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GLYCANS FROM STREPTOCOCCAL CELL-WALLS: THE MOLECULAR STRUCTURE OF AN ANTIGENIC DIHETEROGLYCAN OF D-GLUCOSE AND L-RHAMNOSE FROM *Streptococcus bovis**

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ABSTRACT

The monosaccharide sequence and glycosidic bond-types have been determined for an antigenic diheteroglycan of D-glucose and L-rhamnose from the cell wall of *Streptococcus bovis*, strain C3, by use of an integrated analytical scheme based on methylation analysis, periodate oxidation, oxidation with chromium trioxide, enzymic hydrolysis, and chemical degradation. A typical molecule of the glycan consists of a main chain of L-rhamnosyl residues and isomaltose side-chains, with 16 repetitions of the structure, -α-L-rhamnosyl-(1→3)-[α-D-glucosyl-(1→6)-α-D-glucosyl-(1→2)]-α-L-rhamnosyl-(1→2)-α-L-rhamnosyl-, linked alternately by α-L-(1→3) and α-L-(1→2) linkages. The isomaltose side-chains of the glycan are the immunodeterminant groups. The new antigenic glycan is ideally suited for use in the preparation of anti-isomaltose antibodies, which should be of value in the detection of other antigens having isomaltose determinants.

INTRODUCTION

Complex heteroglycans are important antigenic components in the cell wall of many Gram-positive bacteria, being present in most strains of *Streptococcus pneumoniae*[1] and in many strains of Group A, Group C, and Group D *Streptococci*[2]. The identification of organisms of these groups is possible by serological methods based on the use of antisera specific for the heteroglycans[3]. The specificity of antigen–antibody reaction is due to the type of immunodeterminant groups on the antigen and to the nature of the combining site of the antibodies. Recently, a number of antigenic heteroglycans having different types of carbohydrate immunodeterminant groups have been isolated pure in our laboratory from several strains of group D *Streptococci*.

*Dedicated to Dr. Allene R. Jeanes on the occasion of her retirement.

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Included in the list are glycans having the following immunodeterminants: lactose[4], D-galactose[4], D-glucosyl phosphate[5], 2-acetamido-2-deoxy-D-glucosyl phosphate[5], isomaltose[6], and D-glucuronic acid[7]. In other laboratories, heteroglycans having immunodeterminants of 2-acetamido-2-deoxy-D-glucose[8], 2-acetamido-2-deoxy-D-galactose[9], and L-rhamnose[10] have been prepared from members of the Group A and Group C *Streptococci*[11]. The complete molecular structure of some of these glycans has been elucidated by methylation analysis[7,9,12]. Also, antiglycosyl antibodies specific for a single type of carbohydrate immunodeterminant group have been isolated by affinity chromatography from antisera of animals immunized with vaccines of non-viable cells having the glycan *in situ* in the cell walls[13,14].

Determination of the sugar sequence and the glycosidic bond-types of a diheteroglycan of D-glucose and L-rhamnose having isomaltosyl immunodeterminants has now been completed. This glycan is present in the cell wall of *Streptococcus bovis*, strain C3, and has recently been purified to homogeneity by Bio-gel filtration and DEAE-cellulose chromatography[7]. In an earlier report, a tentative structure for this glycan was proposed on the basis of preliminary work[6]. The complete structure of the glycan has now been determined by methylation analysis of the native glycan and of the glycan that had been oxidized by periodate and reduced with borohydride, modified enzymically, and degraded chemically. Hapten-inhibition studies[6] have shown that isomaltose is the only effective inhibitor of the precipitin reaction between the glycan and the antiserum from rabbits immunized with nonviable cells of the organism. Thus the isomaltosyl groups are the major immunodeterminant groups of this antigenic diheteroglycan, and it should be possible to isolate anti-isomaltose antibodies from such antisera by affinity chromatography on isomaltosyl-Sepharose[15]. Anti-isomaltose antibodies would be valuable for comparative studies on antibodies having specificity for dextrans[16,17], pneumococcal type II and type XX glycans[18,19], and myeloma proteins having anti-isomaltose activity[20].

