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DETERMINATION OF THE SUGAR SEQUENCES AND THE GLYCOSIDIC-BOND ARRANGEMENTS OF IMMUNOGENIC HETEROGLYCANS

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ABSTRACT

The complete sugar sequences and glycosidic-bond arrangements have been determined by a combined analytical scheme for a diheteroglycan of D-glucose and D-galactose and a tetraheteroglycan of 6-deoxy-L-talose, L-rhamnose, D-galactose, and D-glucuronic acid. The analytical scheme included methylation analysis by g.l.c. and mass spectrometry, periodate oxidation followed by borohydride reduction and identification of the residual fragments, enzymic hydrolysis followed by characterization of the modified glycan, and chemical degradation followed by characterization of the resulting fragments. The diheteroglycan and the tetraheteroglycan are immunogenic substances on the cell surface of the organisms, and are the group-specific carbohydrates in the cell walls of *Streptococcus faecalis*, strain N, and *Streptococcus bovis*, strain C3. The combined analytical scheme should be of general applicability for the structural analysis of heteroglycans for which selective-degradation procedures can be devised.

INTRODUCTION

In studies on immunogenic, streptococcal heteroglycans[1,2] it was necessary to determine the sugar sequence of the glycans in order to permit correlations of chemical structure with antibody specificity[3]. Heidelberger and Avery[4] discovered that the sugar sequence and the nature of the sugar moieties of carbohydrate antigens specifies the types of antibodies that will be induced in a host. Subsequently, a scheme for the serological classification and identification of pathogenic microorganisms was developed[5], and this scheme is still very valuable. In the structural studies being reported, a combination of analytical methods, namely methylation analysis, periodate oxidation, enzymic hydrolysis, and chemical degradation has been integrated into a scheme for the determination of the complete sugar-sequence and glycosidic-bond arrangement of heteroglycans. Recently, a similar but less complete approach has been used for the elucidation of the structure of a pneumococcal capsular glycan[6]. The microtechniques of combined g.l.c. and mass spectrometry[7] have been employed

for the identification and the characterization of the methyl derivative from the native, enzymically treated, and chemically modified glycans, and from oligosaccharides derived from the glycan. The application of the method is described for determination of the sugar sequences and glycosidic-bond arrangements of two group-specific, cell–wall carbohydrates, a diheteroglycan of D-glucose and D-galactose from *Streptococcus faecalis*, strain N[8], and a tetraheteroglycan[9] of 6-deoxy-L-talose, L-rhamnose, D-galactose, and D-glucuronic acid from *Streptococcus bovis*, strain C3.

RESULTS AND DISCUSSION

A diheteroglycan of D-glucose and D-galactose from the cell wall of *Streptococcus faecalis*, strain N, is the only known natural polymer that contains numerous lactosyl moieties attached as side chains to the main chain of the glycan. Of biological significance is the fact that the glycan is immunogenic and induces the synthesis of a unique spectrum of antibodies in animals immunized with a vaccine of non-viable *S. faecalis* cells having the glycan *in situ* in the cell wall. These antibodies have been separated by affinity-chromatography methods[3] into two isoantibody sets, anti-galactose isoantibodies and anti-lactose isoantibodies. Many facets of the genetic regulation of the synthesis of such sets of isoantibodies should be investigated.

A tentative structure, comprising a main chain of eighteen repetitions of a glucose–glucose–galactose trisaccharide having a lactosyl, or occasionally cellobiosyl, side chain attached to the central glucose residue, has been proposed earlier for this glycan[1]. On the basis of methylation data alone, it was not possible to establish the exact sequence of monosaccharide residues or the types and arrangement of the glycosidic linkages[1]. New structural information has now been obtained by methylation analysis on the native and enzymically modified glycan, on the oligosaccharide fragment resistant to periodate oxidation, and on acetolysis products. The quantitative methylation data on the native and enzymically modified glycan are recorded in Table I. The enzymic modification was effected with an almond β-D-galactosidase that had been shown earlier to remove terminal D-galactosyl groups from the glycan[1] and, in the present experiments, was used to remove over 70% of the terminal D-galactosyl groups. It may be noted in Table I that the decrease in 2,3,4,6-tetra-*O*-methyl-D-galactose in the enzymically modified glycan was balanced by an increase in 2,3,4,5-tetra-*O*-methyl-D-glucose. As the latter derivative arises from internal glucose residues in the native glycan, a corresponding decrease in 2,3,6-tri-*O*-methyl-D-glucose also occurred. The values for 2,3-di-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-galactose remained the same for both samples.

