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NITROUS ACID DEAMINATION OF AMINO-OLIGOSACCHARIDE ALDITOLS AND THEIR PER-*O*-METHYLATED DERIVATIVES*

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ABSTRACT

Per-*O*-methylated amino-oligosaccharide alditols prepared from lacto-*N*-tetraose, lacto-*N*-fucopentaose I, and the mixed populations of oligosaccharide chains from α_1-acid glycoprotein and hog gastric mucin have been used as model substrates to assess the scope of the reaction sequence, *N*-deacetylation–nitrous acid deamination followed by derivatization, in the fragmentation of complex amino-oligosaccharides. G.l.c.–mass spectrometry has been used as the major tool in the characterization of products.

INTRODUCTION

In two previous papers[1,2] we have examined the use of nitrous acid deamination in the selective cleavage of equatorially oriented 2-amino-2-deoxyglycosidic linkages and in the structural rearrangement of 2-amino-2-deoxyhexitols. In both classes of compound, when a 3-*O*-glycosyl substituent is present, side-reactions occur with the loss of that substituent as a reducing sugar. Reducing groups thus exposed, together with those of 2,5-anhydrohexoses from the cleavage of amino glycosidic linkages and of 2-deoxyhexoses from the rearrangement of 2-aminoalditols, are readily recognized by incorporation of deuterium on reduction with sodium borodeuteride. When the deamination and subsequent treatment with sodium borodeuteride are performed with per-*O*-methylated amino-oligosaccharides and are followed by further substitution, as by trideuteriomethylation or acetylation, the location of substituents in the products serves to characterize both liberated reducing groups and exposed aglyconic hydroxyl groups. We now describe such experiments performed on per-*O*-methylated derivatives of lacto-*N*-tetraitol and lacto-*N*-fucopentaitol I, and on the mixed populations of oligosaccharide alditols formed from asialoglycoproteins in which the characterization of deamination products provides useful evidence on the value of the procedure for the determination of sequence and linkage types of sugar residues in complex amino-oligo- or poly-saccharide chains.

*Amino-oligosaccharides, Part III. For Parts I and II, see refs. 1 and 2.
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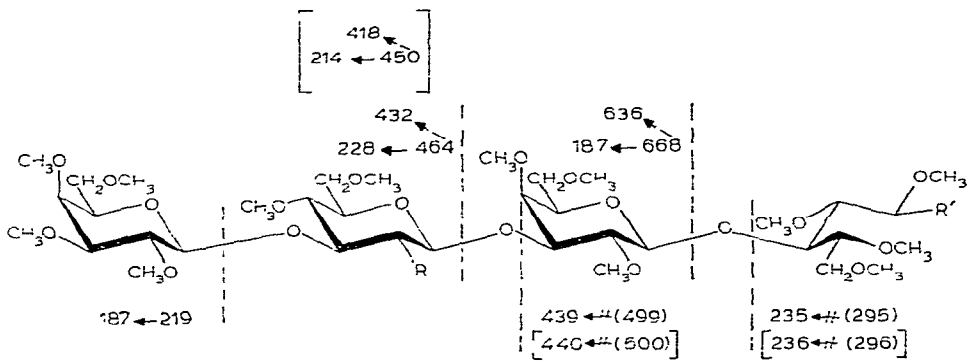
RESULTS AND DISCUSSION

Lacto-*N*-tetraose and lacto-*N*-fucopentaose were isolated from human milk[3] and, in each instance, confirmation of structure was obtained by examination of permethylated derivatives of the oligosaccharide alditols formed by reduction with sodium borodeuteride. Permethylated lacto-*N*-tetraitol-*1-d* (**1**) prepared by the Hakomori alkylation method[4] gave a mass spectrum identical to that reported by Hallgren and Lundblad[5], and analogous mass spectra were obtained for permethylated lacto-*N*-fucopentaitol-*1-d* (**4**) and the corresponding per-*O*-methylated derivatives (**2** and **5**) prepared without significant *N*-methylation. Those mass-spectral features that give evidence for the nature of the sugar residues, their sequence and, in some instances, linkage types, are summarized in this paper. Here and elsewhere in this investigation, information is readily obtained on the environment of 3-*O*-substituted sugar residues[6]. The oxocarbonium ions (A_1) formed on cleavage of the glycosidic linkages of such residues undergo ready loss of the 3-*O*-substituent such that the A_2 ion formed by the further loss of methanol is of low abundance. 3-*O*-Substituted

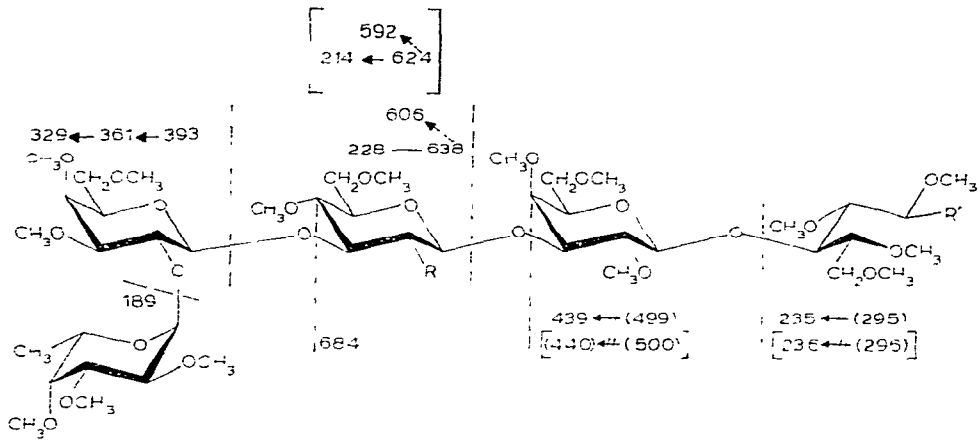


1 R = $-NHCH_3$, $COCH_3$, R' = $-CH_2OCH_3$
2 R = $-NHCOCH_3$, R' = $-CHDOCH_3$
3 R = $-NH_2$, R' = $-CHDOCH_3$

Salient features of mass-spectral fragmentations are shown by the following conventions: (295) → 235 or (295) → 235 indicates that the parent ion of the J series is/is not observed; — → indicates minor degradative pathway. Mass-spectral fragmentations for **2**, where different from those of **1**, are shown in square brackets.

sugar residues are also placed in sequence by the virtual absence of the rearranged ions of the J_t series. Per-*O*-methylated lacto-*N*-tetraitol (**1**) was further characterized by hydrolysis and conversion of the sugars into partially methylated alditol acetates[7]. In the case of per-*O*-methylated lacto-*N*-fucopentaitol-I (**5**), the *N*-deacetylated derivative (**6**) was treated in the following steps: (*1*) hydrolysis of all but 2-amino-2-deoxyglycosidic linkages; (*2*) nitrous acid deamination to cleave the previously intact 2-amino-2-deoxyglycosidic linkages; and (*3*) reduction and subsequent acety-

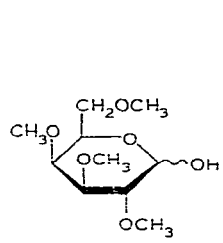
lation to give partially methylated alditol acetates, including 2,5-anhydrohexitol derivatives of the same substitution-pattern as the amino sugars from which they were formed. The mass spectrum of 1,3-di-*O*-acetyl-2,5-anhydro-4,6-di-*O*-methyl-mannitol was not uniquely diagnostic of constitution, but both mass spectrum and retention time served to differentiate this compound from the isomeric 3,6-di-*O*-methyl compound (see later).



