Glycans from Streptococcal Cell Walls

THE MOLECULAR STRUCTURE OF AN ANTIGENIC DIHETEROGLYCAN OF GLUCOSE AND GALACTOSE FROM *STREPTOCOCCUS Faecalis*

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SUMMARY

A complete molecular structure for an antigenic glycan isolated from the cell wall of *Streptococcus faecalis* is being proposed on the basis of analytical data obtained from methylation, acetylation, and periodate oxidation experiments and on the basis of the nature of the oligosaccharide fragments isolated from partial acid hydrolysates of the glycan. The antigen is a diheteroglycan composed of a main chain of trisaccharide units, glucose-β(1,6)-glucose-β(1,4)-galactose, joined by β(1,4) linkages with lactosyl and cellobiosyl side chains attached by β(1,4) linkages at alternate glucose residues of the main chain. The proposed structure for the glycan is consistent with the immunological properties of the polymer. Quantitative precipitin inhibition studies clearly indicate that lactosyl residues are the immunodominant determinants of the glycan.

Heteroglycans are important structural components of microbial cell walls and their unique chemical features are the basis for the serological classification of many bacteria (1, 2) and yeasts (3). Structural information on serologically active glycan is important not only as it relates to classification of microorganisms but also as it pertains to the acquisition of specific immunity against infectious organisms (4–6). Recently, a new type of antigenic glycan was isolated from the cell walls of *Streptococcus faecalis* (7). This glycan was shown to consist of glucose and galactose residues joined by β(1,4) or β(1,6) linkages and to possess lactosyl moieties as the antigenic determinants. In view of the fact that homogeneous antisera against the glycan can be obtained in high concentration, the new diheteroglycan should be useful for preparing mono-specific antibodies that in turn can be used for sequence studies and for elucidating the nature of an antibody-antigen complex.

The complete molecular structure of the diheteroglycan is being proposed on the basis of new information on the nature of the oligosaccharide fragments from the glycan and on the basis of analytical data obtained from methylation, acetylation, and periodate oxidation experiments. The structure is adequately represented by a unit with the accompanying arrangement of monosaccharide residues and linkages.

\[
\begin{align*}
\beta(1,4) \quad \beta(1,6) \quad \beta(1,4) \quad \beta(1,4) \\
-\text{Glc} -\text{Glc} -\text{Gal} - \\
\beta(1,4) \\
\text{Glc} \\
\beta(1,4) \\
\text{Gal}
\end{align*}
\]

In a typical molecule of the glycan 18 such units are present, but a few of the units contain cellobiosyl in place of lactosyl side chains. The proposed structure for the glycan is consistent with immunological properties of the antigen and represents a modification of an earlier suggestion based on preliminary structural data (7).

MATERIALS AND METHODS

**Diheteroglycan** The diheteroglycan was extracted from the cell wall of *S. faecalis* strain N by stirring the cell walls in 10% trichloroacetic acid at 4°C for 24 hours. Purification of the diheteroglycan was achieved by acetone and alcohol precipitation as described earlier (7) and by gel filtration through a Bio-Gel P-60 column, which has been found to be an excellent method for separating cell wall glycans from other streptococcal strains (8, 9). For the present study a sample of 0.38 g of the diheteroglycan was prepared from cells from 20 liters of a 12-hour culture. The purity of the diheteroglycan was checked by density gradient centrifugation, double diffusion in agar gels, and constancy in chemical composition on repeated alcohol fractionation as described earlier (7).