Oxidation of the diheteroglycan with periodate occurred in a unique manner. Fig. 1 shows the results of the periodate-oxidation experiment. It may be noted that, in acid hydrolyzates of the periodate-oxidized and borohydride-reduced glycan, D-galactose was barely detectable, whereas in the hydrolyzate of the native glycan, this sugar was present in high proportion. In contrast, D-glucose was present in high proportion in both hydrolyzates. Apparently, the glucose residues were not oxidized

TABLE I

MOLES OF METHYLATED MONOSACCHARIDES PER MOLE OF GLYCAN IN ACID HYDROLYZATES OF NATIVE AND ENZYMICALLY MODIFIED DIHETEROGLYCAN

Compound	*Native*	*Modified*	*Difference*
2,3,4,6-Tetra-*O*-methylglucose	4.0	15.9	+11.9
2,3,4,6-Tetra-*O*-methylgalactose	17.5	5.0	−12.5
2,3,6-Tri-*O*-methylgalactose	17.6	17.8	+ 0.2
2,3,6-Tri-*O*-methylglucose	35.0	21.9	−13.1
2,3-Di-*O*-methylglucose	17.1	16.8	− 0.3

by periodate, even though they possessed unsubstituted glycol groups. The protection of the glucose residues against periodate oxidation is due to formation of hemiacetal bonds[10], and evidence for the formation of such bonds during oxidation of this glycan has been presented[11].

In the hydrolyzate of the oxidized and reduced glycan, glycerol and D-threitol, the oxidation products expected from terminal and internal galactose residues, were present. Furthermore, a series of oligosaccharides of various degrees of polymerization were present in the acid hydrolyzate of the native glycan, but only oligosaccharides of the disaccharide and trisaccharide types were present in the hydrolyzate of the oxidized and reduced glycan. These results are consistent with a structure proposed earlier[1] for the glycan, having a repeating unit of glucose–glucose–galactose residues

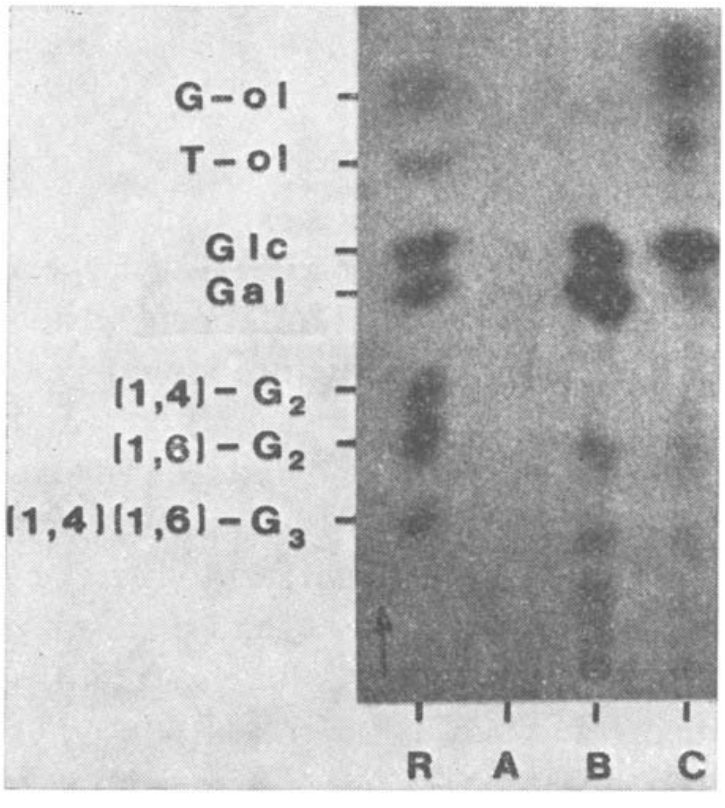


Fig. 1. Paper chromatogram of hydrolyzates of the diheteroglycan and reference compounds: R, reference compounds; A, blank of diheteroglycan; B, hydrolyzate of native glycan, and C, hydrolyzate of glycan oxidized by periodate and reduced by borohydride. G-ol = glycerol; T-ol = D-threitol; Glc = D-glucose; Gal = D-galactose; (1,4)-G_2 = cellobiose; and (1,6)-G_2 = gentiobiose; (1,4)(1,6)-G_3 = α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 4)-D-glucose.

