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# Structure of Seven Oligosaccharides Excreted in the Urine of a Patient with Sandhoff's Disease ($GM_2$ Gangliosidosis-Variant O)

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The urine of a patient with Sandhoff's disease ($GM_2$ gangliosidosis-variant O) contains 10–12 *N*-acetylglucosamine-rich oligosaccharides in high amounts. The structures of seven of these have been determined: β-GlcNAc-(1→2)-α-Man-(1→3)-β-Man-(1→4)-GlcNAc; β-GlcNAc-(1→4)-α-Man-(1→3)-β-Man-(1→4)-GlcNAc; β-GlcNAc-(1→2)-α-Man-(1→6)-β-Man-(1→4)-GlcNAc; β-GlcNAc-(1→4)-α-Man-(1→6)-β-Man-(1→4)-GlcNAc; β-GlcNAc-(1→2)-α-Man-(1→3)-[β-GlcNAc-(1→2)-α-Man-(1→6)]β-Man-(1→4)-GlcNAc; β-GlcNAc-(1→2)-α-Man-(1→3)[β-GlcNAc-(1→2)-α-Man-(1→6)] [β-GlcNAc-(1→4)]β-Man-(1→4)-GlcNAc; β-GlcNAc-(1→2)-α-Man(1)-(1→3) [β-GlcNAc-(1→2)-α-Man(2)-(1→6)]β-Man-(1→4)-GlcNAc, with additional β-GlcNAc-(1→4) on mannose (1) or (2).

An unusual oligosaccharide, with a tri-branched β-mannose, has been characterized as the major component excreted in urine.

$GM_2$ gangliosidosis-variant *O* or Sandhoff's disease is characterized by a defect of both the A and B components of β-*N*-acetylhexosaminidase and the accumulation of $GM_2$-ganglioside and globoside in the tissues [1]. In addition, an important urinary excretion of oligosaccharides containing *N*-acetylglucosamine and mannose has been observed [2]. Recently Ng Ying Kin and Wolfe [3] described the isolation and characterization of a heptasaccharide from the liver of a patient with $GM_2$ gangliosidosis.

In this communication we describe the structure of seven oligosaccharides isolated from the urine of a new patient.

## MATERIALS AND METHODS

### *Fractionation of Urinary Oligosaccharides*

1 l freshly collected urine was demineralized on columns (2 × 40 cm) of Dowex 50 *X* 8, 25–50 mesh, $H^+$; and Dowex 1 *X* 8, 25–50 mesh, $HCOO^-$. The neutral effluent was then analyzed by paper chromatography and electrophoresis, using the following solvents: solvent 1: pyridine/ethyl acetate/acetic acid/water (5/5/1/3); solvent 2: propan-1-ol/ethyl acetate/water (6/1/3); and the buffer: 0.025 M sodium tetraborate in water, pH 9.2. Sugars were stained with aniline oxalate reagent [4]. In the case of paper electrophoresis, borate ions were eliminated by treatment with methanol/acetic acid (5/1), followed by heating at 100 °C during 5 min.

### *Structure of Oligosaccharides*

Monosaccharides were determined by gas-liquid chromatography [5]. The terminal reducing monosaccharide was converted into the alditol by reduction with $KBH_4$ and identified after hydrolysis of the oligomer with 4 M $CF_3COOH$ (100 °C; 4 h) by paper chromatography in solvent 1.

Methylation of reduced oligosaccharides was carried out as described by Hakomori [6] and the methyl ethers of neutral monosaccharides and *N*-methyl-

*Abbreviation.* NMR, nuclear magnetic resonance

glucosamine were identified according to Fournet *et al.* [7].

The nature of linkage of mannose to the *N*-acetylglucosamine in reducing position was established by studying the products obtained after alkali treatment (1 M $Na_2CO_3$ at 100 °C during 10 min) with *p*-dimethylaminobenzaldehyde reagent (indirect Ehrlich reaction) [8]. *β*-*N*-Acetylglucosamine in terminal non-reducing position was hydrolyzed with *β*-*N*-acetylhexosaminidase from *Aspergillus niger*. $^1$H NMR (nuclear magnetic resonance) spectroscopy was performed at 360 MHz on a Bruker HX-360 spectrometer, operating in the Fourier transform mode at probe temperatures of 25 °C and 60 °C. Chemical shifts are given relative to sodium 2.2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in $^2H_2O$: $\delta = 2.225$ ppm).

