The cell wall of Streptococcus faecalis, strain N, has been found to contain two antigenic glycans, a diheteroglycan of glucose and galactose and a tetraheteroglycan of rhamnose, glucose, galactose, and N-acetylgalactosamine. The isolation, purification, and structural and immunochemical characterization of the hitherto unknown diheteroglycan. A tentative structure has been proposed for the new glycan on the basis of structural information on oligosaccharides isolated from acid hydrolysates of the polymer and on the basis of immunological inhibition studies. Of considerable interest is the finding that, of the two major disaccharide moieties (lactosyl and gentiobiosyl) in the glycan, the lactosyl moiety is the immunodominant group.

The cell walls of gram-positive bacteria are heteropolymeric in nature consisting of specific combinations of peptidoglycane, teichoic acids, teichuronic acids, or heteroglycans together with minor amounts of proteins and lipids (1, 2). The heteroglycans, although chemically complex and variable, possess unique structural features which are directly correlatable to the antigenic specificities of bacterial cell surfaces. The relationships between the carbohydrate structure and antigenic specificity, as developed by Heidelberger for the Pneumococci (3), have been employed successfully in the systematic classification of other bacteria and have been especially useful in the classification of Streptococci (4). However, in the case of group D Streptococci, many strains exhibit both group and type specificity and the immunochemical basis for these antigenic specificities is not yet completely known (5). Further, relatively few studies have been published on the isolation and immunochemical characterization of antigens from this group of Streptococci.

A number of years ago, Elliot (6) reported the isolation of type-specific carbohydrates consisting of glucosamine, rhamnose, and glucose from four serological types of group D Streptococci. More recently, a similar carbohydrate antigen of glucosamine, rhamnose, and glucose was isolated from a strain of Streptococcus faecalis by Kame and Karakawa (7). From another type of group D Streptococci (type 1, strain D76), Bleiweis, Young, and Krause (8) isolated a cell wall carbohydrate composed of rhamnose, glucose, glucosamine, galactosamine, ribitol, and phosphorus. This carbohydrate was very reactive serologically but structural data on the glycan or immunochemical correlations were not presented. Studies reported herein show that the cell wall of a group D Streptococcus (strain N of Streptococcus faecalis) contains two carbohydrate antigens, a diheteroglycan of glucose and galactose, and a tetraheteroglycan of rhamnose, glucose, galactose, and N-acetylgalactosamine. The isolation, purification, and structural and immunochemical characterization of the diheteroglycan are described in this report. A tentative structure has been proposed for the glycan on the basis of structural information on oligosaccharides isolated from acid hydrolysates of the polymer and on the basis of immunological inhibition studies.

**EXPERIMENTAL PROCEDURE**

*MATERIALS—The culture of S. faecalis, designated strain N, has been used in studies on rhamnose biosynthesis (9) and was originally obtained from the stock collection of the Microbiology Department, University of Nebraska, Lincoln, Nebraska. Strain N is a nonhemolytic Streptococcus which has been shown to be a group D organism by serological tests in the laboratory of Dr. R. C. Lancefield, Rockefeller University. Cellotriose and cellotetraose (10) were provided by Dr. G. Miller, Department of Biochemistry, University of Miami, Miami, Florida. Gentio-oligosaccharides were prepared from luteose (11) provided by Dr. J. Marshall, Department of Biochemistry, University of Miami, Miami, Florida. The procedure involved partial acid hydrolysis of the luteose and separation and identification of the oligosaccharides by paper chromatographic methods (12). Allolactose (6-O-β-D-galactopyranosyl-D-glucopyranose) was prepared by an enzymic procedure and was available in the laboratory (13). The other oligosaccharides
were laboratory grade reagents from various commercial suppliers. Glucoamylase from \textit{Aspergillus niger} was available in the laboratory (14). It is an \(\alpha\)-glucosidase of broad specificity and acts on \(\alpha\)-glucosides as well as glucosyl oligosaccharides with \(\alpha\)-glucosidic linkages (14). The glucoamylase solutions used in the present study contained 6 mg of protein per ml. \(\beta\)-Glucosidase and \(\beta\)-galactosidase were present in almond emulsion obtained from Nutritional Biochemicals. The solution of emulsin employed in this study contained 10 mg of protein per ml. The \(\beta\)-glucosidase was found to act on \(\beta\)-glucosides and oligosaccharides with \(\beta\)-glucosidic linkages while the \(\beta\)-galactosidase was found to act only on \(\beta\)-galactosides and disaccharides with \(\beta\)-galactosidic linkages.