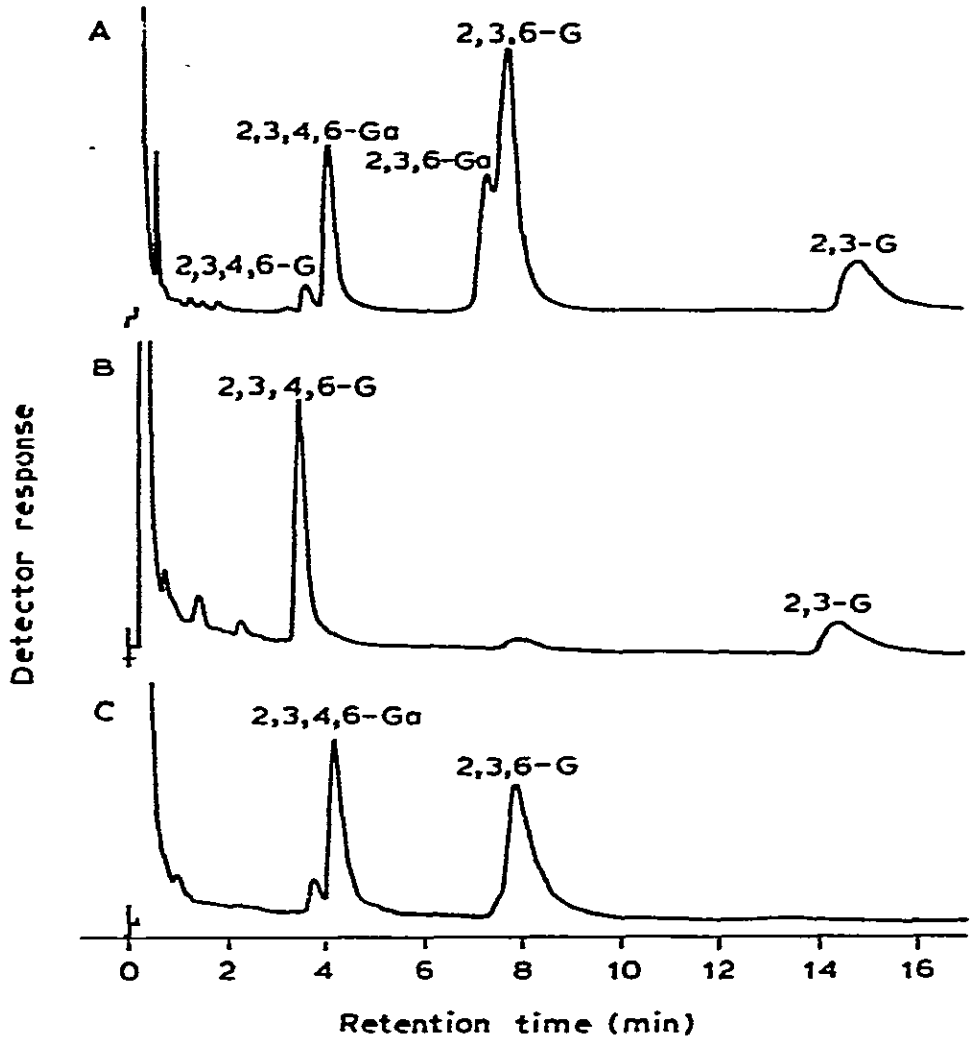


Fig. 2. A photograph of the g.l.c. patterns for the methylated alditol acetates from the native diheteroglycan (A), the oligosaccharide from the periodate-oxidized and borohydride-reduced glycan (B), and the oligosaccharide from the acetolysis of the glycan (C). 2,3,4,6-G = 1,5-di-*O*-acetyl-2,3,4,6-tri-*O*-methylglucitol, 2,3,4,6-Ga = 1,5-di-*O*-acetyl-2,3,4,6-tri-*O*-methylgalactitol, 2,3,6-Ga = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, 2,3,6-G = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, and 2,3-G = 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol.

with a lactosyl side-chain on the central glucose residue, and the present finding that the galactose but not the glucose residues were oxidized by periodate. The largest oligosaccharide that would remain intact after periodate oxidation, borohydride reduction, and mild acid hydrolysis of a glycan having such a structure would be a trisaccharide of D-glucose.

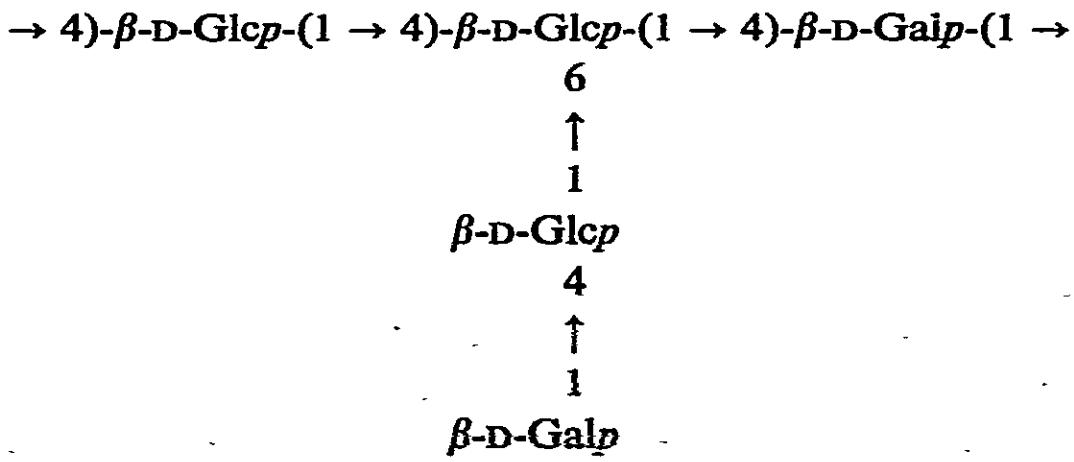
The trisaccharide in the acid hydrolyzate of the oxidized and reduced glycan was isolated by preparative, paper chromatography. Methylation analysis was performed on this compound and the g.l.c. patterns for the methylated alditol acetates from the compound are shown in Fig. 2, frame B. Frame A of the figure shows a g.l.c. pattern for the methylation analysis of the native glycan, whereas frame C shows the g.l.c. pattern for an acetolysis fragment obtained as described later. The major methylated derivatives from the trisaccharide (frame B) were 2,3,4,6-tetra-*O*-methylglucose and 2,3-di-*O*-methylglucose in a molar ratio of 2:1. A small proportion of tri-*O*-methylglucose was detectable in the pattern, but the origin of this derivative is, at present, unknown. These periodate-oxidation results are in harmony with the sugar sequence previously proposed for the diheteroglycan, namely a chain having

glucose–glucose–galactose repeating units with a lactose side chain on the central glucose residue. It should be pointed out that, if the lactose side-chain were on the other glucose residue of the repeating unit, then the oligosaccharide from the periodate-oxidized glycan would not be branched, and could not yield 2,3-di-*O*-methylglucose.