## RESULTS

### *Fractionation of Oligosaccharides*

Oligosaccharides containing *N*-acetylglucosamine were isolated according to the scheme described in Fig. 1. Oligosaccharides 1 and 2 possess the same migration rate in solvent 2. Preparative electrophoresis in buffer pH 9.2 allowed the elimination of fucose-containing oligosaccharides, which contaminated the fraction 1+2. Finally oligosaccharides 1 and 2 were isolated using solvent 2.

Similarly, the mixture of oligosaccharides 3+4 was first isolated using solvent 1. Fractionation of compounds 3 and 4 was carried out using solvent 2; oligosaccharide 4 was still contaminated with oligosaccharides containing fucose. The latter components were hydrolyzed with 0.025 M $H_2SO_4$ (100 °C; 30 min) and oligosaccharide 4, which is stable under these conditions, was isolated by preparative chromatography in solvent 1.

Oligosaccharides 5, 6 and 7 were isolated in solvent 1, developed during 4 days. It was impossible to obtain compounds 6 and 7 in a pure state; oligosaccharide 7 contained a considerable amount of oligosaccharide 6, as we confirmed by methylation analysis. Oligosaccharide 6 contains less than 10% of compound 7.

### *Oligosaccharide Structures*

The molar carbohydrate compositions of the seven oligosaccharides are reported in Table 1. All possess an *N*-acetylglucosamine residue in terminal reducing position. These terminal residues are substituted at C-4 as they do not react with *p*-dimethylaminobenzaldehyde reagent, after alkali treatment (indirect Ehrlich reaction).

The results of exhaustive methylation are given in Table 2 and Fig. 2.

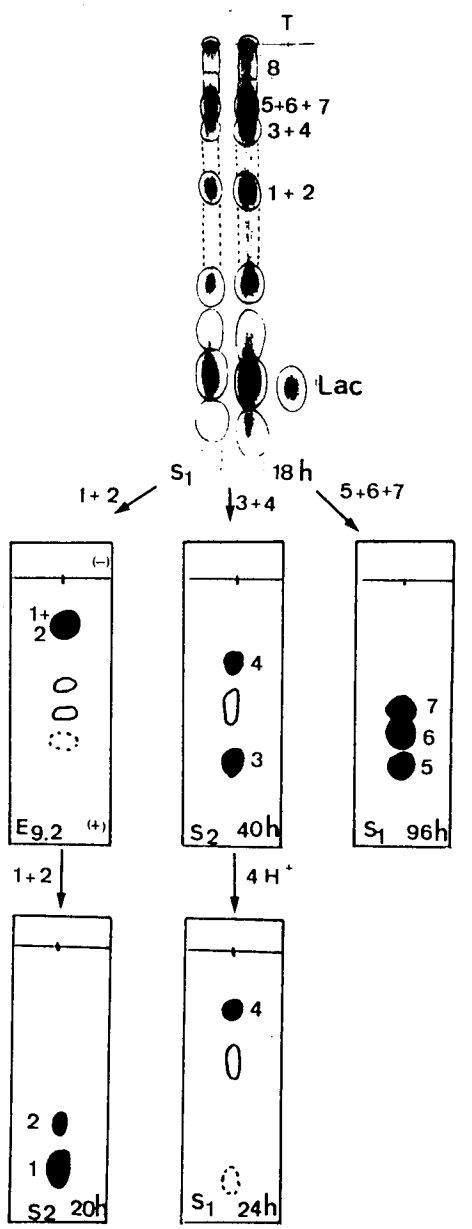


Fig. 1. *Scheme of fractionation of oligosaccharides.* $S_1$: solvent 1; $S_2$: solvent 2; $E_{9.2}$: electrophoresis in buffer sodium tetraborate pH 9.2

Table 1. *Occurrence and composition of oligosaccharides isolated from urine*
Composition was determined on the basis of 2 or 3 mannose residues

| Oligosaccharides | Amount | Molar ratios | |
|---|---|---|---|
| | | GlcNAc | Man |
| | mg/l | | |
| 1 | 16 | 2.00 | 2 |
| 2 | 8 | 1.84 | 2 |
| 3 | 6 | 2.25 | 2 |
| 4 | 3 | 2.30 | 2 |
| 5 | 52 | 2.91 | 3 |
| 6 | 105 | 3.86 | 3 |
| 7 | 16 | 4.00 | 3 |

