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STRUCTURE OF SOME OLIGOSACCHARIDES DERIVED FROM RAT-INTESTINAL GLYCOPROTEINS

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ABSTRACT

Glycoproteins derived from intestinal mucus were isolated by phenol–water extraction of feces from germ-free rats. The water-soluble glycoproteins were subjected to alkaline-borohydride degradation, and three different oligosaccharide alditols were isolated. The structures of these were determined by using methylation analysis and n.m.r. spectroscopy as the principal methods. One disaccharide alditol and one trisaccharide alditol were characterised as β-D-GlcNAc*p*-(1→3)-D-GalNAcol and α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-D-GalNAcol. The third oligosaccharide alditol was a blood-group A-active tetrasaccharide alditol for which the structure α-D-GalNAc*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-D-GalNAcol is proposed.

INTRODUCTION

Mucus in the alimentary tract is rapidly degraded by the intestinal flora[1,2]. Microbial secretions and breakdown products may be difficult to distinguish from material of animal origin, and can complicate analysis of the structure of native mucous secretions. In animals reared under germ-free conditions, microbial contaminants are absent and the mucus passes through the intestine almost unaltered, to be discharged in high amounts in the feces[1,2]. Furthermore, if the animals are fed a semisynthetic diet[3], contaminants of high molecular weight derived from the food are avoided.

Water-soluble glycoproteins, which constitute a major part of the mucus, can be readily obtained from germ-free rat feces by phenol–water extraction. These glycoproteins contain 60–80% of carbohydrate and are polydisperse with respect to molecular size and charge[5,6]. All or most molecular species express blood-group activities A and/or H of the human ABO system to varying degrees[4,5,7]. Organ-specific antigens of carbohydrate nature are also present in the extract[4].

Isolation and analysis of the carbohydrate side-chains from the intestinal glycoproteins of germ-free rats would help to elucidate the structure of native, undegraded mucus.

RESULTS AND DISCUSSION

Glycoproteins were extracted from germ-free rat feces by the phenol–water procedure[4]. The water-soluble glycoproteins contain L-fucose, D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, sialic acid, and small proportions of D-mannose[4,5]. The assignments of the L configuration to fucose and the D configuration to the other sugars were made by analogy with common practice in the field.

The glycoproteins were treated with alkaline borohydride[8], whereby carbohydrate–serine/threonine linkages were cleaved, and the products were subjected to gel filtration on Sephadex G-50. A carbohydrate fraction of low molecular weight (<3000) was isolated. This fraction contained the same sugar components as the undegraded material, except for D-mannose, but only trace amounts of protein and nucleic acid. The mixture of oligosaccharide alditols was subjected to ion-exchange chromatography on Dowex 1 X2, which quantitatively removed sialic acid-containing oligosaccharide alditols (~50% of the total material). The remaining, neutral oligosaccharide alditols were included on gel filtration on Bio-Gel P-2, and three main fractions were eluted in the regions expected for mono-/di-saccharide, tri-/tetrasaccharide, and penta-/deca-saccharide, respectively. These fractions were further fractionated by preparative paper chromatography. Three oligosaccharide alditols (**1**–**3**) were isolated as chromatographically homogeneous products (p.c. and t.l.c.) in amounts sufficient for detailed structural analysis.

Oligosaccharide alditol **1**, isolated from the foregoing mono-/di-saccharide fraction, showed $[\alpha]_{589}$ −28°. When an acid hydrolysate of **1** was acetylated, and investigated by g.l.c.[9]–m.s.[10], 2-acetamido-2-deoxy-D-galactitol was identified as the terminal. When the products of hydrolysis of **1** were converted into alditol acetates and analysed by g.l.c.–m.s., 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactitol were revealed in the ratio 1:1.3. Here, and subsequently, quantitative values for acetamido-sugar derivatives are somewhat uncertain, because of difficulties in obtaining reproducible response factors in g.l.c. The n.m.r. spectrum of **1** showed, *inter alia*, signals at δ 2.03 and 2.07 (2 s, each 3 H, *N*-acetyl groups of 2-acetamido-2-deoxy-D-galactitol and 2-acetamido-2-deoxy-D-glucose), and at δ 4.61, $J_{1,2}$ 7 Hz (1 H, anomeric proton of 2-acetamido-2-deoxy-D-glucose). These data confirm the presence of equimolar amounts of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactitol, as suggested by the sugar analysis. The large $J_{1,2}$ value indicates that the 2-acetamido-2-deoxy-D-glucosyl group is β-linked, which accords with the negative optical rotation. Moreover, the results indicate that **1** is a disaccharide alditol.

