—Tragacanthic acid—contains 4-linked galacturonic acid, D-xylose, D-galactose, L-fucose. Arabinogalactan—highly branched neutral polymer of D-galactose and L-arabinose</td>
<td>Exudate of shrub genus <em>Astragalus</em> (Asia Minor, Syria, Iran, Turkey)</td>
<td>Bifunctional emulsifier—increases viscosity of aqueous phase and lowers interfacial tension without surface active agents, Suspending agent, Stable at low pH, Resistant to microorganisms</td>
</tr>
<tr>
<td>Locust Bean Gum</td>
<td>4-linked 8-D-mannopyranose backbone with non-uniformly spaced side branches of α-D-galactopyranose linked to 6-position of backbone ~ every fourth residue</td>
<td>Extracted from seeds of the carob tree, <em>Ceratonia siliqua</em> (Mediterranean region)</td>
<td>Efficient water-thickening agent, Ether derivatives used in variety of industries</td>
</tr>
<tr>
<td>Pectins</td>
<td>4-linked α-D-galcturonic acid, partially esterified with methyl groups. Two types: &gt;50% esterified (HM) and &lt;50% esterified (LM)</td>
<td>Extracted from apple pomace and citrus peels (by-products of various industries)</td>
<td>HM: Gelling agent LM: Gelling agent in Ca²⁺-containing systems</td>
</tr>
</tbody>
</table>
Table 3, continued

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Structure</th>
<th>Source</th>
<th>Most Desirable Properties or Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>Two components: Amylose: 4-linked α-D-glucopyranose. Amylopectin—Highly branched amylose backbone and other amylose-chains 6-linked to the backbone</td>
<td>Extracted from seeds of cereal grains (corn, sorghum, wheat, rice), and roots (potato, tapioca, arrowroot)</td>
<td>Thickening, Gelling, Adhesive, and Film-forming, Many derivatives</td>
</tr>
<tr>
<td>Tamarind Gum</td>
<td>15—20% protein, 4—7% oil, 65—72% carbohydrate. Polysaccharide: 4-linked β-D-glucopyranose backbone. 3 of 4 residues substituted with α-D-xylopyranose on 6 position. Some xylose residues contain α-D-galactose linked to 2 position. May contain L-arabinose.</td>
<td>Extracted from seeds of the tamarind tree, <em>Tamarindus indica</em> (India)</td>
<td>Thickener, Can replace starch in various applications</td>
</tr>
<tr>
<td>Xanthan Gum</td>
<td>4-linked β-D-glucopyranose backbone. Every second residue has side chain consisting of β-D-mannose (1→4)-β-D-GlcA-(1→2)-α-D-mannose-(1→3)-backbone. 1/2 terminal mannose residues are substituted with 4,6-linked pyruvate acetals. 6-position of α-mannose is acetylated</td>
<td>Exopolysaccharide of bacteria <em>Xanthomonas campestris</em></td>
<td>Very pseudoplastic, Suspending agent, Resistant to microorganisms, Uniform in structure</td>
</tr>
</tbody>
</table>

5. BIOCHEMISTRY AND PHYSIOLOGY OF EXOPOLYSACCHARIDES

5.A. Natural Function of Exopolysaccharides

The natural roles of exopolysaccharides have not been well elucidated. Hypotheses about the functions of capsular and slime polysaccharides have been made with regard to only specific polysaccharides, and it is unclear whether many of these observations are of a general nature. However, the EPS of individual bacteria is thought to act variously as a mask of the bacterium's cell-surface antigens, a steric buffer against the environment, and a modulator of the translocation of solutes into the cell. Each of these proposals will be discussed separately.

EPS may serve many functions with respect to the pathogenicity of the parent bacteria. The vast majority of pathogenic bacteria are capsulated, and it has been shown, in many cases, that the loss of the capsular material leads to a less-virulent bacterial form. The reason for this is not directly apparent, but it may be due in part to the inability of the slime polysaccharide to elicit antigens. For example, it has been noted that a strain of Klebsiella (formerly called Enterobacter) containing a relatively large capsule and a lot of loose slime is less effective at eliciting antibodies than a strain of the same serotype with a smaller capsule and only a little loose slime.

As a general barrier, the exopolysaccharide may act as a diffusion barrier, molecular sieve, and as an adsorbent. The thickness of the bacterial capsules has been estimated to be approximately 0.3–4.5 μm, depending on the strain and cultural conditions. The presence of these capsules leads to an "unstirred" layer, or a layer of material around each bacterium which is
not drawn into the bulk solution, even under vigorous stirring. The concentration of polysaccharide in these layers is estimated to be 1–2% by weight, or about the same as that found in agar used in laboratory media. Drawing an analogy between agar and polysaccharide may be instructive in determining the polysaccharide's function by its properties. Agar contains pores and channels through which solute molecules must diffuse in order to move. Pore sizes in agar gel range from 59–45 nm for 1–2% concentrations, respectively. It has been proposed that bacterial polysaccharides may act in a similar fashion to a molecular sieve in that solutes of higher molecular weights may be selectively excluded from migration to the cell itself, though this mechanism has not been tested directly. Whether or not the mechanism is one of molecular sieve action, or merely a steric barrier, the EPS serves to protect a bacterium against antibacterial serum factors which may not be able to migrate through the viscous outer layer to the site of action at the cell wall.

EPS is very hydrophilic, and binds quantities of water. Because of its hydrophilic nature, it has also been widely assumed that EPS protects bacteria from desiccation, but there is little direct evidence for this. The ability of the EPS to hold water in an ordered structure may decrease the solubility of various other macromolecules in the layer. The decreased solubility may lead to a lower total flux of solute across the polysaccharide layer, due to the lower concentration-gradient present for these solutes.

The role of EPS in a bacterial colony may be somewhat different from that in the individual cells. The EPS may form a protective layer around the colonies to protect the entire colony against predation. The matrix formed by the slime may also provide a micro-environment for these
bacteria, as it has been proposed that the slime allows bacteria to grow in solutions where the concentration of solutes are otherwise too dilute by allowing the bacteria to concentrate the desirable nutrients inside the polysaccharide layer by utilizing the molecular sieve action previously described.\textsuperscript{28,29} Exopolysaccharides themselves, however, are not typically thought to serve a storage or reserve function. Only a few strains of bacteria are known to be able to depolymerize their own EPS, and only a very few have been shown to be able to use their EPS to support growth.\textsuperscript{22}

Especially in aqueous environments, bacteria tend to form shiny adherent plaques on smooth surfaces. Though there are many mechanisms by which bacteria may adhere to surfaces, exopolysaccharides are often involved.\textsuperscript{22} A common example of adherent plaques occurs in dental caries, where bacteria of the strain \textit{Streptococcus mutans} adhere to smooth surfaces (teeth), form dextrans, and produce quantities of organic acids by the fermentation of dietary carbohydrates. These organic acids lead to the demineralization of the tooth and cause decay.\textsuperscript{30}

5.B. Biosynthesis of Bacterial Heteropolymeric Exopolysaccharides

Microbial heteropolysaccharides can be biosynthesized by the bacterium from almost any carbon source, unlike other bacterial homopolysaccharides such as dextrans and levans, which can only be synthesized from sucrose. Exopolysaccharide synthesis is dependent upon the presence of activated monosaccharide units, the monosaccharide nucleotide diphosphates (NDP-sugars).

Some sugar nucleotides such as UDP-GalA and GDP-ManA are precursors solely in the EPS biosynthetic cycle while others, like UDP-Gal,
are in a pool of precursors also used for other carbohydrate-containing bacterial constituents. Several NDP-monosaccharides are derived from other NDP-monosaccharides while at the nucleotide level, such as UDP-Gal, which is derived from UDP-Glc by the action of the enzyme UDP-Glc-4-epimerase.

Most monosaccharide residues present in the mature polysaccharides are introduced by the nucleotide precursor of that specific sugar. However, exceptions occur for some uronic acids. The only truly known exception in microorganisms occurs in the biosynthesis of bacterial alginate. The D-mannuronic acid introduced into the backbone of alginate is partially converted by an extracellular mannuronan 5'-epimerase to form L-guluronic acid\(^{31}\) when the saccharide is at the polymeric level. This may in fact not be the only exception, as in some cases the involvement of the NDP-monosaccharides of some unusual monosaccharides present in bacterial polysaccharides has not yet been experimentally demonstrated. The mechanism of the synthesis of these residues or their incorporation into the polysaccharide has not yet been elucidated. Examples of these unusual monosaccharides include the methylated hexoses\(^ {32}\) and O-methylglycuronic acids.\(^ {33}\)

The NDP-monosaccharides all appear to be polymerized utilizing a lipid primer. Lipid intermediates (undecaprenyl phosphates) have been directly demonstrated to be involved in the synthesis of some EPS heteropolysaccharides. The proposed mechanism involves the initial transfer of a monosaccharide 1-phosphate from a NDP-monosaccharide, followed by the sequential transfer of sugars from other NDP-monosaccharides to the isoprenoid lipid phosphate to form a complete
repeating-unit (before any possible post-polymeric modifications). This is a similar mechanism to that proposed for the biosynthesis of the O-antigen heteropolysaccharides in Salmonella species.

The chain-elongation or polymer-assembly step is proposed to be the transfer of a complete repeating-unit to the reducing end of another lipid-phosphate repeating-unit to form multiples of the repeating-unit attached to the lipid. The evidence for this mechanism includes the isolation of some lipid intermediates containing only integral numbers of repeating-units from Klebsiella species, and from E. coli. This too is similar to the O-antigen mechanism of chain elongation. This isoprenoid lipid-linked mechanism of heteropolysaccharide biosynthesis has not been unequivocally demonstrated in all species of bacteria. However, in any case, the nascent oligosaccharides seem to be transferred to endogenous acceptors consisting of growing EPS chains in the cell wall. Homopolymers such as dextrans, mutans, and levans are very likely biosynthesized by separate mechanisms.

The addition of acetate and pyruvate groups from acetyl CoA and enolpyruvate phosphate, respectively, occurs at the level of the lipid intermediate. In many cases, the levels of the acetate and pyruvate in the repeating-unit are not stoichiometric and these substituents need not always be present for the completion of the assembly of the repeating-unit to occur. In Xanthomonas campestris, for example, the addition of a pyruvate group to a mannose residue is not necessary for the assembly of the repeating-unit, whereas the addition of a pyruvate group to a particular monosaccharide residue was necessary for the completion of the entire repeating-unit in a Klebsiella species EPS. (See Figure 12.)
Figure 12. The Proposed Biosynthetic Cycle for Xanthan Gum
After the putative exopolysaccharide is formed at the cytoplasmic membrane of the bacterium, the polymer must be translocated to the exterior of the cell. The polymer is presumably attached to the isoprenoid lipid as it is being synthesized at the cell membrane (See Figure 13). The lipid is also used in the biosynthesis of other bacterial components and it must be returned to the cell membrane. At the same time, the hydrophilic polysaccharide must be translocated across the hydrophobic outer membrane to the outside of the cell. Authentic polysaccharide accumulates in the periplasm of certain insertion mutants of *E. coli*, indicating that the periplasmic assembly occurs before translocation of the polysaccharide across the outer membrane.44

The most likely mechanism for the polymer translocation seen to date involves the adhesion, or Bayer, sites of the cell.45 Bayer sites are regions where the inner cytoplasmic membrane and outer membrane come into close apposition. These sites have been proposed to be the export sites for lipopolysaccharides and various outer-membrane proteins.46,47 The actual mechanism of export of any type of material through the Bayer sites is not known.
Figure 13. Structures of Some Biosynthetic Intermediates in Heteropolysaccharide Biosynthesis
CHAPTER II

BACKGROUND

1. GENERAL SCHEME OF POLYSACCHARIDE STRUCTURE DETERMINATION

The general scheme for determining polysaccharide structure, once the polysaccharide's chemical homogeneity has been established, is threefold. These three stages are not necessarily chronological in sequence, but are meant to divide the structure determination into three cohesive parts with respect to the goals of the stage.

The first stage is the determination of the quantitative subunit composition of the polysaccharide. This includes the identification and quantitation of each monosaccharide as well as determining the presence of any non-carbohydrate organic substituents present in the polysaccharide.

The second stage involves the elucidation of the structural formula of a possible repeating-unit, the identification of the linkages between each monosaccharide unit, and the location of any organic substituents on the monosaccharide components.

The third stage is the definition of remaining stereochemical features in the macromolecular structure. This involves specifically the determination of the anomeric linkage of each monosaccharide, as well as the absolute configuration of any chiral substituents.
2. INITIAL CHARACTERIZATION OF THE POLYSACCHARIDE

Traditional wet-chemical analyses remain standard methodology in polysaccharide structure determination. Physical methods, and NMR in particular, are now generally employed for initial analyses as well as for more detailed structure elucidation.

2.A. Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton- ($^1$H-) and carbon- ($^{13}$C-) nuclear magnetic resonance spectroscopy (NMR) are the most useful tools for probing several aspects of polysaccharide structure. This technique is most generally useful for polysaccharides in solution, and have been the subject of several reviews. $^{48-53}$ NMR is ideally suited for soluble polysaccharides having a regular repeating-structure.

For such polysaccharides, the NMR spectrum may be first used to determine which major types of monosaccharides are present in the polysaccharide. Each type of sugar has characteristic resonance patterns. Atoms of the sugar backbone bearing primary and secondary hydroxyl groups give resonances in approximately the same region of the spectrum, but well-resolved and characteristic resonances appear for other types of sugars such as 6-deoxyhexoses, uronic acids, and amino sugars.

Second, the NMR spectrum may be used to identify any organic substituents which may be attached to the polysaccharide chain. Characteristic peaks appear in the spectrum for such substituents.

Most importantly, each anomeric center of each monosaccharide component gives characteristic resonances within a clearly identifiable range, which is well separated from the other resonances of the
monosaccharides. Counting of the $^1$H- and/or $^{13}$C- resonances in the anomeric region is the most useful method for determining the number of monosaccharides in the repeating-unit. Thus, the NMR spectrum can be used to determine the size of the repeating-unit.

Fourth, the NMR spectrum may be used to at least tentatively assign anomeric configuration. The anomeric region is loosely divided into the β-region and the α-region in both $^1$H-NMR and $^{13}$C-NMR. Monosaccharides whose anomeric centers resonate in the β-region are typically β-linked and those that resonate in the α-region are α-linked. This aspect will be discussed more fully in Section 4.A.1. of this chapter.

2.B. Infrared (IR) Spectroscopy

Infrared (IR) spectroscopy is less generally useful than NMR, but may be used to provide complementary information about the polysaccharide, and particularly on substituent groups present in the polysaccharide. Although the "fingerprint" region of the spectrum is generally too complex to furnish much useful information, and the strong hydroxyl band in the functional group region does not provide specific discriminatory information about the polysaccharide structure, other functional group absorptions may be of value. The IR spectrum is especially useful for those polysaccharides that contain carbonyl groups. Carbonyl groups give typical, strong absorbptions in the IR functional-group region, but carbonyl-group signals may not be readily seen in many cases in a $^{13}$C-NMR spectrum because of the signals' weak intensities and the typically large background noise in spectra of macromolecules. Esters, acids, and amides show very
strong and characteristic patterns that are readily identified in the IR spectrum.\textsuperscript{54,55}

2.C. Sugar Analysis

Monosaccharide components of polysaccharides are routinely identified and quantitated by two methods. In the first, complete acid hydrolysis of the polymer followed by paper chromatography of the hydrolyzate indicates the major types of monosaccharides present, and provides provisional identification of these monosaccharides. Paper chromatography may also be scaled up on a preparative level to provide recoverable samples whose enantiomeric identity can be established by polarimetry, and for classic characterization through crystalline derivatives.

In the second method, gas chromatography of acyclic derivatives of the acid hydrolyzate is used to quantitate and positively identify the monosaccharides present through calculation of the peak areas and comparison of retention times with known standards, respectively. This method has the advantage of being more sensitive and can be routinely used to identify closely related monosaccharides. However, this method suffers a disadvantage in that it does not detect acidic monosaccharides such as uronic acids, which decompose or do not form volatile derivatives in this procedure, nor does it directly establish the enantiomeric form of the monosaccharides. (The enantiomeric form of the monosaccharides may be established by GC, however, through glycosidation of the monosaccharides present in the acid hydrolyzate with a chiral aglycon such as 2-(\textendash )-octanol, and comparison of the retention times of the glycosides with standards of known configuration.)
In a typical analysis, a combination of paper chromatography and GC is used to ensure the detection of all monosaccharides present.

2.C.1. Total Acid Hydrolysis

The total hydrolysis of a polysaccharide to its component monosaccharides is one of the most basic procedures in structural carbohydrate chemistry. However, acid hydrolysis is a somewhat difficult reaction since the liberated monosaccharides tend to degrade under the severely acidic conditions necessary to cleave the acetal linkages. There is a tradeoff between the complete cleavage of all the glycosidic bonds and the decomposition of the liberated monosaccharides.

In practice, an empirical set of conditions for the hydrolysis of the polysaccharide polymers should be determined. Uronic acids and amino sugars tend to form extremely acid-resistant glycosidic bonds. The drastic conditions necessary to effect cleavage of these bonds degrades the liberated uronic acids and certain other monosaccharides. A thorough review has been written regarding the total acid hydrolysis of polysaccharides, including the application of various types of acids and the relative rates of hydrolysis of different sugar components. Complementary information may be obtained by hydrolysis under both vigorous and mild conditions.

2.C.2. Reduction and Acetylation

Each monosaccharide (or a monosaccharide derivative from the hydrolysis of a polysaccharide) is a complex tautomeric mixture, and is conveniently reduced to an alditol with borohydride, (or in the case of derivatized polysaccharides, borodeuteride), to give single, stable product.
These alditos are then typically converted into derivatives (peracetates or per(trimethylsilyl) ethers) which are sufficiently volatile for gas-chromatographic analysis. (See Figure 14.)

The incorporation of borodeuteride into an alditol allows for the identification of the mass-spectral fragments arising from portions of the alditol containing C-1 ("top" fragments) from those which do not ("bottom" fragments), which can aid in the absolute assignment of the methylation profile (see Section 3. of this chapter) for those alditols which otherwise may be symmetrical.

2.C.3. Gas Chromatography (GC)

In the case of an underivatized polysaccharide, a simple gas-chromatographic analysis is sufficient for the identification of the monosaccharides present as their derived alditol peracetates. In most cases, known standards can be readily obtained for each monosaccharide. The separation of the alditol acetates is performed easily even on standard packed columns of various polarities, and in many cases the use of a ramped temperature program can enhance the separation of closely eluting alditol acetates. Tabulations have been published of the retention times for various alditol peracetates on a wide variety of packed and capillary columns.57,58
Figure 14. Hydrolysis, Reduction, and Acetylation of a Polysaccharide
3. LINKAGE ANALYSIS OF POLYSACCHARIDES

The classic method of per-O-methylation of a polysaccharide followed by acid hydrolysis and identification of the partially methylated monosaccharide fragments remains the basis of modern, micro-scale linkage analysis of polysaccharides. Methylation of polysaccharides is effected by several different types of reactions which can yield complementary results.

3.A. Modified Hakomori Methylation

Older methylation procedures (see Section 3.B.) have been largely supplanted by variations of the procedure introduced by Hakomori. In Hakomori methylation analyses, all hydroxyl groups not involved in base-stable linkages are converted into their methyl ethers by a Williamson etherification reaction. Additionally, carboxylate groups are esterified to methyl esters. Acid hydrolysis of the permethylated polysaccharide, followed by reduction (with borodeuteride) and acetylation of the partially methylated monosaccharides yields partially methylated alditol acetates, which are analyzed using gas chromatography–mass spectrometry (GC/MS). (See Figure 15.) As in the alditol acetate method of sugar analysis, uronic acid residues are not detected directly by this method. Special techniques must be used to quantitate and identify the linkage pattern of these residues, and these techniques are discussed in Section 3.C. of this chapter.
Figure 15. Hakomori Methylation Analysis
3.A.1. The Methylation Analysis

In all forms of the Hakomori methylation, the anion of (di)methyl sulfoxide (dimsyl anion) is used to deprotonate all of the free hydroxyl groups. (One research group has proposed that the dimsyl anion is merely a catalyst for this deprotonation, and that hydroxide ion is actually the active species.\textsuperscript{60}) Reaction of the deprotonated hydroxyl groups with methyl iodide yields the methyl ethers. The modified Hakomori methylation\textsuperscript{59} utilizes the lithium salt of methyl sulfoxide rather than the sodium salt used in the original procedure.\textsuperscript{61} The lithium derivative can be prepared in higher purity with less danger than the sodium or potassium\textsuperscript{62} derivative.

3.A.2. Gas Chromatography–Mass Spectrometry (GC/MS)

The identities of the partially methylated alditol acetates are determined from the comparison of their retention times with those of suitable internal standards. These may be derived conveniently from cellulose or a mannann treated in the same fashion as the polysaccharides under investigation. The substitution patterns of the methyl ethers and the acetate esters on the alditol backbone are determined from the readily predictable and characteristic mass-spectral patterns, which have been carefully catalogued for many common types of partially methylated alditol acetates, and other various types of carbohydrates.\textsuperscript{57,63–66}

3.B. Other Alkaline Methods for the Methylation of Polysaccharides

Other methods for the alkaline methylation of polysaccharides have been devised. The original procedure of Dudman and Woodhouse\textsuperscript{67} for methylating cellulose utilized 30% aqueous sodium hydroxide and
dimethyl sulfate. Haworth applied this reaction to simple sugars and their glycosides.\textsuperscript{68} The original procedure has been subjected to several refinements and improvements.\textsuperscript{69–72} More recently, Ciucanu and Kerek\textsuperscript{60} have utilized an alkali metal hydroxide, sodium hydride, or potassium tert-butoxide in methyl sulfoxide for the deprotonation step in conjunction with methyl iodide to effect methylation. Ciucanu and Kerek maintain that hydroxide ion, hydride ion, or tert-butoxide ion is the active species in these deprotonation procedures,\textsuperscript{60} but it cannot be ruled out that dimsyl anion is at least partially responsible for deprotonation. If this in fact the case, the procedure of Ciucanu and Kerek should be considered a variant of the Hakomori procedure. Some workers have noted a tendency for oligosaccharide alditols to oxidize under Ciucanu and Kerek's conditions.\textsuperscript{73} Sodium hydride in various solvents in conjunction with methyl iodide has also been used to methylate polysaccharides.\textsuperscript{74}

For methylations such as these (including the Hakomori method) which involve the contact of a strong base with the carbohydrate, the predictable $\beta$-eliminative degradation of polysaccharides containing uronic acids has been noted.\textsuperscript{63,74–79} (See Section 3.D.1.) Therefore, a uronic acid-containing polysaccharide should be subjected to several methylation procedures and the method which gives a minimum of degradation products for a specific polysaccharide should be used if possible.

