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# THE CHARACTERIZATION OF HEMIACETAL BONDS FORMED AFTER PERIODATE OXIDATION OF HETEROGLYCANS*

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ABSTRACT

The location of inter-residue, cyclic hemiacetals formed following the periodate oxidation of four representative heteroglycans has been determined by methylation analysis of the periodate-oxidized glycans. The cyclic hemiacetals led to the protection of hydroxyl groups during methylation in methyl sulfoxide, and their positions were located by analysis of the resulting di- and mono-methyl ethers. Such derivatives were not observed upon methylation analysis of the native and the periodate-oxidized–borohydride-reduced glycans. Inter-residue hemiacetals were thus identified in all oxidized glycans, between aldehydic groups at C-2 or C-3 of oxidized residues and hydroxyl groups at C-3 or C-2 of adjacent, unoxidized residues. Selective removal of 6-*O*-substituents from oxidized residues resulted in a decreased ability of the latter to form the inter-residue hemiacetals. Analysis of the types and proportions of the methyl ethers resulting from inter-residue hemiacetal formation may also yield structural information on the glycan.

## INTRODUCTION

The formation of hemiacetal bonds during the periodate oxidation of glycans has been proposed for some time as the reason for "anomalous" periodate uptake by certain glycans, accounting, in part, for the periodate-resistant residues[1]. Subsequent studies[2,3] suggested that such hemiacetal bonds are probably intra-chain, rather than the sterically unfavorable, inter-chain structures originally proposed[1]. Precise measurements of periodate uptake by amylose and guaran, before and after borohydride reduction, have provided indirect evidence for the existence of hemiacetal bonds[4,5]. Definitive information on the exact nature of the hemiacetal bonds has since become available from methylation studies on a periodate-oxidized diheteroglycan of *Streptococcus faecalis* before its reduction with borohydride[6]. The

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results from these studies were the first to describe accurately the types of hemiacetals formed between adjacent residues within a single glycan chain. Here we present comparative data on the nature and extent of formation of the inter-residue hemiacetal bonds formed during the normal analytical conditions of periodate oxidation (20mM, 50 h, 4°) of four representative heteroglycans. The nature of the hemiacetal bonds was deduced from the products of methylation analysis of the native, periodate-oxidized, and periodate-oxidized–borohydride reduced glycans. The glycans used were a guaran from guar gum having a repeating unit of mannose-(1→4)-galactose-(1→6)-mannose[7,8], a glycuronan from *Arthrobacter viscosus* NRRL-B1973 having a repeating unit of mannuronic acid-(1→4)-glucose-(1→4)-galactose[9,10], a carboxyl-reduced glycuronan having a repeating unit of mannose-(1→4)-glucose-(1→4)-galactose, and a glycan from *Streptococcus faecalis*, strain N, having a repeating unit of glucose-(1→4)-lactose-(1→6)-glucose-(1→4)-galactose[11,12].

## RESULTS AND DISCUSSION

The four heteroglycans, a guaran from guar gum, a glycuronan from *Arthrobacter viscosus*, the carboxyl-reduced glycuronan, and a diheteroglycan from *Streptococcus faecalis*, were oxidized in aqueous solution under normal analytical conditions. The concentration of periodate allowed a 3–4 molar excess over the maximum number of possible Malapradian attack-sites in the glycan. The percent of oxidation of the various sugar residues in each glycan (Table I) was determined by analysis of hydrolyzates of the native and the periodate-oxidized glycans by quantitative paper-chromatography[11]. Table I shows that, for each glycan, >90% of the residues of some of the constituent monosaccharides were oxidized, whereas other residues, particularly glucose, were characteristically resistant to periodate oxidation.

Sugar residues that survived oxidation were analyzed by methylation analysis[13,14], and evidence for the complete methylation of all available hydroxyl groups is discussed in the Experimental section. From each oxidized and fully methylated glycan, certain methyl ethers were obtained, indicating that hydroxyl groups other

TABLE I

PERCENTAGE OF MONOSACCHARIDE RESIDUES OXIDIZED BY PERIODATE IN REPRESENTATIVE HETEROGLYCANS

| *Glycan* | *Monosaccharide residue* | | | |
|---|---|---|---|---|
| | *Glc* | *Gal* | *Man* | *ManA* |
| Guaran | | 95 | 60 | |
| Glycuronan | 20 | 80 | | 95 |
| Triheteroglycan[a] | 30 | 75 | 95 | |
| Diheteroglycan | 10 | 90 | | |

[a]The triheteroglycan was prepared by reduction of all of the carboxyl groups of the glycuronan.

