Eur. J. Biochem. 117, 291 – 300 (1981)

Structures of Fifteen Oligosaccharides Isolated from New-Born Meconium

Marie-Claire HERLANT-PEERS, Jean MONTREUIL, and Gérard STRECKER
Laboratoire de Chimie Biologique et Laboratoire Associé 217 du Centre National de la Recherche Scientifique,
Université des Sciences et Techniques de Lille I;
Institut de Recherches sur le Cancer (Institut Jules Driessens) et Unite 124 de l'Institut National de la Santé et de la Recherche Médicale, Lille

Lambertus DORLAND, Herman van HALBEEK, Gerrit A. VELDINK, and Johannes F. G. VLIEGENTHART
Department of Bio-organic Chemistry, University of Utrecht

(Received November 21, 1980 / March 31, 1981)

New born meconium contains at least a hundred oligosaccharides. In this study the isolation and characterization of the major constituents is described. The structure elucidation of 15 neutral and acidic oligosaccharides was carried out by methylation analysis, mass spectrometry and 360-MHz ^{1}H-NMR spectroscopy. The results show that the oligosaccharides accumulating in human meconium are probably products of the catabolism of the O- and N-linked carbohydrate chains of glycoproteins. It is proposed that endo-*N*-acetyl-α-D-galactosaminidase, endo-β-D-galactosidase and endo-*N*-acetyl-β-D-glucosaminidase are involved in the production of these compounds.

Meconium is known to be a rich source of immunologically active glycoproteins and glycolipids [1 – 4]. Furthermore, it contains a wide variety of oligosaccharides which represent partial structures of these glycoconjugates. To gain insight into the metabolic fate of glycoconjugates at the end of embryonal development, an investigation was started on the isolation and characterization of the oligosaccharide constituents of human meconium.

The present paper describes the structure elucidation of 15 oligosaccharides by permethylation analysis, mass spectrometry and 360-MHz ^{1}H-NMR spectroscopy.

MATERIALS AND METHODS

Fractionation of Oligosaccharides [5]

2 kg meconium, collected from 80 new borns, was homogenized with 6 l water and the insoluble material was eliminated by centrifugation. The supernatant was concentrated under reduced pressure to 2 l and macromolecular material was then precipitated by the addition of 3 l ethanol (96%). After centrifugation the supernatant was evaporated to dryness and the residue dissolved in 1 l water. The insoluble material was discarded by filtration and the solution was demineralized by passage through columns (4 × 40 cm) of Dowex 50X8, 25 – 50 mesh, H^+, and Dowex 1X8, 25 – 50 mesh, $HCOO^-$, respectively. The effluent was then submitted to adsorption chromatography on a column (5 × 40 cm) of charcoal-celite. After washing with water (5 l), carbohydrate-containing material was desorbed by 10 l 50% ethanol in water. The latter eluate was evaporated to dryness and the residue dissolved in 1 l water.

The concentrated sugar-containing eluate was desalted on a column (3 × 30 cm) of Dowex 50X2, 200 – 400 mesh, H^+, by washing with water (2 l). Subsequently, fractionation was carried out on a column (3 × 30 cm) of Dowex 1X2, 200 – 400 mesh, CH_3COO^-. Desorption of the acidic compounds was achieved by a discontinuous gradient of pyridine/acetate buffer (pH 5.5) of increasing concentration (1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 mM, respectively).

The neutral fraction was further submitted to adsorption chromatography on a column (3 × 30 cm) of charcoal-celite, and neutral oligosaccharide were desorbed by a discontinuous gradient of ethanol in water (3.5, 5, 7.5, 10, 15 and 20%, respectively).

The carbohydrate material was analyzed by paper chromatography on Whatman no. 3 paper in the following solvents: (1) ethyl acetate/pyridine/acetic acid/water (5/5/1/3, v/v); (2) *n*-butanol/acetic acid/water (4/1/5, v/v), and by paper electrophoresis on Whatman no. 3 paper in 1% sodium tetraborate in water (pH 9.2). Carbohydrates were stained with aniline oxalate reagent [6]. Performing electrophoresis in borate buffer, the paper was impregnated with methanol/acetic acid (5/1, v/v) and dried at 80°C, in order to remove borate ions prior to treatment with the aniline oxalate reagent.

Analytical Methods

The molar ratios of hexoses, *N*-acetylhexosamines and *N*-acetylneuraminic acid were determined by gas-liquid chromatography, according to Zanetta et al. [7], after methanolysis with methanol/0.5 M HCl for 24 h at 80°C. After reduction of the oligosaccharide with sodium borohydride the reducing end residue was identified as its alditol.

Methylation analysis of reduced oligosaccharides was performed according to Hakomori [8]. Methyl glycosides resulting from methanolysis of a permethylated reduced oligosaccharide were analyzed by gas-liquid chromatography before (neutral) and after (*N*-acetylhexosamines) peracetylation as described by Fournet et al. [9, 10]. Gas-liquid chromatography was carried out with an Aerograph 1200 (Varian, Orsay, France), equipped with a glass column (0.3 × 300 cm, Carbowax 6000 on

Abbreviations. Fuc, L-fucose; Gal, D-galactose; Man, D-mannose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; NeuAc, *N*-acetylneuraminic acid.

Chromosorb W-HMDS, 60–80 mesh, N_2, flow rate 30 ml/min, temp. 110–220°C, 2°C/min). The identity of the methyl ethers of the monosaccharides was confirmed by comparison of their mass spectra with those of standards [10]. Gas-liquid chromatography/mass spectrometry was carried out with a Riber-Mag, model 10-10 (Rueil-Malmaison, France), coupled with a capillary glass column coated with SE-30 (110–220°C, 2°C/min).

The permethylated reduced disaccharides and trisaccharides were analyzed also as intact compounds by gas-liquid chromatography/mass spectrometry.

Fucose was split off by mild acid hydrolysis in 25 mM H_2SO_4 for 20 min at 100°C. The resulting afuco compound was isolated by preparative paper chromatography and analyzed as described above.

For NMR analysis the oligosaccharides were repeatedly exchanged in ^2H_2O (Aldrich). The 360-MHz ^{1}H-NMR spectra were recorded on a Bruker HX-360 spectrometer, operating in the Fourier transform mode at probe temperatures of 25°C or 60°C. Chemical shifts are given at 25°C relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in ^2H_2O: δ = 2.225 ppm).