RESULTS AND DISCUSSION

An earlier report[12] presented evidence that antigenic glycans in the cell walls of bacteria are held in the wall matrix by ionic and secondary bond-forces, and can thus be isolated by mild extraction procedures. Extraction of *Streptococcus bovis* (strain C3) cells with dilute potassium chloride–hydrochloric acid solution of pH 2 yielded extracts from which two antigenic glycans have been obtained. These glycans were separated and obtained pure by Bio-gel filtration[6] and by DEAE-cellulose chromatography[7]. One glycan has been shown to be a tetraheteroglycan of 6-deoxy-L-talose, L-rhamnose, D-galactose, and D-glucuronic acid, and its complete structure has been determined[7]. The other one is a diheteroglycan of L-rhamnose and D-glucose, and its structure has now been determined.

Analytical data for the identification and quantitative determination of the hexoses in the glycan were obtained by g.l.c. analysis of the reduced and subsequently acetylated hydrolytic products from the glycan. A typical pattern is reproduced in

Fig. 1. It may be noted that rhamnitol pentaacetate and glucitol hexaacetate were the only products present in the mixture. The ratio of L-rhamnose to D-glucose in the glycan, as calculated from the g.l.c. data, is 3:2 and not 1:1 as previously calculated from data of colorimetric analysis[6]. The 3:2 ratio has been verified by methylation analysis on the native glycan, the results of which are discussed later.

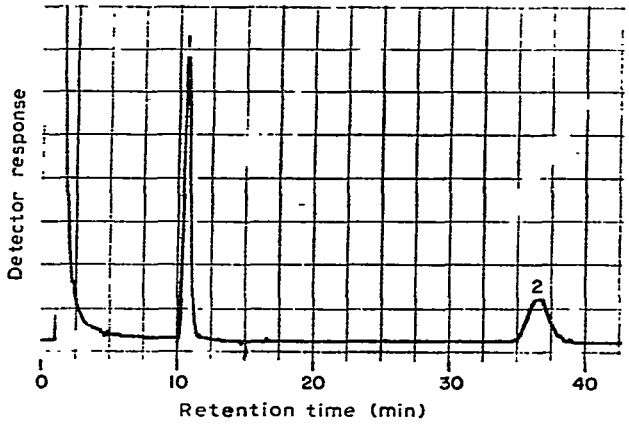


Fig. 1. G.l.c. pattern for the alditol acetates of the reducing sugars in an acid hydrolyzate of the diheteroglycan on an OV-225 column at 190°: 1 = rhamnitol pentaacetate and 2 = glucitol hexaacetate.

In order to determine the total number of residues per mol, it was necessary to determine the molecular weight of the glycan. Such determinations were performed by two different methods. First, Bio-gel filtration of solutions of the *S. bovis* diheteroglycan and a diheteroglycan of D-glucose and D-galactose of known molecular weight from *Streptococcus faecalis*[12] yielded the results shown in Fig. 2. As the *S. bovis* diheteroglycan contains L-rhamnose as a structural unit, the location of the glycan in the fractions from the column was achieved by analysis of the fractions for 6-deoxyhexose by the cysteine–sulfuric acid method[21]. The location of the two glycans was achieved by analysis of the fractions for total hexoses by two colorimetric methods[21,22]. It may be noted in Fig. 2 that the *S. bovis* glycan was eluted from the column after the *S. faecalis* glycan. As the molecular weight of the *S. faecalis* glycan has been determined[12] to be 15,000, the molecular weight of *S. bovis* glycan was calculated from these data to be 12,000. This value was also obtained by density-gradient centrifugation experiments as described in the experimental section. On the basis of the molecular weight and the new ratio for L-rhamnose and D-glucose, it has been calculated that a typical molecule of the glycan consists of 48 rhamnose residues and 32 glucose residues.

