Novel Polysaccharides with Blood Group A, H, X, and Y Determinants from Human Small Intestinal Epithelial Cells*

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Jukka Finne§, Michael E. Breimer§, Gunnar C. Hansson§, Karl-Anders Karlsson§, Hakon Leffler§, Johannes F. G. Vliegenthart§, and Herman van Halbeek**

From the §Department of Medical Biochemistry, University of Turku, SF-20520 Turku, Finland and the **Department of Medical Biochemistry, University of Göteborg, S-40033 Göteborg, Sweden, the §Department of Bio-Organic Chemistry, University of Utrecht, NL-3522 Utrecht AD, The Netherlands, and the **Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30613

A novel type of N-linked glycopeptide representing a major part of the glycans in human small intestinal epithelial cells from blood group A and O individuals were isolated by gel filtrations and affinity chromatography on concanavalin A-Sepharose and Bandeiraea simplicifolia lectin I-Sepharose. Sugar composition, methylation analysis, 1H NMR spectroscopy of the underivatized glycopeptides and FAB-mass spectrometry and electron impact-mass spectrometry of the permethylated glycopeptides indicated a tri- and tetra-antennary structure containing an intersecting N-acetylgalactosamine and an α(1→6)-linked fucose residue in the core unit for the majority of the glycans. In contrast to most glycoproteins of other sources, the intestinal glycopeptides were devoid of sialic acid, but contained 6–7 residues of fucose. The outer branches contained the following structures:

\[
\begin{align*}
\text{Fuco1-2Gal1-3GlcNAc21} & \quad \text{(H type 1)} \\
\text{Fuco1-2Gal1-4GlcNAc21} & \quad \text{(H type 2)} \\
\text{Gal1-4(Fuco1-3)GlcNAc21} & \quad \text{(X)} \\
\text{Fuco1-2Gal1-4(Fuco1-3)GlcNAc21} & \quad \text{(Y)} \\
\text{GalNAc1-3(Fuco1-2)Gal1-3GlcNAc21} & \quad \text{(A type 1)} \\
\text{GalNAc1-3(Fuco1-2)Gal1-4GlcNAc21} & \quad \text{(monofucosyl A type 2)} \\
\text{GalNAc1-3(Fuco1-2)Gal1-4(Fuco1-3)GlcNAc21} & \quad \text{Galfucosyl A type 2)} \\
\text{GalNAc1-3(Fuco1-2)Gal1-4(Fuco1-3)GlcNAc21} & \quad \text{(trifucosyl A type 2)}
\end{align*}
\]

The blood group determinant structures were mainly of type 2, whereas glycolipids from the same cells contained mainly type 1 determinants. The polysaccharides represent a novel type of blood group active glycopeptides. The unique properties of the small intestinal glycopeptides as compared with glycopeptides of other tissue sources may be correlated with the specialized physiological properties of the small intestinal epithelial cells.

The epithelial surface of the small intestine, consisting of a single layer of cells, has many important physiological functions, which may be long-term absorption of nutrients and the formation of a barrier to chemical and infectious agents of the gastrointestinal tract. For such function, the properties of the cell surface components are anticipated to be of major importance. The small intestinal epithelium has been found to be unusually rich in glycosphingolipids (1–3). Moreover, some glycolipids, such as the blood group active glycolipids, seem to be especially characteristic of this tissue (1–3).

In view of the many structural and antigenic similarities between glycoproteins and glycolipids (4), it would be of interest to get information of the properties of protein-bound carbohydrates of the small intestinal epithelium. Such information could also give indications as to the functional properties of these glycans. Although studies have been carried out on the properties of the free mucus or soluble glycoprotein enzymes isolated from the gastrointestinal tract (5, 6), little is known of the properties of the cellular glycoproteins and their carbohydrate units. In the present work we describe the isolation and characterization of human small intestinal epithelial glycoproteins, which appear to represent a novel type of blood group active carbohydrate units of glycoproteins.

**EXPERIMENTAL PROCEDURES**

Isolation of Intestinal Epithelial Cells—Specimens of human small intestine were obtained during surgery. Permission was given by the Ethical Committee, the Medical Faculty, University of Göteborg. A sample of proximal ileum from a patient with the blood group status A, Le(a+b-) secretor and a sample of proximal jejunum from a patient with the blood group status HLe(a+b--) secretor were studied. The blood group A patient was operated on because of a leiomyoma of the small intestine. The blood group O patient was resected ad modum Billroth II 30 years earlier and was now being treated with total gastrectomy due to a ventricular cancer. The intestinal specimens were free of tumor tissue. The blood group status of the patients was established by routine typing of the red blood cells and saliva per-
formed at the Blood Bank, Sahlgrenka Hospital, Göteborg. The total epithelial cells were isolated by incubation in EDTA-containing buffer, as previously described (7).

**Extraction and Analysis of Glycolipids**—The epithelial cells were extracted with methanol and chloroform/methanol 1:2 (v/v) as previously described (7). The total nonacetyl glycolipids were isolated from this extract (8) and analysed by thin layer chromatography, mass spectrometry, and $^1$H NMR spectroscopy as described elsewhere (2, 7, 9, 10). The delipidated cells were further extracted as described by Svennerholm and Fredman (11) to ensure extraction of the more polar glycolipids, but no significant amounts of glycolipids were detected in this extract as analysed by thin layer chromatography, mass spectrometry, and $^1$H NMR spectroscopy.

**Preparation and Fractionation of Glycopeptides**—Aliquots of 200 mg of the dried delipidated cells were subjected to extensive digestion with Pronase as previously described (12). After removal of the material precipitable with cetylpyridinium chloride, the total glyco-epitopes were purified by gel filtration on a column (2 × 75 cm) of Sephadex G-25 Fine eluted with 10 mM pyridine-acetic acid buffer, pH 5.0 (12). The glycopeptides were fractionated by gel filtration on a column (2 × 75 cm) of Sephadex G-50 Fine eluted with 0.1 M pyridine-acetic acid buffer, pH 5.0.

Prior to affinity chromatography, the glycopeptides were passed through columns of Dowex 50W-X16 in order to remove residual free peptides, and N-$^1$H-acetylated in their peptide moiety (12). The glycopeptides were separated on a column (4.0 × 5.2 cm) of concanavalin A-Sepharose by elution with a two-step gradient of methyl-$^1$D-glucoside (12). The glycopeptide fractions were desalted by gel filtration on Sephadex G-50 and subjected to affinity chromatography on a column of Bandeiraea simplicifolia lectin I-Sepharose under conditions described before (14).

**Analytical Methods**—The sugar composition of the glycopeptides was determined by gas-liquid chromatography after methanlysis (15), using myo-inositol as an internal standard. Detection of the glycopeptides in gel filtration was carried out by the anthrone reaction (16) or by radioactivity (12). Permethylation of the glycopeptides was carried out using potassium t-butoxide as a base (17). The permethylated samples were degraded by acetylation/acid hydrolysis and analyzed as their alditol acetates by gas-liquid chromatography using selective ion monitoring as previously described (18). Deacetylation of aliquots (40-50 nmol) of the glycopeptides was carried out for methylation analysis or NMR purposes by treatment with 0.1 M HCl at 100 °C for 1 h; the excess HCl was removed under vacuum overnight (19).

**$^1$H NMR Spectroscopy**—Samples for NMR experiments were repeatedly dissolved in $^2$H$_2$O (99.96 atom % $^2$H, Aldrich) at room temperature and pH 6 with intermediate lyophilization. The deuterium-exchanged glycopeptides were subjected to $^1$H NMR spectroscopy at 500 MHz (Bruker WM-500 instrument equipped with an Aspect-2000 computer, SON NMR facility, Dept. Biophysical Chemistry, University of Nijmegen, the Netherlands). Spectra were recorded at room and elevated probe temperatures (27 and 52 °C, respectively). For solvent peak suppression in the spectra of fraction AS and its deacetylated analogue, a water-eliminated Fourier transform pulse sequence ($\pi$-r-\(\pi/2\)-acquisition, with composite, nonselective $\pi$-pulse) was applied (20). Further experimental details have been described (21). $^1$H chemical shifts are expressed in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate. They were actually measured relative to internal acetone ($\delta$ 2.225) with an accuracy of 0.002 ppm.