\textbf{Isolation of Glycans from Cell Walls—} \textit{S. faecalis} strain N was grown in 8 liters of nutrient broth supplemented with 0.1\% glucose. Cell walls from 80 g of whole cells, wet weight, were prepared by the method described by Bleise, Karaka, and Krause (15). The carbohydrate components were extracted from the cell walls at 4\(^\circ\)C for 24 hours with 10\% trichloroacetic acid following the general procedure of Park and Hancock (16). Protein components in the trichloroacetic acid-soluble fraction were precipitated by the addition of 2 \(\times\) HCl-ethanol mixture (1:20). After removal of this precipitate by centrifugation, the trichloroacetic acid-soluble material was precipitated by the addition of 5 volumes of acetone. The precipitated material was collected by centrifugation, redissolved in water, and dialyzed against distilled water and lyophilized. The yield of white amorphous product was 0.3 g.

\textbf{Streptococcal Vaccines—} Vaccines were prepared from \textit{S. faecalis} essentially according to the method described by McCarty and Lancefield (17). The bacteria were grown in 500 ml of Todd-Hewitt broth at 37\(^\circ\)C for 16 hours and collected by centrifugation. The resulting bacterial sediment was resuspended in 20 ml of 0.2\% formalized NaCl solution and appropriate aliquots of the material were injected into four rabbits. Several different preparations of antisera were obtained from the rabbits by the procedures previously described (18).

\textbf{Immunological Methods—} Qualitative and quantitative precipitin reaction analyses were performed according to the method of McCarty and Lancefield (17). The hapten inhibition studies were performed by a quantitative precipitin inhibition test. In this test the reaction mixture consisted of a total volume of 1 ml, and contained, in addition to 0.8\% NaCl solution diluent, 0.1 ml of antiserum, optimum concentration of carbohydrate antigens, and varying concentrations of monosaccharide and oligosaccharide inhibitors. The resulting precipitates were washed with cold 0.8\% NaCl solution and antibody levels were determined by the method described by Kane and Karaskawa (18). Double diffusion analysis in agar was performed by the method described by Tan and Kunkel (19).

\textbf{Determination of Phosphate and Rhamnose—} Samples, 4 mg, of the various preparations of trichloroacetic acid-soluble cell wall components were dissolved in 2 ml of water. Aliquots of these solutions containing 40 to 200 \(\mu\)g of material, dry weight, were diluted to 0.1 ml and mixed with 0.1 ml of 10 \(\mu\)l sulfuric acid. A drop of hydrogen peroxide was added to this solution and the mixture was maintained at 170\(^\circ\)C until all the organic matter had been oxidized. Phosphate in the mixture was determined by the use of ammonium molybdate reagent (20) and 0.1\% Elon solution for stabilizing the color. Other samples of the original solutions containing 100 to 400 \(\mu\)g were used for the quantitative determination of rhamnose by the Dische-Shettles procedure (21).

\textbf{Acid Hydrolysis of Cell Wall Components—} Samples of 3 to 5 mg of the various preparations of cell wall components were hydrolyzed in 0.15 ml of 0.1 \(\times\) hydrochloric acid in a boiling water bath. Samples of 5 \(\mu\)l were removed after hydrolysis for 0, 0.1, 0.3, 1, 3, and 6 hours and examined for reducing sugars by paper chromatography (9) in a solvent system on n-butyl alcohol-pyridine-water (6:4:3 \(v/v\)). The reducing sugars on the chromatogram were detected by appropriate spray reagents (silver nitrate, aniline oxalate, and acetyl acetone-dimethylaminobenzaldehyde reagents), and identified by their \(R_F\) values and their characteristic reactivity with the above reagents. The identity of glucose, N-acetylgalactosamine, and galactosamine was further verified by use of glucose oxidase and galactose oxidase which were sprayed directly on the chromatograms (22). Other samples of comparable weight were also hydrolyzed in 2 \(\times\) hydrochloric acid for 2 hours and the reducing monosaccharides in the hydrolysate were separated by paper chromatography. The concentrations of the monosaccharides in the hydrolysates were estimated from color intensities of these compounds and appropriate standards on the paper chromatograms.