3.C. Carboxyl Reduction

As previously mentioned, uronic acids are not detected directly by either the alditol acetate method of monosaccharide analysis or the modified Hakomori methylation method of linkage analysis. The most
direct way to identify and quantitate these residues is to reduce the ω-carboxyl group of the hexuronic acid to a primary alcohol, yielding the neutral hexose derived from the uronic acid. A deuterated reducing agent allows the ready differentiation by MS of these reduced derivatives from the corresponding neutral sugars present in the polysaccharide.

Carboxyl reduction of a methylated polysaccharide may be used to reveal the linkage pattern of the original uronic acid, and allow the identity of the uronic acid present to be verified. Carboxyl reduction of an underivatized polysaccharide is used for the quantitation and identification of the neutral hexose derived from the uronic acid.

3.C.1. Carboxyl Reduction of a Methylated Polysaccharide

In order to confirm the identity of the uronic acid and to determine the non-reducing linkage present on the uronic acid, the polysaccharide is subjected to the modified Hakomori methylation followed by carboxyl reduction of the resulting methyl ester of the uronic acid. Hydrolysis of the methylated and borodeuteride-reduced polysaccharide to the constituent partially methylated monosaccharides, followed by the reduction of the anomeric center of each monosaccharide and acetylation, yields a mixture of partially methylated alditol acetates, including the 6-O-acetyl-6,6-dideuterio partially methylated alditol acetate derived from the original uronic acid. The mixture of the partially methylated alditol acetates is then analyzed by GC/MS. (See Figure 16.)
Figure 16. Carboxyl-Group Reduction of a Permethylated Polysaccharide
3.C.2. Carboxyl Reduction of the Native Polysaccharide

The classic procedure of Taylor and Conrad is typically used to reduce the uronic acid groups in an underivatized polysaccharide. Taylor and Conrad react the carboxyl group of the polysaccharide with a water-soluble carbodiimide under carefully controlled pH conditions. The resulting adduct is reduced to the primary alcohol by the addition of sodium borodeuteride, also under carefully controlled pH conditions. The reduced polysaccharide is analyzed by the alditol acetate method or by methylation analysis (see Figure 17).

3.D. Chain Cleavage Reactions

3.D.1. β-Elimination Reaction

In polysaccharides containing uronic acid residues, chain cleavage may be effected by the action of strong base on the permethylated polysaccharide. When a uronic acid methyl ester is present, as in the product of methylation of a polysaccharide, the hydrogen on the carbon α to the carboxyl carbon (H-5) is sufficiently acidic to be removed by a strong base such as methoxide. After, or concurrently with, the deprotonation of C-5, a facile elimination of the leaving group present at C-4 (either methoxide or the reducing terminus of another monosaccharide) occurs. The resulting 4,5-unsaturated uronic acid at the non-reducing terminus of the oligosaccharide fragment is unstable to acid and is decomposed by exposure to mild acid. Methylated oligosaccharide fragments result, including a non-reducing hydroxyl group to which the uronic acid was linked. This free hydroxyl group can be tagged using an ethyl ether or a trideuteriomethyl
Figure 17. Reduction of Uronic Acid Residues by the Taylor-Conrad Method
ether by the modified Hakomori procedure, and readily located using GC/MS. In this fashion, the residue and position to which the uronic acid was linked through its anomeric center can be located. (See Figure 18.)

A similar selective cleavage method for the cleavage at uronic acid residues has been described which involves the decarboxylation and reductive cleavage of the uronic acid residues.


A fragmentation procedure for obtaining quantities of oligosaccharides or degraded polysaccharides formed by the cleavage and loss of the uronic acid unit has been described by Mort and Bauer. The method utilizes lithium metal in ethylenediamine to simultaneously decompose and remove the uronic acid unit in a polysaccharide (see Figure 19). This procedure is advantageous in that the polysaccharide does not need to be derivatized before reaction. The resulting oligosaccharides or degraded polysaccharide is amenable to spectroscopic or other chemical analyses.


Anhydrous hydrogen fluoride (HF) has been used for the total hydrolysis of polysaccharides, for the depolymerization of the polysaccharides into their component repeating-units, and to randomly cleave the polysaccharide into smaller oligosaccharides. Theoretically, oligosaccharide fragments that overlap those prepared by the cleavage of the uronic acid units in a polysaccharide may be obtained. In practice, the anhydrous HF depolymerization works best when the polysaccharide
Figure 18. β-Elimination Reaction of a Methylated Polysaccharide
Figure 19. Degradation of a Uronic Acid-Containing Polysaccharide by Lithium Metal in Ethylenediamine
contains one type of linkage that is more acid-labile than the rest of the monosaccharides, such as a 6-deoxy sugar, and when the repeating-unit contains only a few monosaccharides and is unbranched. In addition, HF depolymerization is of limited value for large repeating-units containing only neutral hexose residues as large amounts of material are required for the generation of enough pure oligosaccharide to enable the analysis of the structure of the fragment. Limited treatment of a complex polysaccharide by HF can, however, facilitate NMR analysis of viscous polysaccharides or complex polysaccharide repeating-units, as the treated polysaccharide solution has a decreased viscosity.

Anhydrous HF is extremely dangerous as it causes severe burns when inhaled or contacts the skin. An apparatus marketed for the safe handling of anhydrous HF exists, but it is expensive and only available in a large size unsuitable for the smaller reactions typically needed in carbohydrate analysis. An alternative microapparatus for the safe handling of anhydrous HF has been proposed.85

3.E. Location of Non-Carbohydrate Substituents

3.E.1. Location of Base-Labile Substituents


Base-labile organic substituents, including acetic and succinic esters, are removed by the strongly alkaline conditions found in the modified Hakomori methylation procedure. A non-alkaline method of methylation analysis can be used to determine the location of these substituents.
Comparison of the modified Hakomori methylation and the non-alkaline methylation profiles indicates the location of the base-labile substituents.

The methyl trifluoromethanesulfonate (methyl triflate) methylation procedure of Prehm\textsuperscript{86} is a neutral or slightly acidic procedure that allows the location of base-labile substituents (see Figure 20). Other classical non-alkaline methylation procedures include the alkyl iodide–silver oxide method of Purdie and Irvine.\textsuperscript{87} Refinements of the Purdie–Irvine procedure to include many sugar types including amino sugars have been proposed.\textsuperscript{88–90}

The methylated polysaccharides obtained from either procedure can be analyzed in the same manner as the polysaccharides methylated by the modified Hakomori procedure.

3.E.1.b. Acyclic Acetal Formation

Acetate and succinate groups may be located by substituting all free hydroxyl groups with removable base-stable protecting groups in such a manner that the base-labile groups remain intact. The base-labile groups are then exchanged for methyl ethers by an alkaline methylation procedure. The protecting groups are removed, and the partially methylated polysaccharide is analyzed by GC/MS. Acyclic acetals introduced by the reaction of methyl or ethyl vinyl ether with the polysaccharide have been used as convenient base-stable protecting groups that are easily removed with acid during the subsequent hydrolysis step.\textsuperscript{91}
Figure 20. Non-Alkaline Methylation by the Methylation of Prehm
3.E.2. Location of Base-Stable Substituents

Pyruvic acetals are located by methylation of the polysaccharide, removal of the pyruvic acetal under weakly acidic conditions, and tagging the liberated hydroxyl groups with trideuteriomethyl or ethyl ethers. The pyruvate acetal is more acid-labile if its free carboxyl group is reduced to the alcohol after the methylation step.

4. DETERMINATION OF THE CONFIGURATION OF THE REPEATING-UNIT

4.A. Anomeric Configuration

4.A.1. NMR Analysis

4.A.1.a. $^{13}$C-NMR Analysis

As mentioned in Section 2.A. of this chapter, $^{13}$C-NMR can be used to determine the configuration of the anomeric centers of the monosaccharides in the repeating unit. For monosaccharides that adopt stable $^4C_1$ conformations, such as glucose and galactose, the anomeric region can be divided into the axial and equatorial regions. In the vast majority of the cases for the D-series, the monosaccharides whose resonances fall into the equatorial region are $\beta$-linked and the monosaccharides whose anomeric center resonance falls into the axial region are $\alpha$-linked. There are many tabulations of data for the $^{13}$C-NMR chemical shifts of the anomeric centers of various mono-," oligo-, and
poly-saccharides. In general, for the D-series of hexopyranosides, the anomeric resonances which fall between 90 and 100 ppm are considered to arise from axial, or α-linkages, and those that fall above 100 ppm are from equatorial or β-linkages.

4.A.1.b. $^1$H-NMR Analysis

When feasible, $^1$H-NMR is perhaps more useful than $^{13}$C-NMR in determining the anomeric disposition of monosaccharide in the polysaccharide repeating-unit. Anomeric signals generally fall between 4.5 and six ppm for glycosides of sugars. The anomeric region is loosely divided into the "equatorial" and "axial" regions with respect to the proton on the anomeric carbon (typically C-1). When the proton on C-1 has an equatorial disposition in the most stable chair conformation, the anomeric resonance tends to come further downfield than when the proton is axial.

Perhaps the most useful feature of $^1$H-NMR spectra, however, is the tabulation of the coupling constants ($J$-values) between the proton on C-1 and its neighbor on C-2 ($J_{H-1,H-2}$). The anomeric proton resonance appears as a doublet for most natural sugars, since it is coupled to a single proton on C-2. The value of the coupling constant is dependent upon the relative orientations of the protons on C-1 and C-2. When the protons are diaxial, coupling constants tend to be between eight and 11 Hz. If one proton is axial and the other equatorial, coupling constants ideally range from three to four Hz, and when the protons are diequatorial, the coupling constant is one to two Hz. Deviations from these values are considered to arise from either a flattening or puckering of the chair shape, which changes the dihedral angle between the protons, or from an equilibrium between the
ring-flipped chair forms of the particular monosaccharide. The latter is typically not seen in the common hexopyranosides. Knowledge of the identity of the sugar residue, i.e., knowing the absolute orientation of the proton on C-2, and its resonance in the $^1$H-NMR spectrum, can allow the anomeric configuration of the residue to be deduced.\textsuperscript{94}

Unfortunately, the residual HOD resonance resulting from the incomplete exchange of the alcohol protons to their deuterated form falls into the anomeric region, and can obscure some of the anomeric peaks. The location of the HOD peak is sensitive to temperature, and, if it is not very large, it can be moved by adjustment of the temperature. $^1$H-NMR spectra at both high and ambient temperatures may be needed for a complete picture of the anomeric region.

4.A.2. Chromium Trioxide Oxidation

For polysaccharides that are not amenable to NMR analysis, or for the verification of the anomeric configuration of a residue, the chromium trioxide oxidation method\textsuperscript{95} may be used. It has been shown that chromium trioxide oxidizes the acetal (glycoside) functionality of suitably protected sugars (both monosaccharide glycosides and polysaccharides) to a keto ester. Furthermore, it has been shown that the H-1–C-1 bond in an equatorial acetal linkage is oxidized much more rapidly than an H-1–C-1 bond which is in an axial acetal linkage. Therefore, the limited oxidation of a suitably protected polysaccharide in the presence of an internal standard (inositol hexaacetate), followed by the decomposition of the resulting keto ester derivative, and analysis of the remaining alditol acetates by GC can
provide information on the anomeric configuration of the monosaccharides in a repeating unit.96,97 (See Figure 21.)

4.B. Configuration of Pyruvic Acetals

The pyruvic acetal is typically linked to the 4- and 6- position of a terminal monosaccharide residue, forming a fused [4.4.0] (cis- or trans-decalin type) ring system. (See Figure 22.) The methyl group of the pyruvic acetal may be oriented axially or equatorially with respect to the pyruvate ring. The chemical shift of the methyl group is more sensitive to its axial or equatorial orientation than to the specific monosaccharide to which it is linked. Therefore, the orientation of the methyl group can be determined solely from its chemical shift.

The chemical shift of the methyl groups for all possible configurations of pyruvic acetals have been tabulated.98 Interestingly, in all naturally occurring hexopyranose 4,6-pyruvate acetals studied to date, the methyl group has been found in the equatorial position. For the gluco- and manno- pyranose 4,6-pyruvate acetals, the pyruvate retains the S-configuration, and in the galactopyranose 4,6-pyruvate acetal the pyruvate has the R-configuration.
Figure 21. Chromium Trioxide Oxidation of a Peracetylated Polysaccharide
Figure 22. The Chirality of Pyruvic Acetal Substituents
Chapter III

THE STATEMENT OF THE PROBLEM

Industrial Polysaccharides

High-field NMR spectroscopy has greatly reduced the need for wet-chemical reactions in determining the structure of all types of carbohydrates. Every resonance of many mono- and di-saccharides can often be assigned, and, utilizing two-dimensional NMR techniques, the spectra of higher-order saccharides may also be worked out. However, there are many underivatized carbohydrates, and in particular, polysaccharides, which are intractable to NMR analysis. This is often caused by their unwillingness to form homogeneous solutions due to a high degree of secondary structure in solution. Attempts at dislodging the higher-order conformational structures have been made; sonication has been and continues to be a most useful method for rendering a polysaccharide soluble in dipolar solvents. However, many of the polysaccharide solutions which contain a large degree of secondary structure in solution are extremely viscous at relatively modest concentrations. The solutions must be made dilute, and these dilute solutions need to be analyzed in the NMR over a long period, and the tendency for the secondary structure to re-form is very high.

Viscous polysaccharides are useful to a wide variety of industries, and interest is being shown in their structures. NMR analysis can be an
informative, inexpensive (if facilities are available), and noninvasive method for yielding a large quantity of information about a carbohydrate in a short period of time. If the sample does not lend itself to NMR analysis, other techniques must be used.

Certainly, the basis for the wet-chemical analysis of polysaccharides has been in place for many years. The recent advances in separation technology, especially for small quantities of samples, and the introduction of several safer and more specific reagents for the derivatization of carbohydrates opens the field for advances in the chemistry of polysaccharide structure determination.

Acidic polysaccharides are, in general, very interesting with respect to their rheological properties. Many of the polysaccharides which are extremely viscous contain acidic residues or substituents. Many of these acidic, viscous polysaccharides have been screened for use as industrial thickeners, and interest has been shown in their structures. These polysaccharides often form gels, are very viscous, or are insoluble at low to modest concentrations in water or methyl sulfoxide, and even high-field NMR analysis does not reveal the desired structural information.

The industrial polysaccharides studied here were supplied by Hamish McArthur of Pfizer, Inc., Groton, CT. The potentially industrially useful exopolysaccharides were collected from a laboratory fermentation of bacteria originally isolated from soils throughout the world, unless otherwise noted. The bacteria have been characterized by the ATCC, and microbiological data on the bacteria may be obtained from ATCC records. The numerical polysaccharide names used here are derived from the
proprietary numbers assigned to each individual bacterial strain by Pfizer, Inc., and have no intrinsic meaning.

Bacteria were grown in minimal media in a laboratory fermentation culture. Polysaccharides were isolated from the media by isopropyl alcohol precipitation. The polysaccharides were dissolved in water and lyophilized. Before use, the polysaccharides were chromatographed on Bio-Gel P-300.

Plant Pathogen Polysaccharides

Many pathogenic bacteria produce exopolysaccharides. Pathogenicity of the bacteria has been related to polysaccharide production. *Erwinia stewartii* is a major bacterial pathogen that infects young sweet corn plants and causes Stewart's Wilt. The bacteria is spread by a flea beetle which feeds on the leaves of the corn plant. The manifestations of Stewart's Wilt are the watersoaking of the leaves and wilting of the plant itself.100

"Watersoaking" is defined as the retention of fluids in the leaves of the plant. The watersoaking symptom of Stewart's Wilt is thought to be caused by the bacteria growing in the intercellular spaces of the leaves of the plant. The cell membranes in this area lose their semipermeability, and fluids accumulate in the affected area. The general wilting of the plant is caused by bacteria which grow in the xylem (water-carrying cells) of the plant. The bacteria plug the xylem with their relatively viscous exopolysaccharides, decreasing or eliminating the flow of water through the plant.

Virulent strains of *E. stewartii* produce a watery, smooth, spreading colony, and copious amounts of an acidic exopolysaccharide. Avirulent strains tend to form smaller, rough, raised, and non-mucoid colonies.100 It
was known that the virulence of a related bacteria, *E. amylovora*, (which causes fireblight of fruit trees), was directly proportional to the strain's exopolysaccharide production. The ability of the exopolysaccharide from *E. amylovora* to act as a wilting agent was related to its polysaccharide's physical and chemical properties. The exopolysaccharides of a virulent (wild-type) and avirulent (mutant) strain of *E. stewartii* which continued to produce exopolysaccharide were to be examined. The polysaccharide of the wild-type and mutant strains of *E. stewartii* were to be compared to determine whether or not they were identical in composition, and to, if possible, obtain methylation analysis data on the polysaccharide toward determining its linkage pattern and structure.

In this portion of the study, the purpose was to:

1. Determine the repeating-unit structure of several viscous, potentially industrially useful bacterial exopolysaccharides which were isolated from bacteria found in soils throughout the world, and
2. Evaluate existing techniques in structural carbohydrate chemistry and determine which are useful for studying viscous polysaccharides, and, if possible, find new procedures to replace those which are not acceptable.
3. Determine whether or not two polysaccharides of two separate strains of *E. stewartii* are identical in chemical structure
CHAPTER IV

RESULTS AND DISCUSSION

Part A. General Methodology

1. GAS CHROMATOGRAPHY-MASS SPECTROMETRY

The derivatives of the constituent monosaccharides of the polysaccharides studied here were analyzed on a capillary GC system equipped with a mass-selective detector. The use of a capillary column greatly enhanced the separation of the partially methylated derivatives encountered in polysaccharide structure determination when compared to traditional packed columns. In addition, capillary columns require less sample than the packed columns, which allows a greater number of manipulations to be done on a small amount of material.

The column used in these studies easily separated mono- from di-, tri-, and tetra-O-methylhexitol peracetate derivatives, and was reasonably effective in separating the individual derivatized monosaccharides within each of these classes. With the use of internal standards, it was possible to quite readily assign identities to each of the partially methylated derivatives.
1.A. Internal Standards

Standards of suitably treated cellulose, amylose, an ivory nut mannan, or synthetically derived monosaccharides were used to calibrate the column with each use. In many cases, these standards were used internally. Internal standards were reduced with borohydride rather than borodeuteride when forming the alditol. Comparison of the retention times of the specific fragment ions (which differ by one mass unit) of the peak in question and of the standard allowed for the comparison of the retention times for the purpose of identification. Since molecular ions of carbohydrates are never seen in electron-impact mass spectrometry, the "primary" fragment ions (ions arising from the scission of the alditol backbone chain) were used for comparison. The GC/MS instrument which was used for these analyses is equipped with software which allowed the ready viewing of specific-ion chromatograms. Alternatively, tabulations of the fragment ions from each scan throughout the standard and the peak in question were obtained and the abundance of the specific ions vs. time were hand-plotted and compared.

Caution must be exercised when comparing internal standards which do not have deuterium incorporated into them versus samples which do. A deuterated compound will elute slightly faster under the conditions used than its non-deuterated counterpart. The ratio of the retention times of nondeuterated to deuterated otherwise-identical hexitols in these analyses was approximately 1.001. This very small difference could be readily seen when utilizing specific-ion chromatograms to compare retention times. This difference in retention time needs to be recognized when comparing elution times for identification purposes.
The retention times of partially methylated alditol acetates encountered in this study appear in Table 4.

1.B. Contaminants and Artifacts

Due to the sensitivity of the system, contaminants and artifacts are easily located in the analyses. Knowledge of the commonly seen artifacts and contaminants is essential when analyzing polysaccharide derivatives.

1.B.1. Contaminants

Plasticizers are very common contaminants in mass-spectral analyses. Avoiding contact between the dichloromethane solvent and the plastic caps and cap liners of the storage vials greatly reduced the amount of contamination.

Phthalate esters were the most common contaminant seen in these analyses. Phthalate esters typically elute typically directly after the hexitol hexaacetates, which were the last relevant peaks to elute, and therefore were not a problem in the analyses. The characteristic peak of phthalate ester mass spectra is an abundant peak at \( m/z \) 149.