**Mass Spectrometry**—100 μg of the OS glycopeptides and 60 μg of the AS glycopeptides were permethylated and LiAlH$_4$-reduced (22, 23). The samples were analysed on a VG ZAB-HP mass spectrometer (Manchester, United Kingdom) equipped with a VG 11.250 data system. Both the electron impact-mass spectrometry (EI-MS) and the fast atom bombardment-mass spectrometry techniques were applied in the positive mode. The calibration was controlled by manually counting peaks in paper spectra. The figures reproduced refer to the nominal masses obtained this way.

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1 The abbreviations used are: EI-MS, electron impact mass spectrometry; Cer, ceramide; Fuc, fucose; GalNAc-ol, N-acetylgalactosaminitol; Hex, hexose; HexNAC, N-acetylatedhexosamine.
The blood group A glycopeptides were retarded and required several column volumes of buffer for elution. In contrast to the blood group A active glycopeptides from human erythrocytes chromatographed under the same conditions (14), only minor amounts of the blood group A small intestinal glycopeptides were bound to the column and eluted in the elution buffer, when galactose was included (Fig. 3).

The blood group O glycopeptides and the retarded fraction of blood group A glycopeptides were finally subjected to gel filtration (Fig. 4). The middle portions of each peak, fractions AS and OS, were subjected to structural characterizations.

Sugar Composition and Methylation Analysis—The sugar composition of the glycopeptides isolated is shown in Table 1. The values are expressed assuming 3 mannose residues/molecule, which is in accordance with the molecular size of the glycopeptides in gel filtration (Fig. 4). The proportions of mannose, galactose and N-acetylgalactosamine were found to be typical of N-glycosidic glycopeptides of various sources. However, the glycopeptides differed from previously studied glycopeptides in their high content of fucose and in the absence of sialic acid residues. In addition, the glycopeptides from the blood group A individual contained N-acetylgalactosamine, a rare constituent of N-glycosidic carbohydrate units.

Methylation analysis (Table II) revealed a substitution pattern of mannose residues suggesting tetra- and triantenary core structures for the glycopeptides. The presence of approximately 1 residue of 3,4,6-tri-substituted mannose concomitant with 1 residue of terminal N-acetylgalactosamine suggested that the chitobiose-linked mannose residue of the core contained an intersecting N-acetylgalactosamine residue.

In AS glycopeptides the presence of terminal N-acetylgalactosamine and 2,3-di-substituted galactose, which were not found in OS glycopeptides (Table II), suggested the presence of the blood group A determinant structure (Scheme I). In accordance with this, defucosylation with mild acid converted most of the 2,3-di-substituted galactose into 3-mono-substituted galactose residues were converted into terminal galactose residues by defucosylation, which suggested the presence of the H determinant structure (Scheme I) in these glycopeptides.

An unusually high proportion of the N-acetylgalactosamine residues was found to be 3,4-di-substituted in both AS and OS glycopeptides (Table II). After defucosylation most of these residues were found to be replaced by 4-monosubstituted N-acetylgalactosamine, which suggested the possible presence of the X antigen structure (Scheme I) in the glycopeptides. The presence of some 3-mono-substituted N-acetylgalactosamine after defucosylation suggested that a small proportion of the blood group determinants could be of type 1.

$^1$H NMR Spectroscopy—To arrive at a more definite eval-

![Fig. 2](image-url)  
**Fig. 2.** Affinity chromatography of glycopeptides from human small intestinal epithelial cells on concanavalin A-Sepharose. The affinity column (4.0 x 5.2 cm) was eluted with the starting buffer and 20 and 200 mM methyl-α-D-glucose was included in the buffer at the positions indicated by arrows. Fractions of 15 ml were collected and analyzed for radioactivity of the N-3H-acetylated glycopeptides. 

![Fig. 3](image-url)  
**Fig. 3.** Affinity chromatography of glycopeptides from human small intestinal epithelial cells on B. simplicifolia lectin I-Sepharose. The affinity column (1.4 x 10 cm) was eluted with the starting buffer, and 5 mM galactose was included in the buffer at the arrow. Fractions of 6 ml were collected and analyzed for radioactivity of the N-3H-acetylated glycopeptides. 

![Fig. 4](image-url)  
**Fig. 4.** Gel filtration of the glycopeptides isolated by affinity chromatography. The glycopeptides were subjected to gel filtration as described in the legend of Fig. 1. The middle part of each peak (A and O), as shown by the bars, was used for structural characterizations.

**Table 1**  
**Monosaccharide composition of glycopeptides from human small intestinal epithelial cells**

| Constituent | Blood group A (AS) | Blood group O (OS) |
|-------------|-------------------|--------------------|
| Fuc         | 7.4               | 5.7                |
| Man         | 3.0               | 3.0                |
| Gal         | 4.0               | 4.0                |
| GalNAc      | 2.9               | 2.9                |
| GlcNAc      | 6.8               | 6.7                |
| NeuAc       |                   |                    |
tection of the heterogeneity that appeared to occur in the small intestinal glycopeptide fractions OS and AS from the data of sugar and methylation analyses (see Tables I and II). 500 MHz 1H NMR spectroscopy was applied, both before and after defucosylation. The relevant parts of the 1H NMR spectra of fraction OS, recorded before and after defucosylation, are compared in Fig. 5. The characteristic features of the 1H NMR spectra of glycopeptides OS and AS and their defucosylated analogues (df-OS and df-AS, respectively) are summarized in Table III. The structures discussed and the coding of residues are indicated in Scheme 2.

The NMR-spectroscopic data confirm that both fractions OS and AS contain N-glycosidic glycopeptides of the N-acetyllactosamine type. The N-type is concluded from the presence of signals that are typical (21) for the structural element \((-\rightarrow4)\text{GlcNAc}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrowN)\text{Asn}\), namely, the H-1 doublet at \(\delta \approx 5.05\) (J1,2 > 9 Hz) and the NAc singlets at \(\delta 2.011\) and \(\delta = 2.094\). The singularity of the signal at \(\delta 2.011\) in the spectra of both (df-)OS and (df-)AS indicates that the residual peptide moiety (resulting after Pronase digestion) of the fractions is homogeneous in composition (21). A Fuc residue was found to occur in \(\alpha(1\rightarrow6)\)-linkage to the Asn-bound GlcNAc residue (GlcNAc-1) of all glycans in fractions OS and AS. This is revealed (21) by the position of the NAc signal of GlcNAc-2, namely, \(\delta 2.095\) (for OS) or \(\delta 2.093\) (for AS). The \(\alpha(1\rightarrow6)\)-linked Fuc has its H-1 signal at \(\delta 4.87\), its H-5 signal at \(\delta 4.12\), and its CH3 signal at \(\delta 1.200\) (see Table III). Only the CH3 doublet at \(\delta 1.200\) could be observed individually (for OS, see Fig. 5C); the H-1 signal of Fuc(1\rightarrow6) coincides with a Man(1\rightarrow4) H-1 signal and with some of the Fucco(1\rightarrow3) H-5 signals, whereas the H-5 signal of Fuc(1\rightarrow6) is found among Man H-2 and Fucco(1\rightarrow2) H-5 signals (see below). The core portion of the glycopeptides in OS and AS is completed by 3 Man residues in the assembly that is common to all \(N\)-type glycans; evidence for the latter statement is outlined below.

It became evident from methylation analysis (see Table II) that the compounds in fractions OS and AS were likely to contain highly branched Man3GlcNAc core units. The number of \(\beta\)-linked GlcNAc residues attached to the core, and their linkage positions, are in general expected (21) to become available from inspection of the Man H-2 region of the 1H NMR spectrum (4.0 < \(\delta < 4.3\) ppm), in conjunction with counting the number of NAc methyl singlets (2.0 < \(\delta < 2.1\) ppm). In the spectrum of OS (see Fig. 5A), the NAc methyl proton region shows signals equivalent to a total of about 7.5 NAc groups (based on the unit intensity of the signals at \(\delta 2.011\) and \(\delta 2.095\); thus, 5–6 peripheral GlcNAc residues occur in the glycans of fraction OS. From integration of the corresponding region of the spectrum of AS, we concluded that a

**TABLE II**

Methylation analysis of native and defucoslated small intestinal glycopeptides

Defucosylation of the glycopeptides was carried out by mild acid treatment. The relative proportions of the hexosamine derivatives were taken from the mass fragmentography scans and combined using the data of the total sugar composition. The proportion of the fucose derivative was not determined due to interfering peaks near the solvent front.