\textbf{Isolation of Oligosaccharides from Acid Hydrolysates of Cell Wall Components—} A sample of 10 mg of the pure glycans was hydrolyzed in 0.5 ml of 0.1 \(\times\) hydrochloric acid in a boiling water bath for 3 hours. Three types of hydrolytic products (disaccharides, trisaccharides, and tetrasaccharides) were isolated from the hydrolysate by paper chromatographic procedures (12). These oligosaccharides were subjected to further hydrolysis in acid and to the action of selective enzymes. Acid hydrolysis of the oligosaccharides was performed in 0.1 \(\times\) hydrochloric acid in a boiling water bath. The enzymes which were used for hydrolyzing the oligosaccharides were glucoamylase (an \(\alpha\)-glucosidase), \(\beta\)-glucosidase, and \(\beta\)-galactosidase. The enzymic hydrolysis was performed by two different procedures; first, directly on the paper chromatogram by placing a solution of an enzyme of known specificity on the compound on the paper chromatogram and detecting the production of glucose or galactose with appropriate oxidases (9, 22). Second, the purified oligosaccharides were mixed with an equal volume of the enzyme and the reaction mixtures were examined for hydrolytic products by paper chromatography after incubation for 3, 6, and 24 hours. The hydrolytic products in these samples were identified by the \(R_F\) values and their reactivity with glucose oxidases.

\textbf{Estimation of Molecular Weight—} Samples of 2 mg of the pure glycans and of insulin were introduced onto different density gradient columns (5 to 25\% glycerol). All samples were then centrifuged at the same time and for 14 hours at 65,000 rpm in a Spinco model L-65 centrifuge. At the end of this time, the density gradient columns were fractionated into 0.2-ml samples by means of an automatic fractionator (23). Samples obtained from the tubes were analyzed for carbohydrate by the orcinol-sulfuric acid method (24). Assuming that sedimentation behaviors for the carbohydrate polymers are comparable, molecular weights for the two glycans were calculated utilizing the empirical relationships developed by Martin and Ames for proteins (25).
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RESULTS

Trichloracetic Acid Extraction of Cell Walls of S. faecalis, strain N—Previous studies have identified a carbohydrate and peptidoglycan as the two major constituents of trypsinized cell walls of Streptococci, including those of group D Streptococci (2). The results of the chemical analyses of the carbohydrate extracted from cell walls of strain N by the cold trichloracetic acid method indicate that the material is composed of rhamnose, glucose, galactose, N-acetylgalactosamine, and phosphorus. On the other hand, the trichloracetic acid-insoluble peptidoglycan has been shown to consist of hexosamines, alanine, glutamic acid, aspartic acid, and lysine (26). Initial immunological analysis indicated that the trichloracetic acid-soluble fraction consists of two distinct antigens. A photograph of a typical double diffusion agar plate is reproduced in Fig. 1. The trichloracetic acid-soluble fraction from the cell walls and rabbit anti-S. faecalis serum yielded two bands (Wells 1 and 3) indicative of two antigenic components in this fraction. However, the purified component yielded a single band (Wells 3 and 8) in this test. The separation of the two antigenic substances has been achieved by an alcohol fractionation method described in the following section.

Separation of Two Antigens—A sample of 0.15 g of trichloracetic acid-soluble material from S. faecalis cell walls was dissolved in 9 ml of 10% solution of trichloracetic acid. After removal of a slight amount of insoluble material by centrifugation, 1 volume of absolute ethyl alcohol was added to the clear supernatant and the mixture was maintained at 4°C for 18 hours. In this period, a white precipitate (Component I) appeared and this precipitate was isolated by centrifugation. The supernatant from the above was saved for subsequent work. Component I was then washed with 3 ml portions of ethyl alcohol and with diethyl ether, collected by centrifugation, and dried in a desiccator. The yield of Component I was 0.05 g. This sample was redissolved in 3 ml of 10% solution of trichloracetic acid and the fractionation with alcohol was repeated. The yield of Component I after the second precipitation was 0.04 g. The supernatant from the first precipitation was stirred with 4 additional volumes of ethyl alcohol and the mixture was stored at 4°C for 18 hours. A second amorphous precipitate (Component II) appeared and this was collected by centrifugation, washed with ethyl alcohol and diethyl ether, and dried in a desiccator. The yield of Component II was 0.07 g. The sample was also subjected to a second fractionation with alcohol and the final yield of Component II was 0.06 g.