**Streptococcal Vaccines**—Vaccines were prepared from *S. faecalis* following a published procedure (10). The *S. faecalis* cells from a 500-ml culture were suspended in 30 ml of phosphate buffer, pH 7.2, containing 0.2% formalin and were maintained at room temperature.
at which point 0.5 ml of methyl iodide was added and agitation in an ultrasonic bath was continued for an additional 1 hour. At the end of this time the reaction mixture was poured into a variety of solvents and the supernatant was used as the enzyme source. The pH of this supernatant was 7, and the protein content as determined by a standard method (11) was 10 mg per ml. Paper chromatograms of this solution were stained with silver nitrate stain (13) showed that neither glucose nor galactose were dissolved in 0.5 ml of dry methyl sulfoxide in a 5-ml serum bottle sealed with a rubber cap. The bottle was flushed thoroughly with nitrogen. To this solution 0.5 ml of freshly prepared 2 M methylsulfonyl sodium in methyl sulfoxide (15) was added dropwise from a syringe and the resulting reaction mixture was agitated in an ultrasonic bath for 1/2 hour. The reaction mixture was then maintained for 8 hours at room temperature at which point 0.5 ml of methyl iodide was added and agitated in an ultrasonic bath was continued for an additional 1/2 hour. At the end of this time the reaction mixture was poured into approximately 10 ml of water, dialyzed, and concentrated to dryness. The methylated compound was suspended in 1 ml of 90% formic acid for 2 hours at 100° in order to effect a dissolution of the methylated glycan. The formic acid was removed by evaporation and the residue was resuspended in 1 ml of 0.13 M sulfuric acid and heated at 100° for 12 hours. The acid was neutralized and the hydrolytic products in the reaction mixture were reduced with excess sodium borohydride at 25° for 12 hours. The solution was acetylated with Dowex 50 (H+) resin and the acid filtrate was concentrated to dryness. The residue was acetylated with a small volume of acetic anhydride-pyridine (1:1 by volume) by maintaining the reaction mixture on the steam bath for 1/4 hour. The cooled reaction mixture was injected directly into a ECNSS-column of a gas chromatograph and analyzed for sugar alditol acetates (16). The gas chromatograph was coupled to a mass spectrometer and mass spectra data were also obtained on the various peaks. The identity of methyl ether hydrolytic products was established by comparison of the retention times on the gas-liquid chromatographic column and the types of fragments on mass spectrometry of the various componenets with the values for standard compounds (17). Quantitative values were also obtained from these data.

Identification of Reducing Residue—A sample of 1.0 mg of the diheteroglycan was dissolved in 1 ml of water and reduced with 10 mg of sodium borohydride in order to label the carbohydrate residue at the reducing end of the glycan by maintaining the solution at 25° for 12 hours. The reaction mixture was then acidified with Dowex 50 (H+) resin, and the filtrate containing the reduced glycan was taken to dryness. The residue was subjected to methanolysis (3% HCl in methanol) at 100° for 4 hours. Silver carbonate was added to the reaction mixture and the resulting precipitate was removed by filtration. The filtrate was concentrated to dryness and the residue consisting of methyl glycosides and the sugar alditol from the reducing end of the glycan was acetylated with acetic anhydride-pyridine (1:1 by volume) for 1/2 hour on a steam bath. An aliquot of the acetylated reaction mixture was analyzed by gas-liquid chromatography on an ECNSS-column (a nitrile silicone polyester copolymer manufactured by Applied Science Labs., State College, Pa.) and the compound containing the deuterium label was identified as galactitol peracetate.