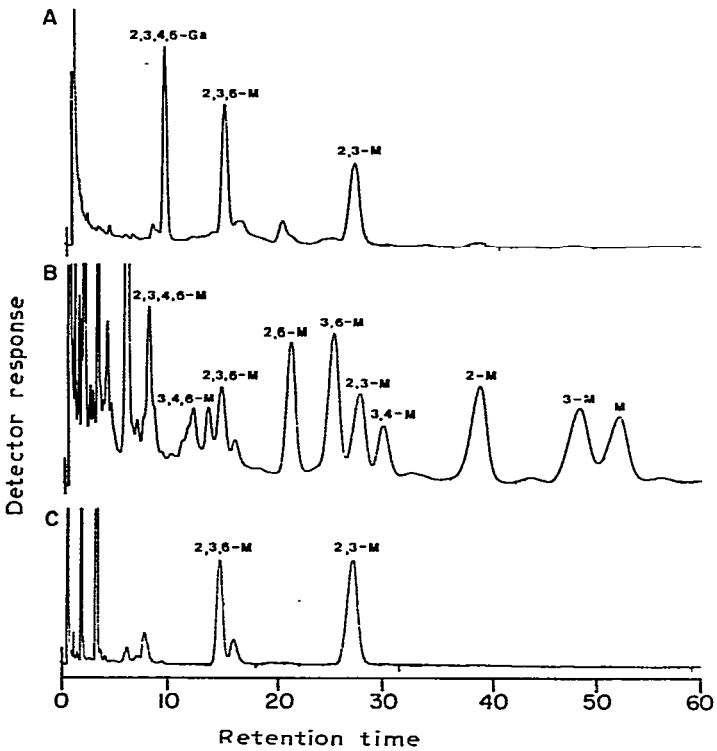


Fig. 1. A photograph of g.l.c. patterns of the methylated alditol acetates of native (A), periodate-oxidized (B), and periodate-oxidized and borohydride-reduced (C) guaran: 2,3,4,6-Ga = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 2,3,4,6-M = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol, 3,4,6-M = 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol, 2,3,6-M = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol, 2,6-M = 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylmannitol, 3,6-M = 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylmannitol, 2,3-M = 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylmannitol, 3,4-M = 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylmannitol, 2-M = 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylmannitol, 3-M = 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methylmannitol, and M = 1,2,3,4,5,6-hexa-*O*-acetylmannitol. Retention times are in minutes.

than those involved in the glycosidic linkages were protected, and accordingly not available for methylation. These methyl ethers were not observed upon methylation analysis of the native and the oxidized–borohydride-reduced glycan preparations, and they arose from protection of hydroxyl groups by inter-residue, hemiacetal formation. The percentage of non-oxidized residues protected by such hemiacetals was determined by integration of the g.l.c. peak areas of the corresponding methyl ethers, and ranged from 30–80% for the four glycans. The remaining, non-oxidized residues in each glycan exist as unprotected residues.

The observed proportions of methyl ethers derive from the oxidized glycans were produced from inter-residue hemiacetals that existed in methyl sulfoxide during methylation, and thus correspond to the potential hemiacetals that may actually affect rates of periodate oxidation in aqueous systems. Methyl sulfoxide has been

TABLE II

METHYL ETHERS OF MONOSACCHARIDES IN ACID HYDROLYZATES OF METHYLATED GUARAN: A = NATIVE, B = PERIODATE-OXIDIZED, AND C = PERIODATE-OXIDIZED AND BOROHYDRIDE-REDUCED

| *Hexoses and location of methyl groups* | $T_R$[a] | *Mole percentage* | | |
|---|---|---|---|---|
| | | *A* | *B* | *C* |
| 2,3,4,6-Man | 0.99 | | 6 | |
| 2,3,4,6-Gal | 1.14 | 32 | | |
| 2,3,6-Man | 1.88 | 34 | 7 | 36 |
| 2,6-Man | 2.72 | | 14 | |
| 3,6-Man | 3.19 | | 17 | |
| 2,3-Man | 3.54 | 34 | 9 | 64 |
| 3,4-Man | 3.87 | | 5 | |
| 2-Man | 5.01 | | 15 | |
| 3-Man | 6.23 | | 14 | |
| Man | 6.67 | | 13 | |

[a]Retention time of corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on a column of 3% OV-225 at a temperature of 190°.

shown to favor hemiacetal formation in model compounds[15], and is thus well suited to analysis of hemiacetal formation in heteroglycans.