4 R = $-N(CH_3)COCH_3$, R' = $-CH_2OCH_3$
5 R = $-NHCOCH_3$, R' = $-CHDOCH_3$
6 R = $-NH_2$, R' = $-CHDOCH_3$

Mass-spectral fragmentations for **5**, where different from those of **4**, are shown in square brackets.

Despite the previously noted[1] extreme resistance of fully substituted 2-acetamido-2-deoxyglycosides to *N*-deacetylation, reasonably complete reaction of per-*O*-methylated lacto-*N*-tetraitol-*l*-*d* (**2**) was achieved by hydrazinolysis for 120 h at 110°, and the resulting amine was treated with nitrous acid. A portion of the mixture was treated with hydroxylamine, followed by acetic anhydride in pyridine. Examination of the derivatives by g.l.c.–m.s. showed the presence of an acetylated aldononitrile formed from 2,3,4,6-tetra-*O*-methylgalactose (**7**), together with a fraction having the retention time of 2,5-anhydro-3-*O*-(2,3,4,6-tetra-*O*-methyl-β-D-galactopyranosyl)-4,6-di-*O*-methyl-D-mannononitrile[1] (**8**) but whose mass spectrum was consistent with the presence in addition of 4-*O*-(3-*O*-acetyl-2,4,6-tri-*O*-methyl-β-D-galactopyranosyl)-1,2,3,5,6-penta-*O*-methyl-D-glucitol-*l*-*d* (**9**). A further portion of the mixture was reduced with sodium borodeuteride and then trideuteriomethylated. Examination of the products by g.l.c.–m.s. showed the presence of two disaccharide derivatives having the retention times of per-*O*-methylated derivatives of 2,5-anhydro-3-*O*-(β-D-galactopyranosyl)-D-mannitol (**10**) and of lactitol (**11**). The relative abundances of fragment ions at *m/e* 158 and 161 in the approximate proportion of 2:1 provided evidence for a (1→3)-linkage in the isotopically labelled compound[1] **10**.



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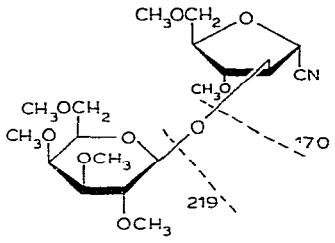

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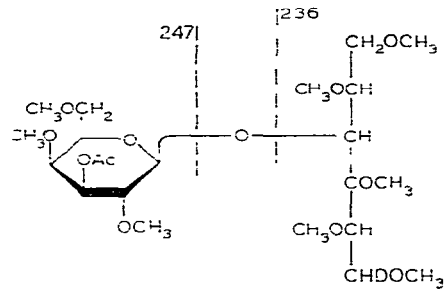

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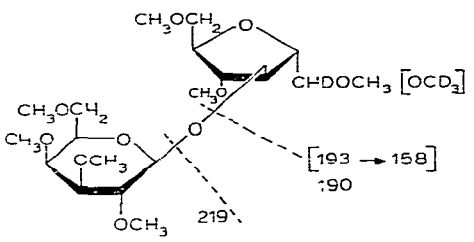
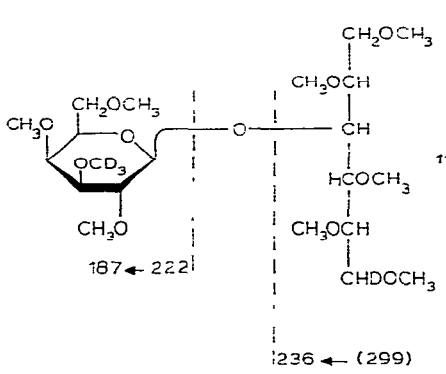


10 [10a]



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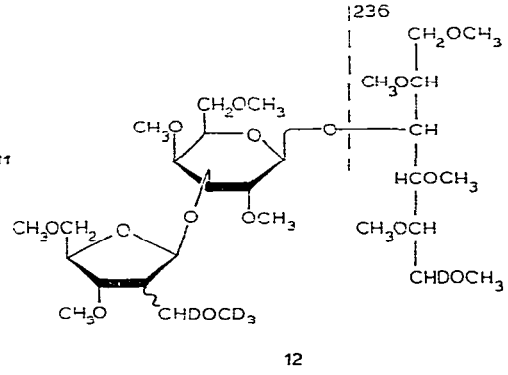
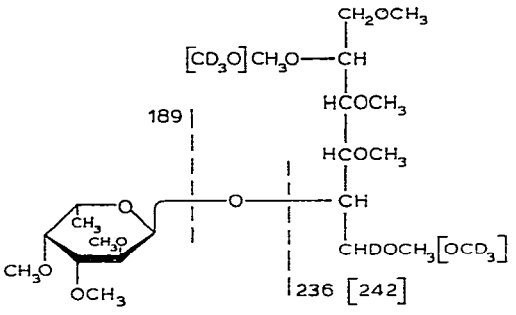


12

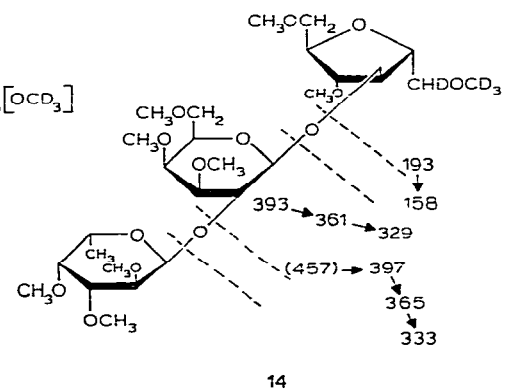
The appearance of a fragment ion at *m/e* 299 (*ab*J_1) and its loss of trideuteriomethyl formate to give the ion at *m/e* 236 in the mass spectrum of the lactitol derivative **11** provided evidence for the location of a trideuteriomethyl group at O-3′, and thus confirmed the (1→3)-linkage between the two central sugar residues in the parent amino-oligosaccharide. The formation of 2,3,4,6-tetra-*O*-methylgalactose (**7**) amongst the deamination products implies that it was liberated as a 3-*O*-substituent from a 2-amino-2-deoxyglucose residue by the alternative deamination reaction-pathway accompanied by the formation of a 2-deoxy-2-*C*-formylpentofuranoside. However, attempts by g.l.c.–m.s. to detect the formation, after reduction and further alkylation, of the trisaccharide derivative (**12**) in the mixture containing the disaccharides **10** and **11** were unsuccessful.

Per-*O*-methylated lacto-*N*-fucopentaitol I (**5**) was similarly *N*-deacetylated by

hydrazinolysis and the resulting amine (**6**) was treated with nitrous acid. The mixture was treated with sodium borodeuteride and then trideuteriomethylated. Examination of the products by g.l.c.–m.s. showed the presence of two disaccharide alditols, the major component of which was indistinguishable from the isotopically labelled permethylated lactitol (**11**) previously formed from methylated lacto-*N*-tetraitol (**2**), and a minor component whose mass spectrum was consistent with that of the 2-*O*-fucopyranosylgalactitol derivative (**13**). Further examination of the mixture revealed a trisaccharide whose mass spectrum was in accord with that anticipated for the 2,5-anhydromannitol derivative **14**. The relative proportions of fragment ions at *m/e* 158 and 161 pointed to the presence of a 3-*O*-substituted 2,5-anhydromannitol



13 [13a]



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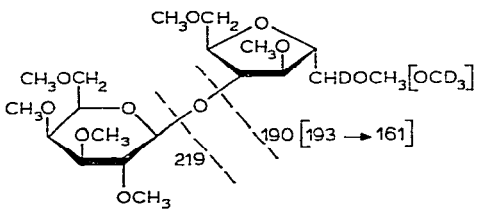