Periodate Oxidation—Periodate oxidations of the diheteroglycan and reference compounds were performed by two different procedures, first by the Smith degradation (18) as described by Sloneker et al. (19) and second by the method of Potter and Hassid (20) in which formic acid produced from end groups is measured. In the first procedure the oxidation of 8 mg of the glycan was effected in 0.02 M sodium periodate at 4° for 48 hours. In the second, samples of 1 to 5 mg of glycan or methyl-α-D-glucoside were oxidized in 0.15 M sodium periodate containing 1.5% sodium chloride in the dark for 1 hour at 4°. In each case the excess periodate was decomposed by addition of ethylene glycol. In the first procedure, the reaction mixture was reduced with sodium borohydride (20%) by weight of the glycan) overnight at room temperature. Excess borohydride was destroyed by acidifying the solution to pH 5 with acetic acid. The solution was adjusted to pH 0.3 with sodium carbonate and dialyzed for several days against distilled water. The material was taken to dryness by lyophilization. Samples of approximately 3 mg of the oxidized and reduced glycan and of the native glycan were subjected to graded acid hydrolysis in 0.2 ml of 0.1 N HCl for varying periods up to 4 hours. The hydrolytic products in the hydrolysate were separated by paper chromatography in an ultrasonic bath was continued for an additional 1 hour. At the end of this time the reaction mixture was poured into approximately 10 ml of water, dialyzed, and concentrated to dryness. The methylated compound was suspended in 1 ml of 90% formic acid for 2 hours at 100° in order to effect a dissolution of the methylated glycan. The formic acid was removed by evaporation and the residue was resuspended in 1 ml of 0.13 M sulfuric acid and heated at 100° for 12 hours. The acid was neutralized and the hydrolytic products in the reaction mixture were reduced with excess sodium borohydride at 25° for 12 hours. The solution was acetylated with Dowex 50 (H+) resin and the acid filtrate was concentrated to dryness. The residue was acetylated with a small volume of acetic anhydride-pyridine (1:1 by volume) by maintaining the reaction mixture on the steam bath for 1/4 hour. The cooled reaction mixture was injected directly into a ECNSS-column of a gas chromatograph and analyzed for sugar alditol acetates (16). The gas chromatograph was coupled to a mass spectrometer and mass spectra data were also obtained on the various peaks. The identity of methyl ether hydrolytic products was established by comparison of the retention times on the gas-liquid chromatographic column and the types of fragments on mass spectrometry of the various components with the values for standard compounds (17). Quantitative values were also obtained from these data.
eleven fractions of the paper chromatogram containing the oligosaccharide fragments were treated with a drop of \( \beta \)-galactosidase solution. After 15 min, the chromatogram was sprayed with glucose oxidase or galactose oxidase followed by \( o \)-toluidine (21). In the second procedure the formic acid produced from the compounds was measured by titration with 0.0045 N barium hydroxide. These values were compared with those expected for methyl glucoside and with those expected for a glycan with 19 terminal residues and 1 reducing residue.

Acid Hydrolysis and Acetolysis—A sample of 30 mg of the pure diheteroglycan was dissolved in 0.5 ml of 0.1 N hydrochloric acid and heated in a boiling water bath for \( \frac{1}{2} \) hour. The oligosaccharides in the hydrolysate were separated on paper chromatograms by five ascents of the solvent system of \( a \)-butyl alcohol-pyridine-water (6:4:3 by volume) and located on the paper by spraying with silver nitrate stain procedure. Identification of the products was achieved by comparison of \( R_f \) values for the products and reference compounds. The identity of the fragments was confirmed by enzymatic methods. The areas of the paper chromatogram containing the oligosaccharide fragments were treated with a drop of \( \beta \)-galactosidase solution. After 15 min, the chromatogram was sprayed with glucose oxidase or galactose oxidase followed by \( o \)-toluidine (21). In the second procedure the formic acid produced from the compounds was measured by titration with 0.0045 N barium hydroxide. These values were compared with those expected for methyl glucoside and with those expected for a glycan with 19 terminal residues and 1 reducing residue.

Controlled acetolysis of 2 mg of native glycan was effected in acetic anhydride-glacial acetic acid-sulfuric acid (10:10:1, v/v) at 30\( ^\circ \)C for 12 hours (22). The acetolysis fragments were deacylated with sodium methoxide and the oligosaccharides in the mixture were separated on a superfine Sephadex G-25 column (23). The optical rotation measurements of the eluates indicated that two major fractions were eluted from the column. On the basis of the elution patterns one fraction was a disaccharide and the other was a higher molecular weight oligosaccharide. The two fractions were maintained separately and concentrated to a small volume. Gas-liquid chromatographic data indicated that the disaccharide fraction contained some monosaccharide contaminants, whereas the oligosaccharide fraction contained some minor components. The disaccharide fraction was subjected to reduction with sodium borohydride as described in a preceding section. The reduced product was hydrolyzed in acid and the resulting hydrolysate was subjected to a second reduction utilizing sodium borodeuteride. After acetylation of the mixture, the monosaccharide derivatives were separated and identified by gas-liquid chromatography and mass spectrometry. The moiety containing the deuteride was easily detectable by this procedure.