G.l.c. patterns for the partially methylated alditol acetates derived from native, oxidized, and oxidized–reduced guaran are shown in Fig. 1. Quantitative values for the derivatives labeled in Fig. 1 were obtained by integration of the peak areas, and these values are recorded in Table II. In agreement with established results[8], native guaran yielded mannose and galactose derivatives in the ratio of 2:1 (Table II, Column A). The oxidized–reduced preparation yielded the two mannose derivatives (Table II, column C), 2,3-di-Me-Man and 2,3,6-tri-Me-Man, indicating a ratio of branch-point residues to non-branched residues of 1.8–1.0. No galactose derivatives were detected. From the foregoing data, it was not possible to determine the proportion of mannose residues oxidized, but quantitative paper-chromatographic analysis showed that 40% of the mannose residues of guaran was not oxidized under the conditions employed. This value is in agreement with the typical, "anomalous oxidation" limit of guaran reported by others[5,16].

The g.l.c. pattern for the methylation analysis of the oxidized (but not reduced) guaran sample is shown in Frame B of Fig. 1. The type of pattern shown in Fig. 1 was obtained with three different preparations of oxidized guaran. Each preparation received two Hakomori methylations as described in the Experimental section to ensure against under-methylation. All of the major peaks in the figure were identified by g.l.c. retention times and by characteristic fragments obtained on mass spectrometry. The cluster of early peaks for the oxidized samples constitute 2-, 3-, and 4-carbon oxidation fragments, as revealed by mass-spectral analysis, but the structure of these compounds was not determined.

Of significance is the finding that the periodate-oxidized guaran yielded numer-

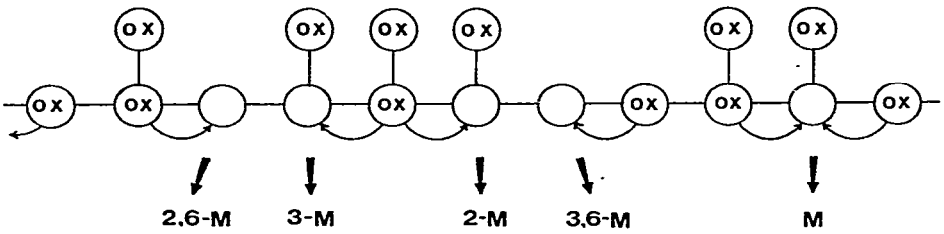


Fig. 2. A diagram of the structure of a segment of a guaran molecule: top row of circles = galactosyl residues, bottom row of circles = mannosyl residues, OX = oxidized residues, vertical lines = $\alpha$-(1→6) linkages, horizontal lines = $\beta$-(1→4) linkages, arrows = hemiacetal bonds. The abbreviations are as in Fig. 1.

ous new peaks on methylation analysis. The five major peaks were identified as the alditol acetates of 2,6-di-Me-Man, 3,6-di-Me-Man, 3-Me-Man, 2-Me-Man, and mannitol, and the minor peaks were identified as derivatives of 2,3,4,6-tetra-Me-Man, 3,4,6-tri-Me-Man, 2,3,6-tri-Me-Man, 2,3-di-Me-Man, and 3,4-di-Me-Man. Integration of the g.l.c. peak areas yielded the data in Table II. As the five major derivatives arise from residues protected by hemiacetal bonds, it may be calculated that ~80% of the unoxidized mannose residues were protected against oxidation by such bonds. The remaining 20% of the mannose derivatives were composed of residues that survived the oxidation, even though not protected (2,3,6-tri-Me-Man and 2,3-di-Me-Man), and from residues liberated by depolymerization during oxidation of the glycan (2,3,4,6-tetra-Me-Man, 3,4,6-tri-Me-Man, and 3,4-di-Me-Man).