A monosaccharide sequence and the glycosidic bond-types of the glycan have been determined by several types of methylation analyses. The g.l.c. pattern for the methylated alditol acetates from the fully methylated, native glycan is reproduced in

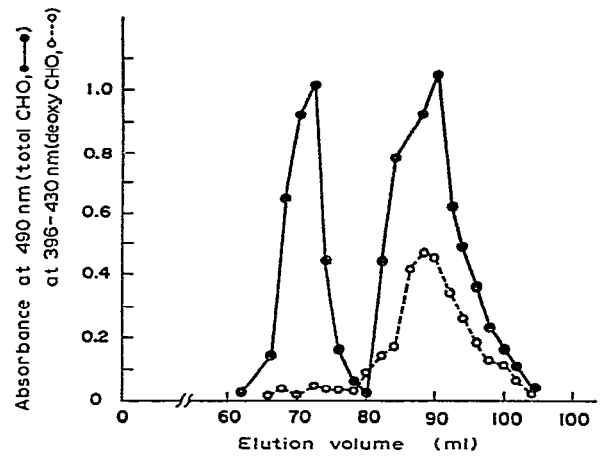


Fig. 2. The concentration of diheteroglycans from *S. faecalis* and *S. bovis* in eluates from a column of Bio-gel P30.

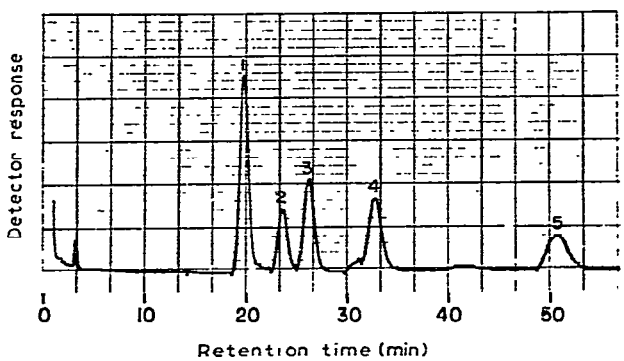


Fig. 3. G.l.c. pattern for the methylated alditol acetates from the methylated diheteroglycan on an OS-138 column at 190°: 1 = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, 2 = 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol, 3 = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 4 = 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylrhamnitol, and 5 = 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol.

Fig. 3. The derivatives were identified by their retention times and mass spectra. Three types of L-rhamnose derivatives, 3,4-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, and 4-*O*-methylrhamnose, and two types of D-glucose derivatives, 2,3,4,6-tetra-*O*-methylglucose and 2,3,4-tri-*O*-methylglucose, were recognized. The ratios of the L-rhamnose derivatives were found to be 3.1:1.0:2.0 and for the D-glucose derivatives the value on the same scale was 2.1:1.9. These ratios, and the molecular weight of the glycan, were used to calculate the number of mol of methylated derivatives per mol of glycan (Table I).

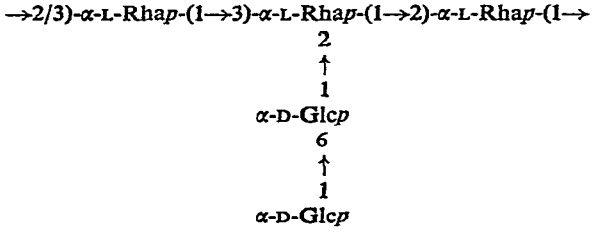
TABLE I

MOLES OF METHYLATED MONOSACCHARIDES PER MOLE OF METHYLATED NATIVE DIHETEROGLYCAN AND ENZYMICALLY MODIFIED DIHETEROGLYCAN

Compound	*Theoretical*[a]	*Native*	*Modified*
2,3,4-Tri-*O*-methylrhamnose	1	1	1
3,4-Di-*O*-methylrhamnose	24	24.8	24.6
2,4-Di-*O*-methylrhamnose	8	8.0	7.6
2,3,4,6-Tetra-*O*-methylglucose	16	16.8	20.2
4-*O*-Methylrhamnose	16	16.0	16.0
2,3,4-Tri-*O*-methylglucose	16	14.8	6.7

[a]On the basis of the proposed structure for the glycan of molecular weight 12,000.