The oligosaccharide fraction from the acetolysis mixture was subjected to methylation analysis following the procedure outlined in an earlier section. The methyl ether hydrolysates from the oligosaccharide were separated and identified.

Methylolysis Analyses—In Table I the nature of the methyl ether fragments and the amounts of each fragment for the native glycan are recorded. In addition, values are recorded for fragments expected from the compound with the formula depicted in Fig. 1. Similar data on the methyl ether fragments from the enzyme-modified glycan are recorded in Table II. In this table the theoretical values are calculated for the compound of the type shown in Fig. 1 but devoid of 7 galactose and 1 glucose residues as indicated by the arrows in the figure.

Periodate Oxidation—The glycan sample which was subjected to the Smith degradation with sodium periodate was hydrolyzed in dilute acid for varying periods of time. Chromatographic analysis of the hydrolysates showed that over 95% of the galactose residues had been oxidized, while only a small percentage of the glucose residues had been oxidized under the reaction conditions. Differences in the susceptibility of galactose and glucose residues to periodate oxidation have been reported earlier (18, 19) and these differences have been attributed to steric factors and the formation of mixed cyclic acetals.

In the partial acid hydrolysates of the oxidized and reduced glycan, compounds corresponding in \( R_f \) values (two ascents of the solvent) to glycerol (0.76), threitol (0.67), glucose (0.55), cellobiose (0.36), gentiobiose (0.29), and a glucosyl trisaccharide

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Found</th>
<th>Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Tetramethylglucose</td>
<td>4.0</td>
<td>3</td>
</tr>
<tr>
<td>2,3,6-Triacetylglucose</td>
<td>35.0</td>
<td>36</td>
</tr>
<tr>
<td>2,3-Dimethylglucose</td>
<td>17.1</td>
<td>18</td>
</tr>
<tr>
<td>2,3,4,6-Tetramethylgalactose</td>
<td>17.5</td>
<td>16</td>
</tr>
<tr>
<td>2,3,6-Triacetylgalactose</td>
<td>17.6</td>
<td>18</td>
</tr>
</tbody>
</table>

* On the basis of the formula depicted in Fig. 1.
with a 1,4 and a 1,6 linkage (0.19) were observed on the paper chromatogram. The identification of the oligosaccharides was further established by enzymic procedures. On a duplicate unstained chromatogram the areas on the chromatogram corresponding to the oligosaccharides were treated with a β glycosidase solution and incubated for 15 min. These chromatograms were then sprayed with glucose oxidase or galactose oxidase followed by o-tolidine. Glucose but not galactose was detected at the area of the chromatogram corresponding in $R_F$ value to cellobiose, gentiobiose, and the glucosyl trisaccharide.

In the second periodate procedure, the formaldehyde produced from 1.10 mg of methyl α-glucoside and 3.42 mg of the glycan (dry weight) was neutralized by 1.22 ml and 0.97 ml of 0.0045 N barium hydroxide. Utilizing the formula of Potter and Hassid (20) and the data in Table II the theoretical values expected for the above samples are 1.26 ml and 1.06 ml, respectively.

**Acid Hydrolytic Products from Diheteroglycan**—The $R_F$ values for nine oligosaccharides isolated from an acid hydrolysate of the glycan and for reference disaccharides are recorded in Table III. Each oligosaccharide was subjected to further hydrolysis in 0.1 N HCl and their monosaccharide constituents were identified by paper chromatography. These results are also recorded in Table III.

A second sample of each oligosaccharide was incubated with a 2% solution of β-glycosidase for 48 hours. The hydrolysate of each oligosaccharide was examined for the presence of glucose and galactose by use of glucose oxidase and galactose oxidase test strips (25) obtained from Ames Company, Elkhart, Indiana. The results of these tests were in agreement with the data in Table III.