A typical segment of a guaran molecule, showing the observed proportion of oxidized residues and the resulting hemiacetal-bond types, is drawn in Fig. 2. In constructing such a model sequence, the participation of oxidized galactose residues in inter-residue hemiacetal formation is considered unlikely, as the resulting, 8-membered cyclic acetals would be thermodynamically unstable. Also, previous kinetic studies of periodate uptake in guaran have shown that the galactosyl side-groups are oxidized rapidly and independently from the mannose chain, with no apparent effect on the rate of oxidation of the latter[5]. For the oxidized mannose residues, hemiacetal bonds can be formed with the aldehyde groups and the available hydroxyl groups on adjacent, unoxidized residues to yield 6-membered structures, which are more stable. It is also assumed that oxidized, unbranched residues tend to participate in only one inter-residue hemiacetal, because of the possible formation of the (more stable) intra-molecular hemiacetals. A further examination of guaran model-sequences shows that the observed hemiacetal bond-types can reasonably be formed only if oxidized residues, serving as branch points, act as 2-way donors of both aldehyde groups to hydroxyl groups on each of their neighbors, in line with the suggestion made in previous studies on guaran[5]. Thus, in oxidized guaran, inter-residue hemiacetals may occur in the mannose chain between positions 2 or 3 of oxidized residues and positions 3 or 2, respectively, of non-oxidized residues. Approximately 25% of the branched mannose residues that are non-oxidized contain both types of hemiacetal bonds, and such residues yield mannitol acetate upon methylation analysis.

Other investigators[17] have interpreted data from methylation analysis of periodate oxidized–borohydride reduced guaran to indicate a random distribution of galactosyl side-groups, in contrast to the block-polymer structure proposed for the locust bean galactomannan[18]. The results from the present study substantiate the random structure for guaran. A random structure for guaran necessitates short regions of adjacent, branched residues as well as some short regions of adjacent, unbranched residues, as shown in the sequence in Fig. 2. It may be noted (Table II) that the five derivatives resulting from inter-residue hemiacetal bond-formation are present in relatively equal amounts. Model sequences for guaran, constructed by using the foregoing guidelines show that, in a block-polymer structure, the long regions of unsubstituted residues, when oxidized, could donate aldehyde groups to the 2-hydroxyl group of adjacent mannose residues, producing the 3,6-di-Me-Man derivative upon methylation analysis. The corresponding branched regions in such a block-polymer would tend to form inter-residue hemiacetals in both directions along the mannose chain, producing all of the observed derivatives except 2,6-di-Me-Man and 3,6-di-Me-Man. On the other hand, a completely ordered polymer, consisting of alternating branched and unbranched residues, would yield only the 3-Me-Man, 2,6-di-Me-Man, and 3,6-di-Me-Man derivatives. Thus, significant amounts of the 2,6-di-Me-Man derivative can only be produced from regions were branch points are relatively evenly distributed among non-branch points. The fully protected mannitol hexaacetate and 2-Me-Man would then arise from the short regions of adjacent, branched residues, which are characteristic of a random guaran structure.

The *Arthrobacter viscosus* B-1973 glycuronan has been shown to consist primarily of a linear chain having the sequence mannuronic acid-(1→4)-glucose-(1→4)-galactose; some of the residues are *O*-acetylated[9,10]. The *O*-deacetylated

TABLE III

METHYL ETHERS OF MONOSACCHARIDES IN ACID HYDROLYZATES OF METHYLATED GLYCURONAN: A = NATIVE, B = PERIODATE-OXIDIZED, AND C = PERIODATE-OXIDIZED AND BOROHYDRIDE-REDUCED

| *Hexoses and location of methyl groups* | $T_R$[a] | *Mole percentage* | | |
|---|---|---|---|---|
| | | *A* | *B* | *C* |
| 2,3,4,6-Glc | 1.00 | | 9 | |
| 3,4,6-Glc | 1.75 | | 5 | |
| 2,3,6-Gal | 2.01 | 27 | 10 | 16 |
| 2,3,6-Glc | 2.12 | 30 | 12 | 73 |
| 2,6-Glc | 3.00 | 4 | 42 | 9 |
| 3,6-Glc; 3,6-Gal[b] | 3.29 | 6 | 15 | 2 |
| 6-Glc | 4.35 | | 7 | |
| 2,3-ManA[c] | | 33 | | |