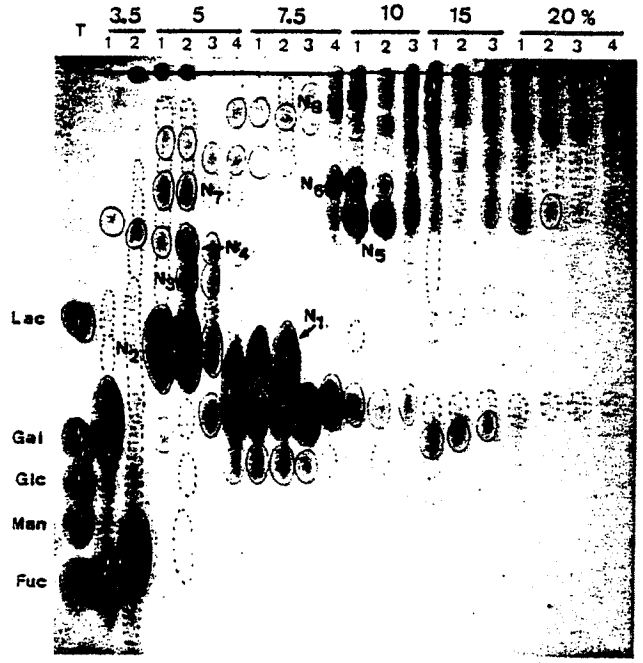


Fig. 1. *Paper chromatography of neutral oligosaccharides eluted from a charcoal-celite column by a discontinuous gradient of ethanol.* Time of migration = 18 h

RESULTS

Fractionation of Neutral and Acidic Oligosaccharides

In Fig. 1 the stained paper chromatogram of neutral oligosaccharides, eluted from the charcoal-celite column by a discontinuous gradient of ethanol in water, is depicted. In Fig. 2 the paper chromatogram of acidic oligosaccharides, eluted from the anion exchanger (Dowex 1X2) by a discontinuous gradient of pyridine/acetate buffer, is shown. The two chromatograms represent an overwhelming amount of oligosaccharides: at least a hundred different components can be distinguished. By preparative paper chromatography and electrophoresis, 12 fractions were isolated. Details upon chromatographic isolation, yield and carbohydrate composition of each of the oligosaccharides present in the fractions mentioned above, are given in Table 1.

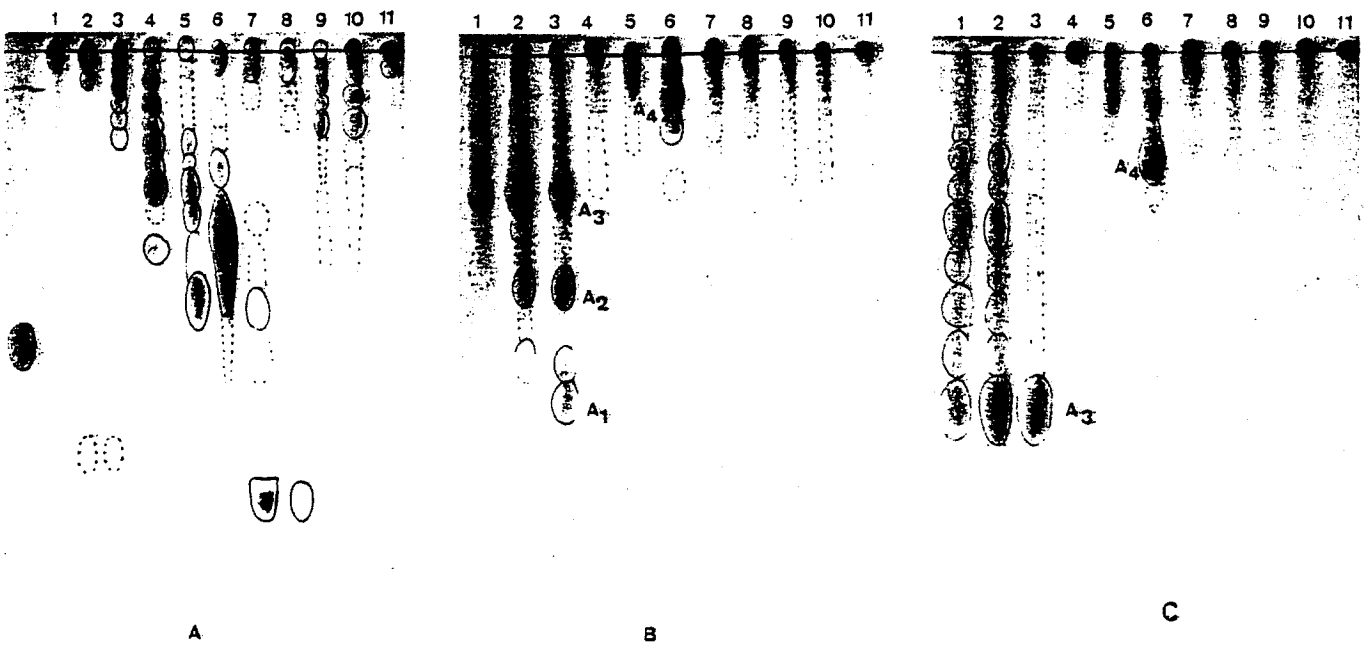


Fig. 2. *Paper chromatography of acidic oligosaccharides eluted from an anion exchanger (Dowex 1X2, 200–400 mesh, CH_3COO^-) by a discontinuous gradient of pyridine/acetate buffer (pH 5.5).* (1) 1 mM; (2) 2 mM; (3) 5 mM; (4) 10 mM; (5) 20 mM; (6) 50 mM; (7) 100 mM; (8) 200 mM; (9) 500 mM; (10) 1000 mM; (11) 2000 mM. Time of migration in solvent 1: (A) 18 h; (B) 2 weeks; (C) 1 month

Table 1. *Characteristics of 12 oligosaccharide fractions isolated from meconium*
Composition of N-1 to N-7 was determined on the basis of 1 or 2 galactose residues molecule, that of N-8, A-1 to A-4 on the basis of 3 mannose residues/molecule

Oligosaccharide fraction	Quantity isolated from 2 kg fresh meconium	Preparative paper chromatography in solvent system(s)	Molar ratios of							Sugar in reducing position
			Gal	Glc	Man	Fuc	GalNAc	GlcNAc	NeuAc	
	mg									
N-1	969	1[a]	1	–	–	–	–	1.02	–	GlcNAc
N-2	122	1[a]	1	–	–	–	1.00	–	–	GalNAc
N-3	16	1, 2[a]	1	–	–	0.88	–	0.97	–	GlcNAc
N-4	36	1[a]	1	1.12	–	1.15	–	–	–	Glc
N-5	45	1, 2	2	–	–	–	–	1.12	–	Gal
N-6	15	1	2	–	–	–	–	1.09	–	Gal
N-7	11	1	1	1.15	–	2.20	–	–	–	Glc
N-8	12	1	1.16		3	0.16	–	4.11	–	GlcNAc
A-1	71	1	1.25	–	3	–	–	2.04	1.11	GlcNAc
A-2	88	1	1.23	–	3	–	–	3.07	1.06	GlcNAc
A-3	296	1	1.89	–	3	–	–	3.22	1.05	GlcNAc
A-4	105	1	2.00	–	3	–	–	3.09	2.01	GlcNAc

[a] Further purified by electrophoresis.