Of the many structures that may be written for the glycan on the basis of the foregoing methylation data, the structure shown in the accompanying formula for a primary unit of the glycan is proposed.



In a typical molecule of the glycan there are 16 such units joined alternately by α-L-(1→3) and α-L-(1→2) linkages. The evidence for the α linkages is discussed in a later section. In addition to the methylation data, three other lines of evidence substantiate such a structure for the glycan.

First, all of the D-glucose residues and one half of the L-rhamnose residues of the native glycan were oxidized by periodate, as would be the case for a molecule having the structure indicated by the formula. Furthermore, Smith degradation[23] of the periodate-oxidized glycan, followed by methylation analysis, yielded 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol, which were identified by g.l.c.–m.s. In the proposed structure, the residues that would yield these derivatives on Smith degradation and methylation analyses are the rhamnose residues at the branch points and those adjacent to the branch points and substituted at position 3.

Second, when the diheteroglycan was treated with a rice α-D-glucosidase, ~50% of the terminal glucose residues were liberated as free D-glucose. Methylation analysis of the enzymic digest yielded data from which the number of residues of the various methylated derivatives could be calculated for the modified glycan (Table I). It may be noted that the values for the L-rhamnose derivatives for the enzyme-treated glycan were, within experimental error, the same as those for the native glycan. However,

the values for the D-glucose derivatives were significantly altered. The amount of the tri-*O*-methylglucose derivative decreased, whereas the amount of tetra-*O*-methylglucose derivative increased. As the α-D-glucosidase removed terminal glucosyl groups from the glycan, the removal of such groups would convert internal glucose residues into terminal residues. As a result, the value for the tetra-*O*-methylglucose derivative would increase by the amount of D-glucose that was liberated, and the value for the tri-*O*-methyl derivative would be decreased by the same amount. Quantitative analysis for the free D-glucose in the mixture showed that the decrease in the value for the tri-*O*-methylglucose was balanced by the amount of free D-glucose that was liberated. As may be noted in Table I, the increase in tetra-*O*-methylglucose was only about half of the expected increase. The latter result is probably due to losses of D-glucose during preparation of the sample for methylation analysis.

Third, acid hydrolysis of the glycan gave an oligosaccharide that migrated on paper chromatography in 6:4:3 (v/v) butyl alcohol–pyridine–water with the same R_F value as isomaltose. Treatment of the oligosaccharide with glucoamylase resulted in the liberation of D-glucose, showing that isomaltose was present in the preparation. However, even prolonged incubation of the preparation with glucoamylase left a compound in the mixture that was not hydrolyzable by the enzyme. Acid hydrolysis of another sample of the oligosaccharide preparation yielded L-rhamnose and D-glucose in about equal amounts. Methylation analysis on the oligosaccharide preparation yielded the following methylated derivatives, 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, and 3,4-di-*O*-methylrhamnose, all of which were identified as their alditol acetates by g.l.c.–m.s. The foregoing findings show that the oligosaccharide preparation is a mixture of two compounds, isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose) and a trisaccharide having the structure glucosyl-(1→2)-rhamnosyl-(1→2)-rhamnose. Apparently, the (1→2) linkage of the glycan is relatively stable to hydrolysis by acids. As recorded in the experimental section, quantitative methylation data are consistent with this interpretation.