**TABLE III**

Constituent Substitution Blood group A (AS) Blood group O (OS)

| Constituent | Substitution | Blood group A (AS) | Blood group O (OS) |
|-------------|--------------|--------------------|--------------------|
| Gal         | Terminal     | 0.4                | 0.8                |
|             | 3            | 0.3                | 0.8                |
|             | 2            | 0.2                | 0.1                |
|             | 6            | 2.3                | 0.9                |
| Man         | 2            | 0.3                | 0.5                |
|             | 4            | 0.9                | 0.8                |
|             | 3            | 0.1                | 0.2                |
|             | 6            | 0.7                | 0.5                |
|             | 3,4,6        | 1.0                | 1.1                |
| GlcNAc      | Terminal     | 0.9                | 0.9                |
|             | 4            | 2.1                | 2.4                |
|             | 3            | 0.2                | 0.7                |
|             | 4,6          | 0.3                | 0.1                |
| GalNAc      | Terminal     | 2.3                | 2.7                |

**SCHEME 1**

Blood-group and differentiation antigenic determinants

| Coding    | Structure                                                                 |
|-----------|---------------------------------------------------------------------------|
| H type-1  | Fuc\(\alpha(1\rightarrow2)\text{Ga1}\beta(1\rightarrow3)\text{GlcNAc}\beta(1\rightarrow\cdot)\) |
| H type-2  | Fuc\(\alpha(1\rightarrow2)\text{Ga1}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow\cdot)\) |
| X         | \text{Ga1}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow\cdot) \text{Fuc}(1\rightarrow3)'/ |
| Y         | \text{Fuc}(1\rightarrow2)\text{Ga1}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow\cdot) \text{Fuc}(1\rightarrow3)'/ |
| B type-1/2 | \text{Fuc}(1\rightarrow2)\text{Ga1}\beta(1\rightarrow3/4)\text{GlcNAc}\beta(1\rightarrow\cdot) \text{Ga1}(1\rightarrow3)'/ |
| Difuosyl B | \text{Fuc}(1\rightarrow2)\text{Ga1}\beta(1\rightarrow3/4)\text{GlcNAc}\beta(1\rightarrow\cdot) \text{Fuc}(1\rightarrow4/3)' |
| A type 1/2 | \text{Fuc}(1\rightarrow2)\text{Ga1}\beta(1\rightarrow3/4)\text{GlcNAc}\beta(1\rightarrow\cdot) \text{Ga1NAc}(1\rightarrow3)'/ |
| Difuosyl A | \text{Fuc}(1\rightarrow2)\text{Ga1}\beta(1\rightarrow3/4)\text{GlcNAc}\beta(1\rightarrow\cdot) \text{Ga1NAc}(1\rightarrow3)'/ \text{Fuc}(1\rightarrow4/3)'/ |
| Trifuosyl A | \text{Fuc}(1\rightarrow2)\text{Ga1}\beta(1\rightarrow3/4)\text{GlcNAc}\beta(1\rightarrow\cdot) \text{Ga1NAc}(1\rightarrow3)'/ \text{Fuc}(1\rightarrow4/3)'/ \text{Fuc}(1\rightarrow3)'/ |
FIG. 5. Structural-reporter group regions of the resolution-enhanced 500 MHz $^1$H NMR spectra of glycopeptide fraction OS, recorded before (trace A) and after (trace B) defucosylation. The anomeric-proton region (3.9 < $\delta$ < 5.6 ppm) of the spectrum of df-OS, recorded at 27 °C is shown on the bottom trace of B; the corresponding region of the spectrum recorded at 52 °C is shown on the top trace of B. Trace C shows the Fuc CH$_3$ region of the spectrum of OS in trace A. For the comprehensive structure of glycopeptides OS, see Scheme 2. The numbering of the core and backbone residues is as indicated in Table III and Scheme 2. Branching patterns are indicated as tri (for tri-antennary), tet (for tetra-antennary), and int (for intersected). GN-ext stands for extra (i.e., additional) GlcNAc residue. Superscripts as in Fuc*, Fuc’, Gal3 indicate the position of the glycosidic linkage in which the residue is involved; *GN indicates that the GlcNAc residue is substituted at the C-3 position. The key to the coding used for blood group and differentiation antigenic determinants is Scheme 1. Remainder in trace B refers to the fact that defucosylation was not complete (see text). Signals marked by 4 stem from a frequently occurring noncarbohydrate, nonprotein contaminant.

total of 9.5 NAc groups is present in compound AS. Sugar analysis (Table I) indicated that 2.5 of these belong to the GalNAc residues that give rise to the blood group A activity of the native AS glycopeptides. Thus, also taking into account that two signals belong to GlcNAc-1 and -2, we arrived at a number of 5 peripheral GlcNAc residues in AS. The spectra of both OS (Fig. 5A) and AS, however, showed highly crowded patterns of partially overlapping resonances in the 4.0 < $\delta$ < 4.3 region; among those, the Man H-2 signals could not readily be located. The signals obscuring this spectral region
### Polyfucosylated Glycopeptides

#### TABLE III

1H chemical shifts of pertinent structural-reporter groups of the blood group active glycopeptides (and their defucosylated analogues) from human small intestinal epithelial cells from individuals with blood group O and A.

| Residue | Reporter group | Chemical shift$^{\text{a}}$ in Chemical shift$^{\text{b}}$ in |
|---------|----------------|-----------------------------------|
|         |                | 27°C OS 27°C df-OS 27°C OS 27°C df-AS* 27°C AS* 27°C |
| GlcNAc-1 | H-1           | 5.055 5.055 5.056 5.056 5.060 5.060 |
|          | NAc           | 2.011 2.014 2.011 2.010 2.011 2.011 |
| GlcNAc-2 | H-1           | 4.629 4.629 ND 4.629 ND |
|          | NAc           | 2.081' 2.079' 2.085 2.083 2.093 |
| Fuc(1→6) | H-1           | 4.875 4.875 |
|          | H-5           | 4.12 4.12 |
|          | CH$_3$        | ND 1.200 |
| Man-3    | H-1           | ND 4.686$^{\text{a}}$ 4.695$^{\text{b}}$ 4.695$^{\text{b}}$ |
|          | H-2           | 4.147 4.145 4.140 4.148 ND |
| Man-4    | H-1           | 5.055$^{\text{a}}$ 5.055$^{\text{b}}$ 5.055$^{\text{b}}$ 5.059$^{\text{a}}$ 5.059$^{\text{a}}$ |
|          | H-2           | 4.286 4.283 ND 4.286 ND |
| Man-4'   | H-1           | 5.003$^{\text{c}}$ 4.995$^{\text{c}}$ 4.995$^{\text{c}}$ 5.06$^{\text{c}}$ 5.06$^{\text{c}}$ |
|          | H-2           | 4.916$^{\text{d}}$ 4.906$^{\text{d}}$ 4.965$^{\text{d}}$ 4.965$^{\text{d}}$ 5.039$^{\text{d}}$ 5.039$^{\text{d}}$ |
| GlcNAc-5 | H-1           | 4.58 4.58 ND 4.52 4.54 4.576 |
|          | NAc           | 2.059' 2.060' 2.051' 2.061' 2.054' |
| GlcNAc-5' | H-1          | 4.86 4.86 ND 4.52 4.54 4.576 |
|          | NAc           | 2.052$^{\text{c}}$ 2.040$^{\text{c}}$ 2.051$^{\text{c}}$ 2.040$^{\text{c}}$ 2.056/2.024 |
| GlcNAc-7 | H-1           | 4.52 4.52 ND 4.52 4.54 4.576 |
|          | NAc           | 2.081 2.079 2.075 2.083 2.074 |
| GlcNAc-7' | H-1          | 4.53 4.53 ND 4.52 4.54 4.576 |
|          | NAc           | 2.039' 2.042' 2.040' 2.040' 2.056/2.024 |
| GlcNAc-9 | H-1           | 4.47 4.47 ND 4.46 4.47 4.45 |
|          | H-4           | 3.283 3.285 3.277 3.28 3.287 |
|          | NAc           | 2.059' 2.060' 2.062' 2.061' 2.070' |
| Gal$^{\text{1}}$ (6*) | H-1          | 4.438* 4.438* 4.45 (1') 4.456 (1') 4.456 (1') |
| Gal-6'/(8/8') | H-1        | 4.46 4.46 ND 4.443 4.459 (3) 4.489 (3) |
| GlcNAc-ext β(1→3) | H-4$^{\text{f}}$ | 4.147 4.145 ND 4.215 (3) 4.198 (3) |
|          | H-1           | 4.70 4.686 ND ND ND |
|          | NAc           | 2.032 2.034 2.037 ND ND ND |
| Gal-ext β(1→4) | H-1          | 4.46 ND 4.45 ND ND ND |
|          | H-1           | 5.188 5.216 |
|          | H-5           | 4.26 4.26 |
|          | CH$_3$        | 1.235 1.235 |
|          | Fuc(1→2)$^1$ | H-1           | 5.300 5.300 (3) |
|          | H-5           | 4.21 4.32 (3) |
|          | CH$_3$        | 1.235 1.235 |
|          | Fuc(1→2)$^2$ | H-1           | 5.278 5.279 (1') 5.279 (1') |
|          | H-5           | 4.25 4.25 |
|          | CH$_3$        | 1.240 1.293 (<1) |
|          | Fuc(1→3)$^3$ | H-1           | 5.120 5.120 |
|          | H-5           | 4.87 4.87 |
|          | CH$_3$        | 1.166 1.166 |
|          | Fuc(1→3)$^4$ | H-1           | 5.102 5.119 (<1) |
|          | H-5           | 4.87 4.87 |
|          | CH$_3$        | 1.277 1.277 |
|          | GalNAc        | H-1           | 5.174 5.200 |
|          | H-2           | 4.315 4.235 |
|          | H-5           | ND ND 4.16 |
|          | NAc           | 2.040 2.036 |