Whereas the crude mixture of glycans yielded two distinct components in the double diffusion agar test, the purified glycans (Components I and II) yielded single bands with the homologous antiserum. It should be mentioned that on the basis of intensities of the bands (Fig. 1), it appears that Component I is a more potent antigen than Component II. Quantitative data on the monomeric constituents in the once and the twice precipitated samples were within experimental error. Thus Component I was found to consist only of glucose and galactose in the ratio of 1:1. Component II was found to consist of N-acetylgalactosamine, galactose, rhamnose, and glucose in the molar ratio of 1:1:2:4. Whereas Component I contained no phosphorus, Component II did contain approximately 2% organic phosphorus. This phosphorus could not be removed by dialysis, reprecipitation, or filtration through Bio-Gel, and it is evidently an integral structural element of Component II.

Components I and II differed also in their sedimentation...
**TABLE I**

*Inhibition of quantitative precipitin reaction between diheteroglycan and homologous antiserum with carbohydrate inhibitors*

| Inhibitor              | Structure         | Concentration (mg/ml) | Percentage inhibition (%) |
|------------------------|-------------------|-----------------------|---------------------------|
| D-Galactose            | gal-(β-1,4)-glc  | 10                    | 38                        |
| D-Glucose              |                   | 10                    |                           |
| p-Nitrophenyl-β-D-galactoside |        | 10                    | 50                        |
| p-Nitrophenyl-α-D-galactoside |            | 10                    | 15                        |
| Lactose                | gal-(β-1,4)-glc  | 0.5                   | 91                        |
| Allo-lactose           | gal-(β-1,6)-glc  | 0.5                   | 49                        |
| Melibiose              | gal-(α-1,6)-glc  | 10                    | 30                        |
| Digalactose            | gal-(β-1,6)-gal  | 10                    | 35                        |
| Cellobiose             | glc-(β-1,4)-glc | 10                    | 32                        |
| Maltose                |                   | 10                    |                           |
| Gentiobiose            |                  | 10                    |                           |
| Isomaltose             | glc-(α-1,6)-glc | 10                    |                           |

Fig. 3. Inhibition of the precipitin reaction of the diheteroglycan and homologous antiserum by lactose and allo-lactose.

rates on density gradient centrifugation. These results are shown in Fig. 2. The top frame of this figure represents the sedimentation rate of inulin which has a molecular weight of 5,000 to 6,000. Component II as shown in the middle frame sediments at the same rate as inulin and therefore its molecular weight is also 5,000 to 6,000. However, Component I, shown in the bottom frame, sediments at a faster rate than inulin and Component II. By use of empirical relationships developed for proteins (29) it is estimated that molecular weight of this component is 15,000 to 16,000.