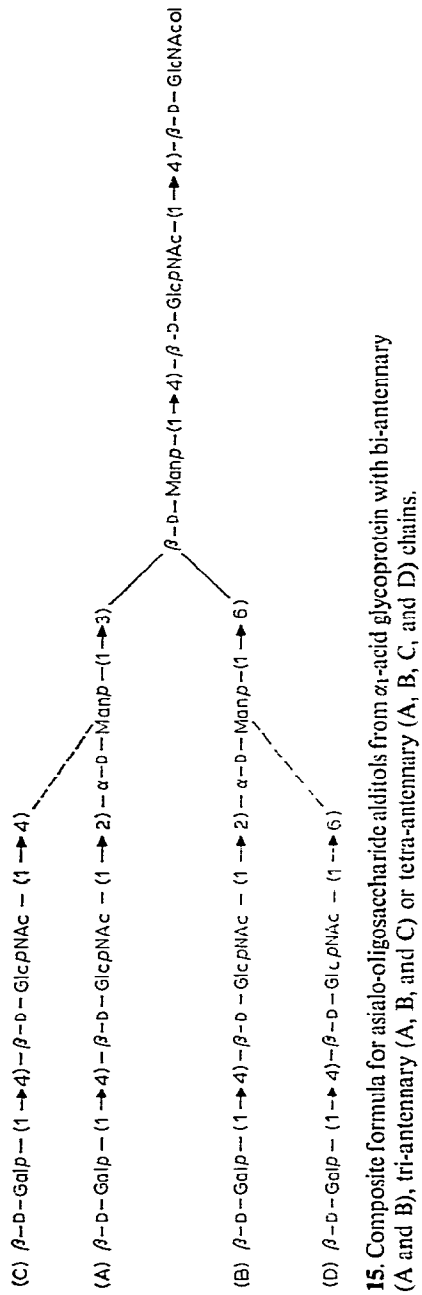
16 [16a]

Formulae within square brackets, such as [13a], carry trideuteriomethyl groups as shown, and *m/e* values for fragment ions incorporating such substituents are likewise in square brackets.

residue. Although not uniquely diagnostic of linkage type, the relatively high abundances of fragment ions of the series baA_{1-3} at *m/e* 393, 361, and 329, of the series bcA_{1-3} at *m/e* 397, 365 and 333, and of the ion at *m/e* 457 ($abcJ_1$) were characteristic of those observed by Kärkkäinen[8] for methylated trisaccharides containing a central

2-*O*-substituted hexopyranose residue, and by Hallgren and Lundblad[5] for methylated oligosaccharides containing terminal 2-*O*-α-L-fucopyranosyl-D-galactopyranose groups. Attempts to detect the trisaccharide derivative **12** were again unsuccessful.

Montreuil, Vliegenthart, Schmid and their collaborators[9] have shown that



15. Composite formula for asialo-oligosaccharide alditols from α_1-acid glycoprotein with bi-antennary (A and B), tri-antennary (A, B, and C) or tetra-antennary (A, B, C, and D) chains.

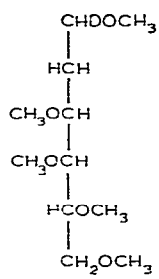
α_1-acid glycoprotein contains a series of structurally related oligosaccharide chains *N*-glycosylically linked to asparagine residues in the protein core. All chains consist of a common pentasaccharide core ("mannotriosylchitobiose") in which the exterior mannose residues carry two, three, or four *N*-acetyllactosamine groups, with a small proportion of such chains containing additional fucose or sialic acid residues. *N*-Deacetylation (by hydrazinolysis) followed by deamination with nitrous acid provided important evidence for this structural formulation[10]. In our experiments, oligosaccharide chains were liberated by treatment with hot alkaline sodium borohydride[11] and the mixed population of chitobiitol-terminated oligosaccharide alditols (**15**) was characterized by the following observations: (*1*) *N*-deacetylation followed by nitrous acid deamination and reduction with sodium borohydride afforded 2-deoxy-*arabino*-hexitol (from the 2-acetamido-2-deoxyglucitol terminus), 2,5-anhydro-(galactosyl)mannitol, and a higher oligosaccharide, which although not fully characterized, was probably the branched mannotriosyl-2,5-anhydromannitol previously characterized by Bayard and Fournet[10]: (*2*) hydrolysis of the permethylated derivatives gave the following neutral sugars that were identified by g.l.c.–m.s. after conversion into their alditol acetates: 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-*O*-methylmannitol, and 2,4- (possibly in admixture with 3,4-)di-*O*-methylmannitol. The methylation data indicated the presence of bi-antennary, tri-antennary and possibly small quantities of tetra-antennary chains in this preparation.

One aspect of the value of deamination of methylated amino-oligosaccharides was shown in the sequence of reactions: (*a*) per-*O*-methylation, (*b*) hydrazinolysis, (*c*) nitrous acid deamination, (*d*) reduction with sodium borodeuteride, and (*e*) trideuteriomethylation. The major product from the oligosaccharide mixture (**15**) was characterized as the disaccharide **16** by g.l.c.–m.s. The presence in the mass spectrum of fragment ions at *m/e* 161 and 158 in the approximate ratio of 3:1 distinguished this compound from the (1→3)-linked disaccharide (**10a**) from which it differed only in isotopic labelling.

Hog gastric mucin is a much studied glycoprotein[12] in which the oligosaccharide chains show considerable heterogeneity. Kochetkov and his collaborators[13–15] have shown that reductive elimination from glycosidic linkage to serine or threonine residues leads to the formation of a complex mixture of structurally related oligosaccharide alditols ranging in degree of polymerization from 2–3 to 15–20, and all terminated by 2-acetamido-2-deoxy-D-galactitol residues. The structures of several penta- and hexa-saccharides, and higher oligosaccharides, have been established[13,14]. These oligosaccharides contain a high proportion of 2-acetamido-2-deoxy-D-glucose residues, and most of the penta- and hexa-, and some of the higher oligo-saccharides contain a common, interior, branched tetrasaccharide unit [residues **A–D** in structure **17**] to which L-fucose and additional D-galactose and 2-acetamido-2-deoxy-D-glucose residues may be attached. The mixture of oligosaccharide alditols formed from commercial hog gastric mucin was shown to be qualitatively similar in respect of sugar residues to that obtained by Kochetkov *et al.*[15] from the degradation of blood-group substance H from pig stomach linings. Methylation analysis was carried out

by hydrolysis of the per-*O*-methylated oligosaccharide mixture after prior *N*-deacetylation, followed by deamination, reduction, and acetylation to give mixtures of partially methylated alditol acetates. All of the neutral sugar residues reported by Kochetkov *et al.*[13,14] were detected. 2,5-Anhydromannitol derivatives arose from the corresponding 2-acetamido-2-deoxyglucose derivatives that were present as non-reducing end-groups or as 4-*O*-substituted residues. In addition, two incompletely characterized 2-deoxyalditol derivatives were present and probably arose from deamination of 2-amino-2-deoxygalactitol residues. The substitution patterns of these terminal residues were established by hydrolysis of the per-*NO*-methylated oligosaccharide mixture to give, *inter alia*, 1,4,5,6-tetra- and 1,4,5-tri-methyl ethers, which were characterized by their mass spectra.