The $R_F$ value of an oligosaccharide is indicative of its molecular size when compared to $R_F$ values of oligosaccharides of known structure (26). On the basis of such relationships it was concluded that the oligosaccharides listed in Table III ranged in molecular size from that of a disaccharide to that of a pentasaccharide. It is noted in the table that with the exception of the disaccharides, all oligosaccharides were found to be composed of glucose and galactose. Furthermore no evidence has thus far been obtained for the presence of high molecular weight oligosaccharides composed solely of glucose in the hydrolysates of the glycan. The latter observation argues against the possibility that long segments of contiguous glucose residues are structural elements of the glycan.

In order to obtain additional evidence for the identity of the disaccharides from the glycan, the osazone derivatives of oligosaccharides 1 and 2 (Table III) were prepared (14). The crystalline osazones were collected on a filter and dissolved in a drop of ethyl alcohol. The solutions of the osazones were subjected to paper chromatographic analysis in the top layer of the solvent system of 5 parts of n-amyl alcohol, 1 part of water, and 1 part of concentrated ammonia. Reference cellobiose osazone, lactose osazone, and gentiobiose osazone were subjected to the same analysis. The osazone of oligosaccharide 1 migrated as a single spot with an $R_F$ value of 0.61 which was also the value for the cellobiose osazone. The osazones preparation of oligosaccharide 2 yielded two spots on chromatography, one with an $R_F$ value of

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>$R_F$ value</th>
<th>Monosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiase</td>
<td>0.75</td>
<td>Glucose</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>0.68</td>
<td>Glucose</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.68</td>
<td>Glucose, galactose</td>
</tr>
<tr>
<td>Allolactose</td>
<td>0.62</td>
<td>Glucose, galactose</td>
</tr>
<tr>
<td>Oligosaccharide 1</td>
<td>0.75</td>
<td>Glucose</td>
</tr>
<tr>
<td>Oligosaccharide 2</td>
<td>0.68</td>
<td>Glucose, galactose</td>
</tr>
<tr>
<td>Oligosaccharide 3</td>
<td>0.02</td>
<td>Glucose, galactose</td>
</tr>
<tr>
<td>Oligosaccharide 4</td>
<td>0.50</td>
<td>2 Glucose, 1 galactose</td>
</tr>
<tr>
<td>Oligosaccharide 5</td>
<td>0.45</td>
<td>2 Glucose, 1 galactose</td>
</tr>
<tr>
<td>Oligosaccharide 6</td>
<td>0.33</td>
<td>3 Glucose, 1 galactose</td>
</tr>
<tr>
<td>Oligosaccharide 7</td>
<td>0.29</td>
<td>2 Glucose, 2 galactose</td>
</tr>
<tr>
<td>Oligosaccharide 8</td>
<td>0.22</td>
<td>3 Glucose, 2 galactose</td>
</tr>
<tr>
<td>Oligosaccharide 9</td>
<td>0.18</td>
<td>3 Glucose, 2 galactose</td>
</tr>
</tbody>
</table>

* On the basis of the formula depicted in Fig. 1.
0.62 and the other an \( R_f \) value of 0.50. The \( R_f \) value of lactose osazone was 0.61 and of gentiobiase osazone was 0.50.

All of the oligoasaccharides isolated from acid hydrolysates of the glycan were susceptible to hydrolysis by \( \beta \)-galactosidase and \( \beta \)-glucosidase. The configuration of the linkages between the monosaccharide units in these compounds is therefore \( \beta \). Methylation results on the native and modified glycan (Tables I and II) establish that the positions of the linkages between the monoasaccharide units are 1, 4, and 6.

**Acetolysis Analysis**—From the acetolysis mixture of the glycan, two fractions, one consisting primarily of a disaccharide component and the other consisting primarily of a high molecular weight oligoasaccharide component, were isolated. Both preparations on hydrolysis yielded glucose and galactose. Data from reduction with borohydride, hydrolysis in acid, and reduction with borodeuteride of the disaccharide fraction showed that galactose was the terminal unit of the disaccharide. The oligoasaccharide in the second fraction from the acetolysis mixture was subjected to methylation analysis. The major products were 2,3,4,6-tetramethylglucose, 2,3,6-trimethylgalactose, and 2,3,6-trimethylglucitol with the ratio of these fragments approximating 1:1:2. Some 2,3-dimethylglucose and 2,3,4,6-tetramethylgalactitol were also detectable in the reaction mixture.