New structural data on the acetolysis products from the glycan have also been obtained. Under mild acetolysis conditions for short periods, the major products obtained from the glycan were a disaccharide, which was isolated by preparative, paper chromatography and a modified glycan isolated from the origin of the chromatogram. D-Galactose and D-glucose were also detectable in the acetolysis mixture, indicating some non-specific cleavage of terminal residues of the glycan. Methylation analysis of the disaccharide from the acetolysis mixture yielded 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylglucose, identified as the alditol acetate in Fig. 2 (frame C) and a trace of 2,3,4,6-tetra-*O*-methylglucose, the latter probably arising from a contaminant in the preparation. As the compound was hydrolyzed by a β-D-galactosidase, and in view of the foregoing results, the structure of the oligosaccharide from the acetolysis mixture is established to be β-D-galactopyranosyl-(1 → 4)-D-glucopyranose.

The major methylation products from the acetolysis fragment isolated from the origin of the chromatogram were 2,3,6-tri-*O*-methylglucose and 2,3,6-tri-*O*-methylgalactose in about a 2:1 molar ratio and comprising >80% of the methylated products. The other products, amounting to <20%, were 2,3,4,6-tetra-*O*-methylglucose, 2,3-di-*O*-methylglucose, and 2,3,4,6-tetra-*O*-methylgalactose. These new acetolysis results, together with the methylation data on the native and enzymically modified glycan and the periodate-oxidation results, establish that the monosaccharide residues of the main chain of the glycan are joined by (1 → 4)-glycosidic linkages and that the lactosyl side-chains are (1 → 6)-linked to the second glucose residue of the repeating unit. In view of the susceptibility of the linkages of the native glycan and numerous oligosaccharides from the glycan to hydrolysis by β-D-galactosidase and β-D-glucosidase[1], the configuration of all of the glycosidic linkages in the native glycan is evidently β-D.

On the basis of the foregoing results, it is now possible to write a complete sugar sequence for the diheteroglycan. The arrangement of the residues and types of glycosidic linkages for a repeating unit of this glycan are shown in the accompanying formula.