The mixture of oligosaccharide alditols (**17**) was submitted to hydrazinolysis,



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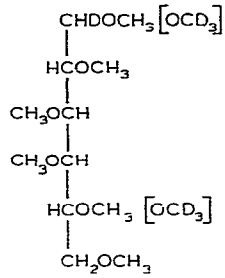
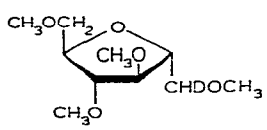


19 [19a]



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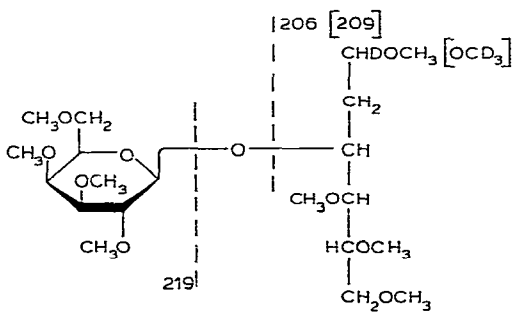
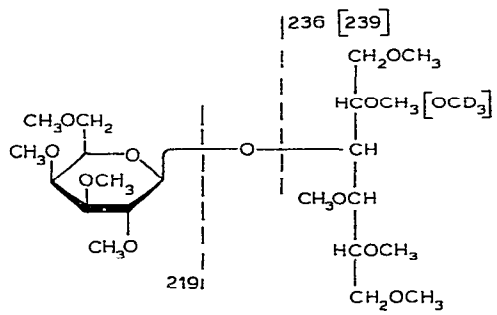
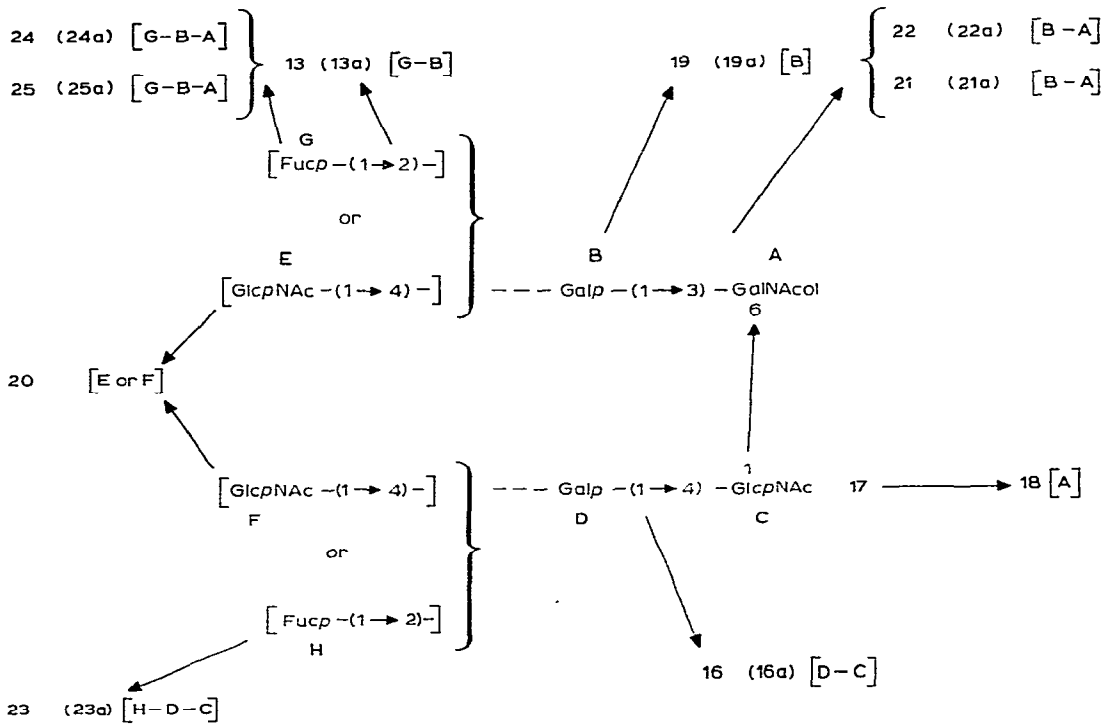


21 [21a]



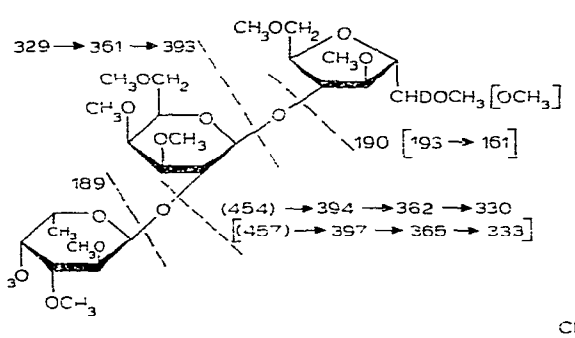
22 [22a]



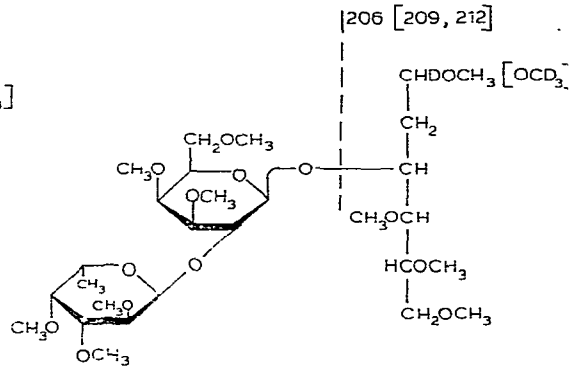
Hydrazinolysis–deamination of oligosaccharide alditols from hog gastric mucin giving degradation products characterized as their permethylated derivatives. Compounds in parenthesis having the suffix a (e.g. **13a**) contain trideuteriomethyl groups introduced as described in the text.

followed by deamination by nitrous acid, and the products were treated with sodium borodeuteride and then methylated[4]. The volatile components were then examined by g.l.c.–m.s. and the following compounds* were recognized as their permethylated derivatives: 2-deoxy-*lyxo*-hexitol-*1-d* (**18**, **A**†), 2,5-anhydromannitol-*1-d* (**20**, **E** or **F**), galactitol-*1-d* (**19**, **B**), 2-*O*-fucopyranosylgalactitol-*1-d* (**13**, **G–B**), 3(or 4)-*O*-galactopyranosylhexitol (**22**, **B-A**), 2,5-anhydro-4(or 3)-*O*-(galactopyranosyl)mannitol-*1-d* (**10** or **16**, **D-C**), and an unresolved mixture of trisaccharides. The more abundant ions of high mass, especially those of the baA series at *m/e* 393, 361, and 329 and of the bcA series at *m/e* 394, 362, and 330, and the very intense abcJ$_1$ ion at *m/e* 454, indicated that the trisaccharide **23** (**H-D-C**) was the major component of the mixture. In addition, however, the detection of a relatively abundant ion at *m/e* 206 pointed to the presence of trisaccharide **24** (**G-B-A**) as a second component in the mixture, but the absence of an ion at *m/e* 235 in appreciable abundance provided no evidence

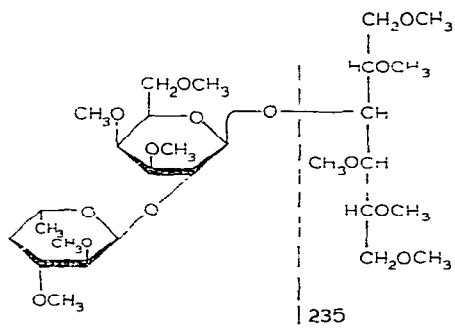
*Where no direct comparison with reference compounds was possible, configurations of sugar residues in di- and tri-saccharides are assumed from the known constituents of the glycoprotein.
†Here and elsewhere, bold-face letters indicate sugar residues in the composite structure (**17**) from which the derivative is formed.