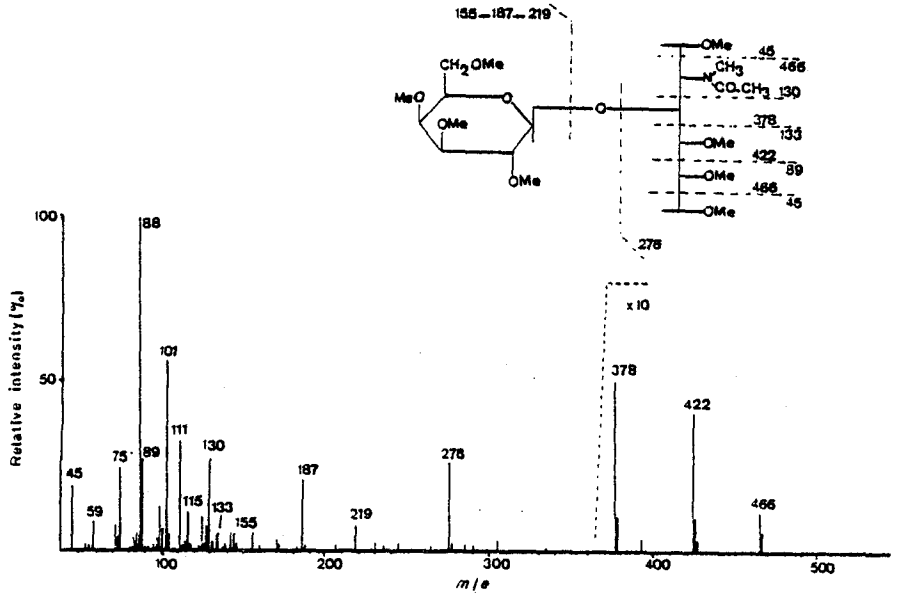


Fig. 3. *Mass spectrum and fragmentation pattern of the permethylated-reduced disaccharide N-1: β-Gal-(1→3)-GlcNAc*

STRUCTURES OF NEUTRAL OLIGOSACCHARIDES (N-1 TO N-8)

N-1

The mass spectrum of the permethylated reduced compound (see Fig. 3) proves it to be a disaccharide having GlcNAc in reducing position (fragment *m/e* 276). The fragments *m/e* 219, 187 and 155 correspond to the terminal Gal. The fragments *m/e* 133, 378 and 422 are characteristic of a (1→3) linkage. The 360-MHz ^{1}H-NMR spectrum of N-1 is given in Fig. 4. The signal at 5.171 ppm ($J_{1,2}$ = 3.1 Hz) and the signal at 4.746 ppm ($J_{1,2}$ = 7.3 Hz) represent the anomeric protons of the α and β anomers of the reducing GlcNAc residue (ratio 0.65:0.35). Due to this anomerisation two signals are also observed for the anomeric proton of the non-reducing Gal residue viz. at 4.460 ppm ($J_{1,2}$ = 7.9 Hz) and at 4.421 ppm ($J_{1,2}$ = 7.3 Hz), also in the ratio 0.65:0.35.

The values of the coupling constants $J_{1,2}$ of the H-1 of Gal define the β-type of linkage. On the basis of chemical, mass spectral and NMR analysis, oligosaccharide N-1 is established to be β-Gal-(1→3)-GlcNAc.

N-2

The mass spectrum of the permethylated reduced oligosaccharide confirms the reducing position of GalNAc in the parent disaccharide (fragment *m/e* 276). The fragments *m/e* 133, 378 and 422 are characteristic of a (1→3) linkage. The 360-MHz ^{1}H-NMR spectrum shows four anomeric signals. The signals at 5.215 ppm ($J_{1,2}$ = 3.3 Hz) and at 4.692 ppm ($J_{1,2}$ = 8.2 Hz) represent the H-1 of the reducing GalNAc in the α and β anomer, respectively (ratio α:β = 0.55:0.45). The H-1 signals of the terminal Gal are found at 4.495 ppm ($J_{1,2}$ = 7.9 Hz) and