Table 2. *Identification and determination of methylated monosaccharides obtained from permethylated oligosaccharides*

| Methylated monosaccharides | Oligosaccharides | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 1[a] | 2 | 2[a] | 5 | 6 | 7 |
| 2,3,4,6-Tetra-*O*-methyl-mannose | | 0.72 | | 0.64 | | | |
| 3,4,6-Tri-*O*-methyl-mannose | 0.68 | 0.18 | 0.74 | 0.27 | 2 | 2 | 1 |
| 2,3,6-Tri-*O*-methyl-mannose | 0.34 | 0.14 | 0.52 | 0.19 | | | |
| 2,4,6-Tri-*O*-methyl-mannose | 1 | 1 | | | | | |
| 2,3,4-Tri-*O*-methyl-mannose | | | 1 | 1 | | | |
| 2,4-Di-*O*-methyl-mannose | | | | | 0.85 | 0.08 | 0.51 |
| 3,6-Di-*O*-methyl-mannose | | | | | | 0.09 | 0.41 |
| 2-Mono-*O*-methyl-mannose | | | | | | 0.72 | 0.58 |
| 3,4,6-Tri-*O*-methyl-*N*-methylglucosamine | + | traces | + | traces | + | + | + |

[a] Oligosaccharides 1 and 2 hydrolyzed with β-*N*-acetylglucosaminidase.