23 [23a]



24 [24a]



25

for trisaccharide **25** (**G-B-A**) whose formation might be expected to accompany that of **24**. Of these products, compounds **20**, **16**, and **23** clearly result from the cleavage of 2-amino-2-deoxyglycosidic linkages, and compounds **18**, **21**, **22**, and **24** originate from the deamination of terminal 2-amino-2-deoxygalactitol groups[2]. As no 3-*O*-substituted 2-acetamido-2-deoxyglucose residues are present in the oligosaccharides from which deamination involving the alternative ring-contraction[1] could result in the liberation of new reducing groups, the only known pathway to account for the formation of the permethylated derivatives of galactitol-*1-d* (**19**) and 2-*O*-fucopyranosylgalactitol-*1-d* (**13**) is by loss of 3-*O*-substituents during the deamination of 2-amino-2-deoxyhexitols[2]. Thus the liberation of galactitol-*1-d* is consistent with the presence of 2-amino-2-deoxy-3-*O*-(galactopyranosyl)galactitol groups (**B-A**) in the oligosaccharide alditol mixture from the mucin. Such groups would also afford the two disaccharide alditols (**21** and **22**) in the deamination sequence of reactions. Similarly, the liberation of 2-*O*-fucopyranosylgalactitol-*1-d* implies that it originates from groups of *O*-fucopyranosyl-(1→2)-*O*-galactopyranosyl-(1→3)-(2-amino-2-deoxygalactitol)(**G-B-A**). The deamination of such groups, followed by reduction

and methylation, would be expected to furnish the permethylated trisaccharide **24** as a major product.

The mucin oligosaccharide alditols were also submitted to the sequence of reactions: (*a*) per-*O*-methylation, (*b*) hydrazinolysis, (*c*) nitrous acid deamination, (*d*) reduction with sodium borodeuteride, and (*e*) trideuteriomethylation. The products, isotopic labelling aside, were shown by g.l.c.–m.s. to be identical to those already described. In addition to confirming the nature of reducing groups exposed during the deamination, the results gave the following new information. The introduction of the trideuteriomethyl substituent into the 2,5-anhydromannitol residues in **16a** and **23a** provided the basis for mass-spectral evidence that these residues were 4-*O*-substituted. The incorporation of one trideuteriomethyl group into approximately two thirds of the galactopyranose residues in the disaccharide derivatives **16a**, **21a**, and **22a*** showed that deamination had resulted in the loss of 2-acetamido-2-deoxyglucose substituents external to these galactose residues. It is apparent from these observations that galactose residues **B** in oligosaccharide groups represented in the composite structure **17** are of three types, (*i*) those carrying no substituents and thus giving rise to disaccharides **21a** and **22a** without incorporation of trideuteriomethyl groups into the galactose residues, (*ii*) those carrying 2-acetamido-2-deoxyglucose substituents (**E**) that are removed during the deamination sequence, and (*iii*) those carrying terminal fucose substituents and thus furnishing oligosaccharides **13a** and **24a**. The mass spectra of the permethylated disaccharides **21a** and **22a** did not provide evidence for the site of attachment of the removed 2-acetamido-2-deoxy-glucose groups (**E**), but (1→4)-linkages are implied since 4-*O*-substituted galactose residues are not otherwise involved in the overall structure **17**. However, insofar as the hexa-*O*-methylgalactitol (**19a**) is also derived from similarly substituted galactose residues **B**, fragment ions in its mass spectrum were consistent with the presence of some additional trideuteriomethyl groups at O-4. Similar arguments place galactose residues **D** in oligosaccharide segments depicted in structure **17** in three environments, (*i*) with no substituents, (*ii*) with removable 2-acetamido-2-deoxyglucose substituents (**F**), and (*iii*) with 2-*O*-fucopyranosyl substituents and thus affording the trisaccharide **23a**.

The nitrous acid deamination of 2-amino-2-deoxyhexose residues in oligo- and poly-saccharides is a well established procedure for effecting cleavage of glycosidic linkages when the amino substituents are in equatorial orientation[16,17]. Useful extensions of the reaction have been described recently in studies on microbial polysaccharides[18,19]. The experiments reported here illustrate some further aspects of the deamination procedure. Although the reaction is more complex than formerly realized, the occurrence of alternative reaction-pathways in the deamination of amino glycosides[1,20] and aminoalditols[2] may be turned to advantage. Taken in conjunction with methylation analysis, the deamination reaction may be used to establish sugar sequences and linkage types in homogeneous amino-oligosaccharides and in poly-

*For clarity, these additional trideuteriomethyl groups are not shown in the structures.

saccharides having regularly repeating structures. Our studies on the mixed population of oligosaccharides derived from hog gastric mucin have also shown that information may be obtained from the characterization of reaction products from a family of structurally related oligosaccharides. Glycoproteins of the mucin type having glycosidic oligosaccharide chains linked to serine or threonine residues[1,2] frequently display microheterogeneity, and an insight into their general structures could suggest suitable methods for separation into individual components. The application of the *N*-deacetylation–deamination reaction to per-*O*-methylated derivatives gives additional information, but the advantages gained must be weighed against the experimental difficulties of achieving complete *O*- with little or no accompanying *N*-methylation and of overcoming the extreme resistance of fully substituted 2-acetamido-2-deoxyglycosides to *N*-deacetylation.

EXPERIMENTAL

General methods. — Evaporations were carried out under diminished pressure at bath temperatures of 40° or less. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter at 20 ±2°. N.m.r. spectra were recorded with a Varian EM-360 spectrometer.

G.l.c. was performed, at temperatures indicated, with Perkin–Elmer 990 and Tracor 560 chromatographs, using S.C.O.T. columns or columns of Gas-Chrom Q coated with the indicated liquid phases: (*A*) OV-225 S.C.O.T., (*B*) Silar 10C S.C.O.T., (*C*) a column of Superpak 20M (Analab), (*D*) 3% of silicone–polyester copolymer ECNSS-M, (*E*) 3% of silicone gum OV-225, (*F*) 3% of silicone gum XE-60, and (*G*) 1% of silicone gum SE-30. For g.l.c.–m.s., columns were attached *via* a Watson–Biemann separator to a Perkin–Elmer–Hitachi RMU-6 mass spectrometer, or *via* a jet separator to a VG Micromass 16F mass spectrometer operated with an inlet temperature of 250°, an ionization potential of 70 eV, and an ion-source temperature of ~250°.

*Isolation and characterization of lacto-*N*-tetraose and lacto-*N*-fucopentaose I from human milk.* — Oligosaccharides from human milk were isolated and fractionated as described by Kobata[3]. Lacto-*N*-tetraose was crystallized from ethanol–water. A sample of the tetrasaccharide was reduced with sodium borohydride and the resulting tetrasaccharide alditol was methylated by the Hakomori procedure[4]. The per-*N*,*O*-methylated derivative (**1**) gave a mass spectrum identical with that reported by Hallgren and Lundblad[5]. Hydrolysis of the methylated derivative (**1**), followed by reduction and acetylation[7], afforded the expected mixture of partially methylated alditol acetates, when analyzed by g.l.c.–m.s. with column *A* (180°, 2 min, 180→220°, 4°/min, hold). Similar treatment of oligosaccharide material isolated from the mother liquor furnished a mixture of partially methylated alditol acetates that included 3,6- and 4,6-di-*O*-methyl-2-acetamido-2-deoxyglucitol acetates. A further sample of lacto-*N*-tetraose was reduced with sodium borodeuteride and the resulting alditol was methylated with successive additions of methyl sulfate and aqueous 30% so-

dium hydroxide to give per-*O*-methylated lacto-*N*-tetraitol-*l*-*d* (**2**) which, after recrystallization from toluene, had m.p. 150–151°, $[\alpha]_D$ −9.5° (*c* 1.0, chloroform): *m/e* (% relative abundance) 450 (33, baA_1), 440 (2, cald), 418 (5, baA_2), 236 (20, ald), 214 (42, bA_2), 219 (3, aA_1), 187 (22, aA_2), 101 (100), and 88 (40).