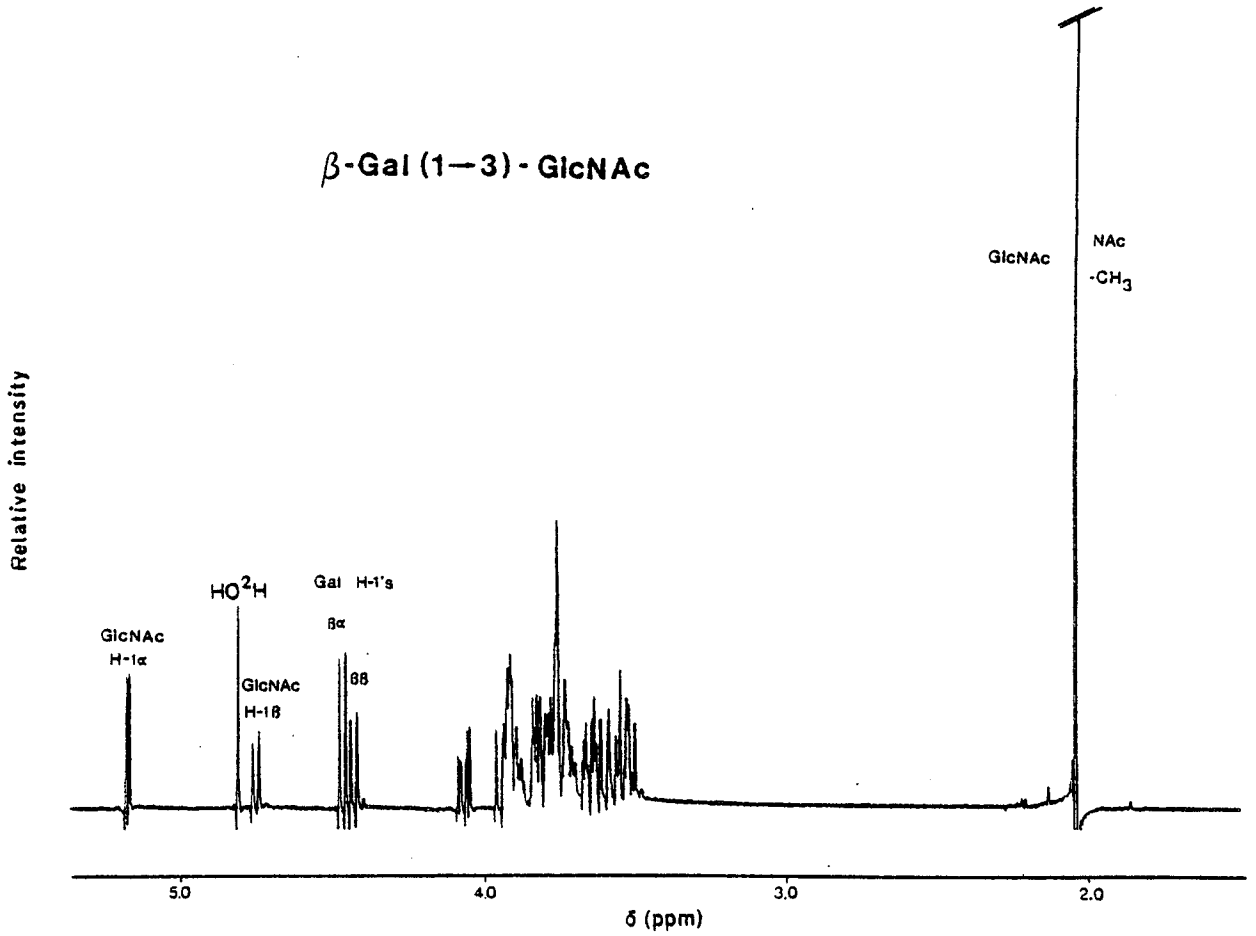


Fig. 4. *360-MHz ^{1}H-NMR spectrum of disaccharide N-1, β-Gal-(1→3)-GlcNAc, in ^2H_2O at 25°C*

at 4.436 ppm ($J_{1,2}$ = 7.9 Hz) in the same ratio. These coupling constants indicate a β-type of linkage between the two sugar residues. Therefore the structure of N-2 is β-Gal-(1→3)-GalNAc.

N-3

The absence of fragment *m/e* 277 in the mass spectrum of the permethylated deuteride-reduced oligosaccharide (Fig. 5) indicates a double substitution of the reducing GlcNAc residue. A branched structure of N-3 is confirmed by the presence of fragments *m/e* 451 and 481, the structures of which are given in Fig. 5. Fragments *m/e* 219 and 189 correspond to terminal Gal and Fuc residues in the parent trisaccharide, respectively.

The reducing *N*-acetylhexosamine is not substituted at C-6 because of the presence of fragments *m/e* 89 and 59 (89 minus HCHO). Furthermore, fragment *m/e* 291 is characteristic of a double substitution involving two adjacent C atoms (so C-3 and C-4) of an *N*-acetylhexosamine. Fragment *m/e* 291 is the analogue of *m/e* 250 in the mass spectrum of a permethylated reduced oligosaccharide, containing a C-2,C-3 or C-3,C-4 disubstituted reducing hexose [11]. The presence of fragment *m/e* 349 shows that Fuc is attached to C-3 of GlcNAc; by consequence Gal is linked to C-4. Further evidence for the location of the terminal residues was obtained from the mass spectrum of the permethylated reduced defucosylated oligosaccharide. The presence of fragments *m/e* 174 and 381 in the latter spectrum are characteristic of a parent disaccharide consisting of a hexose (1→4)-linked to an *N*-acetylhexosamine.

The 360-MHz ^{1}H-NMR spectrum of N-3 shows two partially overlapping doublets at about 5.10 – 5.11 ppm, one of which is attributed to H-1 of the α anomer of the reducing GlcNAc. The H-1 signal of the β anomer arises at 4.733 ppm ($J_{1,2}$ = 8.0 Hz). Due to the anomerisation two Gal H-1 signals are observed at 4.468 ppm ($J_{1,2}$ = 7.9 Hz) and at 4.457 ppm ($J_{1,2}$ = 7.9 Hz), respectively, in a ratio 0.60:0.40. The values of the latter coupling constants indicate that Gal is β-linked to GlcNAc. The signal of the Fuc H-1 is found at about 5.10 ppm. This chemical shift is indicative of an α-type of linkage between Fuc and GlcNAc. The positions of the Fuc H-5 signal (4.824 ppm) and the CH_3 singlet (1.177 ppm) are the same as described in [12, 13], thereby further substantiating the conclusion, based on mass spectrometry, that Fuc is attached at C-3 of the GlcNAc residue. If Fuc is α-(1→4)-linked to GlcNAc in the structural element α-Fuc-(1→4)-[β-Gal-(1→3)]-GlcNAc, its H-5 resonates at 4.87 ppm (L. Dorland et al., unpublished results).

Based on chemical, mass spectral and NMR data, the structure of N-3 is: β-Gal-(1→4)-[α-Fuc-(1→3)]-GlcNAc.

N-4

Methylation analysis of the reduced oligosaccharide N-4 furnished a mixture of methyl-2,3,4-tri-*O*-methylfucoside, methyl-2,3,4,6-tetra-*O*-methylgalactoside and 3,4-di-*O*-acetyl-1,2,5,6-tetra-*O*-methylglucitol in the proportions 0.80 :1.00:0.96. Partial hydrolysis of N-4 by dilute sulphuric acid