* For comprehensive structures of OS and AS and coding of residues, see Scheme 2.

* Chemical shifts were acquired at 500 MHz for neutral H$_2$O solutions at 27°C, and for df-OS also at 52°C (δ HOH 4.509).

* Italicized numbers in parentheses refer to relative-intensity ratios of the signals in the NMR spectra.

* ND, value could not be determined, because signal overlapped with HOH resonance at the applied probe temperature, or with other signals.

* Defucosylation under the applied conditions (see text) was not complete. For example, the Fuc(1→6) residue linked to GlcNAc-1 remained present in at least 35% of OS and AS. Two signals were observed in the spectra of df-OS and df-AS for the NAc methyl protons of GlcNAc-2, at δ 2.081 and δ 2.094, in the intensity ratio 5:1.

* Additional symbols used are: $^{\text{a}}$, tentative assignment; $^{\text{b}}$, for intersected tri-antennary compounds; $^{\text{c}}$, for intersected tetra-antennary compounds; $^{\text{d}}$, for nonintersected tetra-antennary compounds; $^{\text{e}}$, assuming arbitrarily that the β(1→3)-linked Gal is Gal-6* (see text and legend to Scheme 2); $^{\text{f}}$, substituted by the "additional" N-acetyllactosamine unit in β(1→3)-linkage.

* Alternatively, this set of Fuc(1→3) reporter group signals may be assigned to the third Fuc residue in the trifucosyl A determinant (see text).
**Scheme 2**

Comprehensive structures proposed for human small-intestinal glycopeptides OS and AS

| Glycopeptide OS | Peripheral domain | Backbone | Core |
|-----------------|-------------------|----------|------|
| (X)             | Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→6) | Fucα(1→3) |      |
| (Y)             | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→2)Manα(1→6) | Fucα(1→3) |      |
|                 | [GlcNAcβ(1→4)]_α.s |          |      |
| H(1)            | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→2)Manα(1→3) | Fucα(1→6) |      |
| H(2)            | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→4)GlcNAcβ(1→N)Asn |      |      |

Glycopeptide AS

| asfucosyl | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→6) | Fucα(1→3) |      |
| A(2)      | GaNAcα(1→3) |          |      |
| (Y)       | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→2)Manα(1→6) | Fucα(1→3) |      |
| A(1)      | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→4)GlcNAcβ(1→N)Asn | Fucα(1→6) |      |
| trifucosyl | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→4)GlcNAcβ(1→N)Asn |      |      |
| A(2)      | GaNAcα(1→3) |          |      |
| A(2)      | Fucα(1→2)Gaβ(1→4)GlcNAcβ(1→3)Gaβ(1→4)GlcNAcβ(1→4)GlcNAcβ(1→N)Asn | Fucα(1→6) |      |

* The type-1 blood-group determinants are linked directly to the core. Here, they are arbitrarily shown β(1→2)-linked to Man-4; however, their precise location is not known (see text).
* The average number of GalNAc residues per molecule AS is 2.5; this is in agreement with the occurrence of the β(1→6)-linked branch in 60% of the molecules.
* The sum of the indices x + y + z (for OS) and x + v (for AS) = 1, indicating that the glycopeptides contain, on average, one "additional" N-acetyllactosamine unit, which varies in type of terminal antigenic structure.
* The branch β(1→6)-linked to Man-4' is present in 60% of the glycopeptides, the intersecting GlcNAc-9 residue occurs in 80% of the molecules.
were thought to be either Fuc H-5 signals or signals of structural-reporter groups of other monosaccharides that had emerged out of the bulk resonance of skeleton protons \((3.4 < \delta < 4.0)\) due to the presence of the Fuc residues. To enable the determination of the sites of substitution of the peripheral GlcNAc residues to the \(\text{Man}_{n}\text{GlcNAc}_{c}\) core unit, the OS and AS glycopeptides were subjected to mild acid treatment resulting in the removal of most (but not all; see footnote e to Table III) of the Fuc residues.

**Backbone Structure of the Components of Fractions OS and AS**—The chemical shift values of the predominant Man H-1 and H-2 signals in the spectrum of \(\text{df-OS}\) have been included in Table III. At least three different sets of values were observed, pointing to the occurrence of three different types of branching in OS. The set of values \(\delta 5.055, 4.998,\) and 4.686 (measured at \(52 \degree C\)) for H-1 of Man-4, -4', and -3, respectively, suggested the presence of intersected triantennary type glycopeptides (see Table IV; compare Ref. 21). The second set of values, namely, \(\delta 5.122, 4.877,\) and 4.752, pointed to the presence of nonintersected tetra-antennary type of compounds (see Table IV; compare Ref. 21). The third set of values, namely, \(\delta 5.055, 4.906,\) and 4.686 for H-1 of Man-4, -4', and -3, respectively, had not been previously observed. In view of the results of methylation analysis (Table II), this third set of values is attributed to glycopeptides with an intersected tetra-antennary type of branching. The NMR line of reasoning for this assignment is based on the analogy between, on one hand, the chemical shift differences between the new set of Man H-1 (and H-2) chemical shifts and the one previously established for nonintersected tetra-antenna, and, on the other hand, the chemical shift effects observed for the Man H-1 atoms in the step from intersected tri- to nonintersected triantennas (see Table IV). The relative intensity ratio of these three sets of signals (taking into account that also at \(\delta 5.055\) the H-1 signal of GlcNAc-1 is located, that the signal at \(\delta 4.877\) at least in part stems from the remainder of Fuc(1→6), and that the signal at \(\delta 4.686\) partly belongs to the H-1 doublet of one of the peripheral GlcNAc residues, see later) points to the occurrence of intersected tri-, intersected tetra-, and nonintersected tetra-antennary compounds in the ratio 2:2:1. That is to say, Man-4 is substituted by GlcNAc-5 and -7 at C-2 and C-4, respectively, in all components of \((\text{df-})\text{OS}\). Man-4' bears GlcNAc-5' and -7' at C-2 and C-6, respectively, in about 30% of the constituents of \((\text{df-})\text{OS}\); in the remaining 40% of the molecules, Man-4' is substituted only at C-2 by GlcNAc-5'. Finally, Man-3 is substituted at C-4 by a \(\beta\)-linked (so-called intersecting) GlcNAc residue (usually referred to as GlcNAc-9) in 80% of the molecules in \((\text{df-})\text{OS}\) (see Scheme 2). Relatively intense signals in the Man H-2 region of the spectrum of \(\text{df-OS}\) are seen at \(\delta 4.28\) and 4.14-4.12 (Man-4 H-2 signal, and coinciding H-2 signals of Man-3 and -4', respectively) (see Fig. 5B); these values are in agreement with the predominance of intersected type of glycopeptides in \((\text{df-})\text{OS}\) (compare Table IV).