The enzymic experiments with rice α-D-glucosidase and glucoamylase (an α-D-glucosidase) show that the glucose residues are α-D linked. That the configuration of the other glycosidic bonds of the glycan is also α was established by chromium trioxide oxidation of the peracetylated glycan, followed by methylation analysis. Thus, it was found that the peracetylated derivative that had been treated with chromium trioxide yielded the same methylated alditol acetates, in approximately the same ratios, as obtained from the native glycan. Such a result is consistent with the α configuration of the glycosidic bonds and an axial orientation of the aglycons on all of the D-glucose and L-rhamnose residues of the glycan. It has been established[24] that carbohydrate residues of glycans having the foregoing orientation are not degraded by the oxidation with chromium trioxide.

It should be pointed out that the structure proposed for the new glycan comprises a main chain of rhamnose residues joined by α-L-(1→2) and α-L-(1→3) linkages, with isomaltose side-chains on every third rhamnose residue. The sequence of the residues is established, but the possibility of a slightly different order of glycosidic

linkages has not been completely ruled out. A main chain of rhamnose residues appears to be a common structural feature of carbohydrate antigens from other groups of *Streptococci*[8,9]. Thus, the group-specific carbohydrates from Group A and Group C *Streptococci* also contain a main chain of rhamnose residues joined by (1→2) and (1→3) linkages. The side chains of the glycan from Group A organisms are 2-acetamido-2-deoxy-D-glucose residues[8], whereas those of the glycan from the group C organism are 2-acetamido-2-deoxy-D-galactosyl-(1→3)-2-acetamido-2-deoxy-D-galactosyl groups[9].

The hapten-inhibition results reported earlier[6] have shown that isomaltose is the most potent low-molecular-weight inhibitor for the precipitin reaction between the glycan and homologous antiserum. Such an antiserum therefore contains anti-isomaltose antibodies. Work is in progress on the isolation of these antibodies by affinity chromatography[15]. The immunodeterminant groups of the glycan are therefore the isomaltosyl groups, which are also the side chains on the main chain of rhamnose residues comprising the glycan. Side chains acting as the immunodeterminant groups of antigenic glycans appear to be a common structural feature of many antigenic glycans in microbial cell-walls.

EXPERIMENTAL

Preparation of the diheteroglycan. — *Streptococcus bovis*, strain C3, was grown on Todd–Hewett media as previously outlined[6]. The cells from 18 liters of a 24-h culture were collected in a Sharpless centrifuge, washed with saline–0.1M phosphate buffer of pH 7, and suspended in 200 ml of 0.05M potassium chloride and 0.01M hydrochloric acid solution (pH 2). Sequential extraction of the carbohydrate components in the cell walls was effected by heating the suspension for short periods of time (5, 10, 30, and 60 min) in a boiling-water bath. After the first heating period, the suspension was centrifuged and the supernatant was saved for subsequent isolation of the solubilized components. The residue was resuspended in 200 ml of fresh KCl–HCl solution and heated for the next time-interval. This process was repeated for the time periods just listed. The 5-min extract contained a component which, on acid hydrolysis, yielded 6-deoxy-L-talose, L-rhamnose, D-galactose, and D-glucuronic acid. The 60-min extract contained a component that yielded primarily D-glucose and L-rhamnose on acid hydrolysis. The other extracts contained a mixture of the two glycans. The glycans have been purified to homogeneity by Bio-gel filtration[6] or by chromatography on DEAE-cellulose[7]. Criteria of purity have been presented earlier[7]; they included immunodiffusion patterns, gel-filtration behavior, and constancy in composition on repeated purification. The yield of purified glycan, obtained from the cells from the 18-liter cultures by 60 min of extraction, was 0.2 g.

Sugar analysis. — Two procedures were employed for the identification and quantitative determination of the monosaccharide constituents of the glycan. In the first, samples (4 mg) of the glycan were dissolved in 0.15 ml of 0.1M hydrochloric acid and heated in a tightly stoppered tube in a boiling-water bath for 2–3 h. Reducing

sugars in the hydrolyzate were separated by paper chromatography in 6:4:3 (v/v) butyl alcohol–pyridine–water and determined quantitatively by colorimetric methods[21,22]. Specific-rotation measurements[25] and the D-glucose oxidase test[26] have shown that the rhamnose is of the L configuration and the glucose of the D configuration.