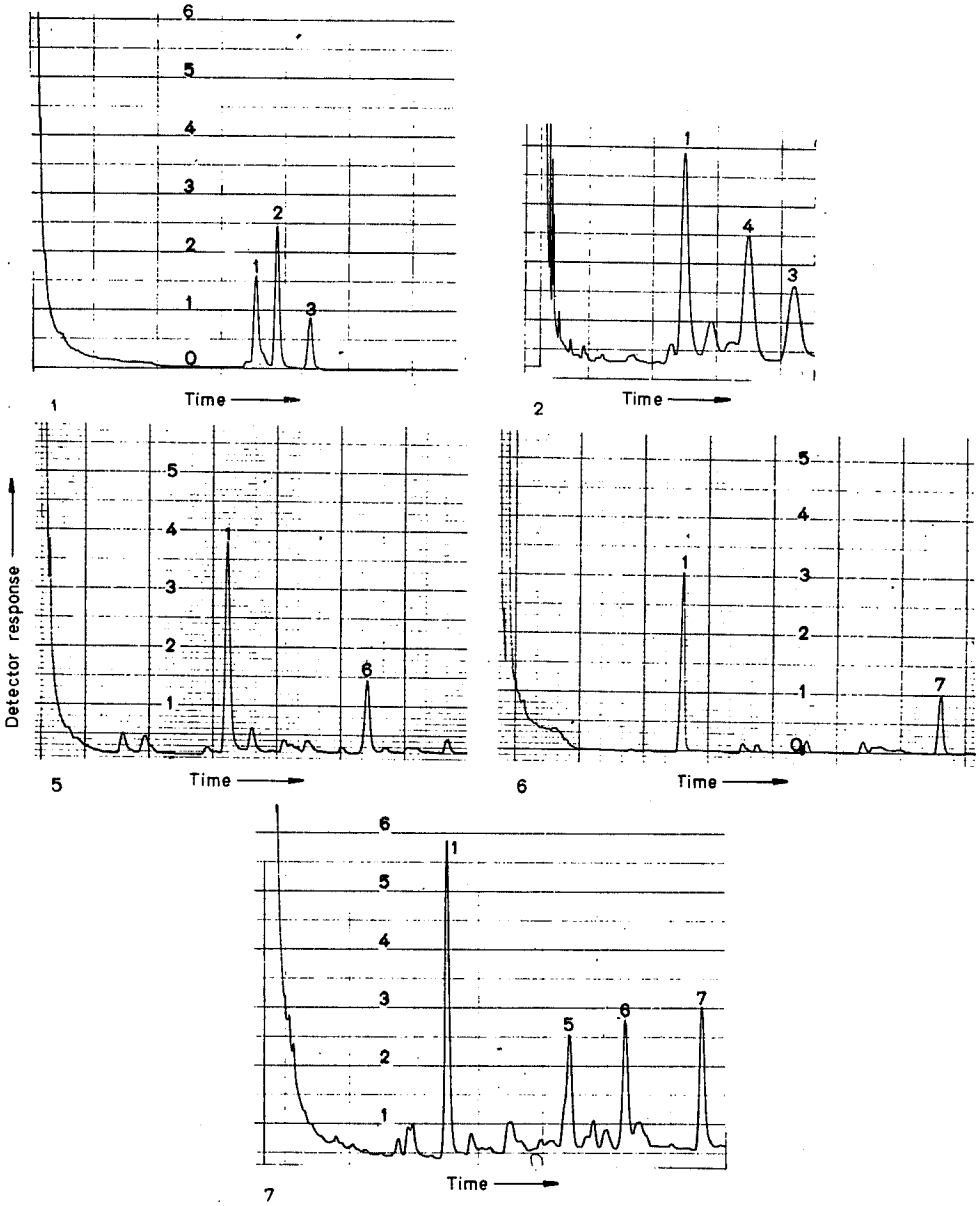


Fig. 2. *Gas-liquid chromatography on Carbowax 6000 of methyl ethers of neutral monosaccharides present in the methanolysates of permethylated urinary oligosaccharides 1,2,5,6 and 7.* Peaks: (1) 3,4,6-tri-*O*-methyl-mannoside; (2) 2,4,6-tri-*O*-methyl-mannoside; (3) 2,3,6-tri-*O*-methyl-mannoside; (4) 2,3,4,tri-*O*-methyl-mannoside; (5) 3,6-di-*O*-methyl-mannoside; (6) 2,4-di-*O*-methyl-mannoside; (7) 2-monomethyl-mannoside. Methyl ethers were analyzed as the methyl glycosides (oligosaccharides 1 and 5), silyl ethers of methyl glycosides (oligosaccharide 2) or acetate esters of methyl glycosides (oligosaccharides 6 and 7)

Table 3. *$^1H$ NMR data at 25 °C of anomeric protons and mannose-H-2 for oligosaccharides 5 and 6*
For coding of monosaccharide residues, see Fig. 3

| Proton | Residue | Oligosaccharide 5 | | Oligosaccharide 6 | |
|---|---|---|---|---|---|
| | | $\delta$ | $(J_{1,2})$ | $\delta$ | $(J_{1,2})$ |
| | | ppm | (Hz) | ppm | (Hz) |
| H-1 of residue | a | 5.21 | (2.6)α | 5.21 | (2.5)α |
| | | 4.72 | (7.7)β | 4.72 | (≈7.5)β |
| | b | 4.78 | (<1) | 4.70 | (<1)[a] |
| | | | | 4.71 | (<1)[a] |
| | c | 5.12 | (1.4) | 5.06 | (1.5) |
| | d | 4.92 | (1.6) | 5.00 | (1.6) |
| | e | 4.56 | (8.1) | 4.56 | (8.3) |
| | f | 4.56 | (8.1) | 4.56 | (8.3) |
| | g | – | | 4.47 | (8.5) |
| H-2 of residue | b | 4.25[a] | | 4.18[a] | |
| | | 4.26[a] | | 4.19[a] | |
| | c | 4.19 | | 4.25 | |
| | d | 4.11 | | 4.15 | |

[a] See text.

Oligosaccharide 1 furnished a mixture of 3,4,6-, 2,3,6- and 2,4,6-tri-*O*-methyl-mannose in the proportions 0.68:0.34:1 besides 3,4,6-tri-*O*-methyl-glucosamine. After removing the terminal *N*-acetylglucosamine residue with β-*N*-acetylhexosaminidase, a trisaccharide was isolated having the same migration rate as the compound α-Man-(1→3)-β-Man-(1→4)-GlcNAc obtained from urine of mannosidosis. Methylation of the product of the enzymic hydrolysis gave 2,3,4,6-tetra-*O*-methyl-mannose and 2,4,6-tri-*O*-methyl-mannose in the proportions 0.72:1, with small amounts of 3,4,6- and 2,3,6-tri-*O*-methyl-mannose (0.18:0.14), resulting from incomplete enzymic digestion. These results point to the conclusion that compound 1 consists of a mixture of two isomers, *viz.* 1a: β-GlcNAc-(1→2)-α-Man-(1→3)-β-Man-(1→4)-GlcNAc; 1b: β-GlcNAc-(1→4)-α-Man-(1→3)-β-Man-(1→4)-GlcNAc.