[a]As in Table II. [b]Represents a mixture of the two derivatives as these were not resolved on OV-225. [c]The methyl ether of mannuronic acid cannot be determined by g.l.c. analysis and the mannuronic acid value, as determined by quantitative paper-chromatography, is recorded in the table and used in calculating the percentage of the other derivatives in column A.

glycuronan was used for all oxidation and reduction experiments in this study. Results of methylation analysis of the native deacetylated, the periodate-oxidized, and the oxidized–reduced preparations are recorded in Table III. The derivatives arising from the native glycan, including the small proportion of dimethyl derivatives, and their ratio, are consistent with previous methylation studies[9,10], mannuronic acid not being detectable. Paper-chromatographic analysis of a hydrolyzate of the native preparation also showed that mannuronic acid, glucose, and galactose were present in equal molar amounts. No attempt was made to verify the sequence of sugar residues, which has been well documented[10]. Data from methylation analysis of the periodate-oxidized–borohydride-reduced preparation (Table III, column C) indicated a 5:1 ratio of glucose to galactose. This ratio is supported by paper-chromatographic data (Table I) which shows that 95% of the mannuronic acid, >80% of the galactose, and <20% of the glucose was oxidized. These values are in agreement with results previously reported[10].

Methylation analysis of the oxidized-only preparation (Table III, column B) revealed a 5:1 ratio of glucose to galactose derivatives, consistent with the observed ratio in the oxidized–reduced preparation. The derivatives that arose from residues protected by inter-residue hemiacetals are 2,6-di-Me-Glc, 3,6-di-Me-Glc, 3,6-di-Me-Gal, and 6-Me-Glc. In view of the nature of the derivatives and the steric considerations already discussed, hemiacetal bonds occurring in the oxidized glycuronan were located between C-2 of oxidized mannuronic acid residues and C-3 of non-oxidized glucose residues, between C-3 of oxidized mannuronic acid residues and C-2 of non-oxidized galactose residues, and between C-3 of oxidized galactose residues and C-2 of non-oxidized glucose residues. It may be seen from the yields of methyl derivatives (Table III) that the hemiacetal between the C-2 aldehyde group of the oxidized mannuronic acid and the 3-hydroxyl group of non-oxidized glucose residues is formed more frequently than the other types of hemiacetal bonds.

Derivatives produced from unprotected residues in the oxidized glycuronan are 2,3,6-tri-Me-Gal and 2,3,6-tri-Me-Glc and those produced from residues liberated by depolymerization reactions during periodate oxidation are 2,3,4,6-tetra-Me-Glc and 3,4,6-tri-Me-Glc. Calculation from the data in Table III shows that derivatives arising from protected residues constitute ~60% of the total sugar residues surviving oxidation.

To study the effect of 6-*O*-substituents on inter-residue hemiacetal formation, the mannuronic acid residues of the glycuronan were reduced to mannose prior to oxidation studies, to yield a triheteroglycan comprised of mannose, glucose, and galactose. The partially methylated, alditol acetates obtained on methylation analysis from the native, oxidized, and the oxidized–borohydride-reduced preparations of this glycan are recorded in Table IV. The derivatives obtained from the non-oxidized glycan showed equal amounts of mannose, glucose, and galactose in the polymer, as verified by paper-chromatographic analysis. The derivatives obtained from the oxidized–reduced preparation indicated a 6:2:1 ratio of glucose–galactose–mannose. Paper chromatograms of a hydrolyzate of the oxidized–borohydride-reduced prepara-