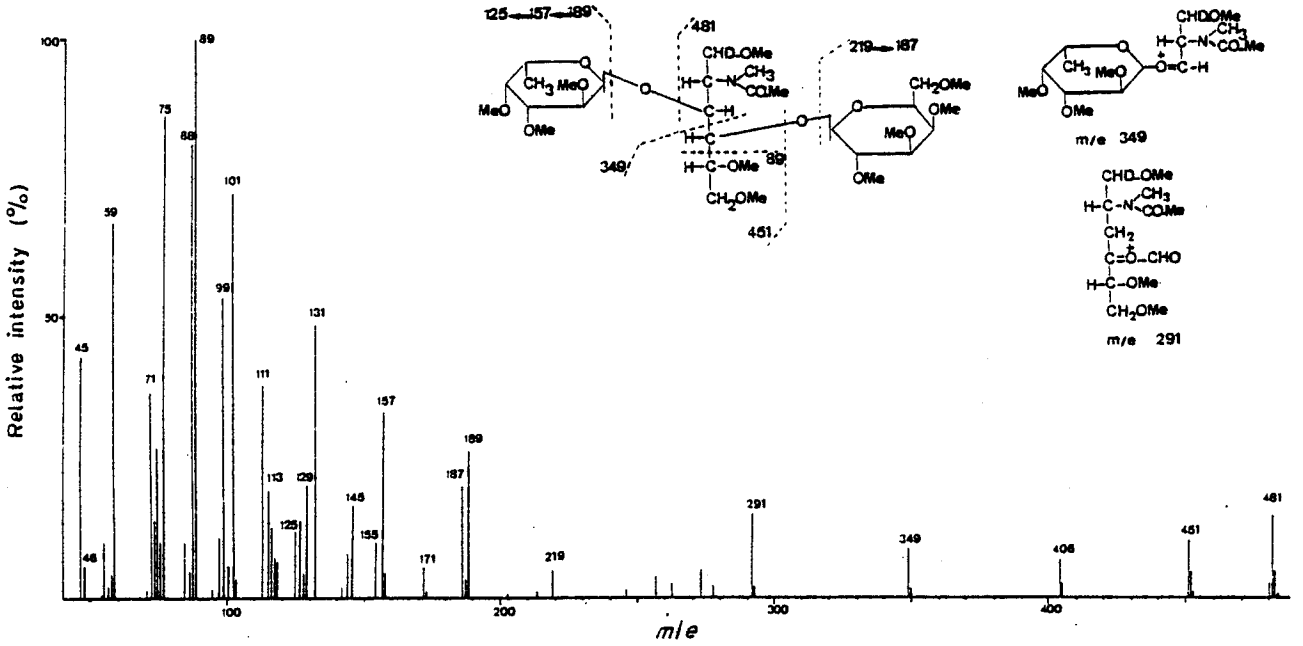


Fig. 5. *Mass spectrum and fragmentation pattern of the permethylated reduced trisaccharide N-3: β-Gal-(1→4)-[α-Fuc-(1→3)-]GlcNAc*

led to the formation of lactose, which was characterized by means of mass spectrometry.

Based on this chemical study, N-4 was established to be a trisaccharide with the following structure: β-Gal-(1→4)-[α-Fuc-(1→3)]-Glc.

N-5

Methylation analysis of the reduced oligosaccharide N-5 yielded methyl-2,3,4,6-tetra-*O*-methylgalactoside, methyl-3-*O*-acetyl-4,6-di-*O*-methyl-*N*-acetyl-*N*-methylglucosaminide and 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol in about equal amounts. The assignment of the position of the *O*-acetyl substituent in the glucosaminide derivative was achieved on the basis of fragments *m/e* 102 ($H_3C-O-CH_2-{}^+CH-{}^-CH-O-CH_3$), *m/e* 71 ($CH_2=CH-CH={}^+O-CH_3$), *m/e* 142 ($H_3C-O-CH=CH-CH=N^+(CH_3)COCH_3$) or ($H_3C-O^+=CH-CH=CH-N(CH_3)COCH_3$) and *m/e* 100 (142 minus $CH_2=C=O$) in its mass spectrum, whereas fragment *m/e* 75 ($CH_3-O^+=CH-OCH_3$) was missing.

In the NMR spectrum of N-5 five anomeric signals are observed. The H-1 resonances of the reducing Gal residue are found at 5.226 ppm ($J_{1,2}$ = 3.1 Hz; α; 0.5 proton) and 4.563 ppm ($J_{1,2}$ = 7.3 Hz; β; 0.5 proton). Due to the anomerisation the H-1 signals of the GlcNAc residue, linked to the reducing Gal, are found at 4.744 ppm ($J_{1,2} \simeq$ 7.5 Hz; 0.5 proton) and 4.718 ppm ($J_{1,2} \simeq$ 7.5 Hz; 0.5 proton) leading to the conclusion of a β-glycosidic linkage between the aforementioned residues. The presence of one doublet at 4.482 ppm ($J_{1,2}$ = 7.9 Hz; 1.0 proton) indicates a β-linked Gal in terminal non-reducing position. It has to be noted that the H-4 signal of the terminal Gal is found at about 3.92 ppm, whereas the H-4 signal of the β-(1→3)-substituted Gal are observed at 4.204 ppm (α) and [redacted] (β). The chemical shift increment for the Gal H-4 is characteristic for its β-(1→3) substitution (H. van Halbeek et al., unpublished results), thereby making the Gal H-4 a structural reporter group [14].

Combination of methylation analysis and NMR data points to the following structure of N-5: β-Gal-(1→3)-β-GlcNAc-(1→3)-Gal.

N-6

The carbohydrate composition and the nature of the reducing end monosaccharide of N-6 are identical to those of N-5 (see Table 1). The only difference concerning the results of methylation analysis of the reduced trisaccharide N-6, compared with those of N-5, is the production of methyl-4-*O*-acetyl-3,6-di-*O*-methyl-*N*-acetyl-*N*-methyl-glucosaminide rather than of its 3-*O*-acetyl-4,6-di-*O*-methyl analogue. Identification of this derivative occurred on the basis of its mass spectral data; the position of the *O*-acetyl group at C-4 appeared from observation of fragments *m/e* 170 ($H_3C-CO-O-CH=CH-CH=N^+(CH_3)COCH_3$), *m/e* 128 (170 minus $CH_2=C=O$) and *m/e* 130 ($H_3C-O-CH_2-{}^+CH-{}^-CH-O-CO-CH_3$).

As in the 360-MHz ^{1}H-NMR spectrum of N-5, the doublets at 5.224 ppm ($J_{1,2}$ = 3.1 Hz) and 4.560 ppm ($J_{1,2}$ = 7.7 Hz) in the spectrum of N-6 belong to the H-1 of the α and β anomer of the reducing Gal residue, respectively. Substitution of GlcNAc residue at C-4 rather than at C-3, is reflected in the chemical shifts of its H-1 signals: 4.761 ppm ($J_{1,2}$ = 7.3 Hz) and 4.740 ppm ($J_{1,2}$ = 7.9 Hz). The values of the coupling constants indicate a β linkage between GlcNAc and the reducing Gal. The resonance positions of H-4 of the reducing Gal, 4.197 ppm (α) and [redacted], define this linkage to be β-(1→3). The H-1 resonance of the terminal non-reducing Gal is found at 4.438 ppm ($J_{1,2}$ = 7.9 Hz). This value is also significantly different from that for H-1 of the non-reducing Gal in N-5, due to the different substitution of the GlcNAc residue.

On the basis of methylation analysis and NMR results the structure of oligosaccharide N-6 must be: β-Gal-(1→4)-β-GlcNAc-(1→3)-Gal.