The methylation analysis of oligosaccharide 2 gave a mixture of 3,4,6-, 2,3,6- and 2,3,4-tri-*O*-methyl-mannose in the proportions of 0.74:0.52:1, besides 3,4,6-tri-*O*-methylglucosamine. After the action of β-*N*-acetylhexosaminidase, methylation analysis furnished 2,3,4,6-tetra-*O*-methylmannose, 3,4,6-, 2,3,6- and 2,3,4-tri-*O*-methyl-mannose in the proportions of 0.64:0.27:0.19:1. Taking into account a possible incomplete enzymic digestion it can be concluded that oligosaccharide 2 also consists of a mixture of two isomers, *viz.* 2a: β-GlcNAc-(1→2)-α-Man-(1→6)-β-Man-(1→4)-GlcNAc; 2b: β-GlcNAc-(1→4)-α-Man-(1→6)-β-Man-(1→4)-GlcNAc.

The methylation analysis of compounds 3 and 4 led to the conclusion that these were mixtures of more than three or four oligosaccharides but assignment of the structures was impossible.

The methylation analysis of oligosaccharide 5 gave 3,4,6-tri-*O*-methyl-mannose and 2,4-di-*O*-methyl-mannose in the proportions 2:0.85, besides 3,4,6-tri-*O*-methyl-*N*-methylglucosamine. The part of the 360 MHz $^1H$ NMR spectrum containing the resonances of the anomeric protons and the mannose H-2 protons of oligosaccharide 5 is presented in Fig. 3A (for $^1H$ NMR data of reference glycopeptides and oligosaccharides see [9]. Based on these data the following structure of the oligosaccharide is deduced: β-GlcNAc-(1→2)-α-Man-(1→3)-[β-GlcNAc-(1→2)-α-Man-(1→6)]-β-Man-(1→4)-GlcNAc. With regard to the assignment of the various NMR signals the following remarks are relevant: The resonance positions of the mannose-H-1 protons and mannose-H-2 protons (Table 3) are identical to those found for the asialo-glycan chains of human serotransferrin [9]. This indicates that the molecule contains the mannotriosido-branching-core, which is surrounded by *N*-acetylglucosamine residues a, e and f (Fig. 3A). Also the anomeric protons of *N*-acetylglucosamine residues e and f resonate at the same position as the corresponding residues of serotransferrin. *N*-acetylglucosamine residue a is reducing as shown by the doublets at $\delta = 5.21$ ppm (0.65 proton, α-anomer) and 4.72 ppm (0.35 proton, β-anomer). There is a small difference in the chemical shift of H-1 of mannose residue b between both anomers of oligosaccharide 5. A similar phenomenon is observed for H-2 of residue b.

The methylation analysis of oligosaccharide 6 afforded the following derivatives: 3,4,6-tri-*O*-methyl-