The mass spectra of many types of contaminants commonly encountered in mass spectrometry has been thoroughly reviewed.\textsuperscript{103}

1.B.2. Artifacts

Several artifacts can arise in the methylation analysis of the polysaccharides. Artifacts are defined here as carbohydrate derivatives seen the GC/MS analysis which do not arise directly from the primary monosaccharide structure in the polysaccharides. Once recognized for what
Table 4. Retention Times of Partially Methylated Alditol Acetates

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Me₄-1,5-Ac₂ Glc</td>
<td>1.00</td>
</tr>
<tr>
<td>2,3,4,6-Me₄-1,5-Ac₂ Man</td>
<td>1.01</td>
</tr>
<tr>
<td>2,3,6-Me₃-1,5-Ac₂ Hex⁴</td>
<td>1.02</td>
</tr>
<tr>
<td>2,3,4,6-Me₄-1,5-Ac₂ Gal</td>
<td>1.05</td>
</tr>
<tr>
<td>2,3,6-Me₃-1,5-Ac₂ Hex⁴</td>
<td>1.06</td>
</tr>
<tr>
<td>2,3,6-Me₃-4-Et-1,5-Ac₂ Glc</td>
<td>1.07</td>
</tr>
<tr>
<td>2,4,6-Me₃-1,3,5-Ac₃ Glc</td>
<td>1.20</td>
</tr>
<tr>
<td>2,3,6-Me₃-1,4,5-Ac₃ Gal</td>
<td>1.21</td>
</tr>
<tr>
<td>2,3,6-Me₃-1,4,5-Ac₃ Man</td>
<td>1.21</td>
</tr>
<tr>
<td>2,3,6-Me₃-1,4,5-Ac₃ Glc</td>
<td>1.24</td>
</tr>
<tr>
<td>2,4,6-Me₃-1,3,5-Ac₃ Gal</td>
<td>1.25</td>
</tr>
<tr>
<td>2,3,4-Me₃-1,4,6,-Ac₄ Glc</td>
<td>1.27</td>
</tr>
<tr>
<td>2,6-Me₂-1,4,5-Ac₃-3-Et Gal</td>
<td>1.27</td>
</tr>
<tr>
<td>2,3-Me₂-1,5,6-Ac₃ Hex</td>
<td>1.34</td>
</tr>
<tr>
<td>2,6-Me₂-1,3,4,5-Ac₄ Gal</td>
<td>1.34</td>
</tr>
<tr>
<td>2,3,4-Me₃-1,4,6,-Ac₄ Gal</td>
<td>1.34</td>
</tr>
<tr>
<td>2,6-Me₂-1,3,4,5-Ac₄ Man</td>
<td>1.35</td>
</tr>
<tr>
<td>2,6-Me₂-1,3,4,5-Ac₄ Glc</td>
<td>1.36</td>
</tr>
<tr>
<td>3,6-Me₂-1,2,4,5-Ac₄ Glc</td>
<td>1.39</td>
</tr>
<tr>
<td>2,3-Me₂-1,4,5,6-Ac₄-6-d₂ Man</td>
<td>1.43</td>
</tr>
<tr>
<td>2,3-Me₂-1,4,5,6-Ac₄-6-d₂ Glc</td>
<td>1.45</td>
</tr>
<tr>
<td>2-Me-1,4,5,6-Ac₄ Hex</td>
<td>1.45</td>
</tr>
<tr>
<td>2,3-Me₂-1,4,5,6-Ac₄ Glc</td>
<td>1.46</td>
</tr>
<tr>
<td>2,4-Me₂-1,3,5,6-Ac₄ Glc</td>
<td>1.48</td>
</tr>
<tr>
<td>6-Me-1,3,4,5,6-Ac₅ Man</td>
<td>1.49</td>
</tr>
<tr>
<td>6-Me-1,3,4,5,6-Ac₅ Glc</td>
<td>1.50</td>
</tr>
</tbody>
</table>

(Table 4 Continued on Next Page)
<table>
<thead>
<tr>
<th>Monosaccharide Derivative&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Retention Time&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Me&lt;sub&gt;2&lt;/sub&gt;-3-(2-Acetoxy-d&lt;sub&gt;2&lt;/sub&gt;-methylethyl)-1,5-Ac&lt;sub&gt;2&lt;/sub&gt; Rha</td>
<td>1.51</td>
</tr>
<tr>
<td>2-Me-1,3,4,5,6-Ac&lt;sub&gt;5&lt;/sub&gt; Man</td>
<td>1.60</td>
</tr>
<tr>
<td>2-Me-1,3,4,5,6-Ac&lt;sub&gt;5&lt;/sub&gt; Glc</td>
<td>1.61</td>
</tr>
<tr>
<td>3-Me-1,3,4,5,6-Ac&lt;sub&gt;5&lt;/sub&gt; Glc</td>
<td>1.64</td>
</tr>
<tr>
<td>2-Me-1,3,4,5,6-Ac&lt;sub&gt;5&lt;/sub&gt; Gal</td>
<td>1.65</td>
</tr>
<tr>
<td>4-Me-3-(2-Acetoxy-d&lt;sub&gt;2&lt;/sub&gt;-methylethyl)-1,2,5-Ac&lt;sub&gt;3&lt;/sub&gt; Rha</td>
<td>1.70</td>
</tr>
<tr>
<td>Ac&lt;sub&gt;6&lt;/sub&gt; Man</td>
<td>1.72</td>
</tr>
<tr>
<td>Ac&lt;sub&gt;6&lt;/sub&gt; Glc</td>
<td>1.74</td>
</tr>
<tr>
<td>Ac&lt;sub&gt;6&lt;/sub&gt; Gal</td>
<td>1.76</td>
</tr>
<tr>
<td>3-(2-Acetoxy-d&lt;sub&gt;2&lt;/sub&gt;-methylethyl)-1,2,4,5-Ac&lt;sub&gt;4&lt;/sub&gt; Rha</td>
<td>1.90</td>
</tr>
</tbody>
</table>

<sup>a</sup>30 m DB-1 capillary column (J&W Scientific), 140 °C, hold for 3 min, and then increase temperature 3 °C/min to 240 °C. See Chapter V, Section 2.B.3. for other details.

<sup>b</sup>For simplicity, monosaccharide derivatives are named in a shorthand abbreviation. For example, 2,3-Me<sub>2</sub>-1,4,5,6-Ac<sub>4</sub>-d<sub>2</sub> Glc stands for 1,4,5,6-tetra-O-acetyl-6,6-dideuterio-2,3-di-O-methylglucitol.

<sup>c</sup>Relative to internal 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol.

<sup>d</sup>Underacetylated artifact, 1,5-di-O-acetyl-2,3,6-tri-O-methylhexitol.
they are, these artifacts can provide clues to the identity of some of the components of the polysaccharides.

The most common artifacts in these methylation analyses were products of underacetylation. The most-encountered underacetylated derivatives were the 1,5-di-O-acetyl-2,3,6-tri-O-methylhexitols, in which O-4 of the hexitol was not acetylated. Surprisingly, this derivative is reasonably volatile even though it contains one free hydroxyl group. The 2,3,6-tri-O-methylglucose derivative is particularly difficult to completely acetylate (see Section 2., this chapter).

The mass spectrum of an underacetylated 1,5-di-O-acetyl-2,3,6-tri-O-methylhexitol is shown in Figure 23. This series of compounds elutes with a characteristically unsymmetrical peak shape shortly after the di-O-acetyl-tetra-O-methylhexitol derivatives, and before the fully acetylated tri-O-methylhexitol derivatives. The location of this peak did not obscure any relevant peaks in the GC trace, and was only a problem when quantitating the methylated hexitol derivatives.

Other underacetylation artifacts are the underacetylated derivatives of the 1,3,4,5,6-penta-O-acetyl-2-O-methylhexitols, in which O-3 is not acetylated, and the 1,5,6-tri-O-acetyl-2,3-di-O-methylhexitols, in which O-4 is not acetylated. These underacetylated partially methylated hexitols tend to form when a sample is stored in the presence of a small amount of acid or base for long periods following the initial acetylation. The resulting peak in the GC trace is easily identified by its characteristic and broad peak shape. The underacetylated 2-O-methyl derivatives tend to coelute with the 1,4,5,6-tetra-O-acetyl-2,3-di-O-methylhexitols and obscure these peaks in the GC spectrum, while the underacetylated 2,3-di-O-methylhexitols tend to
Figure 23. The Mass Spectrum of 1,5-di-\(\text{\text{\text{-}}}\)Acetyl-1-deuterio-2,3,6-tri-\(\text{\text{\text{-}}}\)methylhexitol
coelute with the 1,3,4,5-tetra-O-acetyl-2,6-di-O-methylhexitols. These underacetylated derivatives are readily eliminated by reacetylating the sample.

2. THE REDUCTION AND ACETYLATION REACTION

The hydrolyzate of the methylated polysaccharide was reduced and acetylated prior to analysis by GC/MS (Chapter II, Section 2.C.2., and Chapter V, Section 2.A.1.b.). However, as mentioned in Section 1.B.2. of this chapter, underacetylated artifacts were routinely detected in the analyses. An attempt was made to reduce the amount of the underacetylated partially methylated monosaccharide derivatives.

When using the acetylation procedure of Blakeney et. al.\(^{104}\) to acetylate partially methylated alditols containing 2,3,6-tri-O-methylglucitol, as much as 50% of the 2,3,6-tri-O-methylglucitol derivative which should be present as 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol was present as 1,5-di-O-acetyl-2,3,6-tri-O-methylglucitol. When using the acetylation procedure of Kindel and Cheng,\(^{105}\) however, the amount of the underacetylated derivative was less than 5%.

Blakeney et. al. used a solution of borohydride or borodeuteride in methyl sulfoxide to reduce the monosaccharides to the alditols, and, after elimination of excess reducing agent, acetic anhydride and 1-methylimidazole are added to acetylate the alditols by reaction at ambient temperature for short periods of time. Heating the mixture or utilizing longer reaction times tended only to increase the number of dark
byproducts, and did not significantly decrease the amount of underacetylated alditols present.

Kindel and Cheng used \( N, N \)-dimethylformamide rather than methyl sulfoxide as the dipolar aprotic solvent for the borohydride or borodeuteride reducing agent. After the excess reducing agent is eliminated and the acetic anhydride and 1-methylimidazole are added, the solution is heated to 85 °C for four hours to effect acetylation. Using these conditions, the acetylation reaction is relatively complete, and contains no visible dark byproducts.

The procedure of Kindel and Cheng was used for the reduction and acetylation of the hydrolyzate of all methylated polysaccharides in this study. The procedure of Blakeney \textit{et al.} was used for the reduction and acetylation of the monosaccharides derived from unmethylated polysaccharides, since the reaction on these sugars was complete in a matter of minutes.

3. CARBOXYL REDUCTION OF THE NATIVE POLYSACCHARIDE

As noted previously, carboxyl reductions of underivatized polysaccharides are typically done by the Taylor-Conrad method, in which a water-soluble carbodiimide is reacted with a carboxylic acid group on the polysaccharide and the resulting adduct reduced with borohydride or borodeuteride to form the primary alcohol (Chapter II, Section 3.C.2.).

This procedure, however, did not work well with the viscous polysaccharides studied here. The initial polysaccharide solution which is reacted with the water-soluble carbodiimide must be very concentrated, as
the pH is to be carefully controlled by the addition of acidic solutions throughout the reaction. The viscous polysaccharides were not soluble at the proper concentrations, or they formed a gelatinous mass at concentrations below those necessary for the reaction to occur well. More-dilute solutions were often still too viscous to allow for ready and complete mixing. In addition, the measurement of the pH was not possible using a pH meter since the polysaccharide tended to clump onto the electrode, making the determination of the global pH of the solution impossible.

A procedure which circumvented the solubility and viscosity problems was developed to reduce carboxylic acid moieties to the corresponding primary alcohols.

The new procedure, which is similar in strategy to the Taylor-Conrad method, is adapted from that of Fujisawa et. al., who used it mostly on simple, aromatic carboxylic acids containing several kinds of functional groups. Fujisawa's procedure involves the reaction of $N, N$-dimethylchloromethyleniminium chloride (readily produced \textit{in situ}) with the carboxylic acid in an organic solvent, and then reduction of the adduct with sodium borohydride.

To adapt the reaction for use with carbohydrates, it was necessary to first acetylate the polysaccharide in order to increase its solubility in organic solvents. Acetic esters, unlike methyl ethers, are cleanly removed with base following the reduction reaction to yield NMR-quality polysaccharide, or are removed during the total acid hydrolysis step. It was not necessary for the polysaccharide to be completely soluble for the reaction to proceed, but the reaction worked best when the carboxylic acid groups were present in the protonated form (Figure 24).
This reaction was used to reduce two types of uronic acids occurring in two different viscous polysaccharides, and a lactic acid ether substituent in a third polysaccharide. In each case, the reaction was successful, yielding over 50% of the reduction product after the first reduction reaction. A second reduction, when necessary, was almost quantitative. In contrast, the Taylor–Conrad reduction method yielded, in some cases, no detectable product in the first reduction of uronic acid residues (Table 5).

Table 5. Comparison of the Taylor–Conrad and Fujisawa Methods for Carboxyl Reductions of Unmethylated Polysaccharides

<table>
<thead>
<tr>
<th>Polysaccharide (Acidic Functionality)</th>
<th>Taylor–Conrad Method</th>
<th>Fujisawa Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Reduction, (a)</td>
<td>% Reduction, (a)</td>
</tr>
<tr>
<td>One Reduction</td>
<td>One Reduction</td>
<td></td>
</tr>
<tr>
<td>105-4 (Glucuronic Acid)</td>
<td>None Detected</td>
<td>56</td>
</tr>
<tr>
<td>97-67 (Lactic Acid Ether)</td>
<td>10</td>
<td>76</td>
</tr>
<tr>
<td>21-35 (Mannuronic Acid)</td>
<td>15</td>
<td>81</td>
</tr>
</tbody>
</table>

\(a\) Determined by GC/MS of the alditol acetates, percent of theoretical with respect to glucitol hexaacetate.
Preparation of \( \text{N, N-Dimethylchloromethyleniminium Chloride} \)

\[
\text{Cl} \quad \text{Cl} + \text{H} \quad \text{N} \quad \text{CH}_3 \quad \text{CH}_3 \quad \text{CH}_3 \quad \text{O} \quad \text{C} \quad \text{N} \quad \text{H} \quad \text{Cl} \quad \text{Cl} \\
\text{Cl} \quad \text{N} \quad \text{CH}_3 \quad \text{CH}_3 \quad \text{CH}_3 \quad \text{O} \quad \text{C} \quad \text{N} \quad \text{H} \quad \text{Cl} \quad \text{Cl} \\
\text{O}^\circ \text{C}, 1 \text{ hr} \\
\text{N, N-Dimethylchloromethyleniminium Chloride (DMCMIC)}
\]

Carboxyl Reduction of Acetylated Polysaccharide

\[
\text{PS(OH)COOH} \xrightarrow{\text{Ac}_2\text{O}, \text{pyridine}} \text{RT, 18 hr} \xrightarrow{\text{DMCMIC, CH}_3\text{CN, THF}} \text{PS(OAc)COOH} \xrightarrow{0^\circ \text{C}, 1 \text{ hr}} \text{PS(OAc)COOH} \\
\text{NaBD}_4, \text{DMF} \xrightarrow{-78^\circ \text{C to -20^\circ C}} 2 \text{ hr} \xrightarrow{\text{RT}} \text{PS(OAc)CD}_2\text{OH}
\]

Figure 24. Reduction of Carboxylic Acid Groups by the Fujisawa Method
Part B. Structural Studies of Uronic Acid-Containing Polysaccharides

Polysaccharide 21-35

1. INITIAL CHARACTERIZATION OF THE POLYSACCHARIDE

1.A. General Characteristics

Polysaccharide 21-35 had been isolated from an unidentified bacterial strain found in the soil of Gweru, Zimbabwe. The polysaccharide is supplied as a white fibrous material. The polysaccharide is not completely soluble in water at concentrations greater than 20 mg/mL, but forms a swollen, hydrated mass. Polysaccharide 21-35 is soluble in methyl sulfoxide to form a clear, viscous solution.

1.B. Spectroscopic Characterization

1.B.1. $^1$H-NMR

The 500-MHz $^1$H-NMR spectrum of polysaccharide 21-35 in Me$_2$SO-$d_6$ at 343 K was entirely unresolved except for a large, broad signal centered at 1.5 ppm. No information could be obtained from this spectrum.

1.B.2. $^{13}$C-NMR

The 125-MHz $^{13}$C-NMR spectrum of polysaccharide 21-35 in methyl sulfoxide-$d_6$ at 303 K (Figure 25) contains resonances attributable to two
Frequency: 125 MHz
Solvent: Me$_2$SO-$_d_6$
Concentration: 25 mg/mL
Temperature: 303 K
Number of Scans: 72704

Figure 25. $^{13}$C-NMR Spectrum of Polysaccharide 21-35
carbonyl (169.7 and 170.3 ppm), three anomic (104.0, 102.9, and 100.8 ppm), and two methyl (20.6 and 21.0 ppm) carbons (Table 6, column 1). The three signals in the anomic region above 100 ppm but below 106 ppm indicate that this polysaccharide is entirely β-linked, and contains three pyranose residues per repeating-unit. The methyl and carbonyl carbons resonating at approximately 21 and 170 ppm respectively suggest the presence of acetic esters in the repeating unit. The presence of a second carboxylate carbon resonance at approximately 169 ppm and the lack of any other high-field resonances suggests that one of the carboxylate-carbon resonances is due to a uronic acid.

1.B.3. IR

The functional group region of the infrared spectrum of this polysaccharide (Figure 26) has strong and characteristic absorbances at both 1740 and 1250 cm\(^{-1}\), confirming the presence of acetic ester(s) on the polysaccharide. Strong absorbances at 1620 and 1410 cm\(^{-1}\) indicate the presence of an ionized carboxyl group, which was assigned to the uronic acid residue (Table 7). The remainder of the infrared spectrum contains noninformative characteristic absorbances of polysaccharides including a broad absorbance in the hydroxyl group region and unresolved weak absorbances due to acetal groups between 900 and 1100 cm\(^{-1}\).
Figure 26. Infrared Spectrum of Polysaccharide 21-35
Table 6. Selected $^{13}$C-NMR Resonances of Polysaccharide 21-35

<table>
<thead>
<tr>
<th>Polysaccharide Source:</th>
<th>21-35</th>
<th>NRRL B-1973</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assignment</td>
<td>ppm$^a$</td>
<td>ppm$^a$</td>
</tr>
<tr>
<td>Ester Carbonyl Carbon</td>
<td>170.3</td>
<td>170.2</td>
</tr>
<tr>
<td>Uronic Acid Carboxylate Carbon</td>
<td>169.7</td>
<td>169.1</td>
</tr>
<tr>
<td>Anomeric Carbon</td>
<td>104.0, 102.9, 104.7, 103.0, 100.8</td>
<td>100.3</td>
</tr>
<tr>
<td>Acetate Methyl Carbon</td>
<td>20.6, 21.0</td>
<td>21.5, 22.0</td>
</tr>
</tbody>
</table>

$^a$Downfield from external tetramethylsilane

Table 7. Selected Infrared Absorbances of Polysaccharide 21-35

<table>
<thead>
<tr>
<th>Wavenumber (cm$^{-1}$)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1740</td>
<td>Ester Carbonyl</td>
</tr>
<tr>
<td>1620</td>
<td>Uronic Acid Carboxylate</td>
</tr>
<tr>
<td>1410</td>
<td>Uronic Acid Carboxylate</td>
</tr>
<tr>
<td>1250</td>
<td>Acetate Ester</td>
</tr>
</tbody>
</table>
1.C. Characterization of the Component Monosaccharides

1.C.1. Alditol Acetate Method

An approximate 1:1 mixture of the alditol acetate derivatives of glucose and galactose was obtained from the reduced and acetylated acid hydrolyzate (Table 8, column 1) of polysaccharide 21-35.

In a second analysis, the native polysaccharide was subjected to carboxyl reduction before hydrolysis and derivatization to the alditol acetates. In addition to the glucitol and galactitol hexaacetates seen in the first analysis, a peak corresponding to 6,6-dideuteriomannitol hexaacetate appeared in the GC/MS profile. This derivative arises from a mannuronic acid present in the original polysaccharide (Table 8, column 2).

Table 8. Sugar Analysis of Polysaccharide 21-35

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Ratio(^a)</th>
<th>Ratio(^b)</th>
<th>Ratio(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>1.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Mannuronic Acid</td>
<td></td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Identified as the neutral alditol acetate  
\(^b\)Native polysaccharide  
\(^c\)Carboxyl-reduced polysaccharide
1.C.2. Paper Chromatography

Paper chromatography of the acid hydrolyzate of the polysaccharide demonstrated the presence of glucose, galactose, and a slow-moving component attributable to mannuronic acid, confirming the spectroscopic analysis.

The three individual monosaccharides were isolated by preparative paper chromatography and were confirmed as sugars of the D-series by their optical rotations.

From the initial characterization data, it was determined that the repeating-unit of polysaccharide 21-35 contained β-D-glucopyranose, β-D-galactopyranose, and β-D-mannopyranuronic acid.

2. LINKAGE ANALYSIS

2.A. Hakomori Methylation Analysis

The Hakomori methylation profile of polysaccharide 21-35 consisted of two components (Table 9, column 1) that were shown by their mass spectra to be 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylhexitols. The presence of the acetic ester on O-4 of each hexitol demonstrates that each of these neutral residues is linked in the repeating-unit through the hydroxyl group on C-4.

A second portion of polysaccharide 21-35 was methylated by the Hakomori procedure, and then the resulting mannuronic acid methyl ester was reduced with borodeuteride. The GC/MS analysis of the reduced and acetylated component monosaccharides of the borodeuteride-reduced
polysaccharide showed the previously seen tri-O-methylhexitols and a third peak (Table 9, column 2). The new component of the mixture was identified as 1,4,5,6-tetra-O-acetyl-1,6,6-trideuterio-2,3-di-O-methylmannitol by its mass spectrum (Figure 27 and Table 10) and retention time. This derivative arises from a 4-linked mannuronic acid residue in the original polysaccharide.

**Table 9. Hakomori Methylation Profiles of Polysaccharide 21-35**

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1,4,5,6-tetra-O-acetyl-6,6-dideuterio-2,3-di-O-methylmannitol</td>
<td>—</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Native polysaccharide

<sup>b</sup>Polysaccharide was methylated and then reduced before hydrolysis

From the data presented above, the polysaccharide was determined to consist of a 4-linked trisaccharide repeating-unit of β-D-glucopyranose, β-D-galactopyranose, and β-D-mannopyranuronic acid. The repeating unit is substituted with acetic ester(s). Two structures of the polysaccharide backbone (Figures 28 and 29) are consistent with this data.
Table 10. Mass Spectrum of 1,4,5,6-tetra-$\text{O}$-Acetyl-1,6,6-
trideuterio-2,3-di-$\text{O}$-methylhexitol

$\text{MW}=381$

<table>
<thead>
<tr>
<th>$\text{m/z}$</th>
<th>% Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>19.5</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>36.4</td>
<td>162–60</td>
</tr>
<tr>
<td>118</td>
<td>82.3</td>
<td>Primary</td>
</tr>
<tr>
<td>129</td>
<td>30.1</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>161</td>
<td>3.6</td>
<td>263–60–42</td>
</tr>
<tr>
<td>162</td>
<td>7.8</td>
<td>Primary</td>
</tr>
<tr>
<td>203</td>
<td>6.6</td>
<td>263–60</td>
</tr>
<tr>
<td>263</td>
<td>12.8</td>
<td>Primary</td>
</tr>
</tbody>
</table>

Figure 27. Mass Spectrum of 1,4,5,6-tetra-$\text{O}$-Acetyl-1,6,6-trideuterio-2,3-di-$\text{O}$-methylhexitol
\( \rightarrow^4 \)-\( \beta \)-D-ManA\( p-(1 \rightarrow 4) \)-\( \beta \)-D-Gal\( p-(1 \rightarrow 4) \)-\( \beta \)-D-Glc\( p-(1 \rightarrow \)

**Figure 28.** Possible Backbone Structure of Polysaccharide 21-35

\( \rightarrow^4 \)-\( \beta \)-D-ManA\( p-(1 \rightarrow 4) \)-\( \beta \)-D-Gal\( p-(1 \rightarrow 4) \)-\( \beta \)-D-Glc\( p-(1 \rightarrow \)

**Figure 29.** Possible Backbone Structure of Polysaccharide 21-35
2.B. β-Elimination Reaction

The polysaccharide was subjected to β-eliminative degradation to determine which hydroxyl group was involved in a glycosidic linkage with C-1 of the mannosyluronic acid residue (Table 11). Treatment of the per-O-methylated polysaccharide with sodium methoxide, decomposition of the resulting 4,5-unsaturated uronic acid with weak acid, followed by ethylation of the newly exposed hydroxyl group yielded a 1,5-di-O-acetyl-1-deuterio-4-O-ethyl-2,3,6-tri-O-methylhexitol (Figure 30, Table 12) and a small amount of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol, implying that the mannosyluronic acid residue was linked to the O-4 of the glucosyl residue. The partially methylated, partially ethylated hexitol acetate was positively identified as the glucitol derivative by a parallel reaction in which the newly exposed hydroxyl group was trideuteriometalted rather than ethylated. The 1,5-di-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-4-O-trideuteriometaltedhexitol (Figure 31, Table 13) formed in this reaction coeluted with authentic 1,5-di-O-acetyl-1-deuterio-2,3,4,6-tetra-O-methylglucitol, confirming the identity of the hexitol.
Table 11. β-Elimination Profile of Polysaccharide 21-35

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>1,5-di-O-acetyl-4-O-ethyl-2,3,6-tri-O-methylglucitol</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>1,5-di-O-acetyl-2,3,6-tri-O-methyl-4-O-trideuteriomethylglucitol</td>
<td>—</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Free hydroxyl group etherified with an ethyl ether

<sup>b</sup>Free hydroxyl group etherified with a trideuteriomethyl ether

The fact that only a small amount of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol and no 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol was formed in this reaction further demonstrates that this polysaccharide has a trisaccharide repeating-unit. The partially methylated galactose residue is the reducing end of the disaccharide formed in the β-eliminative degradation. The reducing residue would be expected to be degraded by the strongly basic conditions present in the elimination and re-etherification reactions. The 2,3,6-tri-O-methylglucosyl residue present at the non-reducing end of the disaccharide resulting from the β-elimination forms the 2,3,6-tri-O-methyl-4-O-ethyl-, or 4-O-trideuteriomethyl- ether upon re-etherification with the corresponding alkyl iodide.