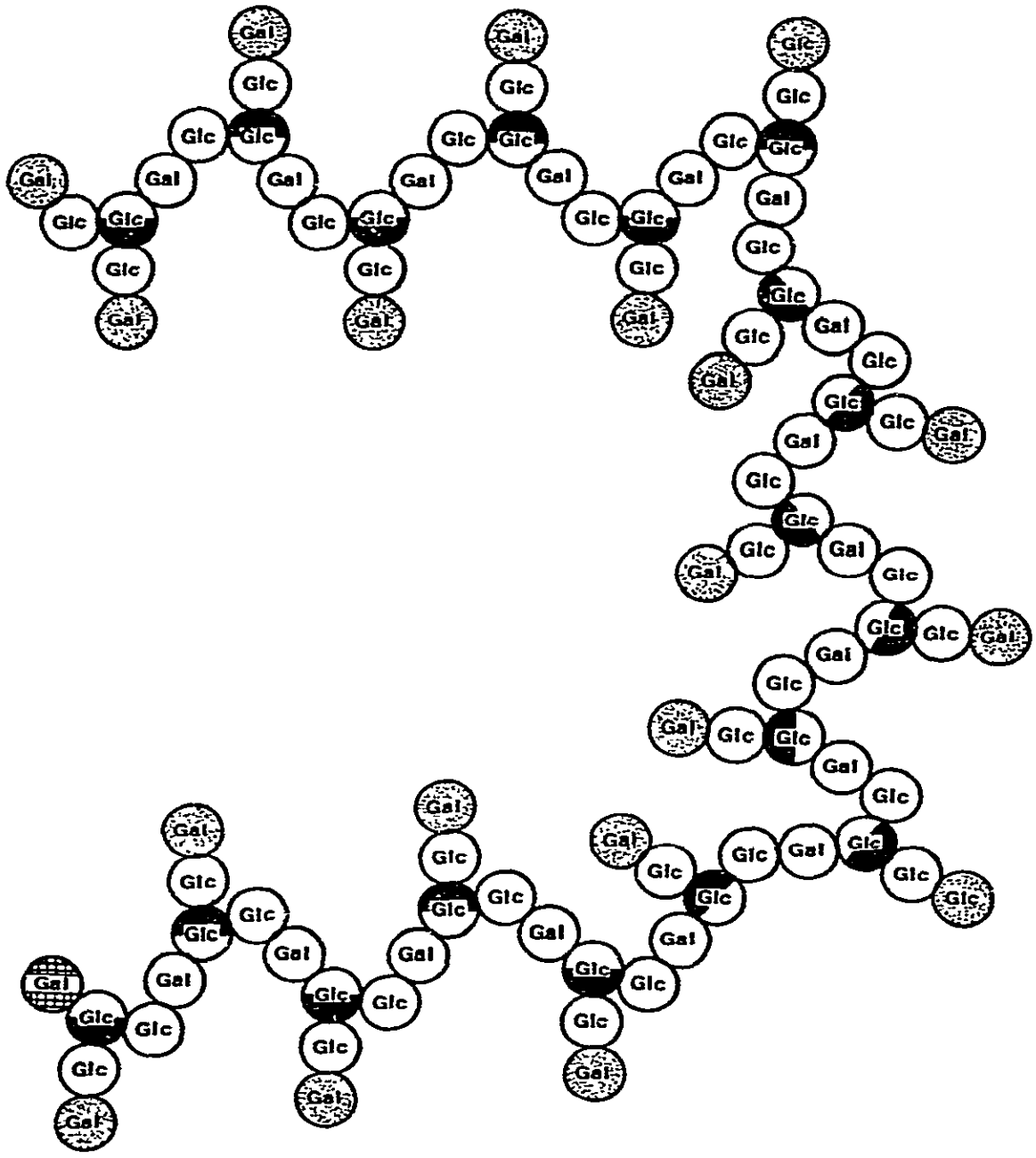


Fig. 3. Diagrammatic representation of the structure of a typical molecule of the diheteroglycan: dotted circles indicate unsubstituted residues, open circles indicate residues substituted at carbon 4, open and dark half-circles indicate residues substituted at carbon 4 (open half) and carbon 6 (dark half); the checked circle indicates the residue having the reducing group and a substituent at O-4.

A typical molecule of the glycan contains eighteen such repeating units, with a molecular weight 14,760, a value in agreement with the value obtained by density-gradient centrifugation[8]. The complete sugar-sequence for the diheteroglycan is shown diagrammatically in Fig. 3. This figure constitutes a correction of the one previously published[1], in that monosaccharide residues of the main chain are all linked by α-D-(1 → 4) bonds, and the side chains are attached to the main chain by α-D-(1 → 6) bonds.

The second glycan for which the complete sugar sequence has been elucidated by the combined analytical scheme is a tetraheteroglycan from *Streptococcus bovis*, strain C3. This glycan is composed of the rare sugar 6-deoxy-L-talose, and of L-rhamnose, D-galactose, and D-glucuronic acid, with the D-glucuronic acid moieties functioning as the immunodeterminant groups[2]. The sugar sequence, a main chain of 6-deoxy-L-talose-(1 → 3)-L-rhamnose-(1 → 3)-D-galactose-(1 → 2)-L-rhamnose