Table 2. *Structures of the neutral oligosaccharides isolated from meconium*

N-1	β-Gal-(1→3)-GlcNAc
N-2	β-Gal-(1→3)-GalNAc
N-3	β-Gal-(1→4) GlcNAc α-Fuc-(1→3)
N-4	β-Gal-(1→4) Glc α-Fuc-(1→3)
N-5	β-Gal-(1→3)-β-GlcNAc-(1→3)-Gal
N-6	β-Gal-(1→4)-β-GlcNAc-(1→3)-Gal
N-7	α-Fuc-(1→2)-β-Gal-(1→4) Glc α-Fuc-(1→3)
N-8	β-GlcNAc-(1→2)-α-Man-(1→3) β-Gal-(1→4) — β-GlcNAc-(1→4) — β-Man-(1→4)-GlcNAc β-GlcNAc-(1→2)-α-Man-(1→6)

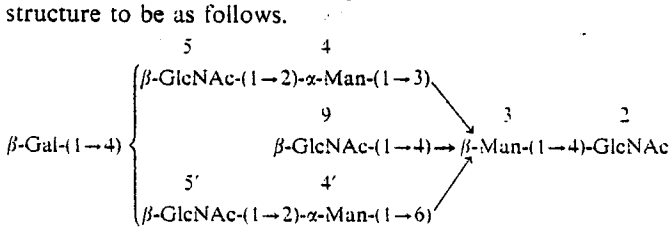
N-7

Methylation analysis of the reduced oligosaccharide N-7 furnished methyl-2,3,4-tri-*O*-methylfucoside, methyl-2-mono-*O*-acetyl-3,4,6-tri-*O*-methyl-galactoside and 3,4-di-*O*-acetyl-1,2,5,6-tetra-*O*-methylglucitol in the proportions 1.62:1.00:0.91. Partial hydrolysis of N-7 liberated lactose, identified by mass spectrometry. Based on these results N-7 is established to be a tetrasaccharide: α-Fuc-(1→2)-β-Gal-(1→4)-[α-Fuc-(1→3)]-Glc.

N-8

The 360-MHz ^{1}H-NMR spectrum of N-8 shows the characteristic features of an asialo-monogalacto-biantennary oligosaccharide structure of the *N*-acetyllactosamine type, bearing an additional β-GlcNAc (1→4)-linked to the inner Man residue of the core (δ for H-1 of Man-4 = 5.058 ppm, of Man-4′ = 5.005 ppm, of Man-3 = 4.707 ppm; δ for H-2 of Man-4 = 4.258 ppm, of Man-3 = 4.189 ppm, of Man-4′ = 4.150 ppm: the H-1 resonance signal of GlcNAc-9 coincides with the signal of the H-1 of Gal-6 or Gal-6′ at 4.470 ppm) [15, 16]. (The numbering of the monosaccharides residues is given below.)

The 360-MHz ^{1}H-NMR spectrum of N-8 revealed its structure to be as follows.



In comparison with the agalacto heptasaccharide isolated from the urine of a patient with Sandhoff's disease [15], the chemical shifts of all structural reported groups are the same, except that of the H-1 of the GlcNAc residue 5 or 5′; upon substitution at C-4 with a β-linked Gal residue, the H-1 of the substituted GlcNAc residue undergoes a shift increment from 4.553 ppm to 4.580 ppm. Location of the additional Gal residue in upper or lower branch could not be established on the basis of the 360-MHz ^{1}H-NMR spectrum.

The structures of the neutral oligosaccharides N-1 to N-8 are summarised in Table 2.

STRUCTURES OF ACIDIC OLIGOSACCHARIDES (A-1 TO A-4)

A-1

The results of the methylation analysis of the chemically homogeneous fraction A-1 (Table 3), together with its sugar composition (Table 1), suggest that A-1 is a partial structure of a biantennary oligosaccharide having GlcNAc-2 in the terminal reducing position (For numbering of monosaccharide residues see Table 5). The three Man derivatives indicate the presence of the trimannosyl core which is characteristic of carbohydrate units linked *N*-glycosidically to asparagine [17, 18]. One of the outer Man residues bears a sialyl-(2→6)-*N*-acetyllactosaminyl unit, the other one occupies a terminal non-reducing positions.

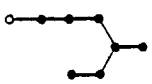
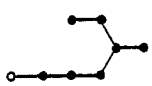
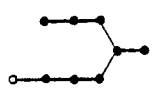
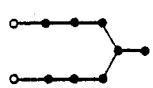
The 360-MHz ^{1}H-NMR data of A-1 are compiled in Table 4. The NMR spectrum reveals that A-1 is in fact a mixture of two isomeric structures, one having the sialyl-(2→6)-*N*-acetyllactosaminyl unit linked to Man-4 (upper branch, A-1 a),

Table 3. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated reduced acidic oligosaccharide fractions from meconium*

Oligosaccharide fraction	2,3,4,6-Me_4-Man	3,4,6-Me_3-Man	2,4-Me_2-Man	2,3,4,6-Me_4-Gal	2,3,4-Me_3-Gal	3,4,6-Me_3-GlcNAcNMe	3,6-Me_2-GlcNAcNMe	1,3,5,6-Me_4-GlcNAcNMe-ol
A-1	0.85	1.05	0.78	–	1.00	–	1.08	1.00
A-2	–	2.00	0.92	–	1.05	1.08	1.06	1.00
A-3	–	2.00	0.92	1.12	0.95	–	2.15	1.00
A-4	–	2.15	0.91	–	2.00	–	2.10	1.00

Table 4. *^{1}H-NMR chemical shifts of anomeric protons, mannose H-2 atoms, sialic acid H-3 atoms and N-acetyl group protons for seven acidic oligosaccharides isolated from meconium*

Chemical shifts in ^2H_2O at 25 °C are given downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate. For numbering of monosaccharide residues and complete structures see Table 5: ●— = neutral or amino sugar residue: ○— = NeuAc-(2→6). The α and β anomeric protons of GlcNAc-2 were in the molar ratio of 0.65:0.35