The β-elimination experiment unequivocally revealed the order of the monosaccharide resonances in the repeating unit, which is shown correctly in Figure 29.
Table 12. Mass Spectrum of 1,5-di-Q-Acetyl-1-deuterio-4-Q-ethyl-2,3,6-tri-Q-methylhexitol

MW=337

<table>
<thead>
<tr>
<th>m/z</th>
<th>% Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>26.5</td>
<td>Primary</td>
</tr>
<tr>
<td>71</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>34.6</td>
<td>162-60</td>
</tr>
<tr>
<td>115</td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>118</td>
<td>29.6</td>
<td>Primary</td>
</tr>
<tr>
<td>143</td>
<td>39.2</td>
<td>175-32</td>
</tr>
<tr>
<td>159</td>
<td>8.1</td>
<td>219-60</td>
</tr>
<tr>
<td>162</td>
<td>11.9</td>
<td>Primary</td>
</tr>
<tr>
<td>175</td>
<td>21.5</td>
<td>Primary</td>
</tr>
<tr>
<td>219</td>
<td>6.5</td>
<td>Primary</td>
</tr>
</tbody>
</table>

Figure 30. Mass Spectrum of 1,5-di-Q-Acetyl-1-deuterio-4-Q-ethyl-2,3,6-tri-Q-methylhexitol
Table 13. Mass Spectrum of 1,5-di-Q-Acetyl-1-deuterio-2,3,6-tri-Q-methyl-4-Q-trideuteriomiethylhexitol

MW =326

<table>
<thead>
<tr>
<th>m/z</th>
<th>% Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td>Primary</td>
</tr>
<tr>
<td>45</td>
<td>29.9</td>
<td>Primary</td>
</tr>
<tr>
<td>71</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>10.4</td>
<td>164-32-42</td>
</tr>
<tr>
<td>102</td>
<td>50.5</td>
<td>162-60</td>
</tr>
<tr>
<td>104</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>118</td>
<td>29.7</td>
<td>Primary</td>
</tr>
<tr>
<td>132</td>
<td>35.0</td>
<td>164-32</td>
</tr>
<tr>
<td>148</td>
<td>32.2</td>
<td>208-60</td>
</tr>
<tr>
<td>162</td>
<td>20.5</td>
<td>Primary</td>
</tr>
<tr>
<td>164</td>
<td>19.1</td>
<td>Primary</td>
</tr>
<tr>
<td>208</td>
<td>11.5</td>
<td>Primary</td>
</tr>
</tbody>
</table>

Figure 31. Mass Spectrum of 1,5-di-Q-Acetyl-1-deuterio-2,3,6-tri-Q-methyl-4-Q-trideuteriomiethylhexitol
2.C. Prehm Methylation

The location of the base-labile acetate group(s) in the repeating unit was accomplished by the non-alkaline Prehm methylation. The base-labile acetate groups are not hydrolyzed by this procedure, and only unsubstituted hydroxyl groups are methylated. In contrast, in the strongly basic medium of the Hakomori methylation, hydroxyl groups which contains base-labile substituents are methylated in addition to the unsubstituted hydroxyl groups.

The Prehm methylation profile of the native polysaccharide consisted of two 1,3,4,5,6-penta-O-acetyl-2-O-methylhexitols (Table 14, column 1) that were identified as the glucitol and galactitol derivatives by authentic standards. This methylation profile demonstrates that naturally occurring acetic esters are present on O-3 and O-6 of both the 4-linked glucose and galactose residues. No other carbohydrate derivatives were seen in this analysis, indicating that the substitution of the acetic esters on the polysaccharide backbone was relatively complete.

In a second analysis, Prehm methylation of the polysaccharide was followed by borodeuteride reduction of the resulting methyl ester of the mannuronic acid. The GC/MS analysis of the reduced and acetylated acid hydrolyzate of the reduced polysaccharide yielded the two previously seen 2-O-methyl hexitols in addition to 1,4,5,6-tetra-O-acetyl-6,6-dideuterio-2,3-di-O-methylmannitol (Table 14, column 2), demonstrating that the mannuronic acid component is not esterified by acetate groups in the natural polysaccharide.
Table 14. Prehm Methylation Profiles of Polysaccharide 21-35

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3,4,5,6-penta-O-acetyl-2-O-methylglucitol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1,3,4,5,6-penta-O-acetyl-2-O-methylgalactitol</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1,4,5,6-tetra-O-acetyl-6,6-di-deuterio-2,3-di-O-methylmannitol</td>
<td>—</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Native polysaccharide

<sup>b</sup>Polysaccharide was methylated and then reduced before hydrolysis

3. THE STRUCTURE OF POLYSACCHARIDE 21-35

From the data above, it was determined that polysaccharide 21-35 has the complete structure shown in Figure 32. The proposed structure of the polysaccharide 21-35 is identical to that produced by *Arthrobacter viscosus* NRRL B-1973.<sup>107</sup> The 125-MHz <sup>13</sup>C-NMR of authentic *A. viscosus* NRRL B-1973 polysaccharide in methyl sulfoxide-<em>d</em><sub>6</sub> (Figure 33 and Table 6, column 2) was identical to that of polysaccharide 21-35. Therefore, the final structure of polysaccharide 21-35 is proposed to be identical to that elaborated by *A. viscosus* NRRL B-1973.
Figure 32. Complete Structure of Polysaccharide 21-35
Frequency: 125 MHz
Solvent: Me$_2$SO-$d_6$
Concentration: 26 mg/mL
Temperature: 343 K
Number of Scans: 87040

Figure 33. $^{13}$C-NMR spectrum of the Polysaccharide from *Arthrobacter viscosus* NRRL B-1973
Polysaccharides From *Erwinia stewartii*

Polysaccharides were supplied by Dr. David Coplin of the Plant Pathology Department at The Ohio State University. The polysaccharides were isolated from two strains of *E. stewartii*. One of the strains was a wild-type, designated DC 283, which was virulent. A second strain, DM 3020, produced exopolysaccharide but was not virulent.

Since the polysaccharides in question were exopolysaccharides, the purification procedure utilized by Coplin’s group for each polysaccharide was rather simple. In short, the bacteria were grown on plates, and, when mature, washed from the plates. The bacterial suspension was centrifuged, the pellet discarded, and the exopolysaccharide-containing supernatant was concentrated. The polysaccharide was precipitated from the supernatant by precipitation with ethanol. The ethanol was removed and exopolysaccharide redissolved in water, dialyzed, and then lyophilized. The crude EPS was purified by passage through a DEAE column, and then lyophilized.

1. **INITIAL CHARACTERIZATION OF THE POLYSACCHARIDE**

1.A. **General Characteristics**

Each of the polysaccharides was provided as a white, fluffy solid. The polysaccharide was soluble in water at concentrations up to about 30 mg/mL to form a moderately viscous solution.
1.B. Spectroscopic Characterization

1.B.1. NMR

1.B.1.a. $^1$H-NMR

The partial 500-MHz $^1$H-NMR spectrum of DC 283 in $D_2O$ at 343 K (Table 15) is shown in Figure 34. The lack of high-field resonances ($\delta < 2.5$ ppm) indicates that the polysaccharide does not contain any acetate or pyruvate substituents, and does not consist of any 6-deoxyhexoses. The abundance of resonances at low field (4.7-6.0 ppm) indicates that the polysaccharide has a complex repeating-unit of six to seven monosaccharides. Because the spectrum is not perfectly resolved, it is difficult to determine whether all of the resonances in this region are doublets, indicative of the proton attached to the anomeric carbon.

The $^1$H-NMR spectra of the polysaccharides (DM 3020 and DC 283) were superimposable. This result immediately suggested that the polysaccharides were identical in the chemical structure of their repeating-units.
Figure 34. The Partial 500-MHz $^1$H-NMR Spectrum of *E. stewartii* Polysaccharide DC 283
Table 15. Selected Low-Field $^1$H-NMR Resonances of the Polysaccharide DC 283 from *E. stewartii*

<table>
<thead>
<tr>
<th>ppm</th>
<th>Tentative Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.80</td>
<td>Anomeric Proton</td>
</tr>
<tr>
<td>5.37</td>
<td>Anomeric Proton</td>
</tr>
<tr>
<td>5.18 (Doublet, $J_{1,2} = 4.5$ Hz)</td>
<td>Anomeric Proton</td>
</tr>
<tr>
<td>5.03$^b$</td>
<td>Anomeric Proton</td>
</tr>
<tr>
<td>4.98$^b$</td>
<td>Anomeric Proton</td>
</tr>
<tr>
<td>4.85 (Doublet, $J_{1,2} = 7.7$ Hz)</td>
<td>Anomeric Proton</td>
</tr>
<tr>
<td>4.80 (Doublet, $J_{1,2} = 7.3$ Hz)</td>
<td>Anomeric Proton</td>
</tr>
<tr>
<td>4.63</td>
<td>HOD</td>
</tr>
</tbody>
</table>

$^a$Downfield from external tetramethylsilane

$^b$Not well resolved

**1.B.1.b. $^{13}$C-NMR**

The partial 125-MHz $^{13}$C-NMR spectrum of DM 3020 in D$_2$O at 303 K (Table 16) is shown in Figure 35. Again, the lack of high field resonances demonstrated the lack of 6-deoxyhexoses, pyruvate groups, or acetate groups. There was, however, a single resonance at 175 ppm, which demonstrates the presence of a carboxylate carbon, implying the presence of a uronic acid in the repeating-unit. There are six resolved signals in the anomeric region, though the high intensity of one of the signals at 104.1 ppm suggests that it may be contributed to by two distinct anomeric carbons. This spectrum also suggests the presence of six to seven monosaccharide pyranoses in the repeating-unit. There are three to four signals for unsubstituted primary alcohols close to 62 ppm, and one or two resonances for substituted primary alcohols at 67 and 69 ppm, indicating the
Frequency: 125 MHz
Solvent: D$_2$O
Concentration: 24 mg/mL
Temperature: 303 K
Number of Scans: 106270

Figure 35. The Partial 125-MHz $^{13}$C-NMR Spectrum of *E. stewartii* Polysaccharide DM 3020
presence of (1→6) linkages in the repeating-unit. The resonances just
downfield from the ring carbons at 83.7 and 80.4 ppm suggest the presence
of (1→3) linkages in the repeating-unit. Often, C-3 of a hexopyranose
monosaccharide which is involved in an acetal linkage will resonate
further downfield from the other carbons bearing secondary alcohols.

Table 16. Selected $^{13}$C-NMR Resonances of Polysaccharide
DM 3020 from *E. stewartii*

<table>
<thead>
<tr>
<th>ppm$^a$</th>
<th>Tentative Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>175.1</td>
<td>C-6, Uronic Acid</td>
</tr>
<tr>
<td>105.7</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>105.4</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>104.1</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>103.6</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>99.6</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>99.5</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>83.7</td>
<td>C-3 of (1→3) Linkage</td>
</tr>
<tr>
<td>80.4</td>
<td>C-3 of (1→3) Linkage</td>
</tr>
<tr>
<td>69.0</td>
<td>C-6 of (1→6) Linkage</td>
</tr>
<tr>
<td>67.0</td>
<td>C-6 of (1→6) Linkage</td>
</tr>
<tr>
<td>62.6</td>
<td>C-6</td>
</tr>
<tr>
<td>62.4</td>
<td>C-6</td>
</tr>
<tr>
<td>62.3</td>
<td>C-6</td>
</tr>
</tbody>
</table>

$^a$Downfield from external tetramethylsilane

The $^{13}$C-NMR spectra of DC 283 and DM 3020 were also
superimposable, further implying that the polysaccharides are identical
with respect to the chemical structure of the repeating-unit.
1.B.2. IR Spectroscopy

The infrared spectra of the *E. stewartii* polysaccharides were superimposable. The IR spectrum of DC 283 is shown in Figure 36. The absorptions in the functional group region of the spectrum at 1635 and 1430 cm\(^{-1}\) indicate the presence of an ionized carboxylic acid group. The lack of ester carbonyl absorptions again demonstrates the lack of organic esters on this polysaccharide. The remainder of the IR spectrum is typical for a polysaccharide, and provides little additional information.

1.C. Characterization of the Component Monosaccharides

1.C.1. Alditol Acetate Method

Sugar analysis of native DC 283 and DM 3020 demonstrated that each polysaccharide consists of glucose and galactose in about a 1.5:1 ratio. A second analysis of DC 283 and DM 3020 which had been carboxyl-reduced before hydrolysis gave a ratio of 2.0:1 glucose to galactose, indicating that there is one glucuronic acid residue present in the repeating-unit (Table 17). Considering that the NMR spectra indicated that there were six to seven monosaccharide residues in the repeating-unit, it is likely that the ratio of the monosaccharides is 3:2:1 glucose–galactose–glucuronic acid or even 3:3:1 glucose–galactose–glucuronic acid.
Figure 36. The Infrared Spectrum of Polysaccharide DC 283
Table 17. Sugar Analysis of Polysaccharides DC 283 and DM 3020

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>DC 283 Native</th>
<th>DC 283 Carboxyl Reduced</th>
<th>DM 3020 Native</th>
<th>DM 3020 Carboxyl Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucitol Hexaacetate</td>
<td>1.5</td>
<td>2.1</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Galactitol Hexaacetate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

1.C.2. Paper Chromatography

The individual monosaccharides were isolated from the acid hydrolyzate by preparative paper chromatography and confirmed as sugars of the D-series by their optical rotations.

2. LINKAGE ANALYSIS OF THE POLYSACCHARIDE

2.A. Hakomori Methylation

The Hakomori methylation of both polysaccharides DC 283 and DM 3020 was difficult. The reaction did not go to completion, as evidenced by the amounts of glucitol and galactitol hexaacetates remaining after the methylation and derivatization to the partially methylated alditol acetates. A second methylation by the Hakomori procedure on the undermethylated polysaccharide resulted in significant degradation of the polysaccharide sample. Nevertheless, Hakomori methylation of native polysaccharides DC 283 and DM 3020 revealed 1,3,4,5,6-penta-O-acetyl-2-O-methylgalactitol,
1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol, 1,5,6-tri-O-acetyl-2,3,4-tri-O-methylgalactitol, and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (Table 18). These monosaccharide derivatives indicate that polysaccharides DC 283 and DM 3020 are doubly branched at a galactosyl residue, and that these branches terminate in glucosyl groups. The polysaccharide contains two (1→6) and at least one (1→3) linkage, in agreement with the NMR interpretation.

Hakomori methylation, followed by reduction of the resulting glucuronic acid methyl ester gave a methylation profile which demonstrated that the glucuronic acid was 4-linked in the polysaccharide repeating-unit. The reduction reaction was not entirely complete, as judged from the IR spectrum of the methylated and reduced polysaccharide. The incompleteness of the reduction is likely due to the incompleteness of the initial methylation reaction.

Table 18. Hakomori Methylation Profiles of DC 283 and DM 3020

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>DC 283 Native</th>
<th>DC 283 Carboxyl Reduced</th>
<th>DM 3020 Native</th>
<th>DM 3020 Carboxyl Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol</td>
<td>1.8</td>
<td>2.1</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methylgalactitol</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>1,3,4,5,6-penta-O-acetyl-2-O-methylgalactitol</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>1,4,5,6-penta-O-acetyl-6,6-dideuterio-2,3-di-O-methylglucitol</td>
<td>—</td>
<td>0.6</td>
<td>—</td>
<td>0.7</td>
</tr>
</tbody>
</table>
2.B. Prehm Methylation Analysis

Methylation by the method of Prehm was explored as an alternative to the Hakomori methylation in an attempt to improve the yield of the reaction. The Prehm methylation of the polysaccharides yielded a product which appeared by IR spectroscopy to be more completely methylated than the polysaccharides which were methylated by the method of Hakomori. The Prehm methylation profiles of DC 283 and DM 3020 were similar to that of the Hakomori methylation profiles, except that there was an additional peak in each profile corresponding to 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol. This derivative arises from a 3-linked galactosyl residue. The reason that this derivative was not detected in the polysaccharides methylated by the Hakomori method is not immediately apparent. On the basis of the $^{13}$C-NMR spectra it appears likely that a second 3-linked galactosyl residue could be present in the polysaccharide repeating-unit. As previously mentioned, resonances found downfield from 80 ppm usually indicate a 3-linked hexose. In the spectra of polysaccharide DC 283 and DM 3020 (Figure 35, Table 16), two resonances were found downfield from 80 ppm. One is assumed to arise from the highly branched galactose residue previously seen in the Hakomori methylation analysis, and the therefore other may in fact arise from this 3-linked galactose residue.
Table 19. Prehm Methylation Profiles of DC 283 and DM 3020

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>DC 283</th>
<th>DM 3020</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methylgalactitol</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>1,3,4,5,6-penta-O-acetyl-2-O-methylgalactitol</td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

2.C. Attempted β-Eliminative Degradation of DC 283

Polysaccharide DC 283 was methylated by the method of Prehm and then subjected to the conditions necessary to effect β-eliminative cleavage. Processing of the reaction in the typical manner (including re-etherification with methylsulfinyl carbanion and ethyl iodide) and analysis by GC/MS did not yield any identifiable products. The reaction was repeated with a fresh sample of DC 283, and trideuteriomethyl iodide instead of ethyl iodide was used to re-etherify the newly exposed hydroxyl group. Once again, the GC/MS analysis did not reveal any identifiable products.

2.D. Reduction of the Native Polysaccharide

To gain further insight into the uronic acid in the repeating-unit, polysaccharide DM 3020 was subjected to carboxyl reduction by the method of Taylor and Conrad. The reduction was repeated twice to ensure reaction. A comparison of the $^{13}$C-NMR spectrum of the reduced polysaccharide with that of the native polysaccharide should reveal if a uronic acid is present, and the comparison of the spectra of the native and reduced polysaccharide
could also yield information as to which free hydroxyl group the reducing end of the glucuronic acid is linked.

The $^{13}$C-NMR (Figure 37) of the reduced polysaccharide demonstrated that, while the reduction did not proceed quantitatively, the anomeric resonance at 105.4 ppm decreased in intensity, with a concomitant formation of a new anomeric peak at approximately 101.3 ppm. This result indicates that the peak at 105.4 ppm corresponds to the glucuronic acid residue. It appears that the glucuronic acid is β-linked, as the glucose formed has an anomeric resonance well downfield of 100 ppm.

The reduction of the glucuronic acid residue did not have a large effect on the other peaks in the spectrum. Neither resonance slightly downfield from 80 ppm appeared to be affected by the reduction, indicating that the uronic acid is probably not linked to the carbon atoms responsible for those peaks. The only significant change in the spectrum occurs close to 65 ppm, where a shoulder had formed on one of the peaks. This could indicate that the uronic acid is involved in one of the (1→6) linkages.

3. STRUCTURE OF THE E. STEWARTII POLYSACCHARIDES DC 283 AND DM 3020

The chemical structures of the repeating-units of the two polysaccharides from E. stewartii are identical. The fact that both the $^{13}$C- and $^1$H- NMR spectra were superimposable in a spectrum this complex immediately indicates that the structures are in the least, very similar. This information, coupled with the identical sugar analyses and Hakomori
Figure 37. The Partial 125-MHz $^{13}$C-NMR Spectrum of Carboxyl-Reduced Polysaccharide DM 3020

Frequency: 125 MHz
Solvent: $D_2O$
Concentration: 27 mg/mL
Temperature: 303 K
Number of Scans: 52224

(Anomeric Region of Unreduced Polysaccharide)
methylolation profiles, demonstrates that the polysaccharides are one and the same with respect to the chemical structure of their repeating-units. The pathogenicity of these E. stewartii bacteria, then is not related to the chemical structure of their polysaccharide repeating-units.

The structures of the polysaccharides were not determined completely. Very limited quantities of this material was available, and so each reaction had to be done on a smaller scale than normally was necessary in order to have enough polysaccharides for repetitive analyses. In addition, this polysaccharide was extremely difficult to work with. The yields of most of the reactions were very low, which made detection of the products even more difficult.

The structure of polysaccharides DC 283 and DM 3020 appears to consist of a heptasaccharide repeating-unit of three glucose, three galactose and one glucuronic acid residues. Two of the residues were α-linked, while the rest were β-linked. The glucuronic acid appears to be β-linked to a position other than C-3 of the 3-linked galactose residues. The polysaccharide repeating-unit is highly branched through a galactose residue. The branch units terminate in a glucose unit.
1. INITIAL CHARACTERIZATION OF THE POLYSACCHARIDE

1.A. General Characterization

Polysaccharide 105-4 had been isolated from an unidentified strain of the *Pseudomonas* species (ATCC 53923) found in the soil of Guigue, Venezuela. The polysaccharide is supplied as a white, fibrous material. The polysaccharide is soluble in methyl sulfoxide and water at concentrations up to 30–35 mg/mL to form a stable, clear, viscous solution.

1.B. Spectroscopic Characterization

1.B.1. $^1$H-NMR

The 500-MHz NMR of sonicated polysaccharide 105-4 in $D_2O$ was unresolved except for a large peak at approximately 1.5 ppm, and provided no useful information.

1.B.2. $^{13}$C-NMR

The 75-MHz $^{13}$C-NMR spectrum of polysaccharide 105-4 (Figure 38) contains resonances attributable to carbonyl (176 and 170 ppm), acetal (98–104 ppm), primary alcohol (60–65 ppm) and methyl (20–25 ppm) carbons (Table 20). The location of the acetal resonances indicated that this polysaccharide contains both β- and α-linked pyranose residues. The methyl-carbon and carbonyl-carbon resonances at approximately 23 and 176
Figure 38. The 75-MHz $^{13}$C-NMR Spectrum of Polysaccharide 105-4
ppm respectively indicate that the polysaccharide contains acetic ester substituent(s). The presence of a second carbonyl-carbon resonance at approximately 170 ppm and lack of other high-field resonances suggests the presence of at least one uronic acid residue.