TABLE IV

METHYL ETHERS OF MONOSACCHARIDES IN ACID HYDROLYZATES OF THE METHYLATED, NEUTRAL TRI-HETEROGLYCAN PREPARED BY REDUCTION OF THE CARBOXYL GROUPS OF THE GLYCURONAN: A = NATIVE, B = PERIODATE-OXIDIZED, AND C = PERIODATE-OXIDIZED AND BOROHYDRIDE-REDUCED

| *Hexoses and location of methyl groups* | $T_R$[a] | *Mole percentage* | | |
|---|---|---|---|---|
| | | *A* | *B* | *C* |
| 2,3,4,6-Man | 0.99 | | 8 | |
| 2,3,4,6-Glc | 1.00 | | | 14 |
| 2,3,6-Man | 1.88 | 31 | | 8 |
| 2,3,6-Gal | 2.01 | 32 | 35 | 24 |
| 2,3,6-Glc | 2.12 | 30 | 28 | 54 |
| 2,6-Glc | 3.00 | 3 | 19 | |
| 3,6-Glc; 3,6-Gal[b] | 3.29 | 4 | 11 | |

[a]As in Table II. [b]Represents a mixture of the two derivatives, as these were not resolved on OV-225.

tion yielded data showing that nearly all of the mannose residues, three quarters of the galactose residues, and one quarter of the glucose residues were oxidized.

Methylation analysis of the preparation oxidized by periodate but not reduced with borohydride showed the presence of two derivatives arising from hemiacetal bond-formation, namely 2,6-di-Me-Glc and 3,6-di-Me-Glc. However, these derivatives account for only 30% of the residues that survived oxidation (Table IV, column B) in the carboxyl-reduced glycuronan. In contrast, >60% of the non-oxidized residues in the native glycuronan were protected by hemiacetal bonds during periodate oxidation. It may be seen that the major difference in the results with these two glycans is the much lower yield of the 2,6-di-Me-Glc derivative (Table IV, column B) from the carboxyl-reduced glycuronan than from the native glycuronan (Table III, column B). As, in the two glycans, the mannose and mannuronic acid residues were almost completely oxidized, it may be calculated from the proportion of the methyl derivatives that only about one quarter of the oxidized mannose residues formed inter-residue hemiacetals in the carboxyl-reduced glycuronan, whereas over three quarters of the oxidized mannuronic acid residues formed hemiacetal bonds in the native glycuronan, showing that inter-residue hemiacetal bond-formation is indeed more likely to occur if the oxidized residue is substituted[2,3] at C-6. The reason for the low level of protection in the former is probably due to the fact that oxidized mannose residues form the intra-residue hemiacetals between the aldehyde group at C-2 and hydroxyl group at C-6 of the same residue, and are, therefore, not available for the formation of inter-residue hemiacetal bonds.

The streptococcal diheteroglycan of glucose and galactose has been shown to consist of a repeating unit of glucose-(1→4)-[lactose-(1→6)]-glucose-(1→4)-galactose connected by (1→4) linkages[12]. Methylation analysis of the oxidized glycan, demonstrating hemiacetal bond-formation, has been reported previously[6], and for comparative purposes the results have been recalculated on a percentage

TABLE V

METHYL ETHERS OF MONOSACCHARIDES IN ACID HYDROLYZATES OF METHYLATED DIHETEROGLYCAN: A = NATIVE, B = PERIODATE-OXIDIZED, AND C = PERIODATE-OXIDIZED AND BOROHYDRIDE-REDUCED

| *Hexoses and location of methyl groups* | $T_R$[a] | *Mole percentage* | | |
|---|---|---|---|---|
| | | *A* | *B* | *C* |
| 2,3,4,6-Glc | 1.00 | 3 | 9 | |
| 2,3,4,6-Gal | 1.14 | 19 | | 4 |
| 2,3,6-Gal | 2.01 | 19 | | |
| 2,3,6-Glc | 2.12 | 38 | 17 | 65 |
| 2,6-Glc | 3.00 | | 42 | |
| 2,3-Glc | 4.15 | 19 | 19 | 31 |
| 6-Glc | 4.35 | | 6 | |
| 2-Glc | 5.95 | | 8 | |

[a]As in Table II.

basis (Table V). The results with the periodate-oxidized preparation (Table V, column B) show the presence of two new derivatives, 2,6-di-Me-Glc and 6-Me-Glc, which arise from non-branched residues protected by hemiacetal bonds, and one new derivative, 2-Me-Glc which arises from protected branch-point residues. Area calculations show that these derivatives constitute 60% of the total glucose that was not oxidized by periodate. The unprotected glucose residues yielded 2,3,6-tri-Me-Glc and 2,3-di-Me-Glc derivatives on methylation analysis and a small proportion of 2,3,4,6-tetra-Me-Glc, which probably arises from depolymerization reactions.