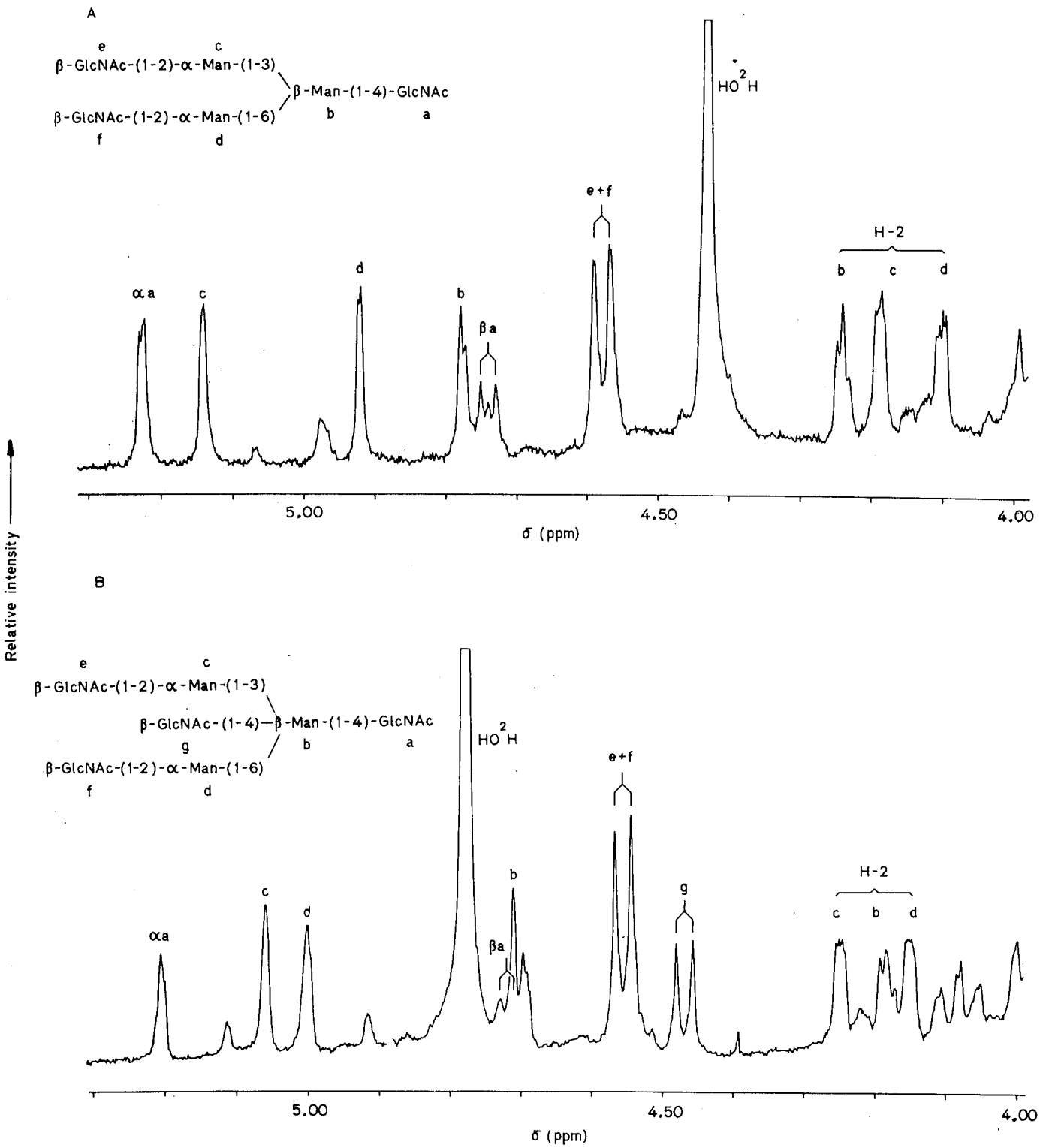


Fig. 3. *360-MHz $^1$H NMR spectra of oligosaccharides 5 and 6.* The region of the anomeric protons and mannose-H-2 protons of oligosaccharide 5 (A) is given at 60 °C to visualize the resonance at 4.78 ppm. The corresponding region of the spectrum of oligosaccharide 6 (B) is given at 25 °C. Change in temperature effects only slightly the resonance positions ($\Delta\delta \leqslant 0.02$ ppm)

mannose, 2,4-di-*O*-methyl-mannose, 3,6-di-*O*-methyl-mannose and 2-*O*-methyl-mannose in the proportions 2:0.08:0.09:0.72, besides 3,4,6-tri-*O*-methyl-*N*-methyl-glucosamine. The partial $^1$H NMR spectrum of oligosaccharide 6 is given in Fig. 3B. The assignment of the resonances of the anomeric protons and the mannose-H-2 protons was made by comparison with the spectrum of oligosaccharide 5 and spectral data of