**Table 20. Selected $^{13}$C-NMR Resonances of Polysaccharide 105-4**

<table>
<thead>
<tr>
<th>ppm$^a$</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>Acetate Methyl Carbon</td>
</tr>
<tr>
<td>98-104$^b$</td>
<td>Acetal Carbon</td>
</tr>
<tr>
<td>170</td>
<td>Carbonyl Carbon</td>
</tr>
<tr>
<td>176</td>
<td>Carbonyl Carbon</td>
</tr>
</tbody>
</table>

$^a$Relative to external tetramethylsilane  
$^b$Not resolved

1.B.2. IR Spectroscopy

The functional group region of the IR spectrum of polysaccharide 105-4 (Figure 39) shows strong and characteristic absorbances at 1740 and 1250 cm$^{-1}$, confirming the presence of acetate group(s) on the polysaccharide. Strong absorbances at 1620 and 1420 cm$^{-1}$ indicate the presence of an ionized carboxyl group possibly due to a uronic acid residue (Table 21). The remainder of the IR spectrum is typical of a polysaccharide and provides little additional information.
Figure 39. The Infrared Spectrum of Polysaccharide 105-4
Table 21. Selected Infrared Absorbances of Polysaccharide 105-4

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1740</td>
<td>Ester Carbonyl</td>
</tr>
<tr>
<td>1620</td>
<td>Uronic Acid Carboxylate</td>
</tr>
<tr>
<td>1420</td>
<td>Uronic Acid Carboxylate</td>
</tr>
<tr>
<td>1250</td>
<td>Acetate Ester</td>
</tr>
</tbody>
</table>

1.C. Characterization of the Component Monosaccharides

1.C.1. Alditol Acetate Method

A 1:4:1 mixture of the alditol acetates of mannose, glucose and galactose was obtained from the reduced and acetylated acid hydrolyzate of the polysaccharide (Table 22, column 1). In a second analysis, the native polysaccharide was subjected to reduction before the hydrolysis and derivatization to the alditol acetates. The ratio of glucitol hexaacetate present increased in proportion to the mannitol and galactitol hexaacetates, indicating that there are glucuronic acid units in the repeating-unit of the polysaccharide (Table 22, column 2).

The integral ratios of the sugars suggest the polysaccharide 105-4 has an octasaccharide repeating-unit consisting of 1:4:1:2 mannose–glucose–galactose–glucuronic acid.

1.C.2. Paper Chromatography

Paper chromatography of the acid hydrolyzate of the polysaccharide again demonstrated the presence of the neutral hexoses mannose, glucose and galactose, and a slow-moving component attributable to glucuronic
acid, confirming the spectroscopic analysis. The individual monosaccharides were isolated and confirmed as sugars of the D-series by their optical rotation.

**Table 22. Sugar Analysis of Polysaccharide 105-4**

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose</td>
<td>1.2</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>4.1</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Identified as the neutral alditol acetate  
<sup>b</sup>Native polysaccharide  
<sup>c</sup>Carboxyl-Reduced Polysaccharide

2. **LINKAGE ANALYSIS**

2. A. Hakomori Methylation

The Hakomori methylation profile of polysaccharide 105-4 consisted of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylmannitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol, and 1,3,4,5-tetra-O-acetyl-2,6-di-O-methylgalactitol (Table 23, column 1). These components suggest that the polysaccharide repeating-unit is branched at the galactose residue, and the branch chains terminate in a glucose residue.
Hakomori methylation followed by reduction of the resulting methyl esters of the uronic acids gave a polysaccharide whose Hakomori methylation profile contained all the previously mentioned peaks in addition to a significant amount of 1,4,5,6-tetra-O-acetyl-1,6,6-trideuterio-2,3-di-O-methylglucitol (Table 23, column 2), which was identified by both its mass spectrum (Figure 27 and Table 10) and retention time. This derivative arises from 4-linked glucuronopyranose residues in the repeating-unit.

Table 23. Hakomori Methylation Profiles of Polysaccharide 105-4

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylmannitol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>1,4,5,6-tetra-O-acetyl-6,6-dideuterio-2,3-di-O-methylglucitol</td>
<td>—</td>
<td>1.7</td>
</tr>
<tr>
<td>1,3,4,5-tetra-O-acetyl-2,6-di-O-methylgalactitol</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Native polysaccharide  
<sup>b</sup>Polysaccharide was methylated and reduced

2.B. β-Elimination Reaction

A β-elimination experiment was performed to identify which hydroxyl groups were involved in glycosidic linkages with the glucuronic acids. Treatment of the permethylated polysaccharide with methoxide to effect β-eliminative cleavage, followed by etherification of the newly released free hydroxyl groups with methyl sulfinyl carbanion and ethyl iodide yielded a degraded polysaccharide, which, upon hydrolysis, reduction
and acetylation, gave two partially methylated partially ethylated derivatives (Table 24).

### Table 24. β-Elimination Profile of Polysaccharide 105-4

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol</td>
<td>0</td>
</tr>
<tr>
<td>1,5-di-O-acetyl-4-O-ethyl-2,3,6-tri-O-methylglucitol</td>
<td>0.6</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylmannitol</td>
<td>1.0</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.8</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-3-O-ethyl-2,6-di-O-methylgalactitol</td>
<td>0.6</td>
</tr>
<tr>
<td>1,3,4,5-tetra-O-acetyl-2,6-di-O-methylgalactitol</td>
<td>0</td>
</tr>
</tbody>
</table>

A 1,5-di-O-acetyl-4-O-ethyl-2,3,6-tri-O-methylhexitol derivative obtained here was shown to be identical to the 1,5-di-O-acetyl-4-O-ethyl-2,3,6-tri-O-methylglucitol formed in the β-elimination experiment of polysaccharide 21-35 (Figure 30 and Table 12). The formation of this 4-O-ethyl derivative of glucose demonstrates that a glucuronic acid unit is linked to the 4-position of a glucosyl residue in the original polysaccharide.

The second partially methylated, partially ethylated alditol acetate, 1,4,5-tri-O-acetyl-3-O-ethyl-2,6-di-O-methylhexitol (Figure 40, Table 25), was formed with a concomitant disappearance of the 1,3,4,5-tetra-O-acetyl-2,6-di-O-methylgalactitol in the methylation profile. This ethylated derivative indicates that a glucuronic acid unit is linked to the 3-position of each branching galactose residue in the repeating-unit.
Table 25. The Mass Spectrum of 1,4,5-tri-O-Acetyl-1-deuterio-3-O-ethyl-2,6-di-O-methylhexitol
MW=365

<table>
<thead>
<tr>
<th>m/z</th>
<th>% Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>31.7</td>
<td>Primary</td>
</tr>
<tr>
<td>59</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>113</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>116</td>
<td>10.5</td>
<td>176-60</td>
</tr>
<tr>
<td>118</td>
<td>24.9</td>
<td>Primary</td>
</tr>
<tr>
<td>127</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>9.0</td>
<td>247-60-42</td>
</tr>
<tr>
<td>176</td>
<td>2.8</td>
<td>Primary</td>
</tr>
<tr>
<td>187</td>
<td>8.0</td>
<td>247-60</td>
</tr>
<tr>
<td>247</td>
<td>13.8</td>
<td>Primary</td>
</tr>
</tbody>
</table>

Figure 40. The Mass Spectrum of 1,4,5-tri-O-Acetyl-1-deuterio-3-O-ethyl-2,6-di-O-methylhexitol
Furthermore, no 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol was noted in this analysis, indicating that this residue was degraded during the strongly basic conditions present in the base-catalyzed elimination and second etherification procedure. The fact that the terminal residues were released upon base-catalyzed elimination suggests that these terminal glucose residues are attached to the non-reducing terminus of a glucosyluronic acid residue. It was established in the original Hakomori methylation profile that the glucosyluronic acid residue were linked at their non-reducing termini through O-4. This data suggests that the structural element Glc-(1→4)-GlcA-(1→ is present in the repeating unit. Figure 41 shows the structural components present in polysaccharide 105-4 as determined from the β-elimination experiment.

2.C. Lithium/Ethylenediamine Degradation

Lithium in ethylenediamine was used to cleave the large repeating-unit into more manageable oligosaccharide fragments by the specific degradation of the glucuronic acid residues. From the β-elimination reaction, it seemed likely that one of the glucuronic acids was in the side chain and involved in the glycosidic linkage with the terminal glucosyl unit. The location of the other glucuronic acid was unknown. If the degraded polysaccharide obtained upon lithium-ethylenediamine treatment was polymeric, it would indicate that both of the uronic acid residues were in the side chain. If the second uronic acid was in the backbone of the polysaccharide, an oligosaccharide would result from the lithium-ethylenediamine degradation.
Figure 41. Structural Components Present in Polysaccharide 105-4
2.C.1. Isolation of the Oligosaccharide Fragment

Polysaccharide 105-4 was treated with lithium metal in ethylenediamine to effect cleavage at each of the glucuronic acid residues. The residue obtained upon the degradation was purified by gel-permeation chromatography on Bio-Gel P-2 resin. A single significant carbohydrate-containing peak eluted at the void volume of the column. To demonstrate homogeneity with respect to molecular weight and to determine whether the fraction contained on oligosaccharide or was a polymeric material, it was subjected to chromatography on Bio-Gel P-6 resin, where it was retained slightly, and eluted as a single sharp peak. This confirmed both the physical homogeneity of the sample and that it was not a high-molecular-weight polymer.

2.C.2. Characterization of the Oligosaccharide Fragment

2.C.2.a. $^{13}$C-NMR

The 125-MHz $^{13}$C-NMR spectrum of the oligosaccharide fragment obtained on lithium–ethylenediamine treatment of polysaccharide 105-4 is shown in Figure 42. The four resolved acetal resonances in the region 101.2–104.7 ppm and the resolved primary alcohol resonances near 62 ppm (Table 26) of this spectrum suggest that it is a linear tetra- or penta-saccharide. All of the monosaccharides appear to be $\beta$-linked and in the pyranose configuration.
\(\beta\)-D-Glcp-(1\rightarrow4)\(-\beta\)-D-Galp-(1\rightarrow4)\(-\beta\)-D-GlcP-(1\rightarrow4)\(-\beta\)-D-GlcP(1\rightarrow4)-Manp

Frequency: 125 MHz
Solvent: D\(_2\)O
Concentration: 16 mg/mL
Temperature: 323 K
Number of Scans: 75776

Figure 42. \(^{13}\)C-NMR of the Pentasaccharide Obtained on Lithium/Ethylenediamine Degradation of Polysaccharide 105-4
Table 26. Selected $^{13}$C-NMR Resonances of Degraded Polysaccharide 105-4

<table>
<thead>
<tr>
<th>ppm</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>104.7</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>103.5</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>101.3</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>101.2</td>
<td>Anomeric Carbon</td>
</tr>
<tr>
<td>62.5</td>
<td>Primary Alcohol</td>
</tr>
<tr>
<td>62.3</td>
<td>Primary Alcohol</td>
</tr>
<tr>
<td>52.0</td>
<td>Primary Alcohol</td>
</tr>
<tr>
<td>61.5</td>
<td>Primary Alcohol</td>
</tr>
</tbody>
</table>

*Downfield from external tetramethylsilane

2.C.2.b. Sugar Analysis

Sugar analysis (Table 27, column 1) of the fragment obtained on the lithium–ethylenediamine degradation of polysaccharide 105-4 yielded a 1:3:1 mixture of mannitol, glucitol, and galactitol hexaacetates, indicating that the oligosaccharide fragment is a pentasaccharide.

To determine the identity of the monosaccharide at the reducing terminus, a sample of the pentasaccharide was reduced to yield a pentasaccharide-alditol, and then this derivative was hydrolyzed and acetylated. Under these conditions, only the reducing terminus of the pentasaccharide will yield an alditol, and the other components will be present as the monosaccharide pentaacetates. The GC/MS profile of the products of this procedure (Table 27, column 2) showed mannitol hexaacetate in a 1:4 ratio with a large unresolved peak of monosaccharide pentaacetates. This data confirmed that the oligosaccharide was a
pentasaccharide, and that it contained a mannose residue at the reducing terminus.

Table 27. Sugar Analysis of Degraded Polysaccharide 105-4

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol Hexaacetate</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Glucitol Hexaacetate</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>Galactitol Hexaacetate</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Monosaccharide Pentaacetates</td>
<td>—</td>
<td>4.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Degraded polysaccharide was successively hydrolyzed, reduced, and acetylated

<sup>b</sup>Degraded polysaccharide was successively reduced, hydrolyzed, and acetylated

2.C.2.c. Hakomori Methylation Analysis

To determine the linkage pattern of the oligosaccharide, it was first reduced to the pentasaccharide-alditol to protect the reducing end from degradation by the strongly basic etherification conditions. Hakomori methylation, hydrolysis, reduction, and acetylation of the oligosaccharide alditol yielded 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol, and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol (Table 28) in a 1:1:2 ratio. This information demonstrates that the pentasaccharide is a linear, 4-linked pentasaccharide containing glucose at the non-reducing terminus.
The oligosaccharide isolated here represents the backbone of the polysaccharide, less the glucuronic acid residues. Since there are four glucose residues present in the entire polysaccharide repeating-unit, and three are present in the oligosaccharide fragment, the side chain of the polysaccharide must contain one glucose unit. In addition, the side chain is attached to O-3 of the branching galactose residue, and a terminal glucose unit is attached to a glucuronic acid unit. The structure of the side chain must be Glc-(1→4)-GlcA-(1→ since, as previously indicated by the β-elimination experiment, a glucuronic acid is attached to O-3 of the galactose residue. The backbone of the polysaccharide must be 4-linked, as shown by the structure of this oligosaccharide. The structure of the backbone from the known data thus far is:

\[ \rightarrow 4\)-GlcA-(1→4)-Glc-(1→[4]-Gal, 4)-Glc, 4)-Glc]-{(1→4)-Man-(1→.\]

2.C.2.d. Base-Catalyzed Degradation of the Pentasaccharide

The previous data has shown that the pentasaccharide contains mannose at the reducing terminus and glucose at the non-reducing terminus. To determine the order of the central three residues of the oligosaccharide, and therefore the order of these residue in the
polysaccharide backbone, the sensitivity of reducing sugars to base-catalyzed
degradation was used. It is known that in the presence of strong base,
oligosaccharides undergo a "peeling" reaction from the reducing end.\textsuperscript{109} It
was expected if this pentasaccharide was subjected to strongly basic
conditions, mannose would be the first sugar to be degraded, followed by
the next sugar in the chain, which would form the new reducing terminus
of the remaining tetrasaccharide. If this next sugar was galactose, after a
period of time, only very small amounts of galactose and mannose would
remain, while significant amounts of glucose would be present, since it
occupies the non-reducing end of the oligosaccharide. If glucose, and not
galactose, was the second sugar in the chain, the ratio of glucose to galactose
would decrease.

To effect the degradation by base, the pentasaccharide was subjected to
pH 10 NaOH at 70 °C for 18 hours. Aliquots were taken after several time
periods. Each aliquot was neutralized with a few drops of acetic acid, and
the solution was evaporated to dryness. The residue was subjected to
hydrolysis, reduction, and acetylation, and the resultant alditol acetates
were analyzed by GC.

The sugar analysis profile (Table 29) of this reaction showed that the
ratio of glucitol to galactitol hexaacetate decreased over the first time period,
indicating that glucose became the next reducing sugar to be degraded after
mannose. The ratio of glucitol hexaacetate to galactitol hexaacetate
continued to decrease until the ratio was approximately 1:1, then the
galactitol hexaacetate began to disappear. Since it was known that glucose
occupied the nonreducing terminus of the pentasaccharide, this result was
expected. It is clear that the order of the residues in the pentasaccharide (from the non-reducing to reducing end) is Glc-Gal-Glc-Glc-Man.

Table 29. Sugar Analysis of the Oligosaccharide from the Lithium Degradation of PS 105-4 Upon Degradation with pH 10 NaOH

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>0 hours</th>
<th>3 hours</th>
<th>6 hours</th>
<th>15 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol Hexaacetate</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucitol Hexaacetate</td>
<td>3.1</td>
<td>2.4</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Galactitol Hexaacetate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

3. ANOMERIC CONFIGURATION OF THE POLYSACCHARIDE

3.A. Anomeric Configuration of the Pentasaccharide

The anomeric configuration of the monosaccharides present in the pentasaccharide was verified by chromium trioxide oxidation. The $^{13}$C-NMR spectrum of the pentasaccharide indicated that all the residues were $\beta$-linked. Therefore, the expectation was that all the sugars would be oxidized by the chromium trioxide, with the exception of mannose, which is the reducing end of the pentasaccharide. This was in fact the case, as shown in Table 30.
Table 30. Ratio of the Monosaccharide Derivatives Obtained on Sugar Analysis of the Acetylated Oligosaccharide After Chromium Trioxide Treatment

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>0 hours</th>
<th>1 hour</th>
<th>2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol Hexaacetate</td>
<td>1.0</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucitol Hexaacetate</td>
<td>2.7</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Galactitol Hexaacetate</td>
<td>1.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Inositol Hexaacetate (Standard)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

3.B. Anomeric Configuration of the Entire Polysaccharide

A 20 mg sample of acetylated polysaccharide 105-4 was oxidized by chromium trioxide. Aliquots were withdrawn after 0, 1, and 2 hours and subjected to sugar analysis by the alditol acetate method. The sugar analysis showed that most of the glucose and galactose was degraded, while the ratio of mannitol hexaacetate to the internal standard remained nearly constant. Therefore, the mannosyl residue in the intact polysaccharide has an axial (α) linkage.

A second set of aliquots were subjected to methylation, reduction, and then hydrolysis, reduction, and acetylation. The products of this reaction sequence (Figure 43) were derived from a terminal glucose residue, a terminal mannose residue, and a large quantity of 4-linked glucuronic acid. This result indicates that the 4-linked mannose, the terminal glucose and the glucuronic acids are axially (α) linked.
Hakomori Methylation Profiles

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Native</th>
<th>Oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4,5-tri-0-acetyl-2,3,6-tri-0-methyl glucitol</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1,4,5-tri-0-acetyl-2,3,6-tri-0-methyl galactitol</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl galactitol</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 43. Determining the Anomeric Configuration of Monosaccharides by Chromium Trioxide Oxidation and Methylation Analysis
4. LOCATION OF THE ACETIC ESTER SUBSTITUENTS ON POLYSACCHARIDE 105-4

4.A. Prehm Methylation

Repeated methylations by the method of Prehm to locate the base-labile acetate ester present on the polysaccharide were un reproducible. Acetate esters appeared to be present most of the time on C-6 of the branching galactose residue, but the substitution did not appear to be stoichiometric. In some lots of polysaccharide 105-4, acetate esters were found on C-6 of the 4-linked glucosyl residues, but again these were not stoichiometric.

4.B. Ethyl Vinyl Ether Method

The ethyl vinyl ether method of locating ester groups also demonstrated that the substitution of esters on the polysaccharide was nonstoichiometric, verifying the result obtained by the Prehm methylation method.

It is well known that ester groups substitution on polysaccharides are often nonstoichiometric and can depend on the conditions used to grow the bacteria. Therefore, the exact locations of the acetate esters on this polysaccharide were not identified.

5. FINAL STRUCTURE OF POLYSACCHARIDE 105-4

The proposed structure of polysaccharide 105-4 is shown in Figure 44. The repeating-unit consists of a 4-linked backbone of glucose, galactose,
mannose and glucuronic acid with a side branch containing glucuronic acid and glucose attached to O-3 of the galactosyl residue. Acetate esters are present of C-6 of some of the galactosyl and glucosyl groups.
Figure 44. The Complete Structure of Polysaccharide 105-4

\[ \rightarrow 4 \text{-GlcA}-(1 \rightarrow 4)-\beta\text{-Glc}-(1 \rightarrow 4)-\beta\text{-Gal}-(1 \rightarrow 4)-\beta\text{-Glc}-(1 \rightarrow 4)-\beta\text{-Glc}-(1 \rightarrow 4)\text{-Man}-(1 \rightarrow)_n \]

\[ \downarrow \]

Glc-(1 \rightarrow 4)-GlcA

+ Acetic Esters
Part C. Structural Studies of Polysaccharides Containing Acidic Substituents

Polysaccharide 97-67

1. INITIAL CHARACTERIZATION OF THE POLYSACCHARIDE

1.A. General Characteristics

Polysaccharide 97-67 had been isolated from a strain of Pseudomonas stutzeri found in the soil of Protrero, Mexico. The polysaccharide was supplied as a stiff white filmy mass. Polysaccharide 97-67 is soluble in water at concentrations up to 20 mg/mL.

1.B. Spectroscopic Characterization

1.B.1. $^1$H-NMR

The 500-MHz $^1$H-NMR spectrum of polysaccharide 97-67 was unresolved except for several broad peaks at high field.

1.B.2. $^{13}$C-NMR

The 125-MHz $^{13}$C-NMR (Figure 45) of native polysaccharide 97-67 at 323 K contained, inter alia, resonances attributable to three methyl (17.0, 20.5, and 21.5 ppm), two primary alcohol (64.0 and 67.9 ppm), a strong secondary alcohol (70.9 ppm), several weak unresolved acetal (100.5–103 ppm), and carbonyl (175.1 and 182.5 ppm) carbons (Table 31). The presence
Frequency: 125 MHz
Solvent: D₂O
Concentration: 25 mg/mL
Temperature: 323 K
Number of Scans: 86016

Figure 45. ¹³C-NMR of Native Polysaccharide 97-67
of the methyl and the carbonyl carbons at 21.5 and 175.1 ppm, respectively, indicated that the repeating-unit contains an acetic ester substituent. The remaining carbonyl and methyl resonances were not initially assigned.