Methylation analysis[11–15] of **1** yielded 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-glucose and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol, identified by g.l.c.–m.s. of their alditol acetates. Thus, the presence of a terminal 2-acetamido-2-deoxy-D-glucosyl group linked to O-3 of a 2-acetamido-2-deoxy-D-galactitol unit is indicated.

From the combined evidence given above, the proposed structure of oligosaccharide alditol **1** is β-D-GlcNAc*p*-(1→3)-D-GalNAcol.

Oligosaccharide alditol **2**, isolated from the tri-/tetra-saccharide fraction, had $[\alpha]_{589}$ −45°. 2-Acetamido-2-deoxy-D-galactitol was found in an acid hydrolysate of **2**. Sugar analysis, by g.l.c.–m.s. of the alditol acetates prepared from an acid hydrolysate of **2**, revealed L-fucose (34%), D-galactose (40%), and 2-acetamido-2-deoxy-D-galactitol (25%). The n.m.r. spectrum of **2** showed, *inter alia*, signals at δ 1.24, $J_{5,6}$ 6 Hz (d, 3 H, methyl group of L-fucose), 2.04 (s, 3 H, *N*-acetyl group of 2-acetamido-2-deoxy-D-galactitol), 4.58, $J_{1,2}$ 8 Hz (d, 3 H, H-1 of D-galactose), and 5.27, $J_{1,2}$ 3 Hz (d, 1 H, H-1 of L-fucose). The equally strong signals in the *N*-acetyl and methyl regions, and in the anomeric region, respectively, suggest equimolar amounts of L-fucose, D-galactose, and 2-acetamido-2-deoxy-D-galactitol. The coupling constants (3 and 8 Hz, respectively) and the chemical shifts observed in the anomeric region (δ 5.27 and 4.58, respectively) indicate the presence of one α-linked and one β-linked sugar unit. From the strong, negative optical rotation, the L-fucosyl unit should be α-linked and the D-galactosyl unit β-linked. The data, furthermore, strongly suggest that **2** is a trisaccharide alditol. Methylation analysis yielded 2,3,4-tri-*O*-methyl-L-fucose and 3,4,6-tri-*O*-methyl-D-galactose in approximately equimolar amounts (0.8 : 1), identified as their alditol acetates by g.l.c.–m.s. The presence of 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol was also observed. These data indicate the presence of one terminal L-fucosyl group, one D-galactosyl residue linked through O-2, and one 2-acetamido-2-deoxy-D-galactitol unit linked at O-3.

The above results together suggest the following structure for oligosaccharide alditol **2**: α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-D-GalNAcol.

Oligosaccharide alditol **3**, obtained in the largest amount and also isolated from the tri-/tetra-saccharide fraction, had $[\alpha]_{589}$ −35° and gave 2-acetamido-2-deoxy-D-galactitol on acid hydrolysis. L-Fucose and D-galactose were found in equimolar proportions (23 and 26%, respectively) in an acid hydrolysate of **3**, with analysis by g.l.c.–m.s. after transformation of the sugars into alditol acetates. A large proportion (42%) of 2-acetamido-2-deoxy-D-galactitol was also present. The n.m.r. spectrum of **3** showed, *inter alia*, signals at δ 1.24, $J_{5,6}$ 6 Hz (d, 3 H, methyl group of L-fucose), 2.06 (overlapping s, 6 H, *N*-acetyl groups of 2-acetamido-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-galactitol), 4.67, $J_{1,2}$ 8 Hz (d, 1 H, anomeric proton), 5.22, $J_{1,2}$ 4 Hz (d, 1 H, anomeric proton), and 5.37, $J_{1,2}$ 3 Hz (d, 1 H, anomeric proton). These n.m.r. data indicate a molar ratio of 1 : 2 for L-fucose to acetamido sugars, and the presence of equimolar amounts of L-fucose and D-galactose. The large coupling constant (8 Hz) and the doublet observed in the high-field part of the anomeric region (δ 4.67) indicate the presence of one β-linked sugar residue. The low coupling constants (4 and 3 Hz, respectively) of the two doublets in the low-field part of the anomeric region (δ 5.22 and 5.37, respectively) indicate the presence of two α-linked units. The data further suggest that **3** is a tetrasaccharide alditol.

Methylation analysis of **3** yielded 2,3,4-tri-*O*-methyl-L-fucose and 4,6-di-*O*-methyl-D-galactose in approximately equimolar amounts (0.8 : 1), identified by g.l.c.–

m.s. of their alditol acetates. Also present were 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactose and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol in approximately equal proportions.

