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Note

Hemiacetal bond-formation during periodate oxidation of a heteroglycan of D-glucose and D-galactose*

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Recently, periodate oxidation, in conjunction with other methods, has been used in our laboratory to elucidate the complete molecular structure of two immunogenic heteroglycans[1,2]. Of particular note was the observation that a diheteroglycan of D-glucose and D-galactose was oxidized exclusively at the galactose residues, notwithstanding the fact that the glucose residues possessed unsubstituted glycol groups[1]. The glycan was isolated from the cell wall of *Streptococcus faecalis*, strain N and its structure was determined to comprise a main chain of β-D-glucose-(1→6)-β-D-glucose-β-D-(1→4)-galactose repeating-units having a lactose side-chain attached to O-4 of the second glucose residue[1]. A typical molecule of the glycan was shown to consist of eighteen such repeating-units linked by β-D-(1→4) linkages; in a few units, the lactose side-chains were replaced by cellobiosyl groups. When the glycan was subjected to sequential periodate oxidation, borohydride reduction, acid hydrolysis, and paper-chromatographic analysis, it was found that >95% of the galactose residues but <5% of the glucose residues had been oxidized by periodate. Glycerol and threitol, the products expected from the galactose residues, were also identified in the hydrolyzates of the oxidized and subsequently reduced glycan.

Methylation analyses[3] on three types of glycan preparations, (1) the native, (2) the oxidized, and (3) the oxidized and subsequently reduced, have now been performed. Gas–liquid chromatographic patterns, showing the partially methylated alditol acetates from the three samples, are reproduced in Fig. 1. Pattern A shows the derivatives from the native glycan; the major components identified by their retention times and mass spectra are: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*C*-methylglucitol. The amount of the trimethyl ether of glucose was twice as much as the amounts of the other components. The small peak migrating ahead of the tetra-*O*-methylgalactose derivative is the tetra-*O*-

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methylglucose derivative from the few cellobiosyl groups also present in the glycan. Pattern B shows that, after periodate oxidation, borohydride reduction, and methylation analysis, only two components are present, namely 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol, in 2:1 molar ratio. Galactose derivatives are absent from or present only in traces in, this mixture, but some highly volatile methyl derivatives of C_3 and C_4 oxidation-fragments are present. Pattern C shows that, after periodate oxidation of the glycan followed by methylation analysis, several new derivatives are present as well as the foregoing two. Thus, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol are present, but in greatly decreased concentration. The new derivatives include 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylglucitol, 1,2,3,4,5-penta-*O*-acetyl-6-*O*-methylglucitol, and 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylglucitol, with the dimethyl ether being present in very high concentration. A small quantity of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and some minor, as yet unidentified, components are also present.

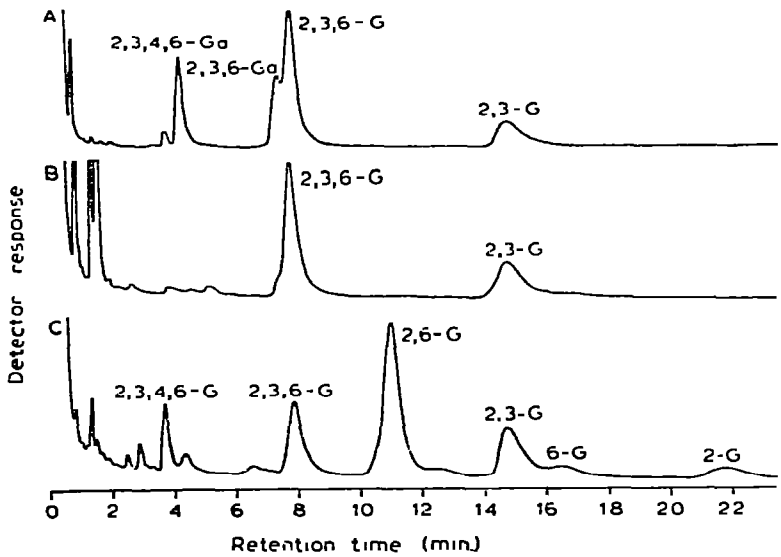

Fig. 1. A photograph of the gas–liquid chromatographic patterns for the partially methylated alditol acetates from the native (A), the periodate-oxidized and borohydride-reduced (B), and the periodate oxidized (C) diheteroglycan: (2,3,4,6-Ga) = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (2,3,4,6-G) = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, (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, (2,3-G) = 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol, (2,6-G) = 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylglucitol, (6-G) = 1,2,3,4,5-penta-*O*-acetyl-6-*O*-methylglucitol, and (2-G) = 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylglucitol.