The branching patterns of the glycopeptides in \(\text{df-AS}\) are proposed to be the same as those in \(\text{df-OS}\), although the ratios in which they occur might be slightly different. Apparently, the exact chemical shift values for H-1 and H-2 of Man-3, -4, and -4' for \(\text{df-AS}\) (Table III) are modified by the presence of the GalNAc(1→3)Gal(1→4) arms, compared with those for \(\text{df-OS}\) and the reference compounds (Table IV).

The presence of the intersecting GlcNAc-9 residue in the majority (=80%) of glycopeptide molecules, both in OS and AS, is corroborated by the occurrence of the H-4 signal of this residue outside the bulk of skeleton resonances, at \(\delta \approx 5.18\).

### Table IV

| Type of branching | Chemical shift of |
|-------------------|-------------------|
| Man-3 H-1         | 4.77 5.12 4.93 4.25 4.19 4.11 |
| Man-4 H-1         | 4.54 4.92 4.15 4.21 4.22 4.11 |
| Man-3 H-2         | 4.76 5.12 4.92 4.21 4.22 4.11 |
| Man-4 H-2         | 4.54 4.92 4.15 4.21 4.22 4.11 |
| Man-4' H-1        | 4.69 5.06 5.00 4.21 4.22 4.14 |
| Man-4' H-2        | 4.54 4.92 4.15 4.21 4.22 4.09 |
| Man-4' H-3        | 4.69 5.05 4.92 4.15 4.28 4.12 |
| Man-4' H-4        | 4.54 4.92 4.15 4.21 4.22 4.09 |

*The values for the intersected tetra-antennary type of branching were inferred from this study.*
and 7') linked to the α-Man residues is substituted by a Gal residue. From methylation analysis (Table II), it was deduced that one of these Gal residues is present in (1→3)-linkage to GlcNAc, whereas the other Gal residues are (1→4)-linked, thus forming part of the N-acetyllactosamine units. The β(1→3)-linked Gal residue in df-OS has its H-1 signal at δ 4.438 (cf. Refs. 29–33); the other Gal residues have their H-1 signals at δ ≈ 4.47. The determination of the site of attachment of the Galβ(1→3)GlcNAcβ(1→-) residue (so-called type-1 unit; see Scheme 1) to the core is, at least in principle, feasible by NMR, for example by comparison with the data published for the biantennary glycopeptide from rat plasma hemopexin (29) having Galβ(1→3)GlcNAcβ(1→-) attached to C-2 of Man-4 and for those in the triantennary glycopeptide obtained from calf fetuin (30, 31) having the Galβ(1→3)GlcNAcβ(1→-) unit attached to C-4 of Man-4. However, the complexity of the β-anomeric proton regions as well as of the NAc methyl proton regions of the spectra of df-OS and df-AS did not permit the deduction of the position of the type-1 unit unambiguously. (In the data listed in Table III, we tentatively assume, for reasons of convenience in tabulating the chemical shifts, that the type-1 unit is linked to C-2 of Man-4.)

The fifth (for tri- or sixth (for tetra-antennary compounds) peripheral GlcNAc residue is not linked to a Man residue but is rather present in β(1→3)-linkage to one of the aforementioned Gal residues. In turn, this GlcNAc bears a Gal residue in β(1→4)-linkage. Typical 1H NMR spectroscopic features of the β(1→3)-linked GlcNAc are its H-1 doublet at δ ≈ 4.69 and its NAc methyl singlet at δ 2.032 (cf. Refs. 21, 25, 33, 34). The H-4 signal of the Gal residue substituted by this β(1→3)-linked GlcNAc residue is found at δ ≈ 4.147 (21, 33, 34), thereby coinciding in the spectrum of (df-)OS with some Man H-2 signals (see Table III). The NMR characteristics of such an extension of a fundamental N-acetyllactosamine branch by an additional N-acetyllactosamine unit have been described before for an intersected biantennary N-type glycopeptide (25) and for some smaller size, mucin-type O-glycosidic oligosaccharide-alditols (33). Owing to the lack of reliable NMR data obtained on higher branched, N-type compounds with repeating N-acetyllactosamine units, it was not possible to determine by 1H NMR spectroscopy the identity of the branch(es) to which this extra N-acetyllactosamine unit may be attached in OS. Due to the complexity of the 1H NMR spectra of AS and df-AS, the occurrence of such an extra N-acetyllactosamine unit could neither be proven nor denied. In methylation analysis, both AS and OS contain a Gal residue monosubstituted at C-3 (Table II), which leads us to assume that the extra N-acetyllactosamine unit is present in AS, too. Mass spectrometry of the permethylated glycopeptides supports this assumption (see below).

**Outer Region Structures of the Glycopeptides in Fractions OS and AS**—With respect to the Fuc residues occurring in the outer regions of the OS glycopeptides, the following details could be elucidated from the 1H NMR spectrum (Fig. 5A). In addition to the core-linked Fucα(1→6) residue mentioned above, Fuc residues occur in OS in two other types of linkages, namely α(1→2) to Gal and α(1→3) to peripheral GlcNAc. Thus, Fuc forms part of either one of the following determinants: H, X, and Y (see Scheme 1). 1H NMR spectroscopic data for Fuc residues forming part of various types of blood group determinants, collected from the literature, have been compiled in Tables V and VI.

Evidence for the presence of the X-antigenic determinant is afforded by the Fuc-H1 signal at δ 5.12, the H-5 signal at δ 4.87, and the CH₃ doublet at δ 1.17 (see Table III) (21, 25, 32, 34–40). A possible reason for the somewhat diffuse shape of the CH₃ doublet (Fig. 5A) is the simultaneous occurrence of peptide signals in this region (cf. Ref. 21). The occurrence

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**TABLE V**

| Residue | Reporter group | H(2) | X | Y | H(2) | X | Y | H(2) | X | Y |
|---------|----------------|------|---|---|------|---|---|------|---|---|
| GlcNAc | H-1 α | 5.210 | 5.106 | 5.080 |
| | β | 4.715 | 4.729 | 4.725 |
| | NAc α | 2.048 | 2.035 | 2.034 |
| | β | 2.045 | 2.031 | 2.032 |
| GlcNAc | H-1 | 4.685 | 4.692 | 4.68 |
| | NAc | 2.043 | 2.032 | 2.028 |
| GlcNAc | H-1 | 4.541 | 4.557 | 4.558 |
| | NAc | 2.064 | 2.056 | 2.057 |
| Gal | H-1 | 4.541 | 4.455 | 4.507 |
| | H-4 | ND | ND | ND |
| Fuc | H-1 | 5.308 | 5.277 | 5.310 |
| | H-5 | 4.226 | 4.249 | 4.226 |
| | CH₃ | 1.224 | 1.235 | 1.237 |
| Fuc | H-1 | 5.098 | 5.080 | 5.138 |
| | H-5 | 4.839 | 4.882 | 4.832 |
| | CH₃ | 1.172 | 1.263 | 1.272 |

Superscripts indicate the position of the glycosidic linkage in which the residue is involved.

Chemical shifts were acquired at 500 MHz for neutral solutions at 27 °C.

For complete structures of determinants, see Scheme 1. Data were compiled from Refs. 32–43 and 48–50.
of one of the Fuc residues in α(1-2)-linkage to Galβ(1-4) (constituting the H type-2 determinant) is revealed by the Fuc H-1 signal at δ 5.185 together with the CH₂ doublet at δ 1.235 (Table III; compare Refs. 32, 34-37). Finally, the combination of Fuc H-1 signals at δ 5.28 (Fucα(1→2)) and δ 5.10 (Fucα(1→3)) together with the CH₂ signals at δ 1.24 (Fucα(1→2)) and 1.28 (Fucα(1→3)) is highly characteristic of the occurrence of the Y determinant (35-38, 42).

The intensity ratio of the various Fuc H-1 signals, along with that of the Fuc CH₂ doublets (1:3:1:0.5) (see Fig. 6C), indicates that, on average, there are 5-6 Fuc residues present/molecule in OS, divided over 6 different positions: Fucα(1→6) to GlcNAc3, Fucα(1→2) to Galβ(1→3) to GlcNAc3 in X determinant; Fucα(1→2) to Galβ(1→4) in H type-2 unit, probably in two different branches: Fucα(1→2) to Galβ(1→4) in H type-1 unit; and two types of Fuc in the Y determinant.