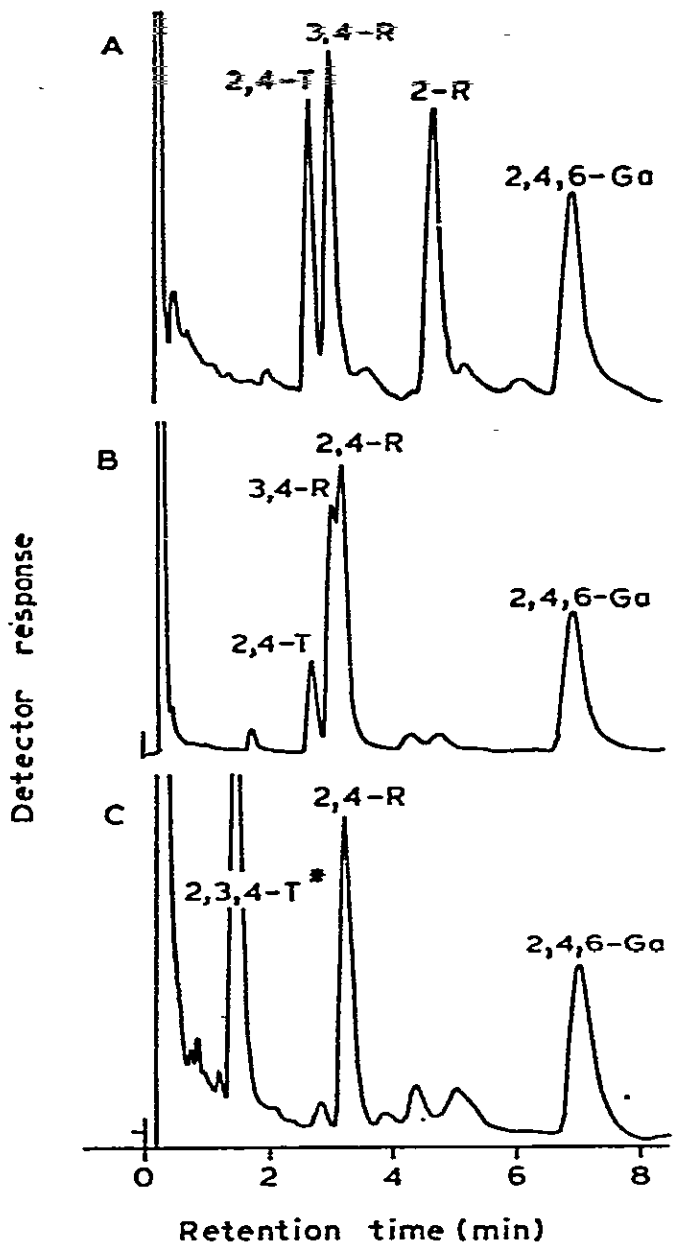


Fig. 4. A photograph of g.l.c. patterns for the methylated alditol acetates from the native tetraheteroglycan (A), the glycan fragment after removal of the D-glucuronic acid residues (B), and the oligosaccharide from the periodate-oxidized and borohydride-reduced glycan (C). 2,4-T = 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-6-deoxytalitol; 3,4-R = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol; 2-R = 1,3,4,5-tetra-*O*-acetyl-2-*O*-methylrhamnitol; 2,4,6-Ga = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; 2,4-R = 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol; and 2,3,4-T* = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-6-deoxytalitol and an acetyl and methyl derivative of a three-carbon product from periodate oxidation and borohydride reduction of the glycan.

repeating units joined by (1 → 3) linkages with D-glucuronic acid residues linked to position 4 of the first rhamnose residue, has been proposed recently for this glycan[2]. This structure has now been shown to be correct by the combined analytical scheme. Fig. 4 shows g.l.c. patterns for methylation analyses of the native glycan (frame A), a glycan fragment from which the D-glucuronic acid residues have been removed by the β-elimination reaction (frame B), and an oligosaccharide isolated from a mild-acid hydrolyzate of the periodate-oxidized and borohydride-reduced glycan (frame C).

It may be noted in frame B that the residual glycan fragment, after β-elimination, no longer yielded 2-*O*-methylrhamnose as did the native glycan (frame A), but instead yielded 2,4-di-*O*-methylrhamnose. This result shows that the D-glucuronic acid was originally linked to position 4 of a rhamnose residue that was also substituted

at position 3. It may be noted in frame C that the oligosaccharide isolated from a mild-acid hydrolyzate of the periodate-oxidized and borohydride-reduced glycan consisted of 6-deoxytalose, rhamnose, and galactose. The products of the methylation analysis of this oligosaccharide were 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-6-deoxytalitol, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol, and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol. The oligosaccharide, on reduction with sodium borohydride, hydrolysis in acid, and acetylation yielded hexa-*O*-acetylgalactitol and a mixture of α and β pentaacetates of L-rhamnose and 6-deoxy-L-talose. These results discussed here establish the structure for the repeating unit of the tetraheteroglycan as that shown in the accompanying formula.

$$\rightarrow 3)\text{-6-deoxy-L-Tal}p\text{-}(1 \rightarrow 3)\text{-L-Rha}p\text{-}(1 \rightarrow 3)\text{-D-Gal}p\text{-}(1 \rightarrow 2)\text{-L-Rha}p\text{-}(1 \rightarrow$$

$$\begin{array}{c} 4 \\ \uparrow \\ 1 \end{array}$$

$$\text{D-GlcA}p$$

Considering a repeating unit having this structure, the D-glucuronic acid and the second L-rhamnose residue were oxidized by periodate and the resulting aldehyde groups were reduced by borohydride. On removal of the reduction fragments by mild-acid hydrolysis, a residual trisaccharide of 6-deoxy-L-talose, L-rhamnose, and D-galactose was obtained. The trisaccharide from such a reaction mixture yielded the methylation products shown in Fig. 4, frame C. As hexa-*O*-acetylgalactitol was identified as the alditol in a hydrolyzate of the reduced oligosaccharide, the sequence of residues in the oligosaccharide is, therefore, 6-deoxy-L-talose, L-rhamnose, and D-galactose. The molecular weight of the tetraheteroglycan was determined earlier[2] to be 6000. Therefore, a typical molecule of the glycan is composed of eight repeating units of the type shown in the diagram.

EXPERIMENTAL

Heteroglycans. — The diheteroglycan of D-glucose and D-galactose was extracted from the cell wall of *Streptococcus faecalis*, strain N with 5% trichloracetic acid at 4° and purified by alcohol fractionation and Bio-gel filtration as previously described[8]. The tetraheteroglycan of 6-deoxy-L-talose, L-rhamnose, D-galactose, and D-glucuronic acid was extracted from the wall of *Streptococcus bovis*, strain C3 with 0.05M potassium chloride and 0.01M hydrochloric acid of pH 2.0 by heating in a boiling-water bath for short periods[9]. Purification of the latter compound was affected by adsorption on DEAE-cellulose and elution with a gradient of sodium chloride in potassium phosphate buffer[2] of pH 7.5. Criteria of purity for both glycans have been presented earlier[1,2].

Methylation. — The methylation of the glycans, modified glycans, and oligosaccharides was performed by the Hakomori method[12] and the analysis was performed by g.l.c.–mass spectrometry as described by Lindberg[7]. The details of the procedure used in our laboratory have been presented in a recent publication[2].