The second procedure for sugar analysis utilized hydrolysis, reduction, and acetylation followed by g.l.c.[27]. In this procedure, 1–2 mg of glycan in 2 ml of 0.1M sulfuric acid was sealed in an ampule under nitrogen and heated for 16 h at 100°. The tube was then opened and the acid neutralized with barium carbonate. Reduction of the hydrolytic products from the glycan was effected with sodium borohydride (5 mg) and acetylation was performed with dry pyridine and acetic anhydride (1 ml of 1:1 mixture). The alditol acetates of the monosaccharides were separated by gas–liquid chromatography on OV-225 on 80–100 Supelcoport (Supelco, Bellefonte, Pa.) at 190°. Fig. 1 is a photograph of a typical g.l.c. pattern. The identification of the components was by retention times and by co-chromatography with derivatives prepared from L-rhamnose and D-glucose. Quantitative values were obtained by integration of appropriate peaks and the ratio of the L-rhamnose to D-glucose in the glycan was calculated to be 3:2. This ratio was confirmed by the methylation analysis data presented in a later section, and is a correction of the earlier[6] value calculated from data of colorimetric analysis[6].

Molecular weight of the glycan. — To obtain data for molecular weight calculations, Bio-gel filtration of the glycan and of a reference glycan was employed. Samples (3 mg) of the diheteroglycan from *S. bovis* and 3 mg of diheteroglycan from *S. faecalis*[12] were placed on a column (1 × 100 cm) of Bio-gel P-30 (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.). The glycans were eluted with a 0.1M sodium phosphate buffer at pH 7 and 2-ml fractions were collected. The total carbohydrate and the 6-deoxyhexose content of each fraction were determined colorimetrically[21,22]. A plot of the data is shown in Fig. 2. From these results, and a molecular weight value of 15,000 for the *S. faecalis* glycan[12], the molecular weight of the *S. bovis* diheteroglycan was estimated to be 12,000. Density-gradient centrifugation data verified the molecular weight assigned. In the latter experiments, samples (2 mg) of the *S. faecalis* diheteroglycan, 2 mg of *S. bovis* diheteroglycan, and 2 mg of inulin were placed on a density gradient of 5–25% glycerol. The samples were centrifuged at 65,000 r.p.m. in a Spinco Model L65 centrifuge for 14 h. At the end of this time, the columns were fractionated into 0.2-ml samples[28] and each fraction was analyzed for carbohydrate by the orcinol–sulfuric acid method[29]. The maximum concentrations of the glycans were in the following fractions: fraction 9 for the *S. faecalis* glycan, fraction 7 for the *S. bovis* glycan, and fraction 4 for the inulin. By utilizing the empirical relationship for calculating the molecular weight from density-gradient centrifugation data[4,30], the molecular weight of *S. bovis* diheteroglycan was calculated to be 12,000, the same value as that obtained by Bio-gel filtration.

Periodate oxidation. — A sample (7 mg) of the glycan was dissolved in 7 ml of 0.025M sodium periodate. Aliquots (1 ml) were removed after oxidation at room

temperature for 0.5, 2, 12, and 48 h and shaken with an excess of ethylene glycol to decompose the remaining periodate. The individual samples were then dialyzed for 24 h against distilled water and taken to dryness by lyophilization. The residue was dissolved in 1 ml of water and analyzed for D-glucose and L-rhamnose by standard methods[21,22]. The analysis showed that D-glucose was oxidized completely within 2 h, but only 50% of the L-rhamnose was oxidized, even in the 48-h period.