In view of the structure of the glycan, the presence of the 2,6-di-*O*-methyl and 2-*O*-methyl derivatives of glucose in the foregoing methylation mixture clearly shows

that O-3 of the glucose residue was substituted in the periodate-oxidized glycan. As this substitution did not persist when the aldehyde groups were reduced with borohydride, it is suggested that this is due to hemiacetal bond-formation between the 3-hydroxyl groups of the glucose residues and the aldehyde groups generated by periodate oxidation of galactose residues. Terminal galactose residues contain *cis*-glycol groups, and such residues are oxidized preferentially by periodate[4]. The internal galactose residues of the glycan are substituted at O-4, and such residues are also oxidized by periodate, whereas glucose residues substituted at C-4 are not, even though both types of residue contain a 2,3-*trans* glycol group. The reason for this difference in oxidation rates is not apparent, but the orientation of the substituents at C-4 of such residues, axial in the galactose and equatorial in the glucose, may be the factor responsible. The results of paper-chromatographic analysis show that the galactose residues were indeed completely oxidized, but the glucose residues remained intact during periodate oxidation. The 2,6-dimethyl ether of glucose from the oxidized glycan most probably arises from 4-substituted glucose residues, whereas the 2-methyl ether arises from glucose residues substituted at positions 4 and 6.

The foregoing suggestions are based on previous observations on the periodate-oxidation reaction. Thus, it is known that *cis*-glycol groups are oxidized by periodate more rapidly than the *trans* isomers[4], that the oxidation of *trans*-glycol groups may be influenced by bulky substituents on adjacent carbon atoms[5] and by strained ring-conformations[6], and that the oxidation of methyl glucopyranosides is accompanied by an intermolecular rearrangement to yield 3-hydroxy-2-(*R*)-methoxy-1,4-dioxane-6-carbaldehyde through formation of a hemiacetal between the aldehyde group derived from C-2 of the glycoside and the 6-hydroxyl group[7,8].

The presence of the 6-methyl ether of glucose in the methylation mixture from the oxidized glycan establishes that two hemiacetal bonds may be formed between a single glucose residue and the two aldehyde groups of the oxidized galactose residue. Such a component could be the internal glucose residue substituted only at O-4. The presence of the tetra-, tri-, and di-methyl ethers indicates that some glucose residues were not oxidized by the periodate, even though they were not protected by hemiacetal bonds. In connection with the latter suggestion, it is also possible that the hemiacetal bonds on some glucose residues may have been broken subsequent to removal of the excess of periodate and prior to methylation. The tetramethyl ether of glucose probably originates from the few terminal glucose units present as cellobiosyl side-chains whereas the tri- and di-methyl ethers arise from internal glucose residues. The glucose residues that yield the foregoing derivatives are resistant to periodate oxidation because of steric hindrance[5,6], presumably caused by the equatorial orientation of the substituents at C-4 of these residues.

A bacterial triheteroglycan of mannuronic acid, glucose, and galactose also contains a β-D-glucose-(1→4)-D-galactose moiety[9]. It has been reported that periodate oxidation of this compound oxidized it preferentially at the galactose residue, with all such residues being oxidized but with >80% of the glucose residues remaining intact[10]. Also, in the oxidation of guaran[11], the galactose residues were

oxidized, whereas 58% of the mannose residues were not oxidized until the oxidized glycan had been reduced and subsequently subjected to further periodate oxidation. In both instances the explanation advanced, but not verified, was that hemiacetal bonds were formed and that these bonds protected the glucose and mannose residues from oxidation. A methylation analysis of the type reported in the present paper should be conducted on these glycans to determine whether hemiacetal bonds are indeed formed during periodate oxidation. Also it would be of interest to study heteroglycans having other monosaccharide constitutents, to determine which combination of monosaccharide residues may lead to hemiacetal bond-formation during periodate oxidation. Results in the literature[12] on differences in the rate of oxidation of the residues of a degradation product from chondroitin sulfate may be explained in the light of hemiacetal bond-formation.