Samples of 0.2–5 mg of the compounds were used for the methylations. The methylated glycans were purified by filtration through Sephadex LH-20. The methylated oligosaccharides were not purified, but were used directly in the reduction and acetylation reactions. The final products, the partially methylated, alditol acetates, were dissolved in chloroform at concentrations suitable for g.l.c.[13]. The chromatographic separations were performed on 3% OV-225 on 80/100 Supelcoport or OS 138 on 100/120 Chromosorb WHP (Supelco, Inc., Bellefonte, PA, 16823) in a Varian Aerograph 1400 gas chromatograph. The mass spectrometry was performed with a Dupont 21–490 mass spectrometer. The chromatographic identifications do not specify chirality of the derivatives; attributions to the D or L series depend on identifications made on the parent monosaccharides[8,9].

Enzymic hydrolysis. — A sample of 60 mg of the diheteroglycan was dissolved in 1.5 ml of water and mixed with 0.75 ml of 4% almond emulsin (Nutritional Biochemicals Co., Cleveland, Ohio) in 0.1M phosphate buffer of pH 6.8. The digest was incubated for 44 h at room temperature and checked periodically by paper chromatography for the liberation of reducing sugars. The qualitative paper chromatograms of the digest revealed that galactose was indeed liberated from the glycan by the enzyme treatment. The enzyme was inactivated and precipitated by addition of an equal volume of 10% trichloracetic acid. The supernatant was dialyzed for 48 h against distilled water and lyophilized to dryness to recover the enzyme-modified glycan. Qualitative capillary precipitin-tests showed that the enzyme-modified glycan still yielded a positive precipitin test with homologous antiserum. The enzyme treatment was repeated two more times as just described. After the third treatment, the yield of enzymically modified glycan was 30 mg, and this preparation gave only a small amount of precipitin complex with the homologous antiserum. The enzyme-modified glycan (2 mg) was subjected to methylation analysis and the products identified by g.l.c. and mass spectrometry. The quantitative values for the products were obtained by integration of the areas on the g.l.c. chart corresponding to the various components. The values for the methylated sugars from the native and enzymically modified glycan are recorded in Table I.

Periodate oxidation. — The diheteroglycan (40 mg) was dissolved in 50 ml of 0.02M sodium periodate (pH 4.5) and maintained in the dark for 18 h at 4°. At this point, the excess of periodate was decomposed by addition of ethylene glycol, materials of low molecular-weight were removed from the mixture by dialysis against distilled water for 48 h, and the oxidized glycan was recovered by lyophilization. The glycan (20 mg) was reduced with sodium borohydride (5 mg) for 24 h at room temperature. The reduced product was purified by dialysis and lyophilization. This oxidized and reduced glycan (10 mg) was dissolved in 1 ml of 0.02M hydrochloric acid and heated for 20 min in a boiling-water bath. A second sample (2 mg) of the modified glycan was dissolved in 0.1 ml of 0.1M hydrochloric acid and heated for 3 h in a boiling-water bath. Analysis of the hydrolyzate for carbohydrates was performed by ascending paper-chromatography in 6:4:3 (v/v) butyl alcohol–pyridine–water. A photograph of the chromatogram of the hydrolyzate in 0.1M acid,

a comparable hydrolyzate of the native glycan, and reference compounds is reproduced in Fig. 1. The glycerol, D-threitol, and D-galactose were also identified by a D-galactose oxidase spray-method[14].

The chromatogram of the 0.02M acid hydrolyzate showed only traces of glucose and galactose, and hydrolytic fragments that migrated on paper in the region of the trisaccharides. The major trisaccharide was isolated by preparative paper-chromatography[15] and subjected to methylation analysis as described in a preceding section. The pattern showing the partially methylated alditol acetates from the oligosaccharide is shown in frame B of Fig. 2. Also shown in Fig. 2 are the patterns for the methylated products from the native glycan (frame A) and an oligosaccharide from an acetolysis mixture (frame C). The *m/e* values for fragments of the methylated alditol acetates obtained from the various compounds were: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol 45 (80), 117 (100), 161 (80), 205 (20); 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol 45 (40), 117 (100), 233 (30); and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol 117 (100), 261 (10). The numbers in parentheses represent relative intensities on a scale of 100 for the most abundant fragment.

The tetraheteroglycan (40 mg) was subjected to periodate oxidation in 20 ml of 0.1M sodium periodate of pH 4.5 for 48 h at 4° in the dark. The oxidized glycan was recovered by dialysis and lyophilization, and subsequently reduced with 10 mg of sodium borohydride. As analysis of the product by the carbazole method[16] revealed some of the glucuronic acid was still unoxidized, the modified glycan was subjected to a second oxidation as already described. Analysis of the resulting product by the carbazole method[16] and the cysteine–sulfuric acid method[17] showed that over 95% of the D-glucuronic acid, and approximately 35% of the 6-deoxyhexose, had been decomposed by the oxidation.

The oxidized and reduced glycan (10 mg) was dissolved in 1 ml of 0.02M hydrochloric acid and heated for 15 min at room temperature. Qualitative paper chromatograms showed that, under the foregoing conditions, the acetal linkages were cleaved but the glycosidic linkages were not hydrolyzed. Two oligosaccharide fragments were detected in the hydrolyzate of the oxidized and reduced glycan, one having R_F 0.32 and the other of 0.23 in 5:1:4 (v/v) butyl alcohol–acetic acid–water (top layer). These oligosaccharides were isolated by preparative paper-chromatography in the foregoing solvent-system. The individual oligosaccharides and the native glycan were subjected to methylation analysis. The g.l.c. patterns obtained for the methylated alditol acetates from the native glycan and the major oligosaccharide (R_F value 0.23) are reproduced in Fig. 3, frames A and C. Also shown in the figure is the pattern obtained for the g.l.c. analysis of the products from the glycan subjected to the β-elimination reaction. The methylated fragments for the compound having R_F 0.32 were comparable to those obtained for the compound having R_F 0.23. It is likely that the former is a glycoside derivative of the latter fragment from which the R group at the reducing end was not removed under the hydrolytic conditions.