The observed proportions of inter-residue hemiacetals formed in oxidized heteroglycans may be attributed to the equilibrium position of the hemiacetal (namely, hydrated, inter-, or intra-residue), which itself must depend on various factors[1,19–22]. Aside from solvent effects and monosaccharide conformation, methylation analysis would appear to be well suited to the elucidation of the role of glycosidic bond-types and the presence of substituted hydroxyl groups in hemiacetal formation in heteroglycans. Significantly, such an analysis may also be used to obtain structural information on the glycan, which, as in the case of guaran, may not be readily obtained by other methods. Analysis of this type should be applicable with other heteroglycans showing transient or permanent hemiacetal formation, including galactomannans, starches[23], xylans[24], and dextrans[25].

## EXPERIMENTAL

*Heteroglycans.* — Guaran was prepared from crude gum guar (Sigma Chemical Co., St. Louis, MO, U.S.A.) by dissolving the gum in water and centrifuging at 13,000*g* to remove insoluble material. The supernatant phase was filtered and lyophilized. The dried product was readily soluble in water and was used in the experi-

ments. The extracellular glycuronan from *Arthrobacter viscosus* NRRL-B1973, composed of mannuronic acid, glucose, and galactose, was supplied by M. E. Slodki, Northern Regional Resarch Center, Peoria, IL, U.S.A. in acetylated and the *O*-deacetylated form[10]; the deacetylated form was used in all experiments. A solution of the glycuronan was dialyzed to remove materials of low-molecular weight and lyophilized to recover the glycan. The cell-wall diheteroglycan of glucose and galactose was prepared from the group D *Streptococcus faecalis* strain N as previously described[26].

*Carbohydrate standards.* — Partially methylated methyl mannosides were provided by C. E. Ballou, Department of Biochemistry, University of California, Berkeley, CA, U.S.A. The glycosides were converted into their alditol acetates for g.l.c.–m.s. analysis by published procedures[13,14]. Methyl 2,6-di-*O*-methyl-3,4-di-*O*-(phenylcarbamoyl)-D-glucopyranoside[27] was provided by R. E. Reeves, Department of Biochemistry, Louisiana State University, New Orleans, LA, U.S.A., and was used to prepare 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-glucitol as already described[6]. Other partially methylated glucose and galactose derivatives were prepared by partial methylation of cellobiose and galactose respectively, as described in a later section.

*Reduction of the uronic acid of the glycuronan.* — The glycuronan (250 mg) was converted into a neutral triheteroglycan by reduction of the carboxyl groups of the mannuronic acid residues by using a water-soluble carbodiimide and sodium borohydride according to the procedure of Taylor and Conrad[28]. The excess of reagents was removed by dialysis and the reduced glycan recovered by lyophilization. The product was subjected to a second reduction and the final product recovered as already described; final yield 85% by weight. The carboxyl-reduced material was analyzed by methods described (Sugar and Methylation analysis), and this product was used for the experiments in this study.

*Periodate oxidation of heteroglycans.* — Glycan samples (30–70 mg) were dissolved in water, with stirring and sonication if necessary. The solution was precooled to 4° and mixed with an equal volume of precooled 0.04M sodium metaperiodate, yielding final concentrations of glycans of 0.1% and of periodate of 0.02M. The pH of the mixture was 4.5 and the reaction was allowed to proceed in the dark for 50 h at 4°. The excess of periodate was decomposed by adding ethylene glycol. The solutions were then made neutral, dialyzed against distilled water for 72 h, and lyophilized. The yields of the periodate-oxidized glycan preparations were 90–98% by weight.

*Reduction of oxidized glycans.* — The oxidized samples were dissolved in wate and adjusted with 0.1M sodium hydroxide to pH 8. Sodium borohydride was adde in amounts to yield a final concentration of 0.1% of hydride, and the reaction wa allowed to proceed for 24 h at room temperature. The mixtures were acidified wit acetic acid, dialyzed against distilled water, and the reduced glycans recovered b lyophilization. For the oxidized preparation of the carboxyl-reduced glycuronar the foregoing steps were conducted at 4° to minimize possible degradation of th glycan.