**Inhibition of Precipitin Reaction**—As previously reported (7), lactose has been found to be the most potent inhibitor of the precipitin reaction between the diheteroglycan and anti-S. faecalis sera. Cellulobiase and allolactose were also inhibitory at high concentrations of the disaccharides. However, gentiobiase was not effective. The values for lactose and cellulobiase for 50% inhibition were in the same range as previously reported (7), 0.03 mg per ml and 12.5 mg per ml, respectively. However, allolactose that had been subjected to further purification showed a much lower potency than previously reported (7). The concentration of allolactose required for 50% inhibition was now 15 mg per ml. The latter value is comparable to the value for \( p \)-nitrophenyl \( \beta \) galactoside. Probably the inhibition observed with the allolactose is due to the terminal \( \beta \)-linked galactose residue in the compound. The high value obtained earlier with allolactose was attributable to lactose which was present as a contaminant in the allolactose preparation used in the earlier studies (7).

**DISCUSSION**

The analytical data on the structure of the antigenic diheteroglycan from S. faecalis suggest the molecular formula for the glycan shown in Fig. 1. Methylation data establish that the glycosidic linkages between the monosaccharide units of the glycan are 1, 4 and 1, 6. The configuration of the linkages is \( \beta \) since glucose and galactose were liberated from the glycan and from oligoasaccharides derived therefrom by an almond \( \beta \)-glycosidase solution. The ratio of galactose to glucose in the formula is 0.50 and agrees with the experimentally determined value of 0.61. The molecular weight of 14,750 is in agreement with the value of 15,000 calculated from sedimentation data (7). The oligoasaccharide types obtained on acid hydrolysis of the glycan and results of periodate oxidation of the glycan are both consistent with this formula.

An almond enzyme preparation containing \( \beta \)-glucosidase and \( \beta \)-galactosidase activity was used to prepare a partially degraded glycan from which 1 glucose and 7 galactose residues were removed per mole of glycan. The methylation data in Table II illustrate a consistency between the experimental and theoretical values for the methyl ether fragments which were derived from the modified glycan. The theoretical values have been calculated for a molecule with a basic structure shown in Fig. 1 but devoid of the glucose and galactose residues as indicated by the arrows.

Controlled acetolysis of the diheteroglycan yielded two fractions, one a disaccharide and the other a low molecular weight oligoasaccharide. Mass spectroscopy data on the methylated and reduced disaccharide fraction showed a galactosylglucose compound, presumably lactose, was the major component in this fraction. The lactose probably arises from a minor amount of acetolysis of the side chains of the glycan (22). In a hydrolysate of the methylated oligoasaccharide fraction, 2,3,4,6-tetramethylglucose, 2,3,6-trimethylgalactose, and 2,3,6 trimethylglucitol were present. These methyl ether monosaccharides were present in the mixture in the ratio approximating 1:1:2. Such a ratio would be obtained for an oligoasaccharide which would be produced from the glycan with the formula shown in Fig. 1 by cleavage of all of the 1,6-bonds and terminal galactose residues (22). The presence of only trace amounts of 2,3-dimethylglucitol in the reaction mixture indicates that indeed a high percentage of 1,6 bonds in the glycan were cleaved during acetolysis. Some 2,3,4,6-tetramethylgalactitol was detected in the methylolation products from the oligoasaccharide fraction.

A Smith degradation (18) of the glycan was performed under conditions which resulted in the oxidation of nearly all of the galactose residues and only a few glucose residues. In hydrolysates of the oxidized and reduced glycan, glycerrut, thestritol, glucose, and glucosyl oligoasaccharides were identified by chromatographic procedures. The oligoasaccharides were further identified as cellulobiase, gentiobiase, and a \((1,4)(1,6)\)-glucosyl trisaccharide by their susceptibility to \( \beta \)-glucosidase. These results of the Smith degradation analysis are consistent with the proposed structure for the glycan and the known differences in rate of periodate oxidation of galactose and glucose residues in glycans of certain sequences (18, 19).