The oligosaccharide (R_F 0.23, 1 mg) in 2 ml of water was mixed with 5 mg of sodium borohydride and maintained for 24 h at room temperature. After removal

of the excess of reagent, the sample was evaporated to dryness and hydrolyzed in 2 ml of 0.1M sulfuric acid for 16 h at 100°. The acid was neutralized with barium carbonate and the barium sulfate was removed by filtration. The filtrate was evaporated to dryness and the product was acetylated with acetic anhydride in pyridine. The reagents were removed by evaporation and the acetylated products were dissolved in chloroform for g.l.c. analysis. The products, identified by their retention time on 3% of OV-225 on 80/100 Supelcoport, were hexa-*O*-acetylgalactitol and tetra-*O*-acetyl rhamnose, and tetra-*O*-acetyl-6-deoxytalose.

Acetolysis. — The diheteroglycan (10 mg) was dissolved in 1 ml of acetolysis mixture (10 parts of acetic anhydride, 10 parts of acetic acid, and 1 part of concentrated sulfuric acid) and heated in a stoppered vessel for 3 h at 40°. The deacetylated products from the acetolysis mixture were obtained by following the directions of a published procedure[18]. The deacetylated products were separated and isolated by paper chromatography[15]. On the qualitative chromatograms, traces of glucose and galactose and a relatively high proportion of a disaccharide that migrated on paper at the same R_F value as lactose were detectable. The disaccharide was isolated by preparative chromatography and subjected to methylation analysis. The g.l.c. pattern for the partially methylated alditol acetates from the compound is reproduced in frame C of Fig. 2.

The deacetylated product from the acetolysis mixture at the origin of the preparative chromatogram was extracted with water. A small sample of this material was hydrolyzed in 0.1M hydrochloric acid for 2 h in a boiling-water bath and the reducing sugars, glucose and galactose, were detected in approximately 2:1 ratio in the hydrolyzate by paper-chromatographic methods. The remainder of the sample was subjected to methylation analysis. The major, methylated alditol acetates identified by g.l.c. and mass spectrometry[7] from this fragment were 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol. The foregoing derivatives accounted for approximately 80% of the methylated products. The remaining 20% was composed principally of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol, and a minor proportion of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol.

β-Elimination. — The tetraheteroglycan (12 mg) was subjected to β-elimination reactions essentially by the procedures developed by Lindberg *et al.*[19,20]. The native glycan was first methylated by the Hakomori procedure[12]. Following purification of the methylated product on Sephadex LH-20, the eluate containing the methylated glycan was transferred to a small flask and evaporated to dryness under nitrogen in a water bath at 35°. In order to remove traces of water, the flask was kept frozen at −78° and evacuated for several h. Subsequently, the flask was fitted with a serum cap and flushed with nitrogen. The reagents, which were then added by syringe through the serum cap, were 2 ml of a solution of dry dimethyl sulfoxide and 2,2-dimethoxypropane (19:1 by volume) containing a trace *p*-toluenesulfonic acid, followed by 0.05 ml of freshly prepared methylsulfinyl carbanion. The mixture was sonicated for 30 min and maintained overnight at room temperature. The mixture

was then placed in an ice bath and the reaction stopped by addition of excess 50% acetic acid. Purification of the glycan was effected by pouring the entire mixture into water and extracting the methylated glycan three times with 10-ml portions of chloroform. The combined extracts were washed three times with water and evaporated to dryness. The resulting residue was hydrolyzed in 2 ml of 10% acetic acid in a sealed ampule under nitrogen for 1 h at 100°. The mixture was lyophilized and the methylated glycan purified by gel filtration on Sephadex LH-20. The product was dried and remethylated by the Hakomori method[12]. The remethylated glycan was hydrolyzed and the products converted into their alditol acetates as described in an earlier section. The g.l.c. pattern for the partially methylated alditol acetates from the degraded glycan is shown in frame B of Fig. 4. Frame A of the figure shows the methylated products from the native glycan and frame C, the methylated products from an oligosaccharide isolated from an acid hydrolyzate of a glycan preparation that had been oxidized by periodate and reduced by borohydride. Identification of the individual components was by retention times and mass spectra. The following *m/e* values for fragments of the methylated alditol acetates were obtained, with the figures in parentheses denoting relative intensities on a scale of 100 for the most-abundant fragment: 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-6-deoxyhexitol 117 (100), 131 (60), 233 (20); 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-6-deoxyhexitol 131 (100), 189 (20); 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl-6-deoxyhexitol 117 (100); and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol 45 (30), 117 (100), 161 (50), 233 (20).

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