As has been outlined previously (43), the chemical shift of a Fuc H-1 signal is not only indicative of the type of linkage in which Fuc itself is involved, and the nature of the residue to which it is attached, but also depends on a number of other structural details in the branch to which it is attached (e.g. the type-1 or type-2 character of the Gal-GlcNAc unit and the type of linkage in which GlcNAc is involved). Thus, the chemical shift of Fuc H-1 is a most sensitive marker of the complete microenvironment of the Fuc residue, but, mutatis mutandis, is also the parameter whose value is the most difficult to predict among the chemical shifts of all ¹H NMR structural-reporter groups. Therefore, it is quite possible that the signal at δ 5.334 in the spectrum of OS, like the more commonly observed one at δ 5.300, belongs to a Fuc residue in an H type-2 determinant. However, that H type-2 unit should then be attached to Man in a type of linkage not observed before (neither β(1→2) to Man (21), nor β(1→3) or β(1→6) to Gal (41)), leading us to propose that this H type-2 unit may be β(1→4)-linked (to Man 4). Alternatively, the presence of the intersecting GlcNAc residue might modify (“finetune”) the chemical shift of H-1 of a Fuc residue in the afucosyl B(2)-structure. Additional symbols used are: *<sup>a</sup>, reducing GlcNAc residue; °<sup>a</sup>, refers to the chemical shift of the reporter-group in the β-anomer of the (reducing) oligosaccharide. °<sup>b</sup>, values predicted from the shift effects observed in the step from B(1) to difucosyl B(1).

### Table VI

| Residue<sup>a</sup> | Reporter group | Chemical shift in<sup>b</sup> | H(1)<sup>c</sup> | H(1) | Le<sup>e</sup> | Le<sup>e</sup> | difucosyl B<sup>c</sup> | B(1) | difucosyl B(1) | A(1) | difucosyl A(1)<sup>c</sup> |
|-----------------|----------------|-----------------------------|-----------------|-------|-------------|-------------|-------------------|-------|-----------------|------|-----------------|
| GlcNAc<sup>d</sup> | H-1            | 4.654                       | 4.70            | 4.665 | 4.727<sup>e</sup> | 4.798        | 4.598             | 4.725 | 4.676          | 4.70 |
|                 | NAc            | 2.111                       | 2.036           | ND    | 2.043<sup>d</sup> | 2.072        |                   |       |                 |      |
| Gal<sup>e</sup>  | H-1            | 4.577                       | 4.581           | 4.43  | 4.582        | 4.546<sup>e</sup> | 4.697             | 4.725 | 4.676          | 4.70 |
|                 | H-4            | 3.889                       | 3.916           | 3.92  | 3.90         | 4.182        | 4.278             | 4.234 | 4.214          | 4.17 |
| Fuc<sup>f</sup>  | H-1            | 5.208                       | 5.268           | 5.153 | 5.549        | 5.232        | 5.378             | 5.29  |                 |      |
|                 | H-5            | 4.273                       | 4.269           | 4.354 | 4.317        | 4.391        | 4.322             | 4.40  |                 |      |
|                 | CH₃            | 1.230                       | 1.235           | 1.252 | 1.227        | 1.274        | 1.292             | 1.29  |                 |      |
| Gal<sup>g</sup>  | H-1            | 5.030                       | 5.026           | 5.041 | 4.834        | 4.880        | 4.880             | 4.83  |                 |      |
|                 | H-4            | 4.88                       | 4.880           | 4.834 | 4.83         | 4.880        | 4.880             | 4.83  |                 |      |
|                 | H-5            | 1.183                       | 1.276           | 1.287 | 1.29         | 1.274        | 1.292             | 1.29  |                 |      |
| GalNAC<sup>h</sup> | H-1    | 5.145                       | 5.259           | 5.165 | 5.158        | 5.185        | 5.18              | 5.18  |                 |      |
|                 | H-2            | 4.621                       | 4.015           | 4.251 | 4.25         | 4.25         |                   |       |                 |      |
|                 | H-4            | 4.020                       |                  |       |              | 4.020        |                   |       |                 |      |
|                 | H-5            | 4.194                       | 4.119           | 4.160 | 4.25         | 4.25         |                   |       |                 |      |
|                 | NAc            | 2.049                       | 2.053           | 2.049 | 2.05         | 2.05         |                   |       |                 |      |

<sup>a</sup> Superscripts indicate the position of the glycosidic linkage in which the residue is involved.

<sup>b</sup> Chemical shifts were acquired at 300 or 500 MHz for neutral solutions at 22 or 27 °C.

<sup>c</sup> For complete structures of determinants, see Scheme 1. Data were compiled from Refs. 20, 36, 37, and 44-47.

<sup>d</sup> Here, the superscript 3 indicates the position of the linkage in which GlcNAc is involved: Galβ(1→4)GlcNAcβ(1→3) (usually to Galβ(1→4)). When no values are listed for the GlcNAc reporter groups for a certain determinant, the gal residue is β(1→3)-linked to GlcNAc-ol. The two columns of chemical shifts for H(1) illustrate that the chemical shifts of the Gal and Fuc reporter groups are more or less strongly dependent on the nature of the aglycone.

<sup>e</sup> In the afuco B-determinant, β-Gal is actually (1→4)-linked to GlcNAc, so the values refer to the afuco B(2)-structure. Additional symbols used are: °<sup>a</sup>, reducing GlcNAc residue; °<sup>a</sup>, refers to the chemical shift of the reporter-group in the β-anomer of the (reducing) oligosaccharide. °<sup>b</sup>, values predicted from the shift effects observed in the step from B(1) to difucosyl B(1).
of the core branching pattern, the impossibility of even deter-
mining the position of the core to which the type-1 unit is
linked, the nature of the Gal residue to which the extra N-
acetyllactosamine unit is attached, etc., prevented determi-
nation of the precise distribution of the various blood group
determinants over the branches of the various OS compo-
ents, except that the H type-1 unit seems to be directly attached
to one of the core α-mannose residues. If this unit were the extra Gal-GlcNAc residue linked β(1→3) to a fun-
damental N-acetyllactosamine branch, its NAc signal would be expec-
ted to be observable at δ = 2.11 (34). A similar
chemical shift (δ ≈ 2.10–2.11) would be predicted for the NAc
signal of the H type-1 unit if it were attached β(1→4) to Man-
4 (39). Since we do not observe a NAc singlet at that position,
we conclude that the H type-1 unit is directly attached to
the core but not to the C-4 position of Man-4 (see Scheme 2).

As to the occurrence of blood group A and other determin-
ants in the various glycopeptide components of fraction AS,
the 1H NMR spectra of AS and df-AS permitted the following
conclusions. The major difference between the spectra of
preparations of df-AS and df-OS is in the occurrence of a
large signal at δ = 5.174 (ν12, 4 Hz), dominating the anomeric
proton region of the spectrum of df-AS (not shown). This
signal is attributed to H-1 of α(1→3)-linked GalNAc attached
to Gal and/or GlcNAc residues (cf. Refs. 36, 37, 44, 47). The relative
intensity of the signal is in agreement (compare Tables I and
II) with the occurrence of 2 to 3 GalNAc residues/molecule of
AS glycopeptides. The other structural-reporter group reso-
nces of α-linked GalNAc are its H-2 (at δ ≈ 4.22) and H-5
(at δ ≈ 4.16, both contributing to the “Man H-2 region” of
the spectrum, and therefore hampering deduction of the core
branching patterns of AS components even more than the
situation with OS). The dominant NAc methyl singlet in the
spectrum of df-AS at δ 2.040 (Table III) is also ascribed to
the terminal GalNAc residues.