*Sugar analysis.* — Samples (~2 mg) of all heteroglycan preparations were hydrolyzed in 2 mL of 0.1M sulfuric acid for 15 h at 105° in sealed ampules. Hydrolyzates were made neutral with barium carbonate, centrifuged, filtered, and lyophilized. The residues were dissolved in 0.1 mL of water and analyzed for carbohydrate components by ascending paperchromatography on Whatman No. 1 paper in 6:4:3 (v/v) butyl alcohol–pyridine–water[29]. Reducing sugars and alditols were detected on the paper by the alkaline silver nitrate method[30].

*Methylation analysis.* — All carbohydrate samples were methylated by the Hakomori procedure[13] and converted into their alditol acetates by published procedures[14,31]. As the guaran and glycuronan did not dissolve readily in dimethyl sulfoxide as used for methylation, these samples were subjected to preliminary acetylation in 1:1 (v/v) pyridine–acetic anhydride in sealed ampules for 1 h at 100° and then maintained in a sonicator for 15 min. Insoluble material was removed by filtration and the solvents were evaporated off. The residual, acetylated glycans dissolved readily in dimethyl sulfoxide, and methylation by the Hakomori procedure caused complete replacement of *O*-acetyl groups by *O*-methyl groups. The preparation of oxidized guaran was essentially insoluble in dimethyl sulfoxide, and produced turbid suspensions under normal procedures of sonication at room temperature. Solubilization of this sample was effected by heating in dimethyl sulfoxide under nitrogen for 15 min at 105°. The resulting, clear solution was methylated conventionally. To avoid possible decomposition of hemiacetal bonds, an alternative procedure was employed with the oxidized guaran: a suspension of oxidized guaran in dimethyl sulfoxide was methylated, and the partially methylated product was recovered by gel filtration on Sephadex LH-20 with 2:1 (v/v) chloroform–acetone[31]. The recovered material was readily soluble in dimethyl sulfoxide and was then remethylated and converted into the alditol acetates. All other polysaccharide preparations required only one methylation. Partially methylated carbohydrate standards were prepared by using a suitable excess of the carbohydrate over the methylation reagents.

G.l.c.–mass spectrometry was performed with a Varian Aerograph series 1400 chromatograph on line with a Dupont 21-490 mass spectrometer. In the analysis, a 6 ft × 1/8 in. column of 3% OV-225 on 80–100 Supelcoport was used with helium carrier gas at 170 or 190°. Mass spectrometry was conducted with a source temperature of 250° and an ionization potential of 70 eV. The following fragments, having the relative abundance indicated in parentheses, were observed for the partially methylated alditol acetates: mannitol hexaacetate 115 (100), 127 (38), 128 (43), 139 (57), 145 (48), 157 (43), 170 (33), 187 (62), 217 (43), 259 (33), 289 (38), and 361 (9); 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylhexitol, 45 (10) and 117 (100); 1,2,4,5,6-penta-*O*-acetyl-3-*O*-methylhexitol, 189 (100) and 261 (44); 1,2,3,4,5-penta-*O*-acetyl-6-*O*-methylhexitol, 45 (100); 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol, 117 (100) and 261(20); 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylhexitol, 45 (30) and 117(100); 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylhexitol, 189 (100); 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol, 45 (45), 189 (100), and 233 (45); 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol, 117 (100), 161 (33), 189 (20), and 233 (15); 1,4,5-tri-*O*-acetyl-2,3,6-

tri-*O*-methylhexitol, 45 (30), 117 (100), and 233 (30); 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol, 45 (65), 161 (100), and 189 (75); 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol, 45 (80), 117 (100), 161 (80), and 205 (20).

*Criteria of complete methylation.* — Infrared spectra of the oxidized glycan samples were recorded, before and after methylation, as potassium bromide pellets or as solutions in chloroform. The oxidized and methylated samples showed the absence of O-H absorbance (3200–3600 $cm^{-1}$) as compared with the native patterns, which showed broad absorption-bands in this region. Spectra were recorded on a Perkin–Elmer Model 21 dual-beam spectrophotometer. Criteria for complete methylation of all other glycan samples included complete solubility of the native or modified samples in methyl sulfoxide prior to methylation, and the absence of unexplained di- and mono-methyl ethers, except for those consistent with structures previously proposed.

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