Anal. Calc. for $C_{40}H_{74}DO_{21}N$: N, 1.54. Found: N, 1.55.

Methylated tetrasaccharide **2** (10 mg) was boiled for 10 days under reflux with hydrazine (3 mL) containing 6% of hydrazine sulfate. Hydrazine was removed by evaporation under diminished pressure in the presence of toluene, and the residue in dichloromethane was passed through a column of Sephadex LH-20 to remove acetylhydrazide. After removal of solvent, the resulting syrup in water (1 mL) was adsorbed on a column of Amberlite resin IR-120(H^+), and the column was eluted with water to remove unchanged acetamido sugar. Elution with 10% aqueous ammonia afforded the amino tetrasaccharide **3**, whose i.r. spectrum showed no carbonyl absorption. Per-*O*-methylated aminotetrasaccharide (**3**) was hydrolyzed with M hydrochloric acid for 16 h at 100°. The cooled solution was concentrated, sodium nitrite (25 mg) was added to the residue in water, and M sulfuric acid was added dropwise to bring the pH of the solution to 3.5. The solution was kept for 45 min at room temperature, excess of nitrous acid was decomposed by the addition of urea, and the solution was taken to dryness. The residue was treated with sodium borohydride (10 mg) in water (1 mL) overnight, the mixture was processed conventionally, and the residue was acetylated with acetic anhydride and pyridine. Examination of the products by g.l.c.–m.s. with column *B* at 180° showed the presence of derivatives of 1,2,3,5,6-penta-*O*-methylglucitol-*l*-*d*, 2,3,4,6-tetra-and 2,4,6-tri-*O*-methylgalactose, and 2,5-anhydro-4,6-di-*O*-methylmannose. The retention time of the latter derivative was different from that of the 3,6-di-*O*-methyl isomer, although the mass spectrum was not uniquely indicative of the substitution pattern.

The final step in the purification of lacto-*N*-fucopentaose I was a chromatographic separation on filter sheets with 12:5:4 ethyl acetate–pyridine–water as the solvent system. A sample of the pentasaccharide was reduced with sodium borohydride, and the resulting pentasaccharide alditol methylated by the Hakomori procedure[4]; the per-*N*,*O*-methylated derivative (**4**) gave a mass spectrum which showed significant fragment ions at *m/e* 684 (4, cdald), 638 (4, $bcaA_1$), 439 (18, dald), 393 (4, baA_1), 361 (5, baA_2), 329 (3, baA_3), 235 (60, ald), 228 (88, cA_2), 189 (30, aA_1), 157 (57, aA_2), 101 (90), 88 (82), and 71 (100). A further sample of lacto-*N*-fucopentaose I was reduced with sodium borodeuteride and the resulting alditol was treated with methyl sulfate and aqueous sodium hydroxide to give per-*O*-methylated lacto-*N*-fucopentaitol-*l*-*d* (**5**), $[\alpha]_D$ −51° (*c* 1.0, chloroform), whose mass spectrum included fragment ions at *m/e* 935 (0.5, $bcdaldJ_1$), 875 (0.5, bcdald), 828 (0.5, $dabcA_1$), 671 (1, cdald), 624 (10, $cabA_1$), 440 (1.5, dald), 393 (1.5, baA_1), 361 (3, baA_2), 236 (21, ald), 214 (36, cA_2), 189 (21, aA_1), 157 (35, aA_2), 101 (99), and 88 (100). Compositional analysis of methylated pentasaccharide **5** was conducted as for methylated tetrasaccharide **2** and furnished derivatives of 1,2,3,5,6-penta-*O*-methylglucitol-*l*-*d*,

2,3,4-tri-*O*-methylfucose, 2,4,6- and 3,4,6-tri-*O*-methylgalactose, and 2,5-anhydro-4,6-di-*O*-methylmannose.

*N-Deacetylation-deamination of per-*O*-methylated lacto-*N*-tetraitol-*1-d (**2**) and laco-*N*-fucopentaitol-*1-d (**5**).* — Per-*O*-methylated tetrasaccharide (**2**, 18 mg) was *N*-deacetylated as already described. The resulting amine (**3**) and sodium nitrite (0.5 g) were dissolved in water, and *M* sulfuric acid was added dropwise to adjust and maintain the pH of the solution at 3.5–4 for 2 h at room temperature. The solution was extracted with dichloromethane, and the extract was dried and evaporated to a syrup (16 mg). A portion (8 mg) of the mixture and hydroxylamine hydrochloride (5 mg) were heated in pyridine (1 mL) for 1 h at 85°, acetic anhydride (1 mL) was added, and heating was continued for a further 0.5 h. Water was added to the cooled solution to decompose acetic anhydride, the aqueous solution was extracted with dichloromethane, and the organic layer was washed with water, dried, and evaporated. The products were examined by g.l.c.–m.s. (column *A*; 155°, 5°/min→210°, hold) and showed (*i*) a minor component (eluted at 170°) whose mass spectrum and retention time were indistinguishable from a derivative similarly prepared from 2,3,4,6-tetra-*O*-methyl-D-galactose, and (*ii*) a major peak (eluted at 210°) having the retention time of 2,5-anhydro-3-*O*-(2,3,4,6-tetra-*O*-methyl-*β*-D-galactopyranosyl)-4,6-di-*O*-methyl-D-mannononitrile[1] (**8**). The mass spectrum showed, *inter alia*, fragment-ions at *m/e* 230, 219, and 170, which were characteristic of this compound, but in addition fragment-ions at *m/e* 247 and 236 indicative of the presence of the permethylated disaccharide alditol acetate (**9**). A further portion (8 mg) of the deamination mixture was treated with sodium borodeuteride and the products were alkylated with trideuteriomethyl iodide and sodium hydride in methyl sulfoxide. Examination of the disaccharide products by g.l.c.–m.s. (column *A*; 210°), showed two components which were characterized, by identity of retention times with non-isotopically labelled compounds and by the relevant fragment ions in the mass spectra, as 2,5-anhydro-3-*O*-(2,3,4,6-tetra-*O*-methyl-*β*-D-galactopyranosyl)-4,6-di-*O*-methyl-1-*O*-(^{2}H)$_3$methyl-D-mannitol-*1-d* (**10a**) (mass spectrum identical to that of a previously prepared sample[1]) and 4-*O*-[2,4,6-tri-*O*-methyl-3-*O*-(^{2}H)$_3$methyl-*β*-D-galactopyranosyl]-1,2,3,5,6-penta-*O*-methyl-D-glucitol-*1-d* (**11**) [*m/e* 299 (8, abJ$_1$), 236 (63, bA$_1$), 222 (25, aA$_1$), 187 (51, aA$_2$), 101 (93), and 91 (100)]. Attempts to detect a trisaccharide component (such as **12**) in the mixture were unsuccessful.