EXPERIMENTAL

Periodate oxidation. — The diheteroglycan from *Streptococcus faecalis*, strain N[13], (50 mg) was dissolved in water (20 ml), mixed with 20 ml of 0.04M sodium periodate, and maintained in the dark for 48 h at 4°. The excess of periodate was then decomposed by addition of ethylene glycol. Materials of low molecular weight were removed from the clear solution by dialysis and the oxidized glycan was recovered by lyophilization; yield 45 mg. This glycan (30 mg) in water (5 ml) was reduced with sodium borohydride (15 mg) for 24 h at room temperature. The remainder of the oxidized sample was saved for subsequent methylation analysis. The mixture containing the reduced product was neutralized with acetic acid, and the reduced glycan was purified by dialysis and then lyophilized; yield 20 mg.

Paper-chromatographic analysis. — Samples (2 mg) of the native, and the oxidized and subsequently reduced, glycan were 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[14], with two ascents of the solvent system of butyl alcohol–pyridine–water (6·4:3 by volume). Standards of galactose, glucose, glycerol, threitol, cellobiose, gentiobiose, and panose were used on the same chromatogram to afford reference R_F values. The developed chromatogram was stained with the silver nitrate reagent[15] to locate reducing products and sugar alcohols. The hydrolyzate of the native glycan contained glucose (R_F 0.63), galactose (R_F 0.56), and a series of oligosaccharides (R_F 0.41 to 0). The hydrolyzate of the oxidized and subsequently reduced glycan contained only traces of galactose (< 5% of the original) and large amounts of glucose (> 95% of the original), glycerol (R_F 0.88), threitol (R_F 0.78), and three oligosaccharides (R_F 0.41, 0.33, and 0.21). The R_F values of the oligosaccharides are characteristic of disaccharides and trisaccharides of (1→4) and (1→6) linked glucose residues. The quantitative values for glucose and galactose were obtained by comparisons of color intensities of the compounds in the hydrolyzates with the intensities of glucose and galactose standards (2.5, 5, 10, 20, 50, and 100 μg). Dilutions of the hydrolyzate were used, if necessary, to ensure that concentrations of the hydrolytic products fell into this range

Methylation analysis. — Samples (5 mg) of the native, the oxidized, and the oxidized and subsequently reduced glycan were employed for methylation analysis[3], following the procedure recently described[3]. Gas–liquid chromatography was performed in a Varian 1400 unit on a column of 3% of OV-225 on 80–100 mesh Supelcoport (Supelco, Bellefonte, PA). The g.l.c. patterns for the three samples are reproduced in Fig. 1. The retention times for corresponding reference standards prepared from glucose, methyl 2,6-di-*O*-methyl-α-D-glucopyranoside[16] (provided by R. E. Reeves, Louisiana State University, New Orleans, LA), 4-*O*-β-D-glucopyranosyl-D-galactose[10] (provided by J. H. Sloneker, Northern Regional Laboratory, Peoria, IL), lactose, and glycogen were identical to those for the compounds shown in Fig. 1. Mass-spectral data were obtained with a DuPont 21-490 mass spectrometer. The following *m/e* fragments characteristic of partially methylated derivatives of the alditol acetates[3] were obtained: 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 (30), 117 (100), 233 (30); 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylhexitol, 45 (30), 117 (100); 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol, 117 (100), 261 (20); and 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylhexitol, 45 (20), 117 (100). The figures in parentheses denote relative intensities, with the most prominent fragment (117) being assigned a value of 100.

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