Table 31. Selected $^{13}$C-NMR Resonances of Polysaccharide 97-67

<table>
<thead>
<tr>
<th>ppm</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.0</td>
<td>C-6 of 6-Deoxyhexose</td>
</tr>
<tr>
<td>20.5</td>
<td>Lactic Acid Methyl Carbon</td>
</tr>
<tr>
<td>21.5</td>
<td>Acetate Methyl Carbon</td>
</tr>
<tr>
<td>64.0</td>
<td>Carbon Bearing Primary Alcohol</td>
</tr>
<tr>
<td>67.9</td>
<td>Carbon Bearing Primary Alcohol</td>
</tr>
<tr>
<td></td>
<td>Carbon Bearing Secondary Alcohol</td>
</tr>
<tr>
<td>70.9</td>
<td>(Lactic Acid)</td>
</tr>
<tr>
<td>100.5–103.0</td>
<td>Acetal Carbon</td>
</tr>
<tr>
<td>175.1</td>
<td>Acetate Carbonyl Carbon</td>
</tr>
<tr>
<td>182.5</td>
<td>Lactic Acid Carbonyl Carbon</td>
</tr>
</tbody>
</table>

*Downfield from external tetramethylsilane

1.B.2. IR Spectroscopy

The infrared spectrum (Figure 46) of polysaccharide 97-67 contained strong absorbances at 1735 and 1250 cm$^{-1}$, again indicating the presence of an acetic ester substituent. There were also strong absorbances at 1645, 1590, 1450, and 1410 cm$^{-1}$ which indicated the presence of a carboxylate or carboxylic acid group (Table 32). The remainder of the spectrum was typical for a polysaccharide and provided little additional information.
Figure 46. The Infrared Spectrum of Polysaccharide 97-67
Table 32. Selected IR Absorbances of Polysaccharide 97-67

<table>
<thead>
<tr>
<th>cm⁻¹</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1735</td>
<td>Acetate Ester</td>
</tr>
<tr>
<td>1645</td>
<td>Carboxylic Acid</td>
</tr>
<tr>
<td>1590</td>
<td>Carboxylate</td>
</tr>
<tr>
<td>1450</td>
<td>Carboxylate</td>
</tr>
<tr>
<td>1410</td>
<td>Carboxylic Acid</td>
</tr>
<tr>
<td>1250</td>
<td>Acetate Ester</td>
</tr>
</tbody>
</table>

1.C. Characterization of the Component Monosaccharides

1.C.1. Alditol Acetate Method

Hydrolysis, followed by the reduction and acetylation of the hydrolyzate and analysis by GC indicated the presence of two neutral hexoses, which were identified as mannose and glucose by their retention times. An unexpected peak was also present which eluted in the 6-deoxyhexitol and pentitol region. This peak did not have the same retention time as any pentitol or 6-deoxyhexitol peracetate standard available. In addition, the ratio of the unknown peak to the mannitol and glucitol hexaacetates was dependent upon the severity of the hydrolysis conditions used. In general, the highest ratio of unknown peak to mannitol and glucitol was obtained under the harshest conditions, and the final ratio was approximately 1:1:1 (Table 33). It was proposed that the unknown component was a degradation product of an acidic substituent.

A peak with nearly the same retention time was also observed by a German group, who attempted an initial characterization of the exopolysaccharide which was a contaminant in their studies of an
extracellular enzyme produced by a strain of *Pseudomonas stutzeri*. The composition of the exopolysaccharide in their study appeared to be similar to the polysaccharide characterized here.

Table 33. Products of Sugar Analysis Under Various Hydrolysis Conditions at 100 °C as Detected by GC

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>10% HCl 8 h</th>
<th>2 M TFA 8 h</th>
<th>2 M TFA 2 hr</th>
<th>Anhydrous HF, 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>1</td>
<td>0.8</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Mannitol Hexaacetate</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Glucitol Hexaacetate</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

1.C.2. Paper Chromatography

The paper chromatographic analysis in 5:5:1:3 ethyl acetate–pyridine–acetic acid–water of polysaccharide 97-67 demonstrated the presence of the neutral sugars mannose and glucose, and a component which migrated slightly faster than the neutral sugars. The glucose and mannose were isolated and identified as sugars of the D-series on the basis of their optical rotations.
2. IDENTIFICATION OF 3-O-[(R)-1-CARBOXYETHYL]-L-RHAMNOSE AS THE UNKNOWN COMPONENT OF POLYSACCHARIDE 97-67

2.A. Identification of a 3-O-[1-Carboxyethyl]-6-deoxyhexose by GC/MS

2.A.1. GC/MS of the Alditol Acetate Hydrolyzate

The sample which had originally been analyzed by GC alone was analyzed by GC/MS. The mass spectrum of the unknown component was unlike that of any sugar, and had very little fragmentation (Figure 47 and Table 34, columns 1 and 2). A second analysis of a borodeuteride-reduced hydrolyzate was performed to demonstrate whether deuterium could be incorporated into the unknown component. A series of peaks did increase in mass by 1 unit, indicating that the unknown component contained a reducible group (Figure 48 and Table 34, columns 3 and 4). No positive identification could be made of this unknown component, though it is likely to be a degradation product of one of the sugar components.

2.A.2. Identification of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,5-di-O-acetyl-6-deoxy-1-deuterio-2,4-di-O-methylhexitol by GC/MS

Carboxyl reduction of the methylated polysaccharide, followed by hydrolysis, reduction with borodeuteride, and then acetylation to form the partially methylated alditol acetates of the component monosaccharides, gave, inter alia, a partially methylated 6-deoxyhexitol which contained an unknown substituent. The unknown substituent must be base-stable, since it was not removed under the highly basic conditions of Hakomori methylation, and it must be acid-stable since it was not removed upon
mineral acid hydrolysis. The stability of this substituent suggested that it was attached through an ether linkage to the 6-deoxyhexose.

The gross features of the mass spectrum of this 6-deoxyhexose derivative were similar to that of a 1,3,5-tri-O-acetyl-6-deoxy-2,4-di-O-methylhexitol, except that the spectrum contained a very abundant peak at \( m/z \) 103, and several peaks were shifted to higher masses than would be expected for a 6-deoxyhexose derivative (Figure 49 and Table 35). Through further analysis of the mass spectrum, it became apparent that the substituent was attached through O-3 of the 6-deoxyhexose, and that this substituent was responsible for the abundant peak at mass 103. It was proposed that the reduced substituent was a 2,2-dideutero-2-acetoxy-1-methylethyl ether (reduced lactic acid ether), which was disubstituted with deuterium atoms from the reduction of an originally ether-linked lactic acid methyl ester moiety which would have been formed in the Hakomori methylation of this polysaccharide.

If the reduced substituent responsible for the peak at \( m/z \) 103 was in fact derived from a lactic acid ether substituent in the original polysaccharide, a similar reduction of the methylated polysaccharide with borohydride rather than borodeuteride would shift the very abundant peak in the mass spectrum at \( m/z \) 103 to \( m/z \) 101, and would result in a concomitant shift in mass of the fragments which include the proposed substituent on O-3 from \( m/z \) 250 and 263 to \( m/z \) 248 and 261, respectively. This was the case upon reduction, as shown in Figure 50 and Table 36. The borodeuteride-reduced component was thereby identified as a 3-O-(2-acetoxy-2,2-dideutero-1-methylethyl)-1,5-di-O-acetyl-6-deoxy-1-deuterio-2,4-di-O-methylhexitol.
Figure 47. Mass Spectrum of the Unknown Component in the Sugar Analysis of Polysaccharide 97-67 (Reduced with Borohydride)

Figure 48. Mass Spectrum of the Unknown Component in the Sugar Analysis of Polysaccharide 97-67 (Reduced with Borodeuteride)
Table 34. Mass Spectra of the Unknown Derivative in the Sugar Analysis of Polysaccharide 97-67

<table>
<thead>
<tr>
<th>m/z</th>
<th>% of Base Peak</th>
<th>m/z</th>
<th>% of Base Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>55</td>
<td>4.0</td>
<td>55</td>
<td>4.3</td>
</tr>
<tr>
<td>56</td>
<td>3.8</td>
<td>56</td>
<td>5.2</td>
</tr>
<tr>
<td>71</td>
<td>3.6</td>
<td>71</td>
<td>4.7</td>
</tr>
<tr>
<td>83</td>
<td>4.1</td>
<td>83</td>
<td>4.9</td>
</tr>
<tr>
<td>85</td>
<td>5.8</td>
<td>86</td>
<td>7.2</td>
</tr>
<tr>
<td>87</td>
<td>4.2</td>
<td>87</td>
<td>6.9</td>
</tr>
<tr>
<td>96</td>
<td>3.4</td>
<td>97</td>
<td>4.0</td>
</tr>
<tr>
<td>112</td>
<td>9.4</td>
<td>113</td>
<td>13.9</td>
</tr>
<tr>
<td>127</td>
<td>3.1</td>
<td>128</td>
<td>4.5</td>
</tr>
<tr>
<td>128</td>
<td>3.6</td>
<td>129</td>
<td>4.9</td>
</tr>
<tr>
<td>141</td>
<td>11.5</td>
<td>141</td>
<td>23.4</td>
</tr>
<tr>
<td>156</td>
<td>2.5</td>
<td>157</td>
<td>3.5</td>
</tr>
<tr>
<td>171</td>
<td>1.6</td>
<td>172</td>
<td>2.3</td>
</tr>
<tr>
<td>183</td>
<td>1.6</td>
<td>184</td>
<td>2.7</td>
</tr>
<tr>
<td>184</td>
<td>2.0</td>
<td>185</td>
<td>2.3</td>
</tr>
<tr>
<td>198</td>
<td>3.4</td>
<td>199</td>
<td>5.1</td>
</tr>
<tr>
<td>201</td>
<td>1.6</td>
<td>201</td>
<td>2.6</td>
</tr>
<tr>
<td>226</td>
<td>5.0</td>
<td>227</td>
<td>5.7</td>
</tr>
<tr>
<td>259</td>
<td>0.23</td>
<td>260</td>
<td>0.22</td>
</tr>
<tr>
<td>304</td>
<td>0.23</td>
<td>305</td>
<td>0.32</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Native polysaccharide was hydrolyzed with mineral acid, reduced with borohydride, acetylated, and then analyzed by GC/MS.

\textsuperscript{b} Native polysaccharide was hydrolyzed with mineral acid, reduced with borodeuteride, acetylated, and then analyzed by GC/MS.
Table 35. Mass Spectrum of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,5-di-O-acetyl-6-deoxy-1-deuterio-2,4-di-O-methylhexitol

<table>
<thead>
<tr>
<th>m/z</th>
<th>% of Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>1.2</td>
<td>Primary</td>
</tr>
<tr>
<td>89</td>
<td>10.9</td>
<td>131-42</td>
</tr>
<tr>
<td>101</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>55.6</td>
<td>Primary</td>
</tr>
<tr>
<td>118</td>
<td>14.4</td>
<td>Primary</td>
</tr>
<tr>
<td>119</td>
<td>1.2</td>
<td>Primary</td>
</tr>
<tr>
<td>131</td>
<td>16.0</td>
<td>Primary</td>
</tr>
<tr>
<td>250</td>
<td>1.6</td>
<td>Primary</td>
</tr>
<tr>
<td>262</td>
<td>0.4</td>
<td>M+–75–15</td>
</tr>
<tr>
<td>263</td>
<td>1.3</td>
<td>Primary</td>
</tr>
<tr>
<td>306</td>
<td>0.5</td>
<td>M+–75</td>
</tr>
</tbody>
</table>

MW = 381

Figure 49. Mass Spectrum of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,5-di-O-acetyl-6-deoxy-1-deuterio-2,4-di-O-methylhexitol
A small amount of an undermethylated derivative of the 6-deoxyhexose was formed in the initial methylation reaction. The mass spectrum of the undermethylated, reduced, and acetylated derivative is shown in Figure 51 and tabulated in Table 37.

2.A.3. Identification of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,2,4,5-tetra-O-acetyl-6-deoxy-1-deuteriohexitol by GC/MS

To verify that none of the methyl groups were endogenous, the native polysaccharide was subjected to carboxyl reduction and hydrolysis, and the neutral monosaccharides in the hydrolyzate were converted to the alditol acetates. Analysis by GC/MS showed a component which eluted shortly after the previously identified mannose and glucose peracetates. The mass spectrum of this component was identical to that proposed for a 3-O-(2-acetoxy-2,2-dideuterio-1-methylethyl)-1,2,4,5-tetra-O-acetyl-6-deoxy-1-deuteriohexitol (Figure 52 and Table 38), confirming the identification made from the previous methylation and reduction. From this data, it was proposed that polysaccharide contained a terminal 3-O-[1-carboxyethyl]-6-deoxyhexose.

Carboxyethyl substituents have been reported as components of several polysaccharides. The glucose derivatives 4-O-[(S)-1-carboxyethyl]-D-glucose\textsuperscript{111} and 4-O-[(R)-1-carboxyethyl]-D-glucose\textsuperscript{112} are found in the exopolysaccharide of the Gram-positive bacteria \textit{Aerococcus viridans} var. \textit{homari} and the Gram-negative bacteria \textit{Shigella dysenteriae} type 3 lipopolysaccharide, respectively. A corresponding glucuronic acid derivative, 4-O-[(S)-1-carboxyethyl]-D-glucuronic acid\textsuperscript{113} is a component of the \textit{Klebsiella} type 37 capsular polysaccharide. The only reported
Table 36. Mass Spectrum of 3-O-(2-Acetoxy-1-methylethyl)-1,5-di-O-acetyl-6-deoxy-1-deuterio-2,4-di-O-methylhexitol  
MW=379

<table>
<thead>
<tr>
<th>m/z</th>
<th>% of Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>2.1</td>
<td>Primary</td>
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<td>58.2</td>
<td>Primary</td>
</tr>
<tr>
<td>102</td>
<td>5.2</td>
<td>Primary</td>
</tr>
<tr>
<td>117</td>
<td>4.0</td>
<td>Primary</td>
</tr>
<tr>
<td>118</td>
<td>13.0</td>
<td>Primary</td>
</tr>
<tr>
<td>131</td>
<td>20.1</td>
<td>Primary</td>
</tr>
<tr>
<td>248</td>
<td>2.0</td>
<td>Primary</td>
</tr>
<tr>
<td>261</td>
<td>1.4</td>
<td>Primary</td>
</tr>
<tr>
<td>262</td>
<td>0.7</td>
<td>M⁺-117</td>
</tr>
<tr>
<td>291</td>
<td>2.0</td>
<td>M⁺-73-15</td>
</tr>
<tr>
<td>306</td>
<td>1.1</td>
<td>M⁺-73</td>
</tr>
</tbody>
</table>

![Mass Spectrum Diagram]

Figure 50. Mass Spectrum of 3-O-(2-Acetoxy-1-methylethyl)-1,5-di-O-acetyl-6-deoxy-1-deuterio-2,4-di-O-methylhexitol
Table 37. Mass Spectrum of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,2,5-tri-O-acetyl-6-deoxy-1-deuterio-4-O-methyl Hexitol
MW=409

<table>
<thead>
<tr>
<th>m/z</th>
<th>% of Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td>Primary</td>
</tr>
<tr>
<td>87</td>
<td>6.2</td>
<td>Primary</td>
</tr>
<tr>
<td>89</td>
<td>15.6</td>
<td>131-42</td>
</tr>
<tr>
<td>102</td>
<td>6.0</td>
<td>Primary</td>
</tr>
<tr>
<td>103</td>
<td>45.0</td>
<td>Primary</td>
</tr>
<tr>
<td>116</td>
<td>3.1</td>
<td>Primary</td>
</tr>
<tr>
<td>131</td>
<td>34.5</td>
<td>Primary</td>
</tr>
<tr>
<td>278</td>
<td>1.5</td>
<td>Primary</td>
</tr>
<tr>
<td>290</td>
<td>1.2</td>
<td>M+–119</td>
</tr>
<tr>
<td>322</td>
<td>0.2</td>
<td>Primary</td>
</tr>
<tr>
<td>334</td>
<td>0.4</td>
<td>M+–75</td>
</tr>
</tbody>
</table>

Figure 51. Mass Spectrum of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,2,5-tri-O-acetyl-6-deoxy-1-deuterio-4-O-methylhexitol
deoxyhexose derivative to date is 3-O-[{(R)-1-carboxyethyl}]-L-rhamnose, which is a component of both the *Shigella dysenteriae* type 5 and *Escherichia coli* type 58 lipopolysaccharides, which have identical structures.

2.B. Identification of the 6-Deoxyhexose as L-Rhamnose

2.B.1. De-etherification with BCl₃

Boron trichloride is known to remove ether, acetal and ester substituents from monosaccharides. Treatment of the intact polysaccharide 97-67 with BCl₃ as previously described (Chapter V, Section 2.B.2.a.) yielded, as expected, a mixture of the constituent monosaccharides which were tentatively identified by paper chromatography. Reduction and acetylation of the monosaccharides and analysis by GC demonstrated the presence of glucitol, mannitol, and rhamnitol peracetates, positively identifying the 6-deoxy sugar as rhamnose.

2.B.2. De-etherification with Lithium in Ethylenediamine

The previously described GC method (Section 2.B.1) cannot differentiate between sugars of the D- and L-series. Quantities of the monosaccharide in question must be isolated and identified by a separate means. The BCl₃ reaction was not convenient on a larger scale. Lithium in ethylenediamine has been known to remove methyl ethers from monosaccharides in polysaccharides, and, this reaction is conveniently done on a large scale. Therefore, this set of reagents was employed to de-etherify polysaccharide 97-67.
Table 38. Mass Spectrum of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,2,4,5-tetra-O-acetyl-6-deoxy-1-deuterio Hexitol
MW=437

<table>
<thead>
<tr>
<th>m/z</th>
<th>% of Base Peak</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>1.8</td>
<td>Primary</td>
</tr>
<tr>
<td>96</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>59.5</td>
<td>Primary</td>
</tr>
<tr>
<td>146</td>
<td>0.7</td>
<td>Primary</td>
</tr>
<tr>
<td>159</td>
<td>0.6</td>
<td>Primary</td>
</tr>
<tr>
<td>176</td>
<td>0.2</td>
<td>278-60-42</td>
</tr>
<tr>
<td>189</td>
<td>0.3</td>
<td>291-60-42</td>
</tr>
<tr>
<td>218</td>
<td>0.3</td>
<td>278-60</td>
</tr>
<tr>
<td>231</td>
<td>0.2</td>
<td>291-60</td>
</tr>
<tr>
<td>278</td>
<td>1.2</td>
<td>Primary</td>
</tr>
<tr>
<td>291</td>
<td>2.1</td>
<td>Primary</td>
</tr>
<tr>
<td>318</td>
<td>4.9</td>
<td>M+–119</td>
</tr>
</tbody>
</table>

Figure 52. Mass Spectrum of 3-O-(2-Acetoxy-2,2-dideuterio-1-methylethyl)-1,2,4,5-tetra-O-acetyl-6-deoxy-1-deuteriohexitol
Intact polysaccharide 97-67 was treated with lithium in ethylenediamine as previously described (Chapter V, Section 2. C.2.b.). A polymeric material was isolated by gel permeation chromatography. The degraded polysaccharide was hydrolyzed, and the hydrolyzate was subjected to preparative paper chromatography. The region of the chromatogram corresponding to a 6-deoxyhexose was identified, and the corresponding monosaccharide was isolated. The optical rotation of the isolated monosaccharide corresponded to that of L-rhamnose.

2.B.3. Identification of 3-O-[(R)-1-Carboxyethyl]-L-rhamnose

The unusual monosaccharide had been identified as 3-O-[1-carboxyethyl]-L-rhamnose. However, the carboxyethyl group (lactic acid ether) is chiral, and the absolute stereochemistry remained to be determined. 3-O-[(R)-1-Carboxyethyl]-L-rhamnose had been isolated from naturally occurring lipopolysaccharide by fractionation on ion-exchange resin, and characterized by Kochetkov, Dmitriev, and Backinowsky. The absolute stereochemistry of the lactic acid moiety was confirmed by synthesis. In order to compare the properties of the anionic fraction of polysaccharide 97-67 to that of the previously isolated 3-O-[(R)-1-carboxyethyl]-L-rhamnose, the acidic fraction of the hydrolyzate of polysaccharide 97-67 was isolated in a similar fashion.

The residue obtained by the treatment of polysaccharide 97-67 with anhydrous HF was fractionated on a cationic ion-exchange resin into neutral and anionic fractions (Chapter V, Section 4.F.). The optical rotation of the isolated anionic monosaccharide of polysaccharide 97-67 was in
agreement with the published optical rotation of the naturally occurring 3-O-[(R)-1-carboxyethyl]-L-rhamnose (Table 39).

Table 39. Optical Rotations of 3-O-[1-Carboxyethyl]-L-rhamnose from Various Sources in H₂O

<table>
<thead>
<tr>
<th>Component</th>
<th>[α]₀</th>
<th>c (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic Fraction of Polysaccharide 9767</td>
<td>+11.9°</td>
<td>0.93</td>
</tr>
<tr>
<td>Natural 3-O-[(R)-1-Carboxyethyl]-L-rhamnose</td>
<td>+12°</td>
<td>1.13</td>
</tr>
<tr>
<td>Synthetic 3-O-[(R)-1-Carboxyethyl]-L-rhamnose</td>
<td>+20°</td>
<td>1.1</td>
</tr>
<tr>
<td>Synthetic 3-O-[(S)-1-Carboxyethyl]-L-rhamnose</td>
<td>-14.5°</td>
<td>0.9</td>
</tr>
</tbody>
</table>

2.C. ¹³C-NMR Analysis of 3-O-[(R)-1-Carboxyethyl]-L-Rhamnose-Containing Polysaccharide

The ¹³C-NMR of the O-deacetylated polysaccharide (Figure 53) is consistent with the proposed structure of a 1-carboxyethyl-6-deoxyhexose. The methyl group resonating at 17.9 ppm is typical of C-6 of rhamnose. The signal at 20.5 ppm, which does not disappear upon O-deacetylation, is attributable to the methyl group of the lactic acid substituent. The strong alcohol resonance at 68.5 ppm is assigned to the secondary alcohol of the lactic acid ether, and the resonance at 182.0 is assigned to the carboxylate group. The presence of the strong resonance at 68.5 ppm and the lack of any strong resonances in the acetal region rules out the presence of a pyruvic acetal, which would have similar resonances.
Frequency: 125 MHz
Solvent: Me₂SO-d₆
Concentration: 20 mg/mL
Temperature: 343 K
Number of Scans: 67584

Figure 53. ¹³C-NMR of O-Deacetylated Polysaccharide 97-67
Table 40. Selected 13C-NMR Resonances of O-Deacetylated Polysaccharide 97-67

<table>
<thead>
<tr>
<th>ppm</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.2</td>
<td>C-6 of 6-Deoxyhexose</td>
</tr>
<tr>
<td>19.5</td>
<td>Lactic Acid Ether Methyl Carbon</td>
</tr>
<tr>
<td>59.5, 60.5</td>
<td>Carbon Bearing Primary Alcohol</td>
</tr>
<tr>
<td>68.5</td>
<td>Carbon Bearing Secondary Alcohol</td>
</tr>
<tr>
<td>99.8–103.0</td>
<td>Acetal Carbon</td>
</tr>
<tr>
<td>177.5</td>
<td>Lactic Acid Carbonyl Carbon</td>
</tr>
</tbody>
</table>

*Downfield from external tetramethylsilane*
3. LINKAGE ANALYSIS OF POLYSACCHARIDE 97-67

3.A. Hakomori Methylation Analysis

The Hakomori methylation profile of native polysaccharide 97-67 contains an approximate 1:1 mixture of 1,4,5-tri-0-acetyl-2,3,6-tri-O-methylglucitol and 1,3,4,5-tetra-0-acetyl-2,6-di-0-methylmannitol (Table 41, column 1). The presence of the branching residue (a 3,4-linked mannose residue) and the lack of a permethylated terminal residue further indicates that the 3-O-[1-carboxyethyl]-L-rhamnose occupies the terminal position in the branch chains of the polysaccharide.