Per-*O*-methylated pentasaccharide (**5**, 18 mg) was *N*-deacetylated and the resulting amine (**6**) deaminated as described for the tetrasaccharide (**3**). The resulting mixture was treated with sodium borodeuteride and the products were alkylated with trideuteriomethyl iodide. The mixture was analyzed by g.l.c.–m.s. on column *C*. Under isothermal conditions at 200° two disaccharide alditols were detected, (*i*) a minor component, $T_{\text{nona-}O\text{-methyllactitol}}$ 0.75, whose mass spectrum showed fragment ions characteristic of 2-*O*-(2,3,4-tri-*O*-methylfucopyranosyl)-3,4,6-tri-*O*-methyl-1,5-di-*O*-(^{2}H)$_3$methylgalactitol-*1-d* (**13a**) at *m/e* 302 (3, abJ$_1$), 242 (20, bA$_1$), 189 (29, aA$_1$), 180 (6), 136 (7), 92 (20), and (*ii*) a major component which was indistinguishable from the isotopically labelled permethylated lactitol (**11**) similarly derived from

per-*O*-methylated lacto-*N*-tetraitol-*l-d* (**2**). Examination of the mixture at 230° showed one major component, which was assigned structure **14** on the basis of fragment ions at *m/e* 457 (37, $abcJ_1$), 397 (3, bcA_1), 393 (1, baA_1), 365 (78, bcA_2), 361 (5, baA_2), 333 (8, bcA_3), 329 (1, baA_3), 253 (86, bcJ_1), 193 (64, cA_1), 189 (73, aA_1), 161 (31, cA_2'), and 158 (74, cA_2). Minor peaks were scanned, but none showed a fragment ion at *m/e* 236 which might have arisen from the trisaccharide **12**.

Asialo-oligosaccharide alditols from α_1-acid glycoprotein. — Asialoglycoprotein (200 mg), prepared by the procedure of Schmid *et al.*[21], was treated with *M* sodium borohydride–M sodium hydroxide for 6 h at 100°[11]. The mixture was made neutralized by the addition of acetic acid and desalted by passage through a column (25 × 2 cm) of Sephadex G-25. Carbohydrate-containing fractions were pooled and concentrated, and the oligosaccharide alditols in dilute sodium hydrogencarbonate solution were treated with acetic anhydride to ensure complete *N*-acetylation. A portion of the oligosaccharide preparation was methylated by the Hakomori procedure[4], the methylated oligosaccharide was purified by chromatography on a column (15 × 1 cm) of Sephadex LH-20 using 1 : 1 chloroform–acetone as eluant, and the neutral sugars formed on hydrolysis were analyzed after conversion into partially methylated alditol acetates by g.l.c.–m.s. on column *D* at 170°. The following sugars were identified [mol.(%) of total neutral sugars]: 2,3,4,6-tetra-*O*-methylgalactose (60%), 3,4,6-tri- (17%), 3,6- (7%), and 2,4- (possibly with some 3,4-)-di-*O*-methylmannose (16%). A further portion (20 mg) of the oligosaccharide preparation was *N*-deacetylated by hydrazinolysis, and the resulting amino-oligosaccharide was deaminated with nitrous acid as described previously. The solution was carefully made neutral with sodium hydroxide, sodium borohydride (100 mg) was added, and the solution was kept overnight. Excess of borohydride was decomposed and sodium ions were removed by the addition of Amberlite resin IR-120(H^+), and the solution was filtered and evaporated to dryness twice with methanol containing 2% of acetic acid. A portion of the residue was acetylated with acetic anhydride and pyridine, and g.l.c.–m.s. on column *E* at 200° showed a single monosaccharide component, indistinguishable from 2-deoxy-D-*arabino*-hexitol pentaacetate. The remainder (10 mg) of the residue was methylated[4] and the product purified on a column (30 × 3 cm) of Sephadex LH-20 by elution with 1 : 1 dichloromethane–acetone. The permethylated oligosaccharide-mixture (the relatively volatile monosaccharide component was lost during processing) was chromatographed on a column of Bio-Gel P-4 (400 mesh) with water as eluant to give: (*i*) a permethylated oligosaccharide whose mass spectrum (direct insertion) showed fragment ions at *m/e* 219 (aA_1), 187 (aA_2), and 155 (aA_3) from the non-reducing termini and at *m/e* 189 from the 2,5-anhydromannitol residue, but none at *m/e* 249 (J series) indicating an adjacent 3-*O*-substituted residue; and (*ii*) a permethylated disaccharide which was identified by g.l.c.–m.s. on column *E* at 210° as 2,5-anhydro-3(4)-*O*-(2,3,4,6-tetra-*O*-methylgalactopyranosyl)-1,4(3),6-tri-*O*-methylmannitol[1].

N-*Deacetylation–deamination of per*-O-*methylated asialo-oligosaccharide alditols.* — The oligosaccharide preparation was methylated with methyl sulfate and

aqueous 30% sodium hydroxide and completeness of methylation was checked by hydrolysis of a portion of the methylated derivative and analysis of the neutral sugars by g.l.c.–m.s. of the derived partially methylated alditol acetates on columns *D* and *E* at 170°. The permethylated oligosaccharide was *N*-deacetylated and the products were deaminated as described previously. The resulting syrup was treated with sodium borodeuteride and the reduction product was alkylated[4] with trideuteriomethyl iodide. This product was purified by chromatography on Sephadex LH-20 (1:1 dichloromethane–acetone); examination by g.l.c.–m.s. on column *E* at 210° showed a single disaccharide, which was indistinguishable from 2,5-anhydro-4-*O*-(2,3,4,6-tetra-*O*-methyl-β-D-galactopyranosyl)-3,6-di-*O*-methyl-1-($^2H)_3$methyl-D-(1-$^2H)_1$mannitol[1] (**16a**).

Isolation of oligosaccharide alditols from hog gastric mucin. — Hog gastric mucin (Sigma Chemical Co., St. Louis, MO 63178; 10 g) was stirred for 2 h in water (200 mL) containing 0.88% of sodium chloride and 0.02% of sodium azide, insoluble material was removed by centrifugation, and the supernatant liquid was dialyzed and freeze-dried to give purified mucin (4.5 g), which contained 28% of hexose[22] (as galactose) residues. In a typical small-scale preparation[23], purified mucin (45 mg) was heated in aqueous 0.05M sodium hydroxide (45 mL) containing M sodium borohydride (1.7 g) for 18 h at 55°. Excess of borohydride was decomposed by the addition of Dowex 50(H^+) resin and the filtrate was chromatographed on a column of the resin (200 mesh). Carbohydrate-containing fractions that emerged just after the void volume were combined and were repeatedly concentrated with methanolic 2% acetic acid to remove boric acid, and removal of residual salts was achieved by passage through a column of Bio-Gel P-2. The resulting oligosaccharide alditol preparation showed signals in the ^{1}H-n.m.r. spectrum (D_2O) at δ 1.3 (d, CH_3-CH of fucose residues) and 2.0 (s, NDAc), characteristic ring-proton signals in the range 3.2–5.0, but no signals for aromatic (δ 5–10) or aliphatic (δ 0–1) substituents, indicating absence of amino acid residues.