In a second periodate-oxidation experiment, a sample (8 mg) of the diheteroglycan was oxidized in 0.2M sodium periodate for 24 h in the dark at room temperature. The excess of periodate was decomposed with ethylene glycol and the mixture was dialyzed for 48 h. The sample was isolated and subjected to Smith degradation[23]. Reduction was effected in 1% sodium borohydride for 24 h at room temperature. The borohydride was decomposed by addition of acetic acid, and the low-molecular-weight products were removed by dialysis. The oxidized and reduced product was recovered by lyophilization. This product was then subjected to hydrolysis with dilute (0.02M) hydrochloric acid for 30 min in a boiling-water bath. The hydrolyzate was taken to dryness in a vacuum desiccator. The dried residue was subjected to methylation analysis as described in the next section. Two methylated alditol acetates were detected in the hydrolyzate by g.l.c. analyses and were identified by their retention times and mass spectra as 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *m/e* 117 (100), 131 (50), 161 (10), and 175 (5), and 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol, *m/e* 117 (100), 131 (40), and 233 (10). The values in parentheses denote relative abundance, the most abundant fragment being assigned a value of 100. By integration of the peaks on the g.l.c. pattern, the ratio of the two derivatives was found to be 2.9 to 1.0. This ratio is higher than that expected for a glycan having the proposed structure, which would yield these derivatives in 2:1 ratio.

Methylation analysis. — Samples (2–5 mg) of the native and periodate oxidized–borohydride-reduced glycan were subjected to methylation analysis by published procedures[31,32]. An improved separation of the methylated alditol acetates of L-rhamnose was obtained using 5% of OS-138 on 100–120 Chromosorb WHP (Supelco, Bellefonte, Pa.)[7]. A typical separation is shown in Fig. 3. The peaks were identified by retention times and mass-spectral data as: peak 1, 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, *m/e*, 131 (100) and 189 (50); peak 2, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylrhamnitol, *m/e* 117 (100), 131 (40), and 233 (15); peak 3, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *m/e* 45 (40), 117 (100), 161 (40), and 205 (20); peak 4, 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylrhamnitol, *m/e* 131 (100) and 261 (30); and peak 5, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, *m/e* 117 (100), 161 (70), 189 (30), and 233 (10). In some of the g.l.c. patterns, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, which would arise from the terminal rhamnosyl groups of the main chain, was detectable in trace amounts. The various derivatives were quantitated by integration. From the ratios and the molecular weight of the glycan, the number of residues of each derivative per mol of glycan were calculated (Table I).

Oxidation with chromium trioxide. — The diheteroglycan (5 mg) was dissolved in 1 ml of *N,N*-dimethylformamide, and 1 ml of an equal mixture of pyridine and

acetic anhydride was added. This solution was maintained overnight at room temperature. The acetylated glycan was recovered by chromatography on a column (1.5 cm × 15 cm) of Sephadex LH-20, with acetone as the eluant. Fractions containing the glycans were combined and evaporated to dryness under a stream of nitrogen. The dried product was then subjected to oxidation[24] in 0.2 ml of glacial acetic acid by 25 mg of finely powdered chromium trioxide for 1 h at 50° with sonication. The glycan was re-isolated from the mixture by chromatography on a column of Sephadex LH-20 as already described. The chromium trioxide-treated glycan was subjected to methylation, hydrolysis, reduction, acetylation, and then g.l.c. analysis. The g.l.c. pattern obtained on this material contained the same derivatives as those obtained from the native glycan, and in the molar amounts listed in Table I.

Oligosaccharides from acid hydrolyzates of the glycan. — A sample (10 mg) of the glycan was hydrolyzed in 0.5 ml of 0.1M hydrochloric acid for 1.5 h in a boiling-water bath. Paper chromatography of this hydrolyzate in 6:4:3 (v/v) butyl alcohol–pyridine–water showed that L-rhamnose, D-glucose, and an oligosaccharide having the same R_F value as isomaltose were present in highest concentration. The R_F values for one ascent of the solvent for these compounds were 0.54 and 0.32, and 0.14, respectively. The oligosaccharide was isolated by preparative paper-chromatography. Further acid hydrolysis in 2M hydrochloric acid for 1 h at 100° converted the compound completely into L-rhamnose and D-glucose. However, enzymic hydrolysis with glucoamylase[33] gave only D-glucose, and a compound with the same R_F value as the original sample remained.