The methylation profile for methylated then reduced polysaccharide 97-67 is shown in Table 41, column 2. As expected, the 3-O-(2-acetoxy-2,2-dideuterio-1-methylethyl)-1,5-di-0-acetyl-2,4-di-0-methylrhamnitol was seen, as previously described (Section 2.A.2.).
Table 41. Hakomori Methylation Profiles of Polysaccharide 97-67

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio, Native Polysaccharide</th>
<th>Ratio, Carboxyl-Reduced Polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>1,3,4,5-tetra-O-acetyl-2,6-di-O-methylmannitol</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3-O-(2-acetoxy-2,2-dideutero-1-methylethyl)-1,5-di-O-acetyl-2,4-di-O-methylrhamnitol</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

*Polysaccharide was successively methylated, hydrolyzed, reduced, and acetylated

bPolysaccharide was successively methylated, reduced, hydrolyzed, reduced, and acetylated

3.B. Connectivity of the Backbone of Polysaccharide 97-67

Structures consistent with the data presented above include a polysaccharide with a backbone consisting of the mannose units containing side chains of 4-linked glucose and the terminal 3-O-[1-carboxyethyl]rhamnose units. Alternatively, the backbone of the polymer may be alternating glucose and mannose residues, with the side chains consisting solely of 3-O-[1-carboxyethyl]rhamnose units. In addition, in either of the proposed structures, the side chains may be linked to either O-3 or O-4 of the mannosyl residues. To differentiate between these possibilities, the resistance of the rhamnosyl and mannosyl residues in the polysaccharide to periodate oxidation was used to cleave the polysaccharide by the specific degradation of the glucosyl residues (Figure 54).
Treatment of the polysaccharide with periodate, reduction of the resulting aldehyde groups, and cleavage of the acyclic acetals with mild acid should yield an polymeric product if the glucose is present in the side chain, or a oligomeric product if the glucose is present in the backbone chain (Figure 55). In addition, the Hakomori methylation of the degraded polysaccharide should reveal which hydroxyl group is involved in the anomeric linkage with the glucose, since upon cleavage of the acyclic acetals a free hydroxyl group is released.


Polysaccharide 97-67 was treated with periodate, reduced and hydrolyzed under mild conditions to cleave the resultant acyclic acetals formed by the oxidized glucose residue. The degraded product was isolated from the residual salts by gel permeation chromatography. The degraded product eluted in the di- and tri- saccharide region on Bio-Gel P-2, indicating that an oligosaccharide was the major product of the reaction. A polymeric product was not obtained, therefore, glucose must be present in the backbone chain.


The Hakomori methylation of the oligosaccharide yielded 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylmannitol, indicating that the glucosyl residue must have been linked to O-4 of the mannose. Therefore, the 3-O-[1-carboxyethyl]rhamnosyl residue must be linked to O-3 of the mannosyl residue in the intact polymer.
Figure 54. Specific Degradation of the Possible Backbone Structures of Polysaccharide 97-67 by Periodate Oxidation, Reduction, and Mild Acid Hydrolysis
3.C. Prehm Methylation Analysis

The Prehm methylation profile of the polysaccharide 97-67 contained 2,3,6-tri-O-methyl-1,4,5-tri-O-acetylglucitol and 1,3,4,5,6-penta-O-acetyl-2-O-methylmannitol (Table 42), indicating that the acetic ester is located on O-6 of the branching mannose residue.

Table 42. Prehm Methylation Profile of Polysaccharide 97-67

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.4</td>
</tr>
<tr>
<td>1,3,4,5-tetra-O-acetyl-2,6-di-O-methylmannitol</td>
<td>0</td>
</tr>
<tr>
<td>1,3,4,5,6-penta-O-acetyl-2-O-methylmannitol</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Taking into account the methylation and periodate oxidation data, the linkage pattern of polysaccharide 97-67 must be as shown in Figure 55.

4. ASSIGNMENT OF ANOMERIC CONFIGURATION

The anomeric configurations of the monosaccharide components of polysaccharide 97-67 were assigned by chromium trioxide oxidation. In chromium trioxide oxidation, residues which have an equatorial anomeric linkage are more easily oxidized than those having an axial anomeric linkage.
Figure 55. Backbone Structure of Polysaccharide 97-67 as Determined by Smith Degradation and Hakomori Methylation
The anomeric configuration of the glucose and mannose residues were assigned by the typical chromium trioxide oxidation reaction described in Chapter V, Section 5.A. The ratio of mannitol to glucitol hexaacetate decreased steadily over the reaction period, indicating that mannose is β-linked and glucose is α-linked.

The anomeric configuration of the acidic component 3-O-[1-carboxyethyl]-L-rhamnose was assigned by the procedure described in Chapter V, Section 5.B. The ratio of the partially methylated rhamnose derivative to the glucose derivative remained nearly constant over the oxidation period, indicating that the rhamnose derivative in the intact polysaccharide has an axial configuration (Table 43).

### Table 43. Ratio of Monosaccharide Derivatives Obtained From Polysaccharide 97-67 On Oxidation With Chromium Trioxide in Acetic Acid

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>0 Hours</th>
<th>1 Hour</th>
<th>2 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol Hexaacetate</td>
<td>0.8</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucitol Hexaacetate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3-O-(2-acetoxy-1-methylethyl)-1,5-di-O-acetyl-6-deoxy-2,4-di-O-methylrhamnitol*</td>
<td>1.4†</td>
<td>—</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Obtained by: Acetylation → CrO₃/AcOH → Methylation → Ester Reduction → Hydrolysis → Reduction → Acetylation → GC/MS Analysis

†(Normalized to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol = 1.0)
5. FINAL STRUCTURE OF POLYSACCHARIDE 97-67

The final structure of polysaccharide 97-67 is shown in Figure 56. The branched trisaccharide repeating-unit contains glucose and mannose in the backbone chain, and a carboxyethylrhamnose unit as a side chain. An acetate ester is present on C-6 of the branching mannosyl residue.
Figure 56. The Complete Structure of Polysaccharide 97-67

\[
\left(\rightarrow 4\right)-\alpha-D-Glc-(1\rightarrow 4)-\beta-D-Man-(1\rightarrow)_n
\]

3-O-[(R)-1-carboxyethyl]-L-Rha
Polysaccharides 75-39, 91-94, and N-8

Succinoglycans

Polysaccharides 75-39, 91-94, and N-8 contain both succinic ester and pyruvic acetal substituents. Polysaccharides of this type are known by the general term "succinoglycan". However, the term "succinoglycan" has also been used to refer to a specific polysaccharide, Succinoglycan 10C3 from *Alcaligenes faecalis* var. *myxogenes* (Figure 57). Succinoglycans of the same structure as Succinoglycan 10C3 are also elaborated by several species of bacteria including *Agrobacterium, Rhizobium*, and other species of *Alcaligenes*. From this point on, "succinoglycan" will refer to the general class of polysaccharides which contain both succinic esters and pyruvic acetals, unless otherwise stated.

1. INITIAL CHARACTERIZATION

1.A. General Characterization

Polysaccharide 75-39 had been isolated from a bacteria found in the soil of Seese, Hungary, and had been identified as a strain of *Agrobacterium radiobacter*. The polysaccharide was supplied as a white, fibrous material. Polysaccharide 75-39 is sparingly soluble in methyl sulfoxide, but is soluble in water at concentrations of less than 30 mg/mL to form a stable, clear, viscous solution. Solution is best achieved by placing the polysaccharide in

Figure 57. The Structure of Succinoglycan 10C3
water and stirring vigorously while under vacuum until the polysaccharide absorbs the water to form a clear, gelatinous mass. At this stage, solution is easily achieved by sonication.

Polysaccharide 91-94 had been isolated from a strain of bacteria found in the soil between the cities of Libano and Armero, Colombia. The bacteria had been thought to of the Agrobacterium species. The polysaccharide was supplied as a white, fibrous material. Polysaccharide 91-94 is somewhat soluble in methyl sulfoxide, and is soluble in water up to a concentration of about 30 mg/mL. A procedure similar to that used for 75-39 is used to dissolve 91-94 in water.

Polysaccharide N-8 had been isolated from a culture mutant strain of Agrobacterium radiobacter IFO 3058. N-8 was supplied as a white, fibrous material. N-8 is relatively insoluble in methyl sulfoxide, and is sparingly soluble in water in a nongelatinous form only with difficulty in concentrations above 10-15 mg/mL. Aqueous solutions of N-8 are somewhat milky in appearance.

1.B. Spectroscopic Characterization

1.B.1. $^1$H-NMR

The 500-MHz $^1$H-NMR of these succinoglycans was unresolved except for a broad peak at high field.

1.B.2. $^{13}$C-NMR

The 125-MHz $^{13}$C-NMR of polysaccharide 75-39 at 343 K (Figure 58) contains, *inter alia*, resonances attributable to one ester carbonyl (182.0
ppm), two carboxylate carbonyl (176.5, 176.7 ppm), several unresolved acetal (102.5–105.5 ppm), two methylene (33.2, 31.7 ppm) and one methyl (26.0 ppm) carbon (Table 44, column 1).

Methylene carbons near 32 ppm and the ester and carboxylate carbonyl resonances are indicative of a succinate ester. The remaining carboxylate resonance and the methyl group at 26 ppm, downfield from most methyl resonances, is especially typical of a 4,6-linked pyruvate acetal. The acetal carbon of the pyruvate substituent is buried in the group of acetal resonances found between 102.5 and 105.5 ppm. The position of these acetal signals indicate that the polysaccharide is entirely β-linked and contains only pyranose residues.

The 125-MHz $^{13}$C-NMR spectra of 91-94 (not shown) was superimposable on that of 75-39, and is tabulated in Table 44, column 2.

The 125-MHz $^{13}$C-NMR spectrum of polysaccharide N-8 (not shown) contains, inter alia, resonances corresponding to several unresolved acetal carbons (101.9–105.2 ppm), two methylene carbons (32.0–33.5 ppm), and a methyl carbon (25.8 ppm) (Table 44, column 3). The background in this spectrum was very large due both to the viscosity of the solution and the insolubility of N-8 in D$_2$O. Therefore, none of the usually less-intense carbonyl resonances which are assumed to be present could be seen over the background. The gross features of the spectra of polysaccharides 75-39, 91-94 and N-8 are essentially identical.
Figure 58. $^{13}$C-NMR of Succinoglycan 75-39

Frequency: 125 MHz
Solvent: $D_2O$
Concentration: 20 mg/mL
Temperature: 323 K
Number of Scans: 69632
Table 44. Selected $^{13}$C-NMR Resonances of Polysaccharides 75-39, 91-94, and N-8

<table>
<thead>
<tr>
<th></th>
<th>75-39, ppm$^a$</th>
<th>91-94, ppm$^a$</th>
<th>N-8, ppm$^a$</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>182.0</td>
<td>181.5</td>
<td>b</td>
<td></td>
<td>Ester Carbonyl Carbon</td>
</tr>
<tr>
<td>176.5</td>
<td>176.5$^c$</td>
<td>b</td>
<td></td>
<td>Acid Carbonyl Carbon</td>
</tr>
<tr>
<td>176.7</td>
<td>176.5$^c$</td>
<td>b</td>
<td></td>
<td>Acid Carbonyl Carbon</td>
</tr>
<tr>
<td>102.5–105.5$^c$</td>
<td>102.5–105.4$^c$</td>
<td>101.9–105.2$^c$</td>
<td></td>
<td>Acetal Carbon</td>
</tr>
<tr>
<td>33.2, 31.7</td>
<td>33.4, 31.9</td>
<td>32.0–33.5$^c$</td>
<td></td>
<td>Succinate Methylene Carbon</td>
</tr>
<tr>
<td>26.0</td>
<td>25.9</td>
<td>25.8</td>
<td></td>
<td>Pyruvate Methyl Carbon</td>
</tr>
</tbody>
</table>

$^a$ Downfield from external tetramethylsilane

$^b$ Not resolved over background

$^c$ Not resolved

1.B.2. IR Spectroscopy

The infrared spectra of polysaccharides 75-39, 91-94 and N-8 (Figure 59, Table 45) are superimposable, and show several absorbances attributable to carboxylate or carboxylic acid groups, and the characteristic set of absorbances at 1730 and 1200 cm$^{-1}$ attributable to a succinate ester. The remainder of each of the spectra is typical for a polysaccharide and provides little additional information.
Figure 59. The Infrared Spectrum of Succinoglycan 91-94
Table 45. Selected Infrared Absorbances of Polysaccharides 75-39, 91-94, and N-8

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1730</td>
<td>Ester Carbonyl</td>
</tr>
<tr>
<td>1620</td>
<td>Carboxylic Acid</td>
</tr>
<tr>
<td>1580</td>
<td>Carboxylate</td>
</tr>
<tr>
<td>1450</td>
<td>Carboxylate</td>
</tr>
<tr>
<td>1410</td>
<td>Carboxylic Acid</td>
</tr>
<tr>
<td>1200</td>
<td>Succinic Ester</td>
</tr>
</tbody>
</table>

1.C. Characterization of the Component Monosaccharides

1.C.1. Alditol Acetate Method

Reduction and acetylation of the acid hydrolyzate of polysaccharides 75-39, 91-94 and N-8 yielded an approximate 7:1 mixture of glucitol hexaacetate to galactitol hexaacetate, as shown in Table 46. This ratio implies an octasaccharide repeating-unit, as has been found in the known succinoglycans.

Table 46. Sugar Analysis of Polysaccharides 75-39, 91-94, and N-8

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>75-39</th>
<th>91-94</th>
<th>N-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>7.3</td>
<td>6.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>
1.C.2. Paper Chromatography

Paper chromatography of the acid hydrolyzate of the polysaccharides 75-39, 91-94, and N-8 confirmed that only neutral hexose sugars were present in the repeating-unit. The individual monosaccharides were isolated and confirmed as sugar of the D-series by their optical rotation. In each case, a component was detected which was determined to be pyruvic acid by comparison of its $R_f$ with authentic pyruvic acid.

2. LINKAGE ANALYSIS

2.A. Hakomori Methylation

The Hakomori methylation profiles of native 75-39, 91-94 and N-8 were complex. Evidence of disubstituted, or branching, residues (1,4,5,6-tetra-O-acetyl-2,3-di-O-methylglucitol) were seen, but no terminal residues were detected, suggesting that the pyruvate acetal was linked to a terminal glucose residue in each polysaccharide (Table 47).

The Hakomori methylation profile of depyruvated 75-39, 91-94, and N-8 contained a peak derived from a terminal glucose residue, and a concomitant decrease in the amount of 1,4,5,6-tetra-O-acetyl-2,3-di-O-methylglucitol formed, demonstrating that a pyruvic acetal was linked to O-4 and O-6 of a terminal glucose residue (Table 48).
Table 47. Hakomori Methylation Profiles of Native Polysaccharides 75-39, 91-94, and N-8

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>75-39</th>
<th>91-94</th>
<th>N-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol</td>
<td>1.6</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.9</td>
<td>2.1</td>
<td>1.7</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol</td>
<td>1.3</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>1,4,5,6-tetra-O-acetyl-2,3-di-O-methylglucitol</td>
<td>2.4</td>
<td>2.2</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 48. Hakomori Methylation Profiles of Depyruvated Polysaccharides 75-39, 91-94, and N-8

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>75-39</th>
<th>91-94</th>
<th>N-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol</td>
<td>0.7</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol</td>
<td>2.2</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.2</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol</td>
<td>1.6</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>1,4,5,6-tetra-O-acetyl-2,3-di-O-methylglucitol</td>
<td>0.6</td>
<td>1.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

2.B. Prehm Methylation

The base-labile succinate ester was located by the Prehm methylation. The Prehm methylation profile of polysaccharide 75-39 and 91-94 contained a new component, 1,3,5,6-tetra-O-acetyl-2,4-di-O-methylglucitol, and a
concomitant decrease in the amount of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol (Table 49), indicating that the succinate ester is present on C-6 of a 3-linked glucose unit.

Table 49. Prehm Methylation Profiles of Native Polysaccharides 75-39, 91-94, and N-8

<table>
<thead>
<tr>
<th>Monosaccharide Derivative</th>
<th>75-39</th>
<th>91-94</th>
<th>N-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol</td>
<td>0.9</td>
<td>0.7</td>
<td>1.3</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol</td>
<td>1.7</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1,5,6-tri-O-acetyl-2,3,4-tri-O-methylglucitol</td>
<td>1.2</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>1,4,5,6-tetra-O-acetyl-2,3-di-O-methylglucitol</td>
<td>1.8</td>
<td>1.6</td>
<td>16</td>
</tr>
<tr>
<td>1,3,5,6-tetra-O-acetyl-2,4-di-O-methylglucitol</td>
<td>1.3</td>
<td>1.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

3. THE FINAL STRUCTURE OF POLYSACCHARIDES 75-39, 91-94, AND N-8

The structure of the polysaccharides 75-39, 91-94 and N-8 appeared to be identical to that of Succinoglycan 10C3. The structure of this succinoglycan, which is also found in other species of bacteria, is well known and is the subject of several papers. Therefore, these polysaccharides were not pursued further.
CHAPTER V

EXPERIMENTAL

1. SPECTROSCOPIC TECHNIQUES

1.A. Nuclear Magnetic Resonance Spectroscopy (NMR)

1.A.1. $^{13}$C-Nuclear Magnetic Resonance Spectroscopy ($^{13}$C-NMR)

$^{13}$C-Nuclear magnetic resonance spectra were obtained at 125 MHz in 5 mm tubes using a Bruker AM-500 spectrometer. Spectra of native polysaccharides were obtained by first dissolving polysaccharide (10–15 mg) samples in 99.8% D$_2$O (2 mL), using sonication under argon (Section 4.C.) if necessary, and then lyophilizing the solution and redissolving at a concentration of 20 mg/mL in D$_2$O. Spectra were obtained immediately at 323 K, unless otherwise stated.

1.A.2. $^1$H-Nuclear Magnetic Resonance Spectroscopy ($^1$H-NMR)

$^1$H-Nuclear magnetic resonance spectra were obtained at 500 MHz in 5 mm tubes using a Bruker AM-500 spectrometer. Spectra of native polysaccharides were obtained by dissolving the polysaccharide in 99.8%
\( \text{D}_2\text{O} \) using sonication under argon (Section 4.C.) if necessary, and then lyophilizing the solution. This procedure was repeated two more times to ensure exchange of the alcohol protons to the deuterated form, and then the polysaccharide was dissolved at a concentration of 10–20 mg/mL in 99.8% \(\text{D}_2\text{O} \). Spectra were obtained at 343 K, unless otherwise stated.

1.B. Infrared (IR) Spectroscopy

Polysaccharide (2–3 mg) and IR-grade KBr (300 mg) were ground together and made into a KBr pellet using a hydraulic press. IR spectra were obtained on a Mattson "Polaris" Fourier-transform infrared spectrometer. The IR spectrum of a blank pellet consisting of 300 mg KBr was electronically subtracted from the spectrum of the sample.

When desired, the polysaccharide was recovered by dissolving the pellet in water (5 mL) and dialyzing (3500 molecular weight cutoff) the solution against portions of distilled water for 8–10 h, and then lyophilizing the retentate.

1.C. Optical Rotation

Optical rotations of the constituent monosaccharides isolated by preparative paper chromatography (Section 2.A.2.b.) were taken using a Perkin-Elmer 141 Polarimeter in a 1.00 mL cell. Solutions of \(c = 0.5–1.3\) (0.5 g/100 mL–1.3 g/100 mL) were made in degassed, deionized water. Rotations were measured after 5 h at 589 nm at 20–23 °C. The equilibrium rotations were compared to literature values.\textsuperscript{122, 123}
2. CHEMICAL ANALYSES

2.A. Analysis of Monosaccharides

2.A.1. Alditol Acetate Method

2.A.1.a Total Acid Hydrolysis of Polysaccharides

Polysaccharide (3–10 mg) samples and 7% HCl (2 mL) are placed in 5-mL screw-top vials equipped with Teflon-lined caps. The vials are heated for 3–5 h at 100 °C. The contents are transferred to round-bottom flasks with some deionized water, and the solvent removed in vacuo with a bath temperature of <40 °C.

2.A.1.b. Reduction and Acetylation of Monosaccharides

The residue from the foregoing acid hydrolysis is transferred to a 5-mL screw-top vial using M NH₄OH (0.5 mL). (If paper chromatography is also to be done on the sample, a small portion of this solution is retained.) The round-bottom flask is rinsed with two 0.5-mL portions of a 2% (w/v) solution of NaBH₄ or NaBD₄ in DMF to remove any residue remaining in the flask, and these solutions are also added to the vial. The reduction is allowed to proceed for 60 min at 40 °C. The excess borohydride is decomposed with AcOH (0.1 mL). Acetic anhydride (2 mL) and 1-methylimidazole (0.2 mL) are added, the sample mixed, and then heated for 4 h at 80 °C, or overnight (8–10 h) at 60 °C. After acetylation is complete, the solution is poured into water (5 mL) in a 30-mL separatory funnel and the vial rinsed with an additional portion of water (5 mL). The aqueous
solution is extracted with CH$_2$Cl$_2$ (2 x 4 mL), and the combined organic phases are washed with water (3 x 5 mL). The organic layer is concentrated to 3 mL, dried with anhydrous Na$_2$SO$_4$, and filtered through a disposable pipet containing a small cotton plug into a vial (~ 4 mL). The pipet is rinsed with CH$_2$Cl$_2$ (1 mL), and the combined eluants are concentrated to dryness.

2.A.1.c. Gas Chromatography

Peracetylated alditols were analyzed on a Varian 3300 gas chromatograph equipped with a flame-ionization detector linked to a Varian 4290 integrator. The injector temperature was 200 °C, and the detector temperature 240 °C. Helium carrier gas at 30 mL/min through a 1.83-m by 2-mm (I.D). packed column of 3% OV-225 on 100-200 mesh Gas Chrom Q was used to separate and quantitate the hexoses present in the polysaccharides. A ramped temperature program was used to enhance the separation of the alditol acetates. The temperature was held at 170 °C for 8 min, and then increased by 3 °C/min to 220 °C. Quantitation was achieved by comparison of the peak area values calculated by the integrator.