**Characterization of Type-Specific Determinant of Component I—**

In an attempt to determine the immunochemical basis for the antigenic specificity of Component I, galactose, glucose, and a variety of oligosaccharides were tested as potential inhibitors of the precipitation reaction. Some of these data are recorded in Table 1. It will be noted that galactose is a moderate inhibitor of the precipitin reaction between Component I and its antiserum. Approximately 38% of the Component I precipitin reaction was inhibited by 10 mg per ml of galactose. On the other hand, glucose was ineffective as an inhibitor at concentrations of 10 mg per ml of glucose. In an extension of these observations, it is also noted that p-nitrophenyl-β-D-galactopyranoside at a concentration of 10 mg per ml was able to inhibit approximately 50% of the Component I precipitin reaction. However, p-nitrophenyl-α-D-galactopyranoside at the same concentration was able to inhibit only 15% of the Component I precipitin reaction. In view of these findings, it would appear that a β linked galactosyl unit is the terminal and major feature of the antigenic determinant of Component I. This suggestion was substantiated by the fact that lactose, 4-β-D-galactopyranosyl-D-glucose, inhibited approximately 91% of the Component I precipitin reaction at very low concentration (0.5 mg per ml). Other disaccharides were much less effective and with the exception of allo-lactose (6-β-D-galactopyranosyl-D-glucose), high concentrations of the oligosaccharides were required in order to obtain a measurable inhibition. To determine the immunochemical importance of the β-(1,4)- and β-(1,6)-galactosidic linkages, quantitative inhibition studies were performed which employed varying concentration of lactose and allo-lactose. These results are illustrated in Fig. 3. It is noted that lactose at a concentration as low as 100 μg per ml inhibited nearly 90% of the precipitin reaction between Component I and homologous antiserum. Allo-lactose at the same concentration inhibited less than 15% of the same precipitin reaction. These results establish that the crucial chemical feature of the antigenic site of Component I consists of a β-galactosyl-(1,4)-glucosyl moiety. Such a moiety is apparently a frequent structural unit of the glycan. The inhibition which
was observed with allolactose is probably attributable to the β linked galactose units linked to glucose residues which in some respects, approximates the structure of a lactosyl moiety.

Determination of Terminal Sugars of Component I—Immunological analysis focused attention on the possibility that β-D-galactosyl residues occupy terminal positions on a main chain of glucosyl units of Component I. Upon mild hydrolysis of Component I in 0.1 N HCl at 100° for varying time intervals, monosaccharides and oligosaccharides were released from Component I at varying rates. Results of the chromatographic analyses of such hydrolysates are shown in Fig. 4. It should be noted that galactose was the first hydrolytic product liberated from the glycan. On further hydrolysis, glucose appeared as well as a series of oligosaccharides in the di-, tri-, and tetrasaccharide range. On prolonged hydrolysis, other disaccharide fragments also appeared. These results further document the suggestion that galactose residues occupy a terminal position in the glycan.

Structural Analysis of Component I—In order to obtain information on the detailed structure of Component I, oligosaccharides were isolated from the partial acid hydrolysates of Component I and their structures were examined. As illustrated in Fig. 5, several oligosaccharides are separable in acid hydrolysates of Component I by the paper chromatographic methods based on the multiple ascent technique (9). These oligosaccharides have been designated A1, A2, A3, B, and C with Fragments A corresponding to disaccharides, Fragment B to trisaccharides, and Fragment C to tetrasaccharides. Further, it is noted that Fragment A1 has a RF value identical with that for lactose and gentiobiose. Since acid hydrolysis of Fraction A2 yielded glucose and galactose in the molar ratio of 3:1, it would seem that this component may be a mixture of the two disaccharides. On hydrolysis of A3 with β-galactosidase, glucose and galactose were detectable, but on hydrolysis with a β-glucosidase only glucose was obtained. The latter observations are consistent with the suggestion that Fraction A3 is a mixture of lactose and gentiobiose. Efforts are being continued to find methods for separating these two disaccharides and for characterizing the compounds structurally. Disaccharides A1 and A3 appeared in much lower concentration than A2. Results of the enzymatic hydrolysis of these fragments indicate that these disaccharides are cellobiose and allolactose, respectively.

Fragment B on complete hydrolysis yielded glucose and galactose in the ratio of 2:1. Chromatographic analysis of partial acid hydrolysates showed the presence of disaccharides of lactose and gentiobiose types in addition to glucose and galactose in such hydrolysates. On the basis of the RF values for Fragment B and the nature of the hydrolytic products from this material, it is likely that Fragment B represents a mixture of isomeric trisaccharides composed of 2 residues of glucose and 1 residue of galactose. These residues are linked by β-D-1,6-glucosidic and β-D-1,4-galactosidic linkages. In light of these observations, a schematic diagram of a structural unit of the diheteroglycan is illustrated in Fig. 6. Further work is in progress on the elucidation of complete structure of this antigen of a group D Streptococci.}