Illustrated in Table III are data on the various oligoasaccharides which were isolated from a partial acid hydrolysate of the diheteroglycan. It should be noted that oligoasaccharides of various combinations of glucose and galactose were obtained. Significantly oligoasaccharides other than disaccharides composed solely of glucose were not detectable in the hydrolysate of the glycan. In view of the differences in rate constants for the hydrolysis of glucosidic and galactosidic bonds (27), it would be expected that if long segments of contiguous glucose units were present in the diheteroglycan, glucosyl oligoasaccharides of the tri-, tetra-, and penta- types would be present in hydrolysates of the glycan.

The immunological data on the inhibition of the precipitin reaction of the antigen with its homologous antiserum are consistent with the structure proposed for the glycan (Fig. 1). Lactosyl side chains of the glycan were found to be the major immunological determinants, and lactose is a very effective inhibitor of the precipitin reaction of the antigen with its homologous antiserum. Cellulobiase, a minor terminal unit of the glycan, was also slightly reactive as an inhibitor of the precipitin reaction. Of the other disaccharide fragments obtained from the glycan, allolactose (\( \beta \)-galactopyranosyl(1–6)-glucose) was effective as an inhibitor, but gentiobiase was ineffective. The moderate inhibition observed with allolactose is most likely due to the terminal \( \beta \)-galactosyl residue of the compound.

It should be noted that the structure (Fig. 1) proposed for the diheteroglycan is of a defined sequence and chain array. It is well known that biological macromolecules with highly specialized
functions possess a uniform structure (28, 29), and it is therefore not surprising that the antigenic diheteroglycan also possesses such a structure. The latter may well be of paramount importance for immunological specificity. The biosynthesis of a glycan of uniform structure can be achieved by known pathways involving glycosyltransferases, glycosyl donor compounds (sugar nucleotides or glycosyl lipid carriers), and appropriate acceptor molecules (30–32). Enzyme specificity for both donor and acceptor molecules is a primary regulatory mechanism for specifying monosaccharide sequences and chain arrays in compounds synthesized by these pathways (33, 34). However regulation of the chain elongation process is less rigid and accordingly chains of different molecular sizes can be obtained. Such may well be the case with the antigenic diheteroglycan from S. fuecalis in which molecules of varying chain lengths can occur but the sequence of the residues and chain arrays are maintained.

The cell wall of S. fuecalis is heteropolymeric in nature consisting of peptidoglycans (35), teichoic acids (36), and heteroglycans (37). As in other gram-positive microorganisms (38, 39) it is likely that the peptidoglycan forms a rigid framework for the cell wall of S. fuecalis. The teichoic acids in group D organisms are associated with the inner membranes of the wall (40, 41), and these antigens are generally not active immunogens of the intact cell in the host animal. In contrast, the cell wall glycans are very antigenic and high titer antibody preparations directed at these polymers are readily obtained from immunized animals (42, 43). The immunogenicity of the diheteroglycan of S. fuecalis indicates that this antigen is located on the cell surface. In view of the earlier observation (44) that highly acid-labile linkages, such as the glycosyl-phosphoryl linkage in another antigen of this organism, are not hydrolyzed under the extraction conditions, it is unlikely that the diheteroglycan is chemically linked to another component of the cell wall matrix. Rather, it is more probable that the glycan is held in the wall matrix by hydrogen bonds between the interior residues of the glycan and certain functional groups of the peptidoglycan of the cell wall. Such an association is substantiated by the observation that the glycan is retained in the cell wall matrix during the preparation of cell walls but can be extracted from the cell walls or intact cells by the trichloroacetic acid procedure. In this complex the lactosyl and cellulosyl side chains of the diheteroglycan will be on the cell surface and available for binding to appropriate antibody-producing cells and initiating the reactions which ultimately lead to synthesis of immunoglobin with specific affinity for the side chain moieties of the antigen.

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