The anomeric configuration of the L-fucose, 2-acetamido-2-deoxy-D-galactose, and D-galactose residues cannot be unambiguously determined from the values given above. Likewise, the position of the terminal L-fucosyl group and the terminal 2-acetamido-2-deoxy-D-galactosyl group cannot be assigned. However, the negative optical rotation suggests that the D-galactosyl residue is β-linked and that the L-fucosyl group is α-linked. The 2-acetamido-2-deoxy-D-galactosyl group should then be α-linked. The A-hemagglutinin (HPA) from the snail *Helix pomatia* reacts specifically with terminal 2-acetamido-2-deoxy-D-hexosyl groups[16]. The affinity of HPA for the α anomer is considerably stronger (>tenfold) than that for the β anomer[17]. About 7.5 nmol of **3** were needed to inhibit 50% of the precipitation reaction between HPA and guaran from *Cyamopsis tetragonolobus*. In the same experiment, 2.5 and 35 nmol of methyl 2-acetamido-2-deoxy-α-D-galactoside and the corresponding β anomer, respectively, were needed for 50% inhibition. The weaker inhibiting capacity of **3**, relative to methyl 2-acetamido-2-deoxy-α-D-galactoside, was expected, as an L-fucosyl group bound to D-galactose in blood-group substance A impairs the binding of HPA[16]. These inhibition experiments thereby support the assumption of an α-linked, terminal 2-acetamido-2-deoxy-D-galactosyl group in **3**. Moreover, 250 μg of **3** completely inhibited the reaction between four hemagglutinating units of a human anti-A anti-serum and A red-blood cells. This confirms the presence of a terminal, α-linked 2-acetamido-2-deoxy-D-galactosyl group and suggests that it is linked to O-3 of the D-galactosyl residue.

Based on the foregoing results, the following structure is proposed for **3**: α-D-GalNAc*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-D-GalNAcol.

Our findings, which confirm and extend those of Wold and co-workers[18], indicate that the majority of the carbohydrate side-chains of glycoproteins from rat-intestinal mucins are glycosidically linked through 2-acetamido-2-deoxy-D-galactosyl residues to the polypeptide. This finding is also in agreement with the analysis of the intact glycoprotein, showing that the polypeptide moiety contains ~50% of threonine and serine[5,6].

Oligosaccharide alditols **2** and **3** have previously been isolated from pig submaxillary gland, after degradation with alkaline borohydride[19]. Other mucins, *e.g.*, from canine submaxillary gland[20], equine gastric mucosae[21], and human ovarial-cyst fluid[22], contain oligosaccharides having identical or similar sequences. Moreover, Wold *et al.*[18] isolated two oligosaccharides after alkaline-borohydride degradation of intestinal mucin from germ-free rat. One of these was a trisaccharide alditol identical to our alditol **2**, and the other was the corresponding disaccharide alditol lacking the L-fucosyl group. These oligosaccharide alditols may constitute parts of oligosaccharide alditol **3**, the corresponding oligosaccharide chains giving rise to blood-group A activity in the glycoprotein[4]. Similarly, chains corresponding to **2** may be responsible for the blood-group H activity in the glycoprotein[4]. The immuno-

dominant sugars and their anomeric configurations are identical to those[22,23] of human blood-group substance A and H. The finding of oligosaccharide alditol **1**, in this study, and the disaccharide *O*-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucose, isolated after acetolysis by Wold *et al.*[18], suggests the presence of at least one more type of carbohydrate chain.