**DISCUSSION**

The complexity of the cell wall carbohydrates of group D Streptococci (5) is further exemplified by our observation that the cell wall of S. faecalis, contains two distinct antigenic glycans; a diheteroglycan of glucose and galactose and a tetraheteroglycan of rhamnose, glucose, galactose, and N-acetyl-galactosamine. The glycans were extracted from the cell wall by a trichloroacetic...
acid procedure and were separated by fractional precipitation with ethyl alcohol. Criteria for purity of the two glycans included constancy in composition on reprecipitation, immunodouble diffusion patterns, and density gradient sedimentation rates. From the sedimentation data, a molecular weight of the diheteroglycan was calculated to be 15,000 to 16,000. This value is several-fold greater than the value of 5,000 to 6,000 for the tetraheteroglycan. Whereas the average degree of polymerization of the tetraheteroglycan is around 30 residues, the value for the diheteroglycan is in the neighborhood of 100 residues. This difference in molecular size may account for the differences in solubilities of the glycans in alcohol-water mixtures and for the difference in the antigenic potencies of the two glycans.

Structural information has thus far been obtained only for the diheteroglycan. This information is derived from identification of hydrolytic fragments from the polymer and from immunological studies. On complete acid hydrolysis, the glycan was converted to glucose and galactose in equivalent molar concentrations. On graded acid hydrolysis, galactose appeared as the initial low molecular weight product, while glucose and low molecular weight oligosaccharides were produced subsequently (Fig. 4). The disaccharides from the glycan have been identified to be lactose, gentiobiose, celllobiose, and allolactose. The trisaccharides and the tetrasaccharides from the glycan were also shown to be composed of glucose and galactose and to contain some of the disaccharide moieties indicated above.

It should be noted in Fig. 5 that gentiotriose and gentiotetraose were not detectable in acid hydrolysates of the glycan and hence such units are not structural components of the polymer. Also, of importance is the observation that glucose or fructose trisaccharides or tetrasaccharides hydrolyzable by β-glucosidase or α-glucosidase were not detectable in hydrolysates of the glycan by the two-dimensional "oligosaccharide-mapping" technique (27). This finding rules out cellobiose, cellotetraose, and α-linked glucosyl oligosaccharides as structural units of the glycan. Since β-glucosidic linkages are most stable of the glycosidic linkages to acid hydrolysis (28), it would be expected that fragments with these linkages would accumulate if they were structural elements of the native glycan.

In view of the foregoing and the immunological results presented in the preceding section, it is concluded that the main chain of the diheteroglycan is a mixed chain of glucosyl and galactosyl residues with no more than two glucosyl units being contiguous. These glucosyl units are linked by β-(1,6) linkages to form gentiobiose units which are, in turn, linked to galactosyl residues by β-(1,6) linkages. Branch units of galactosyl residues linked by β-(1,4) linkages to glucosyl units of the main chain are numerous. A few glucosyl residues are also linked to glucosyl units of the main chain by β-(1,4) linkages. Such a structural unit for the diheteroglycan is shown in Fig. 6. The proposed structure for the new glycan is consistent with the idea that terminal units attached to a main chain of an antigen are the dominant immunogens of antigenic cell wall glycans (29, 30). Methylation and enzyme degradation studies are in progress on the determination of the sequence of monosaccharide residues and the identity of all glycosidic linkages in this antigenic glycan.

The results presented in this study emphasize the view that a direct relationship exists between the structural features of cell wall carbohydrates and antigenic specificity. Further, the finding of a lactosyl unit as an immunodominant group of the antigenic glycan from S. faecalis may be related to the type of infection elicited by the organism in the host. Infections caused by group D Streptococci are more common than once suspected and women in the latter stages of pregnancy frequently develop urinary infections caused by these organisms. In view of the high degree of antibiotic resistance which these organisms frequently exhibit and the observation that lactose is a potent inhibitor of the precipitin reaction of the cell wall glycan from this organism, successful means for active immunization would be a major advance in the control of group D Streptococcus infections. However, before active immunization can be attempted, it is necessary to examine, in detail, the antigenic mosaic of these organisms.

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Glycans from Streptococcal Cell Walls: IMMUNOLOGICAL AND CHEMICAL PROPERTIES OF A NEW DIHETEROGLYCAN FROM STREPTOCOCCUS FAECALIS

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