Compound	Schematic structure	δ for H-1 of residue									δ for H-2 of Man			δ for H-3 of NeuAc		δ for NAc of residue			
		2α	2β	3	4	4′	5	5′	6	6′	3	4	4′	ax	eq	2	5	5′	NeuAc
		ppm																	
A-1a		5.212	≈4.72	4.782	5.137	4.918	4.604	–	4.444	–	4.260	4.195	<4	1.723	2.668	2.058	2.065	–	2.030
A-1b		5.212	≈4.72	4.782	5.103	4.947	–	4.604	–	4.444	4.260	4.068	4.115	1.723	2.668	2.058	–	2.065	2.030
A-2a		5.213	4.719	4.779	5.135	4.919	4.605	4.553	4.444	–	4.260	4.194	4.113	1.725	2.670	2.061	2.071	2.055	2.030
A-2b		5.213	4.719	4.779	5.119	4.948	4.553	4.605	–	4.444	4.260	4.194	4.113	1.725	2.670	2.061	2.055	2.071	2.030
A-3a		5.212	4.716	4.783	5.136	4.928	4.605	4.581	4.444	4.469	4.260	4.195	4.113	1.723	2.668	2.061	2.069	2.051	2.029
A-3b		5.212	4.716	4.783	5.122	4.948	4.581	4.605	4.469	4.444	4.260	4.195	4.113	1.723	2.668	2.061	2.051	2.069	2.029
A-4		5.213	≈4.72	4.781	5.136	4.952	4.606	4.606	4.443	4.443	4.265	4.200	4.119	1.721[a]	2.667[a]	2.063	2.070	2.070	2.029[b]

[a] Signal of two protons
[b] Signal of two methyl groups

the other possessing it linked to Man-4′ (lower branch, A-1b). Compound A-1a is identical to oligosaccharide III, isolated from the urine of a sialidosis patient [19]. From the relative intensities of the resonances of H-1 of Man-4 at 5.137 ppm (A-1a) and 5.103 ppm (A-1b) respectively, as well as from these of Man-4′, it can be derived that the A-1 mixture is composed of 60% A-1a and 40% A-1b.

As previously described [12], in the spectrum of A-1a the H-2 resonance of Man-4′ is buried in the bulk of skeleton protons. However, it has to be noted that the H-2 signal of the terminal Man-4 in A-1b can clearly be distinguished at 4.068 ppm.

The NMR data indicate immediately that the sialic acid residue is linked (2→6) to Gal-6 [19]; however, besides the long-distance effects of an α-(2→6)-linked sialic acid residue on the H-1 protons of Gal, GlcNAc and Man in the branch to which it is attached, a shift increment of the NAc signal of the GlcNAc in this antenna is observed from about 2.050 ppm in the asialo branch [13] to about 2.070 ppm in A-1. The NAc signal found at 2.030 ppm belongs to the sialic acid residue. This is a revision of the assignment given before [19].

A-2

The carbohydrate composition of A-2 (Table 1) shows the presence of an additional GlcNAc residue when compared with that of A-1. Methylation studies (Table 3) provide strong

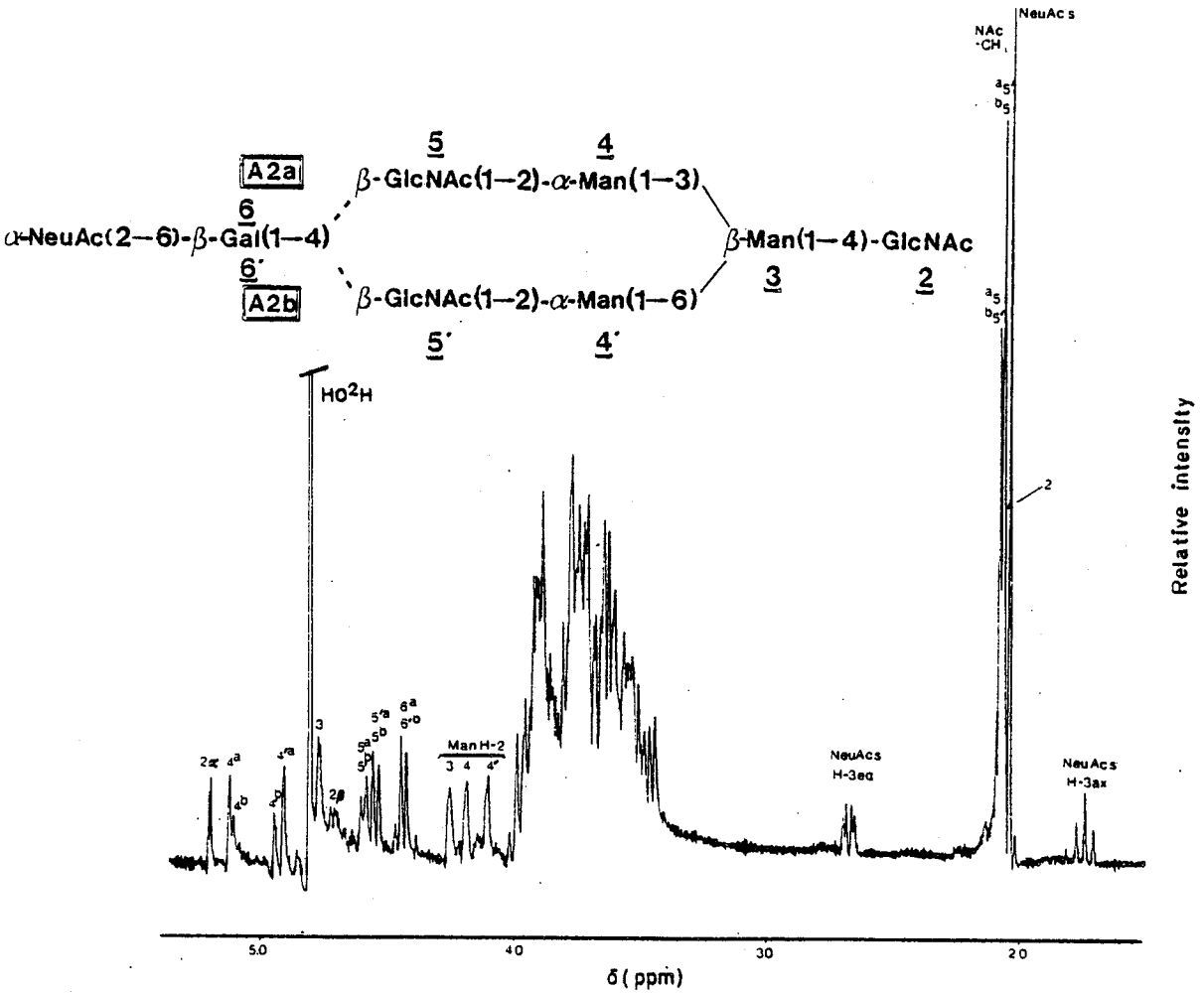


Fig. 6. *360-MHz ^{1}H-NMR spectrum of the mixture of acidic oligosaccharides A-2a and A-2b, in the proportions 2:1, in ^2H_2O at 25°C*

evidence that this GlcNAc residue is in terminal position and attached to an outer Man residue.