EXPERIMENTAL

General methods. — Sugars were analysed as their alditol acetates by g.l.c. on a Perkin–Elmer F-30 instrument (flame-ionisation detector), with arabinose as the internal standard. Separations were performed on glass columns (180 × 0.2 cm) with Gas Chrom Q (100–120 mesh) as support material carrying 3% of ECNSS-M (sugar alditol acetates and partially permethylated, neutral-sugar alditol acetates) and 3.8% of UCW-98 (partially permethylated, acetamido-sugar alditol acetates). For identification of the alditol acetates, g.l.c.–m.s. was performed on a Perkin–Elmer 270 instrument fitted with the above-mentioned columns. N.m.r. spectra were obtained at 100 MHz (for solutions in D_2O at 85°, with sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulphonate as the internal standard) with a Varian XL-100 instrument operated in the PFT-mode. Optical rotations were measured on a Perkin–Elmer 141 instrument (100-mm semi-micro cells). Thin-layer chromatography (t.l.c.) was performed on cellulose plates (included fluorescence indicator; DC-Fertigplatten Cellulose F, Merck) with 1-butanol–pyridine–water (35:39:26). The plates were examined under u.v.-light, and developed with iodine vapour. Paper chromatography (p.c.) was performed on Whatman No. 1MM paper (analytical) and No. 3MM paper (preparative). The following solvents were employed: *A*, ethyl acetate–acetic acid–water (3:1:1); *B*, 1-butanol–pyridine–water (6:4:3); and *C*, 1-butanol–acetic acid–water (44:16:40). Sugars were located with alkaline silver nitrate[24]. Gel filtrations were performed on a column (2.5 × 100 cm) of Sephadex G-50 Fine irrigated with 0.05M pyridinium acetate buffer (pH 5.5), and on a column (1.5 × 90 cm) of Bio Gel P-2 (200–400 mesh) irrigated with distilled water. Ion-exchange chromatography was performed on a column (3 × 30 cm) of Dowex 1 X2 (Cl^-) resin. The effluent from gel-filtration and ion-exchange chromatography was monitored for protein at 280 nm, for hexoses by the orcinol–sulfuric acid method[25], and for sialic acid as described by Warren[26].

Isolation of oligosaccharide alditols. — Water-soluble, intestinal glycoproteins from germ-free rats were obtained by phenol–water extraction of feces and purified as described earlier[4]. Degradation with alkaline borohydride was performed as described by Carlsson[8]. The glycoprotein (10 g) was dissolved in 0.05M NaOH containing M $NaBH_4$ (200 ml) and maintained at 45° for 16 h. The excess of borohydride was decomposed by neutralization with 4M acetic acid to pH 5, and borate was removed by repeated evaporation with methanol. The material was dialysed (Union Carbide dialysis bag) twice (30 min) against water (4 litres) to remove the bulk of the remaining salt. The dialysate was concentrated by evaporation and subjected to gel filtration

on Bio Gel P-2. The majority of the material appeared as one peak in the monosaccharide region. Sugar analysis (g.l.c.–m.s. of alditol acetates) of this material yielded ~70% of 2-acetamido-2-deoxy-D-galactitol. No further purification of monosaccharide alditols was performed.

The content of the dialysis bag was concentrated by evaporation and subjected to gel filtration on Sephadex G-50. Two main fractions were obtained (orcinol–sulphuric acid reaction); the excluded fraction (1.0 g) contained most of the remaining protein, and a fraction (3.3 g) of low molecular weight (with low absorption at 280 nm) was obtained at the end of the fractionation range. Between these fractions was an area of unresolved material (2.4 g). The fraction of low molecular weight (<3000) containing 12.5% of sialic acid, was subjected to ion-exchange chromatography on Dowex 1 X2 (Cl^-) resin. The sample (1.5 g) was applied in water, and the column was eluted until the effluent was negative in the orcinol–sulphuric acid reaction. Carbohydrate-containing fractions were combined and lyophilized; yield, 563 mg (<2% of sialic acid). The adsorbed material was eluted with 0.5M NaCl and subjected to gel filtration on Bio Gel P-2 to remove salt; yield, 543 mg (22% of sialic acid). The sialic acid-containing material was eluted with the void volume of the P-2 gel; since it could not be sufficiently resolved in analytical p.c. with solvents *A-C*, no further attempts at purification were made.

Gel filtration of the neutral oligosaccharide alditols on Bio Gel P-2 yielded three main fractions. Two were eluted in the mono-/di-saccharide (57 mg) and the tri-/tetra-saccharide (136 mg) regions, respectively, and one broader, unresolved fraction was eluted in the penta-/deca-saccharide region (148 mg). Solvent *A* was used for further separation by preparative p.c. The presence of several compounds was revealed by the silver nitrate–hydroxide reaction. Compound **1** was isolated from the mono-/di-saccharide fraction, and compounds **2** and **3** from the tri-/tetra-saccharide fraction. The constituents of the penta-/deca-saccharide fraction had very low mobilities and were poorly resolved. No homogeneous compound was isolated from this fraction. Compounds **1–3** were chromatographically homogeneous in p.c. (solvents *A–C*) and in t.l.c., and they all appeared as narrow, single peaks on concomitant gel-filtration on Bio Gel P-2.

Sugar and methylation analysis. — Methylation of oligosaccharide alditols was carried out according to the method of Hakomori[27]. The products were recovered by partition between water and chloroform, and purified on a Sephadex LH-20 column. Sugars and methylated sugar derivatives were analysed by g.l.c.–m.s. as their alditol acetates[11–15]. To avoid loss of amino sugars after the reduction step, the excess of sodium borohydride was decomposed by the addition of 50% aqueous acetic acid. After acetylation in acetic anhydride–pyridine and evaporation of the reagents, the alditol acetates were extracted into chloroform.