In the spectrum of native AS, the chemical shifts of the
above mentioned GalNAc protons are found to be slightly
altered (see Table III). These alterations are obviously due to
the presence of Fuc residues in α(1→2)-linkage to the same
Gal residue as to which GalNAc is attached, completing the
blood group A sequence (see Scheme I) (compare Refs. 36,
37, 44, 47). The reporter-group resonances of the Fuc(1→2)
residues involved in these A determinants were assigned on
the basis of their relatively large intensity (nearly equal to
that of the GalNAc reporter group signals); they are found at
δ 5.50 (H-1), δ 4.32 (H-5), and δ 1.293 (CH3) (see Table III).
In particular the chemical shift of the CH3 protons differs
dramatically from that known to be characteristic for blood
group A determinants (δ ≈ 1.23, see Table VI). A possible
explanation for the apparent downfield shift might be the presence of an additional Fuc residue in α(1→3)-linkage to
GalNAc: of the N-acetyllactosamine unit to which the A deter-
mnant is attached (together withα(1→2) difucosyl A type-2 determinants, see Scheme 1). NMR evidence
for the presence of this type of determinant (in at least two
branches of the AS molecules, by virtue of the relative inten-
sity of the CH3 doublet at δ 1.29) stems from the fact that,
going from the H type-2 to the Y determinant by means of a
similar attachment of Fuc(1→3) to the GlcNAc residue, a
similar downfield shift of the CH3 group of Fuc(1→2) is
observed (Δδ ≈ 0.04 ppm; see Table V). A similar effect on
the chemical shift of the Fuc CH3 doublet in the step from
a type-2 monofucosyl A to a difucosyl A (so-called AY) deter-
mnant has been observed for glycolipids containing these
structural elements (51). In spite of the fact that the NMR
data on the glycolipids were obtained in Me2SO/D2O solu-
tions, drawing such an analogy to the NMR data reported
here for glycopeptides in D2O is meaningful because it has
been shown (52) that the conformations of such blood group
determinants do not change significantly from one solvent
system to another. Furthermore, the shift effects on the Fuc
structural-reporter groups observed in the step from df-AS to
AS (Table III) are essentially analogous to those observed
going from an αfucosyl-B to a difucosyl-B determinant (Scheme
1; Table VI). In addition to these two difucosyl A type-2
(and/or perhaps one difucosyl A type-1) determinants, some
branches in lower amount glycopeptide components of AS
might terminate in H or Y determinants. However, the com-
plexity of the mixture of AS glycopeptides, both with respect
to heterogeneity of branching pattern and to the occurrence
of various outer determinants, did not permit us to prove the
latter statement, let alone to localize any of the determinants
in any particular branch. The tentative comprehensive struc-
ture of glycopeptides AS has been included in Scheme 2.

Mass Spectrometry—The permethylated glycopeptide frac-
tions OS and AS were analyzed by EI and fast atom bom-
bardment (FAB) mass spectrometry, both in the positive
mode. No molecular ions could be recorded by the FAB
technique. Ions up to about 2000 mass units were recorded by
the EI technique. They correspond to the outer regions of the
OS and AS glycopeptides, with cleavage preferentially occur-
going at glycosidic linkages involving C-1 of HexNAc residues.

Fig. 6 shows the mass spectrum of the permethylated OS
glycopeptides recorded at 360 °C. The intersecting GlcNAc-9
was found as a terminal HexNAc at m/z 260. Terminal
trisaccharides Fuc-Hex-HexNAc were found at m/z 638 (and
606 = 638-32) corresponding to a blood group H sequence
(type-1 and -2 according to NMR), and an X determinant
(Hex-[Fuc]HexNAc) (supported by the peak at m/z 432). The
difucosyl Y determinant found in the NMR spectra is cor-
roborated by the peaks at m/z 812 Fuc-Hex-[Fuc]HexNAc and
780 (812-32). The extension of this structure by a Hex-
HexNAc unit is verified by the peaks at m/z 1261 and 1229.
An extension of the II or X terminals by a Hex-HexNAc unit
can be seen at m/z 1087 and 1055. The mass spectrum thus
supports the interpretation of NMR spectra regarding outer
region structures and once again shows the extent of hetero-
genety. The relative intensities of the different ions in
Scheme 2 is also in rough accordance with the intensities of
the different ions in the mass spectra. Higher molecular
weight ions were also recorded up to m/z 2433, but of low
intensity. This is only half-way up since one of the isomeric
sequences has a calculated nominal mass of 4981. The inter-
pretation of these is difficult as they probably are the result
of both pyrolysis and electron-induced fragmentation.

The mass spectrum of the permethylated AS glycopeptides
is shown in Fig. 7. Only fragments corresponding to terminal
sequences could be recorded. Terminal fragments similar to
the ones obtained for the OS glycopeptides are seen at m/z
638 and 812. The ions at m/z 812 are an indication that all
branches were terminated by HexNAc. Blood group A
terminal sequences with one Fuc are found at m/z 883 and
851, and at 1332 and 1300 for a structure with an additional
N-acetyllactosamine unit. Corresponding structures with one
additional Fuc can be seen at m/z 1057 and 1506, respectively.
Significant peaks at m/z 1680 and 1648 must be due to a
structure within 3 fucose residues, the additional one probably
located at the second internal HexNAc. The amount of this
latter structure is probably almost as large as that of the
difucosyl compound. The presence of the mono- and trisac-
charides A sequences in addition to the difucosyl sequence is in agree-

1 H. van Halbeek, unpublished results.
FIG. 6. Electron impact mass spectrum of permethylated glycopeptides OS (2 nmol) from the blood group O individual. The ion source was held at 360 °C, the electron energy was 40 eV, trap current 500 µA, and accelerating voltage 8 kV. The figures given refer to nominal masses.

FIG. 7. Electron impact mass spectrum of permethylated glycopeptides AS (2 nmol) from the blood group A individual. The ion source was held at 360 °C, the electron energy was 40 eV, trap current 500 µA, and accelerating voltage 8 kV. The figures given refer to nominal masses.

ment with the NMR data, although the presence of the entire sequences could not be specifically pointed out with NMR due to the restrictions of this methodology. For example, the innermost fucose residue in the trifucosyl A structure would be ranked among the NMR-determined sequences as an X determinant. The peak at m/z 182 is the base peak in the OS and AS mass spectra. This ion is found in samples containing type-2 sequences (53) and is also in accordance with the
and the results are described in detail elsewhere (3). The total nonacid glycolipids of the two cases (about 8 µg of glycolipid/mg cell protein) were analyzed by thin layer chromatography including overlay with monoclonal antibodies (Fig. 8). For structural information the total fractions were separated on silicic acid chromatography into two subfractions containing monoglycosylceramides and more polar glycolipids, respectively. The polar fractions were converted to permethylated and LiAlH₄-reduced permethylated derivatives, and these were analyzed in detail by NMR spectroscopy and by mass spectrometry using the direct inlet and a fractional distillation from the probe. The sequences concluded and their relative abundances are summarized in Table VII. Both samples contained monoglycosylceramides and blood group type glycolipids with 5–7 sugar residues as major glycolipids. In the blood group A sample, Le⁶ hexaglycosylceramides and type 1 A (ALe⁶) heptaglycosylceramides were present in about equal amounts. The blood group H sample had type-1 H pentaglycosylceramides as major complex glycolipid. Globotriaosylceramides and globotetraosylceramides, which are major glycolipids of the nonepithelial part of the small intestine, were absent from the epithelial cells. In both cases traces of more complex glycolipids were detected by mass spectrometry (Table VII) and by overlay with antibodies on the thin layer plate (Fig. 8). Blood group X determinants were absent from both glycolipid samples, while the blood group H sample contained a series of bands reacting with the anti-Y antibody (Fig. 8). Thus, the glycolipids had mainly type-1 chains and the glycopeptides mainly type-2 chains.

FIG. 8. Thin layer chromatogram of total nonacid glyco-
 sphingolipid fractions from small intestinal epithelial cells of human blood group A,Le(a–b+) secretor (lane A) and blood group O,Le(a–b–) secretor (lane O) individuals. Detection was done with a chemical reagent, anisaldehyde (top chromatogram) and by autoradiography after labeling with monoclonal anti-X antibodies (bottom chromatogram, left), and anti-Y antibodies (bottom chromatogram, right). In the top chromatogram, bands marked + in lane O are nonglycolipid contaminants and the designation to the right indicates number of sugar residues in each glycolipid band. A reference glycolipid, reacting with the antibody used, was applied to the left of the bottom chromatograms and the short hand designations, X-5 and Y-6, stand for the type-2 chain isomers of the Le⁶ and Le⁶ penta- and hexaglycosylceramides, respectively. Arrow indicates sample origin. The amount of glycolipid applied was 20 µg (top chromatogram) and 10 µg (bottom chromatograms) for lanes A and O and 2 µg for the reference compounds. Monoclonal antibodies used were anti-X, D,56-22 (13) and anti-Y, F-3 (55). Solvent was chloroform/methanol/water, 60:35:8 (by volume). Further details about the thin layer chromatography and the binding of antibodies to the plates are described in detail elsewhere (10, 13).

interpretation of the NMR spectra. No peaks giving evidence for the peptide portion were found, a result in accordance with EI-mass spectra of glycopeptides derived from human transferrin (54).