A sample of the oligosaccharide preparation was then subjected to methylation analysis. The methylated alditol acetates identified by g.l.c.–m.s. were: 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol. The molar ratios of these derivatives were 1.12:1.44:0.90. On the basis of the foregoing results, it was concluded that the oligosaccharide preparation was a mixture of the disaccharide, isomaltose (6-*O*-α-D-glucosyl-α-D-glucose) and a trisaccharide of D-glucose and L-rhamnose having the structure D-glucosyl-(1→2)-L-rhamnosyl-(1→2)-L-rhamnose. In view of the sugar ratios, the yield of tetra-*O*-methylglucose from the trisaccharide would be half that of the rhamnose derivative, or 0.56. This value, when added to the yield of the tetra-*O*-methylglucose derivative from isomaltose (0.90, the same as the value for tri-*O*-methylglucose), gives a value of 1.46, which is in good agreement with the experimentally determined value of 1.44.

Hydrolysis of diheteroglycan with α-D-glucosidase. — Many types of α-D-glucosidases (*A. niger* glucoamylase[33], *Candida* α-D-glucosidase[34], *A. niger* glucosyltransferase[33], glucodextranase[35], and rice α-D-glucosidase, a product of Makor Chemical Ltd., Jerusalem, Israel, and provided by E. J. Hehre, Albert Einstein Medical College, New York, N.Y.) were tested on the glycan with a view to removing terminal glucose groups. Only the rice α-D-glucosidase hydrolyzed the glycan with the liberation of D-glucose. The hydrolysis occurred at a very low rate and, accordingly, a prolonged incubation-period was employed. For methylation

analysis of the enzymic digest, a 3-mg sample of the diheteroglycan was dissolved in 0.3 ml of rice α-D-glucosidase (24 units of total activity, with a unit being the amount of enzyme that hydrolyzes 1 μmol of maltose per min) in 0.2M acetate buffer of pH 5 and was incubated at room temperature for 21 days. At the end of the incubation period, analysis of the digest by paper chromatography in 6:4:3 (v/v) butyl alcohol–pyridine–water showed that free D-glucose was present in the digest. D-Glucose was not present in a blank of the substrate nor in an enzyme solution maintained for 21 days as a control. Quantitative determination of the D-glucose[12] revealed that 0.6 mg of glucose, representing about 50% of the terminal glucose groups, was obtained from 3 mg of the glycan. As tests[22] on a solution of the enzyme showed that the enzyme was devoid of carbohydrate, a methylation analysis was performed on the total enzymic digest. The methylated alditol acetates obtained from this digest were the same as those obtained from the native glycan (Fig. 3). However, the peak for the tri-*O*-methyl-glucose derivative was significantly decreased, whereas the peak for the tetra-*O*-methylglucose derivative was increased. Integration of the peaks on the g.l.c. patterns gave values for the ratio of the derivatives from the enzymically degraded glycan. These values, and the molecular weight of the glycan, were used to calculate the mol of each derivative per mol of modified glycan (Table I).

Serological studies. — The procedures for the preparation of vaccine of non-viable cells of *S. bovis* and the regime for immunizing rabbits have been described earlier[6]. The diheteroglycan *in situ* in the cell wall elicits an immune response leading to the synthesis of antibodies directed against the glycan. Agar-diffusion patterns with the glycan and the antisera have been published[6]. Hapten-inhibition studies with a variety of compounds showed that isomaltose is a strong inhibitor of the precipitin reaction between the glycan and homologous antiserum. D-Glucose, L-rhamnose, maltose, and other derivatives of D-glucose were weak or ineffective inhibitors, even at high concentrations. These results have been interpreted as evidence that the isomaltosyl groups are the immunodeterminant groups of the glycan. Experiments are underway to isolate anti-isomaltose antibodies from the antiserum by affinity chromatography on isomaltosyl-Sepharose[15].

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