Precipitation inhibition. — The ability of oligosaccharide alditols to inhibit the reaction between *Helix pomatia* A-hemagglutinin (HPA) and guaran was investigated by a microprecipitin technique[28]. Serial dilutions of inhibitor (1–100 nmol) in physiological buffered (pH 7.3) saline (PBS) were mixed with 20 µl of a solution of guaran

(1 mg/ml) from *C. tetragonolobus* purified by ethanol precipitation. To this mixture was added 20 µl of HPA (1.3 mg/ml) to give a final volume of 200 µl. The samples were incubated in the cold for 3 days to allow complete precipitation. The precipitates were washed once by centrifugation, and analysed for nitrogen by the ninhydrin procedure of Schiffman[29].

Hemagglutination inhibition. — Hemagglutination inhibition was performed as described earlier[4]. A high-titered anti-A serum (1/128) obtained from a woman of blood-group O was utilized. To two-fold dilution series of inhibitor (0.5–1000 µg/ml) in 0.1 ml of PBS was added an equal volume of antiserum diluted to contain four hemagglutinating units (1/32). After incubation for 30 min at room temperature (25°), 0.1 ml of a 1% suspension of A-erythrocytes was added. Hemagglutination was recorded after incubation overnight (18 h) in the cold. Controls without antiserum or antigen were always included. All determinations were repeated once in independent experiments, with identical results.

Analysis of oligosaccharide alditols **1–3.** — Of each compound, ~0.5 mg was used for sugar and methylation analysis, respectively. Alditol **1** (2.2 mg) had $[\alpha]_{589}$ −28° (*c* 1.3, water), R_{Gal} 0.96 (p.c., solvent *A*), and R_{Lac} 1.15 (t.l.c.). After hydrolysis, reduction, and acetylation, the derived alditol acetates were analysed by g.l.c.–m.s. (ECNSS-M column, 170→210°, 3° per min). Two peaks were obtained with relative retention times corresponding to those of 2-amino-2-deoxyglucitol and 2-amino-2-deoxygalactitol, in the molar ratio 0.9:1. After conversion into alditol acetates, methylated, hydrolysed **1** gave two peaks in g.l.c. (UCW-98 column, 190°), identified by m.s. as 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucitol and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)galactitol.

Oligosaccharide alditol **2** (3.0 mg) had $[\alpha]_{589}$ −45° (*c* 1.7, water), R_{Gal} 0.60, R_{Mal} 1.36 (p.c., solvent *A*), and R_{Lac} 1.05 (t.l.c.). The alditol acetates obtained from **2** by sequential hydrolysis, reduction, and acetylation gave three peaks in g.l.c. (ECNSS-M column 170→210°, 3° per min). Their relative retention times corresponded to fucitol, galactitol, and 2-amino-2-deoxygalactitol (molar ratios, 0.9:1:0.5). Methylation of **2**, followed by hydrolysis and conversion into alditol acetates, yielded three peaks in g.l.c. (ECNSS-M column, 170°). Two of these were identified from their relative retention times and mass spectra as 2,3,4-tri-*O*-methylfucitol and 3,4,6-tri-*O*-methylgalactitol. The third peak (UCW-98 column, 190°) had a retention time and mass spectrum identical to those of 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)galactitol (*cf.* **1**).

Oligosaccharide alditol **3** (23.9 mg) had $[\alpha]_{589}$ −35° (*c* 10, water), R_{Gal} 0.42, R_{Mal} 0.91 (p.c., solvent *A*), and R_{Lac} 0.87 (t.l.c.). After hydrolysis of **3** and conversion of the products into alditol acetates, three peaks were obtained in g.l.c. (ECNSS-M column, 170→210°, 3° per min). Their relative retention times corresponded to fucitol, galactitol, and 2-amino-2-deoxygalactitol (molar ratios, 0.9:1:1.6). Alditol acetates derived from methylated and hydrolysed **3** gave four peaks in g.l.c. (ECNSS-M column, 170°). Two of these were identified as 2,3,4-tri-*O*-methylfucitol and 4,6-di-*O*-methylgalactitol from their relative retention times and mass spectra (*cf.* **2**).

The other two peaks (UCW-98 column, 190°) showed retention times and mass spectra indicative of 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)galactitol and 2-deoxy-1,4,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)galactitol (*cf.* **1** and **2**).

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