Glycolipid Composition of the Small Intestinal Epithelial Cells—The nonacid and acid glycosphingolipid fractions isolated from the epithelial cells were structurally characterized

DISCUSSION

Previous studies on human intestinal glycoproteins have focused on the properties of soluble mucins (5, 56). These molecules contain carbohydrate chains mainly of the O-glycosidic type. In contrast, the bulk of the carbohydrate in the small intestinal cells was found to be of the N-glycosidic type. The polyfucosylated glycopeptides isolated from human small intestinal epithelium display structural features distinct from glycopeptides isolated from several other sources. In contrast to glycopeptides of human erythrocytes (14, 57), human brain (58), rat plasma (59), rat kidney, liver and brain (60), or different types of cells grown in culture (19, 61, 62), the small intestinal glycopeptides are almost devoid of sialic acid, which is usually the most common terminal sugar in the carbohydrate units of glycoproteins. Sialic acid is present in the minor fraction of glycopeptides precipitable with cetylpyridinium chloride, but the sugar composition of these glycopeptides suggests that it consists mainly of O-glycosidic sugar chains.³ The main glycopeptide fraction, not precipitable with cetylpyridinium chloride, consists for the major part of N-glycosidic chains and is characterized by an unusually high content of fucose. Asparagine-linked glycopeptides with several fucose residues have previously been described from human granulocytes (63, 64). They differ from the small intestinal glycopeptides in that they are of the poly-N-acetyllactosamine type, contain fewer fucose residues, and also contain sialic acid. A striking feature of the intestinal glycopeptides is that the majority of potential fucosylation sites are in fact occupied by fucose residues.

³ J. Finne, unpublished results.
The glycolipids of the two human individuals were characterized by thin layer chromatography, mass spectrometry, $^1$H NMR spectroscopy and antibody binding. Note that several glycolipid components are present in the fractions as seen by antibody binding in Fig. 8. The amount of substance given (+++++ to + and trace (+)) is a semiquantitation from the thin layer plate and the ion intensity of mass spectra.

| Glycolipid structure | A$_{(a-b+)}$ secretor | H$_{(a-b-)}$ secretor |
|----------------------|----------------------|----------------------|
| Galβ1-1"Cer,Glcβ1-1"Cer | ++++ | ++++ |
| Hex-Hex-Cer | + | + |
| Ga1β1-3GlcNacβ1-3Galβ1-4Glcβ1-1"Cer | ++++ | + |
| Fucα1-2Galβ1-3GlcNacβ1-3Galβ1-4Glcβ1-1"Cer | + | (+) |
| Ga1β1-3(Fucα1-4)GlcNacβ1-3Galβ1-4Glcβ1-1"Cer | ++ | + |
| Ga1Nacβ1-3(Fucα1-2)Galβ1-HexNac-Hex-Cer | ++ | (+) |
| Ga1Nacβ1-3(Fucα1-2)Galβ1-3(Fucα1-4)GlcNacβ1-3Galβ1-4Glcβ1-1"Cer | ++++ | (+) |
| (Fuc)$_n$, HexNac, (Hex)$_n$, Cer | (+) | (+) |
| (Fuc)$_n$, (HexNac)$_n$, (Hex)$_n$, Cer | (+) | (+) |
| (Fuc)$_n$, (HexNac)$_n$, (Hex)$_n$, Cer | (+) | (+) |

are of the tri- and tetra-antennary type, and most of them contain an intersecting N-acetylgalactosamine residue as well as an α(1→6)-linked fucose residue attached to the core unit. The Gal-GlcNac backbone units are mainly type-2 chains, but on average one type-1 chain/core unit is present. Glycopeptides from the blood group A individual contain the A determinant structure, whereas those from the O individual contain the corresponding H structure. In addition α(1→3)-linked fucose residues are present in the X (or SSEA-1), Y and di-, and trifucosylated A determinant structures. The exact localization of the different terminals to the various antennae was not possible with the methods available. Sequential digestions with exo- and endoglycosidases (65) could potentially be used to study this question, but the limited amounts of glycopeptides available, as well as the inhibitory effects of the multiple fucose residues on among others endo-β-galactosidase cleavability prevented the use of this approach for the structural analyses.

The type-2 blood group A, H, and X determinant structures are known to occur in asparagine-linked sugar chains of human glycoproteins (14, 57, 63, 64, 66). The occurrence of the Y determinant structure has been reported recently (42, 67), whereas the type-1 H and the difucosyllated A structures seem to be novel for human N-linked glycans. All these structures are known constituents of glycolipids and O-linked chains of glycoproteins (2, 3, 10, 68), and thus give further evidence for the similarity of different classes of protein- and lipid-linked glycans (4).

Although FAB-mass spectrometry has previously been successfully used to characterize glycopeptides (69), more comprehensive results were obtained for the permethylated glycopeptides with EI-mass spectrometry. Like with large glycolipids studied before with the same technique (70), no peaks containing all the sugars were obtained. However, fragments corresponding to the outer branches confirmed the results obtained by NMR, and in addition clearly indicated the presence of a blood group A trifucosylated terminal. This type of structure has not been described for protein-linked glycans before but is analogous to the trifucosylated glycolipid described by Nudelman et al. (71).

The polyfucosylated glycopeptides represent a novel type of blood group active glycans of glycoproteins. The previously characterized glycans include the O-glycosidic series of structures of the blood group substances of ovarian cysts and other mucins (72, 73), and the N-glycosidic polyglycosyl (poly-N-acetyllactosamine) peptides of human erythrocytes (14, 57). The small intestinal glycopeptides differ from the latter by their much smaller size and their higher content of fucose. In the erythrocyte polyglycosylpeptides the blood group determinants only occur in part of the glycans, and these glycans seem to be of the largest size among these glycans (14, 57). In contrast to this, the small intestinal glycopeptides contain a high content of blood group determinant structures bound to a normal small-size backbone structure, which indicates that there are different factors regulating the expression of blood group determinants on the erythrocytes and the small intestinal cells.

The present report together with the analyses of the glycolipids of the same source (10) represents the first cell type, besides erythrocytes, where both major types of blood group active glycoconjugates have been characterized in detail in order to get a more complete picture of the cell surface. Therefore, it is of special interest to compare the results with those of erythrocytes. Table VIII shows that the concentration of blood group A,H determinants on the small intestinal cells is 20–50 times that on erythrocytes, as estimated from the amounts of the determinants in the glycopeptides and their approximate yields during the purification. If the carbohydrate is found mainly on the cell surface, as is generally

| Cell type               | Blood group A, H determinants Total | Ratio protein/lipid | Sphingolipids Total | Sphingolipids: protein/lipid |
|------------------------|-------------------------------------|---------------------|-------------------|-----------------------------|
| Small intestine        | 1000$^a$                           | 15                  | 1000$^a$          | 110$^d$                     |
| Erythrocytes           | 20–60                               | 2                   |                   |                             |

$^a$ Sphingolipids (glycosphingolipids and sphingomyelin) are localized to the outer leaflet of the plasma membrane and make up 1/4–1/5 of the lipid molecules (2). The sphingolipid value thus gives a rough measure of the plasma membrane surface area.

$^b$ The estimate of the A or H determinants was based on the amounts of N-acetylgalactosamine and 2-substituted galactose in blood group A and O glycopeptides, respectively, taking into account the amounts of glycopeptides containing these structures and their yields during the isolation procedure.

$^c$ From Ref. 74.

$^d$ Calculation based on Refs. 2 and 75.
believed (2), and if the sphingolipids are found in the outer part of the plasma membrane lipid bilayer making up 15–15% of the lipid molecules, then the density of blood group determinants on the small intestine epithelial cells is very high indeed (compare first and last columns of Table VIII). The greater contribution of A, H determinants by glycoproteins versus glycolipids is similar for the two sources. On the other hand, it is striking that the majority of the determinants of the small intestinal glycopeptides are of type 2, whereas the major blood group glycolipids are of type 1.

An unusual feature of the small intestinal glycopeptides is also the low proportion of concanavalin A-binding glycopeptides. The fraction bound most strongly to this lectin, comprising as binding sites for gluten 25%, is in accordance with the VZZZth International Symposium on Glycoconjugates (Davidson, E. A., Williams, J. C., and DiFerrante, N. M., eds) Vol. 1, 123–124.

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