2.A.2. Paper Chromatography

2.A.2.a. Analytical Paper Chromatography

Analytical paper chromatography was performed by the descending-solvent method. The chromatograms consisted of a 14- by 35-cm strip of Whatman No. 1 filter paper on which references and samples had been spotted. The samples consisted of acid hydrolyzates of polysaccharides dissolved in M NH$_4$OH. The chromatograms were developed with 5:5:1:3
EtOAc–pyridine–water–AcOH. Monosaccharides were detected by alkaline silver nitrate spray.124

2.A.2.b. Preparative Paper Chromatography

Preparative paper chromatography was performed on strips of Whatman No. 3 MM filter paper by the descending-solvent method. The residue from the hydrolysis of 20–30 mg polysaccharide was dissolved in a minimum amount of deionized water and spotted onto the paper. The solvent system was the same as used for analytical paper chromatography (Section 2.A.2.a.). To identify the location of the monosaccharides on the chromatogram, narrow guide strips were cut from each side of the chromatogram. Alkaline silver nitrate spray124 was used to detect the positions of the monosaccharides on the strips. The strips were matched up with the original chromatogram, and the position of the monosaccharides were marked. The chromatograms were cut into sections and the monosaccharides eluted with deionized water. The monosaccharides were recovered by lyophilization.

2.B. Linkage Analysis of Polysaccharides—The Modified Hakomori Methylation

2.B.1. Preparation of Lithium Methylsulfinyl Carbanion

Lithium methylsulfinyl carbanion (dmsyl anion) was prepared by the method of Blakeney and Stone59 with the following modifications. Dry methyl sulfoxide (40 mL) is transferred to a 500-mL three-necked flask equipped with an argon purge, vacuum source, and magnetic stir-bar. The
flask is alternately subjected to vacuum and argon purges to degas the liquid. Cold \( n \)-butyllithium (50 mL, 1.6 M in hexanes) is added dropwise during a 10-min period to the methyl sulfoxide with gentle stirring and slow argon purge. After the addition is complete, the solution is stirred for 10 min at 15–20 °C. Two layers form. No attempt is made to remove the hexanes (upper layer). The very pale yellow lower layer is drawn off in 5-mL aliquots via syringe and placed in argon-flushed glass vials equipped with Teflon-lined caps. The vials are stored at -20 °C. The reagent is stable for several months, especially when a small amount of hexanes is allowed to cover the surface of the reagent.

2.6.2. Modified Hakomori Methylation

Polysaccharide (10 mL) is suspended in dry methyl sulfoxide (5 mL) in a 25-mL Erlenmeyer flask equipped with a Teflon-lined screw-top cap and magnetic stir-bar. The stirring solution is placed under vacuum for ~10 minutes to degas the solution and to aid in the solubilization of the polysaccharide. Dimsyl anion (5 mL) is added under argon purge, and the resulting green-yellow solution is stirred for 18 h at ambient temperature. Methyl iodide (5 mL) is slowly added dropwise with external cooling (ice bath), and the solution stirred for 18 h at ambient temperature. Water (10 mL) is then added to the brownish-red solution to stop the reaction. The suspension is poured into dialysis tubing (3500 molecular weight cutoff) and dialyzed against several changes of deionized water for 48 h. The permethylated polysaccharide is recovered by lyophilization.
The methylated polysaccharide is sequentially hydrolyzed, reduced with NaBD$_4$ and acetylated as previously described (Sections 2.A.1.a. and 2.A.1.b.).

2.B.3. Gas Chromatography/Mass Spectrometry

Partially methylated alditol acetates were analyzed on a Hewlett-Packard 5890 GC/MS system with a model 5870 mass-selective detector. Helium carrier gas at a rate of 0.8 mL/min through a DB-1 methyl silicone capillary column (J & W Scientific) was used to separate the partially methylated alditol acetates. A ramped temperature program was used to enhance the separation. The temperature was kept constant at 140 °C for 3 min, and then increased at a rate of 3 °C/min to 240 °C. The injector and the transfer-line temperatures were kept at 250 °C. The splitless time was 0.1 min, and the mass spectrometer was turned on after a delay of 4 min. Mass ions 41–500 were extracted for analysis.

2.B.4 Analysis of Polysaccharides

In a typical procedure, the partially methylated alditol acetates derived from 10 mg of polysaccharide were dissolved in CH$_2$Cl$_2$ (0.2 mL). Approximately 0.8 μL was injected using a gas-tight 10-μL syringe. Under these conditions, tetra-O-methylhexitol acetates began to elute in ~13 min. The hexitol peracetates are eluted in ~25 min.
2.C. Special Techniques Used For Uronic Acid-Containing Polysaccharides

2.C.1. Carboxyl Reduction Methods

2.C.1.a. Carboxyl Reduction on the Native Polysaccharide

A sample of polysaccharide (15–20 mg) is acetylated by suspending it in 1:1 pyridine–Ac₂O (20 mL) for 18 h. All traces of the reagents are removed in vacuo, and the polysaccharide is scraped into a powder. In a separate flask, oxalyl chloride (0.953 mL of a 2 M solution in CH₂Cl₂) is added to DMF (51.7 μL) at 0 °C with stirring under argon purge. The flask is closed to the atmosphere and the reaction allowed to proceed for 1 h. The CH₂Cl₂ is removed under reduced pressure without the use of heat. The resultant white powder (N, N-dimethylchloromethyleniminium chloride) is cooled to 0 °C and the powdered polysaccharide is added to it. Any remaining polysaccharide is removed from the acetylation flask with the aid of MeCN (1 mL) and THF (3 mL) at 0 °C. The suspension is stirred for 2 h, then cooled to -78 °C (CO₂–acetone). Cold NaBD₄ (2 M in DMF, 0.8 mL) is added dropwise to effect reduction. The temperature of the solution is allowed to rise slowly to -20 °C, and then the solution is stirred for 2 h. The reaction is terminated by the dropwise addition of AcOH. The reduced polysaccharide is purified by dialysis (3500 molecular weight cutoff) against several changes of deionized water for 24 h. The reduced polysaccharide is recovered by lyophilization. The reduced polysaccharide is hydrolyzed (Section 2.A.1.a.) and converted into the alditol acetates (Section 2.A.1.b.) for analysis by GC/MS as previously described (Sections 2.B.3.and 2.B.4.).
2.C.1.b. Carboxyl Reduction of Methylated Polysaccharide

Sodium borodeuteride (18 mg) is dissolved in 95% EtOH (2.1 mL) in a 15-mL Erlenmeyer flask fitted with a Teflon-lined screw-top cap. THF (5.1 mL), and then the methylated polysaccharide (10 mg) are added. The suspension is stirred for 18 h at room temperature, and then heated to 70 °C for 1 h. Acetic acid (0.2 mL) is added dropwise to decompose the reducing agent. The solvent is evaporated under a stream of dry air. The residue is transferred to a 5-mL cone-shaped vial equipped with a Teflon-lined screw-top cap with the aid of the MeOH (1 mL). To remove the borate salts, methanol (1 x 1 mL) and AcOH (2 x 0.5 mL) are added to the vial and evaporated to dryness. This operation is repeated several times. The methylated, reduced polysaccharide is hydrolyzed (Section 2.A.1.a.) without isolation. The hydrolyzate is reduced and acetylated (Section 2.A.1.b.), and then analyzed by GC/MS as previously described (Sections 2.B.3. and 2.B.4.).

2.C.2. Chain Cleavage Reactions

2.C.2.a. β-Elimination Chain Cleavage

The procedure used is basically that of Lindberg et. al.\textsuperscript{76} Permethylated polysaccharide (10–15 mg) is suspended in 18:1:2 MeOH–2,2-dimethoxypropane–CH₂Cl₂ (21 mL) which also contains a trace of p-toluenesulfonylic acid. The suspension is refluxed for 30–40 min and then cooled. Several pieces of freshly cut sodium (250 mg total) are added and the resulting solution is refluxed for 1 h. The pH of the cooled solution is adjusted to 6 by the addition of 50% aqueous AcOH (~2 mL) and poured into a separatory funnel containing deionized water (50 mL). The aqueous
solution is extracted with CH$_2$Cl$_2$ ($3 \times 25$ mL), the combined organic phases are washed with water (25 mL), and concentrated to dryness. The residue is suspended in 10% aqueous AcOH (10 mL) and heated for 1 h at 100 °C. The degraded polysaccharide is recovered by lyophilization. The degraded polysaccharide is successively hydrolyzed (Section 2.A.1.a.), reduced and acetylated (Section 2.A.1.b.), and then analyzed by GC/MS as previously described (Sections 2.B.3. and 2.B.4.).

2.C.2.b. Lithium-Ethylenediamine Degradation

Polysaccharide (50 mL) is vigorously stirred into dry ethylenediamine (7 mL) at ambient temperature under argon purge for 1 h. Small pieces of freshly cut lithium wire (3, each 4 by 3 mm) which have been washed in hexanes, and, immediately before use, dipped in MeOH, then washed again with hexanes, are added to the polysaccharide suspension and the flask is closed to the atmosphere. After the contents turn deep blue, small pieces of lithium are added at 15-min intervals in order to maintain the blue color for 1 h. The reaction is terminated by the dropwise addition of MeOH (1–2 mL) with cooling (ice bath). The solvents are removed in vacuo. Acetic acid (1–2 mL) is added dropwise to the white powdery residue with cooling (ice bath) to neutralize the LiOMe, and then an equal volume of water was added to solubilize the remaining residue. Once solution has been achieved, it is passed through Dowex 50W (50–100 mesh) 8% cross-linked (H$^+$ form) ion-exchange resin. The volume is reduced to 5 mL, and the resulting oligosaccharides are separated by gel-permeation chromatography on a Bio-Gel P-2 column (Section 4.A.). Fractions containing sugar (phenol–sulfuric acid assay, Section 4.B., or refractive-index detection) are
isolated by lyophilization. The oligosaccharides obtained are analyzed by
the same procedures used for the polysaccharides.

3. LOCATION OF SUBSTITUENTS

3.A. Location of Base-Labile Substituents

3.A.1. Methyl Triflate (Prehm) Methylation

Polysaccharide (5–10 mg) is suspended in dry trimethyl phosphate (1–
2 mL) in a 4-mL vial with sonication (Section 4.C.). Under argon purge, 2,6-
di-tert-butylpyridine (150 µL) and methyl trifluoromethanesulfonate
(methyl triflate, 100 µL) are added to the vial. The resulting suspension is
sonicated for 10 min and allowed to react for 2 h at 50 °C. The reaction is
terminated by the addition of water (1 mL) and the milky suspension is
placed into dialysis tubing (3500 molecular weight cutoff) to dialyze against
several changes of deionized water for 6–8 h. The methylated
polysaccharide is recovered by lyophilization. The methylated
polysaccharide is successively hydrolyzed (Section 2.A.1.a.), reduced and
acetylated (Section 2.A.1.b.) for analysis by GC/MS (Sections 2.B.3. and
2.B.4.), or carboxyl-reduced (Section 2.C.1.b.) as previously described.

3.A.2. Ethyl Vinyl Ether Method

Polysaccharide (15 mg) is dissolved in dry Me₂SO (5 ml) using
sonication (Section 5.C.). Ethyl vinyl ether (2 mL) and p-toluenesulfonic
acid (trace) are added under argon purge. The initially clear solution is
stirred for 4 h at room temperature. Methyl sulfinyl carbanion (5 mL, Section 2.B.1.a.) is added to the resulting brick-red solution with cooling (cold water, 15-18 °C) under argon purge. The resulting black-purple solution is stirred overnight at room temperature. Methyl iodide (5 mL) is added dropwise with cooling (ice bath) under argon purge. The brown solution is stirred overnight at room temperature. Water (10 mL) is added and excess volatile reagents are removed in vacuo, and the remaining solution is dialyzed (8000 MWCO) against running tap water for 18 h. The aqueous solvent is removed in vacuo, and the syrupy residue is hydrolyzed in 7% HCl (5 mL) for 4 h at 100 °C. The suspension is vacuum filtered through a Hirsch funnel to remove any black polymeric particles. The filtrate is evaporated to dryness, and then successively reduced and acetylated (Section 2.A.1.b.) and analyzed by GC/MS (Section 2.B.3. and 2.B.4.).

3.B. Location of Base-Stable Substituents

3.B.1. Pyruvic Acetals

Polysaccharide (10-15 mg) is reduced as previously described (Section 2.C.1.a.). The reduced polysaccharide is dissolved in 15 mM oxalic acid and refluxed for 4 h. The solution is dialyzed (3500 MWCO) against several changes of distilled water for 6-8 h. The retentate is lyophilized. To verify the complete removal of the pyruvate groups, the IR spectrum of the acid-treated polysaccharide is obtained and the polysaccharide recovered as described (Section 1.B.). If the IR spectrum indicates that pyruvate groups still remain, the acid-treatment procedure is repeated. The depyruvated
polysaccharide is sequentially methylated (Section 2.B.1.b.), hydrolyzed (Section 2.A.1.a.) reduced and acetylated (Section 2.A.2.b.) as described, then analyzed by GC/MS (Sections 2.B.3. and 2.B.4.).

3.B.2. Naturally Occurring Ethers

Naturally occurring ether substituents were located by inspection of the mass spectrum of the methylated derivative of the component containing the ether substituent. If the ether group contained an acidic functionality, it was reduced to the primary alcohol before formation of the alditol acetate.

3.B.2.a. De-etherification with BCl₃

De-etherification was achieved by the method of Bonner and Bourne¹²⁵ with the following modifications. The polysaccharide (10–30 mg) was cooled to -78 °C in acetone–CO₂. Boron trichloride (4–6 mL of a 1 M solution in CH₂Cl₂) was added under argon purge, and the mixture allowed to stir for 3 h. The suspension was capped with a tube containing CaCl₂ and allowed to warm to room temperature overnight. The tube was removed and the remaining MeCl₂ and BCl₃ were removed with a stream of dry argon. The residue was analyzed by paper chromatography, or reduced and acetylated and analyzed by GC as previously described (Sections 2.A.1.b.–2.A.1.c.).
3.B.2.b. De-etherification with Lithium in Ethylenediamine

De-etherification was also achieved by reaction of the polysaccharide with lithium in ethylenediamine as described previously (Section 2.C.2.b.).

4. OTHER TECHNIQUES

4.A. Gel-Permeation Chromatography

Oligosaccharide fragments obtained from the lithium degradation (Section 3.C.2.b.) or hydrogen fluoride hydrolysis (Section 4.D.) of various polysaccharides were separated on a 90- by 1.5-cm column of Bio-Gel P-2 (Bio-Rad). Fractions which eluted in the void volume of P-2 were chromatographed on a 90- by 1.5-cm column of Bio-Gel P-6 to ensure homogeneity. Aqueous AcOH (0.1 M) was used as an eluant in all cases. Five-mL fractions were collected from the P-2 column and 10-mL fractions were collected from the P-6 column. A refractive-index (RI) detector monitored the column effluents. The peaks indicated by the RI detector were checked for the presence of sugar by the phenol-sulfuric acid assay (Section 4.B.). Carbohydrate-containing fractions were pooled and the carbohydrate recovered by lyophilization.

4.B. Phenol-Sulfuric Acid Assay

Test solution (0.25 mL) was placed in a 4-mL test tube. Liquified phenol (18 µL) was added, and then 1.25 mL of concentrated sulfuric acid was added rapidly to the aqueous phenol solution to maximize the production of heat. The color of each tube was compared to a blank of 0.1 M
AcOH treated in the same manner. A qualitative comparison of color was made, with a medium yellow to dark red color indicating a positive test.

4.C. Sonication

Polysaccharides in organic or aqueous solutions were sonicated in sealed containers under argon in a Branson-12 bath-type sonicator. Even when sonication was done over an extended period, no effort was made to control the bath temperature, since the sonicator shut itself off when the bath temperature exceeded 60 °C.

4.D. Hydrogen Fluoride (HF) Depolymerization

4.D.1. Preparation of Liquid HF

Liquid HF was carefully prepared by the condensation of gaseous HF (Linde) at -78 °C using the apparatus shown in Figure 60.

4.D.2. Depolymerization of Polysaccharides by Liquid HF

Polysaccharide (20–50 mg) was added to liquid HF (10–20 mL) which had been condensed at -78 °C in a 30-mL polyethylene vial as previously described (Section 4.D.1.). The polysaccharide was stirred in the HF for 5–30 min, and then the septum was removed from the vial and the HF was carefully removed under a stream of dry argon in the back of a hood. After all the HF was judged to be evaporated, the vial was placed under vacuum for 10 min. A 5-mL portion of deionized water was added to the vial and the oligosaccharides were separated by gel-permeation chromatography (Section 4.A).
Figure 60. Apparatus for Preparation of Liquid HF.
4.E. Deesterification of Polysaccharides

Deesterification of polysaccharides is achieved by dissolving the polysaccharide in an aqueous solution of NaOH of pH 11 with vigorous stirring at ambient temperature for 3 hours. The solution is adjusted to pH 6–7 by the addition of 50% aqueous AcOH. The polysaccharide is purified by dialysis (3500 molecular weight cutoff) against several changes of deionized water for 6–8 h, and then recovered by lyophilization. The completeness of deesterification is determined by IR spectroscopy as previously described (Section 1.B.).

4.F. Isolation of Acidic Monosaccharide Components Using Cationic Ion-Exchange Resin

Polysaccharide (20–30 mg) was treated with HF for 1 h as previously described (Section 4.D.). The hydrolyzate was immediately applied to a column (8 x 1 cm) of Bio-Rad AGX-2 strongly basic anion-exchange resin (OH⁻ form). The neutral monosaccharides were washed through with boiled and cooled deionized water (~ 30 mL). The anionic fraction was eluted with 0.1 M NaCl in boiled and cooled deionized water. The strongly basic eluant was immediately neutralized with AcOH and evaporated to dryness. The residue was passed through a column of Bio-Gel P-2 resin (Section 4.A.) to remove residual salts. The carbohydrate-containing fractions were lyophilized, and the resulting residue dried under vacuum over P₂O₅ for several hours before use.
5. ASSIGNMENT OF ANOMERIC CONFIGURATION

5.A. Configuration of Neutral Sugars — Chromium Trioxide Oxidation

Polysaccharide (10 mg) was acetylated by treatment with a 1:1 mixture of pyridine--Ac₂O (10 mL) overnight at room temperature. After evaporation of the liquid at reduced pressure, the polysaccharide was dissolved in CH₂Cl₂ (1 mL), and a small portion (0.1 mL) was removed and saved for later analysis. The remaining CH₂Cl₂ was evaporated and the residue was treated with CrO₃ (30 mg) in AcOH (0.3 mL) with sonication at 50 °C for 2–3 h. After each hour, a portion of the suspension was withdrawn. Each aliquot was treated with H₂O (to make total volume to 5 mL). The oxidized polysaccharide was recovered by extraction of each aliquot with CH₂Cl₂ (3 x 2 mL).

The oxidized polysaccharide was sequentially hydrolyzed, reduced and acetylated for analysis by GC as previously described (Sections 2.A.1.a.–2.A.1.c.).

5.B. Configuration of Acidic Sugars — Chromium Trioxide Oxidation and Methylation

The acidic polysaccharide was subjected to chromium trioxide oxidation as previously described (Section 5.A.). Oxidized polysaccharide was methylated by the method of Hakomori as previously described (Section 2.B.2.). The resultant methyl esters of the carboxylic acids were reduced as previously described (Section 2.C.1.b.). The oxidized, methylated and reduced polysaccharide was sequentially hydrolyzed, reduced and
acetylated as previously described (Sections 2.A.1.a. and 2.A.1.b.) and analyzed by GC/MS (Section 2.B.3.).
CHAPTER VI

CONCLUSIONS

General Methodology

Part of the purpose of this study was to evaluate the existing methods for the structural determination of polysaccharides with respect to their ability to function in the structure determination of viscous polysaccharides. In general, with a few minor modifications, the existing methods were adequate for use with viscous polysaccharides. Some techniques, however, had severe, distinct problems associated with their use.

The techniques which had the most problems associated with them, such as NMR spectroscopy and the Taylor–Conrad method of the carboxyl reduction of acidic polysaccharides, were those which required a polysaccharide to be quite soluble in a particular solvent. NMR spectroscopy, while extremely informative, is not absolutely necessary in determining the structure of a polysaccharide. The carboxyl reduction of an acidic functionality, however, is extremely important in several aspects of structure determination. A method for the carboxyl reduction of polysaccharides was developed here which was successful in avoiding the solubility and viscosity problems associated with the Taylor–Conrad reduction of acidic polysaccharides.
The second technique which presented problems was that of the peracetylation of the partially methylated alditols. Obviously, the problems associated with this reaction are by no means limited to viscous polysaccharides. An alternative method for the acetylation of alditols was introduced in 1990.\textsuperscript{105} This method produced superior results when compared to the former standard method of reduction and acetylation,\textsuperscript{104} as it greatly reduced the amount of underacetylated artifacts detected in the analyses.

Capillary GC/MS proved to be an extremely sensitive and useful method for detecting and quantitating the products of these derivatization reactions on a small scale. Many more analyses can be conducted on the same amount of polysaccharide when compared to traditional packed GC columns. In addition, the separation of the derivatives is much better, due in part also to the narrow peak width caused by using a very small amount of sample. This increase in separation allows for a more ready quantitation of the components, which is only limited by the quantitativeness of the derivatization reactions and workup.

Potentially Industrially Useful Bacterial Polysaccharides

The second aim of this study was to identify the repeating-unit structures of several viscous, potentially industrially important bacterial exopolysaccharides. A general description of each polysaccharide is listed below.

Polysaccharide 21-35 is a heavily acetylated, uronic acid-containing polysaccharide consisting of a linear trisaccharide repeating-unit. This
polysaccharide is identical in chemical structure to that of the polysaccharide produced by *Arthrobacter viscosus* NRRL B-1973.

Polysaccharide 105-4 is a complex, nonstoichiometrically acetylated, uronic acid-containing polysaccharide consisting of a branched octasaccharide repeating-unit.

Polysaccharide 97-67 is an acetylated polysaccharide consisting of a branched trisaccharide repeating-unit which contains the unusual sugar component 3-O-[(R)-1-carboxyethyl]-L-rhamnose.

Polysaccharies N-8, 75-39, and 91-94 are complex succinylated and pyruvated polysaccharides consisting of a branched octasaccharide repeating-unit. Each of the three polysaccharides are identical in the chemical structure of their repeating-units, and, in addition, the repeating-unit structure is identical to the repeating unit of Succinoglycan 10C3, produced by *Alcaligenes faecalis* var. *myxogenes*.

**Bacterial Plant Pathogens**

The third aim of the study was to identify whether or not two polysaccharides produced by two separate strains of *E. stewartii* are identical.

Two polysaccharides produced by two separate strains of *E. stewartii* are complex, uronic-acid containing polysaccharides consisting of a doubly branched heptasaccharide repeating-unit. The chemical structures of the repeating-units of the two polysaccharides appear to be identical.
LIST OF REFERENCES