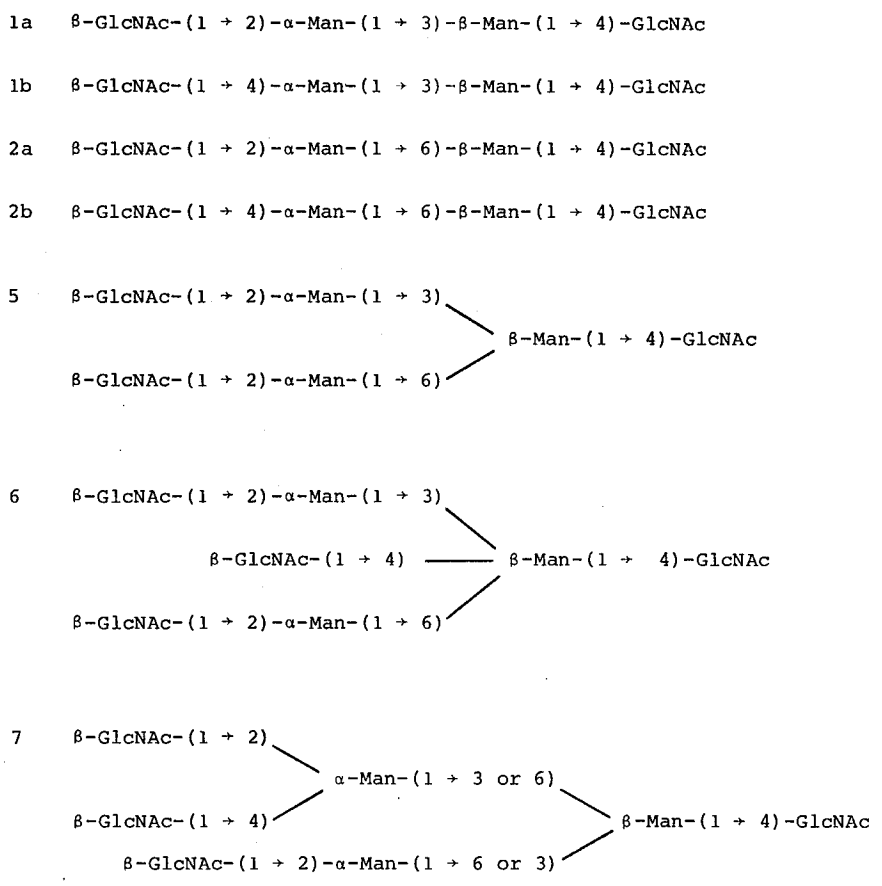


Fig. 4. *Structure of the seven oligosaccharides isolated from urine of Sandhoff's disease*

reference oligosaccharides obtained from ovomucoid acetolysates, respectively, and by selective irradiation experiments (NMR data of reference oligosaccharides will be published in detail elsewhere).

Based on methylation analysis and NMR data it is concluded that compound 6 has the following structure: β-GlcNAc-(1→2)-α-Man-(1→3)[β-GlcNAc-(1→4)][β-GlcNAc-(1→2)-α-Man-(1→6)]-β-Man-(1→4)-GlcNAc. In the NMR spectrum of compound 6 a number of resonance positions are unchanged with respect to compound 5, *viz.* the anomeric proton of residues a, e and f (see Table 3). Owing to the presence of the additional *N*-acetylglucosamine residue g linked to the 4-position of the mannose residue b, the chemical shifts of the H-1 and H-2 protons of the mannotriosido-branching-core are all significantly changed.

The methylation analysis of oligosaccharide 7 gave 3,4,6-tri-*O*-methyl-mannose, 2,4- and 3,6-di-*O*-methyl-mannose and 2-*O*-methyl-mannose in the proportions of 1:0.51:0.41:0.58 besides 3,4,6-tri-*O*-methylglucosamine. These data suggest that oligosaccharide 7 consists of a mixture of oligosaccharide 6 and an isomer there of, *viz.*: β-GlcNAc-(1→2)-α-Man*-(1→3)[β-GlcNAc-(1→2)-α-Man*-(1→6)]β-Man-(1→4)-GlcNAc, with an additional residue of β-GlcNAc-(1→4) on α-Man*.

The latter structure is based on analogy reasoning since the limited amount of oligosaccharide 7 did not allow any further structural characterization.

## DISCUSSION

Deficiency in both hexosaminidases A and B lead to incomplete catabolism of *N*-acetyllactosamine-type glycoproteins [10], giving rise to accumulation in urine of oligosaccharides. These oligosaccharides result from the action of an endo-β-*N*-acetylglucosaminidase, which remains to be characterized in human tissues since the known enzyme of this type is only active on glycopeptides which contain a terminal mannose residue [11].

The origin of these incompletely degraded glycoproteins must be sought in all tissues and body fluids, since the mannotriosido-di-*N*-acetylchitobiose core is widely distributed in glycan structures [10].

Recently Ng Ying Kin and Wolfe [3] described the accumulation of oligosaccharides in liver of a patient with Sandhoff's disease. They found oligosaccharide 7 as a major component. However, in our case, oligosaccharides 5 and 6 appeared to be the major constituent and oligosaccharide 7 was a minor product

which represents less than 5% of the accumulated material. Structures of the type of oligosaccharide 6, with a tri-branched β-mannose residue, have been described [12] in human myeloma immunoglobulins $IgA_1$ and IgG. It is interesting to note that this unusual type of structure was only recognized in a few cases in which glycoproteins were isolated from one single subject. It has been discussed by Kornfeld and Kornfeld [12] that this may reflect genetic control of the specificity of *N*-acetylglucosaminyltransferases.

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