The oligosaccharide alditol preparation was methylated with methyl sulfate and aqueous 30% sodium hydroxide. A sample of per-*O*-methylated oligosaccharide was hydrolyzed and the hydrolyzate deaminated as described for per-*O*-methylated lacto-*N*-tetraitol (**2**). Analysis of the resulting sugars by g.l.c.–m.s. (column *B* at 180°) of derived partially methylated alditol acetates (reduction with sodium borodeuteride) showed the presence of (*i*) 2,3,4-tri-*O*-methylfucose, 2,3,4,6-tetra-, 2,3,6-, 2,4,6- and 3,4,6-tri-, and 2,4-di-*O*-methylgalactose, (*ii*) 3,4,6-tri- and 3,6-di-methyl ethers of 2,5-anhydromannose from the corresponding 2-acetamido-2-deoxyglucose residues, and (*iii*) two incompletely identified components, one a di-*O*-acetyl-2-deoxy-tri-*O*-methylhexitol, and probably formed from 2-amino-2-deoxygalactitol residues. A further portion of the oligosaccharide alditol preparation was methylated by the Hakomori procedure[4]. Hydrolysis[7] of the per-*N*,*O*-methylated derivative, followed by g.l.c.–m.s. analysis (column *A*; 170→210°, 4°/min, hold) of the derived partially methylated alditol acetates showed the presence of neutral sugars as described already in (*i*), 3,4,6-tri- and 3,6-di-*O*-methyl derivatives of 2-deoxy-2-*N*-methylacetamido-

TABLE I

G.L.C.–M.S. OF PERMETHYLATED DISACCHARIDE ALDITOLS FROM *N*-DEACETYLATION–DEAMINATION OF OLIGOSACCHARIDE CHAINS IN HOG GASTRIC MUCIN

Compound no.	**13**	**13a**	**21**	**21a**	**22**	**22a**	**16**	**16a**
Relative retention time[a]	2.78		2.92		3.05		3.80	
Procedure[b]	A	B	A	B	A	B	A	B
Fragment ions (m/e) *and relative abundance* (%)								
aA_1	189 (30)	189 (20)	219 (14)	222 (28) 219 (8)	219 (55)	222 (23) 219 (7)	219 (10)	222 (2) 219 (1)
aA_2	157 (21)	157 (15)	187 (54)	190 (76) 187 (61)	187 (98)	190 (56) 187 (31)	187 (45)	190 (10) 187 (7)
bA_1	236 (24)	242 (30)	206 (78)	212 (89) 209 (73)	235 (72)	241 (22) 238 (16)	190 (45)	193 (78)
bA_2			174 (38)	180 (76) 177 (56)	203 (10)	209 (3) 206 (3)	158 (60)	161 (63) 158 (18)
abJ_1	296 (6)	302 (15)	266 (5)		295 (10)	301 (3) 298 (2)	250 (92)	253 (100)
Ions from C–C bond cleavage in alditol residues	177 (6) 133 (7) 89 (52) 46 (14) 45 (58)	180 (6) 136 (9) 92 (15) 46 (15) 45 (60)	133 (7) 89 (67) 46 (30) 45 (100)	136 (6) 133 (6) 92 (100) 89 (98) 49 (49) 48 (39) 45 (64)	133 (26) 89 (94) 45 (100)	136 (15) 133 (12) 92 (66) 89 (39) 48 (31) 45 (100)		

[a]Retention times are relative to hexa-*O*-methylgalactitol on column *B* (temperature programmed as stated in the Experimental section). [b]A. *N*-Deacetylation–deamination of oligosaccharide alditol preparation from hog gastric mucin, followed by reduction with sodium borodeuteride and methylation[4]. B. *O*-Methylation of oligosaccharide alditol preparation, *N*-deacetylation–deamination of methylated derivatives, followed by reduction with sodium borodeuteride and trideuteriomethylation.

glucose, and 1,4,5,6-tetra- and 1,4,5-tri-methyl ethers of a 2-deoxy-2-*N*-methylacetamidohexitol, presumably formed from 2-acetamido-2-deoxygalactitol termini.

N-*Deacetylation–deamination of oligosaccharide alditols.* — The oligosaccharide alditol preparation (102 mg) was boiled under reflux in hydrazine (5 mL) containing hydrazine sulfate (48 mg) for 14 h. The cooled solution was concentrated by evaporation with toluene. Acetic acid (3 mL) was added dropwise with cooling to the residue in water (2 mL) containing sodium nitrite (0.5 g). After 45 min, urea was added to decompose the excess of nitrous acid, the solution was desalted by passage through a column (30 mL) of Bio-Gel P-2, and the carbohydrate-containing fractions were concentrated. This material was treated with sodium borodeuteride overnight and, after normal processing, furnished a mixture (40 mg) of alditols whose ^{1}H-n.m.r. spectrum (D_2O) showed, *inter alia*, a signal at δ 1.1 (d, CH_3-CH of fucose residues), a weak multiplet at δ 2.0 (CH_2 of deoxyhexitol residues), but no sharp singlets assignable to residual acetamido substituents. A sample of alditols and oligosaccharide alditols was methylated[4] and the permethylated derivatives were examined by g.l.c.–m.s. under the following conditions: (*1*) for alditols and disaccharide alditols, column *B*, 140°, 4 min, 4°/min→210°, hold [for comparative purposes, relative retention-times are quoted for the disaccharide alditols in Table I], or column *A*, 100→210°, 6°/min, and then hold; (*2*) for the most complete separation of disaccharide alditols, column *F*, at 200°; (*3*) for the trisaccharide alditol fraction, column *G* at 235°, or column *C*, 200°, 5 min, 4°/min→240°, hold. Under (*1*), monosaccharide alditols detected were a 2-deoxypenta-*O*-methylhexitol-*1-d* (**18**), *T* 0.66, *m/e* 160 (5), 148 (15), 133 (6), 104 (30), 89 (12), 46 (62), and 45 (100), and 2,5-anhydro-1,3,4,6-tetra-*O*-methylmannitol-*1-d* (**20**), *T* 0.96, and hexa-*O*-methylgalactitol-*1-d* (**19**), *T* 1.00, both of which gave mass spectra identical to those of synthetic samples. Disaccharide alditols **13** and **16** had retention times identical to those of previously characterized samples. The important fragment ions used in the assignment of structures for permethylated disaccharide alditols are summarized in Table I. G.l.c. of the trisaccharide fraction gave a broad peak with no clear resolution into separate components. The major component was assigned structure **23** on the basis of fragment ions at *m/e* 454 (20, abcJ$_1$), 394 (2, bcA$_1$), 393 (4, baA$_1$), 362 (10, bcA$_2$), 361 (12, baA$_2$), 330 (10, bcA$_3$), 329 (3, baA$_3$), 250 (50, bcJ$_1$), 206 (65), 190 (100, cA$_1$), 189 (65, aA$_1$), and 158 (92, cA$_2$). The presence of an abundant ion at *m/e* 206 (65) pointed to the possible existence of methylated trisaccharide alditol **24**. The fragment ion at *m/e* 235 was of too low abundance to provide convincing evidence for the presence of methylated trisaccharide alditol **25**.

Per-*O*-methylated oligosaccharide alditol preparation was boiled under reflux in hydrazine (3 mL) containing 6% of hydrazine sulfate for 12 days. The cooled solution was concentrated by evaporation with toluene and the residue was dissolved in water (3 mL) containing sodium nitrite (250 mg). M Sulfuric acid was added to maintain the pH of the solution at 3.5–4.0 for 1 h. Urea was added to the solution to decompose excess of nitrous acid, sodium borodeuteride (50 mg) was added, and the solution was kept overnight. The solution was processed conventionally and the

residue alkylated[4] with trideuteriomethyl iodide. The mixture of permethylated alditols and oligosaccharide alditols, which was examined by g.l.c.–m.s., was shown to contain components having the same retention-times as those already described, and the mass-spectral data for structural assignments of the methylated disaccharide alditols are summarized in Table I. The mass spectrum of the hexa-*O*-methylgalactitol-*1-d* (**19a**) showed the expected fragment-ions for a compound having trideuteriomethyl groups at O-1 and O-5, but additional ions at *m/e* 183 and 184 indicated that some molecules also contained a trideuteriomethyl group at O-4. The methylated trisaccharide alditol fraction showed, *inter alia* fragment ions at *m/e* 457 (6, $abcJ_1$), 397 (1, bcA_1), 393 (1, baA_1), 365 (16, bcA_2), 361 (2, baA_2), 333 (3, bcA_3), 329 (1, baA_3), 253 (16, bcJ_1), 212 (10), 209 (4), 193 (36, cA_1), 189 (47, aA_1), 161 (31, cA_2), and 158 (16, cA_2), consistent with the presence of two components **23a** and **24a**.

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