The 360-MHz ^{1}H-NMR spectrum of A-2 is given in Fig. 6. The NMR spectral data (Table 4) show that A-2 is a mixture of two isomeric partial biantennary oligosaccharides with GlcNAc-2 in terminal reducing position. Component A-2a can be conceived as an extension of A-1a with GlcNAc-5′, β-(1→2)-linked to Man-4′ (Table 5). The terminal position of GlcNAc-5′ comes to expression in the chemical shift of its H-1 signal (δ = 4.553 ppm) (cf. [16] and compound N8, *vide supra*). The H-2 resonance of Man-4′ appears well resolved from the bulk of the non-anomeric protons, pointing to glycosylation of this residue at position C-2, whereas its H-1 chemical shift is not affected by the β-(1→2)-linked GlcNAc residue. The NAc signal of the terminal GlcNAc-5′ arises at 2.055 ppm.

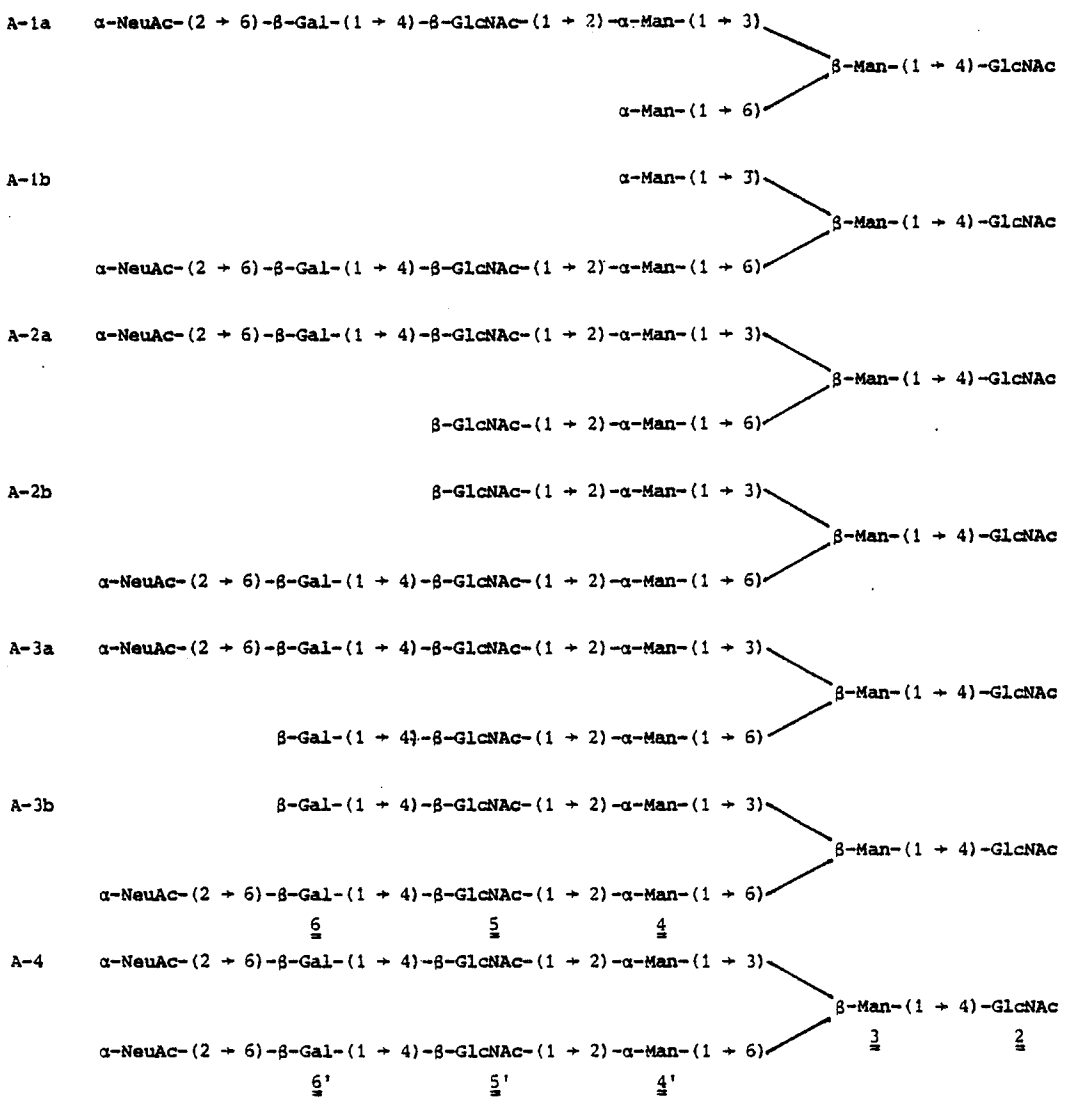
Analogously component A-2b can be conceived as derived from A-1b by attachment of GlcNAc-5 to C-2 of Man-4 (Table 5). The A-2 mixture contains 65% A-2a and 35% A-2b, as can be derived from the relative intensities of the H-1 signals of Man-4 and/or Man-4′ (*vide supra*). The presence of GlcNAc-5 in terminal position is reflected by its H-1 and NAc signals. Due to the attachment of GlcNAc-5 in a β-(1→2) linkage to Man-4, the H-2 resonance of the latter residue undergoes a shift increment to 4.194 ppm, in comparison to A-1b.

A-3

The carbohydrate composition of A-3 (Table 1) as well as the data of methylation analysis (Table 3) suggest that A-3 is a monosialo-biantennary oligosaccharide having GlcNAc-2 in the terminal reducing position, while the sialic acid resid is (2→6)-linked to one of the Gal residues; by consequence the other one occupies a terminal position.

360-MHz ^{1}H-NMR spectroscopy of A-3 reveals that in fact this acidic fraction contains both the biantennary structure with NeuAc α-(2→6)-linked to Gal-6 (upper branch) (A-3a), as well as its isomeric structure bearing NeuAc α-(2→6)-linked to Gal-6′ (lower branch) (A-3b) (Table 5), in about equal amounts. In accordance with earlier observations, [16, 20] extension of an A-2 structure with a Gal residue a Gal residue β-(1→4)-linked to the terminal non-reducing GlcNAc influences the chemical shift of H-1 of this GlcNAc residue, whereas the resonance position of its NAc signal is hardly affected (see Table 4). The sialo and the asialo branch can be distinguished on the basis of the chemical shifts of the H-1 resonances of Gal, GlcNAc and Man residues in that branch [19], as well as on the basis of the chemical shift of the NAc signal of the peripheral GlcNAc residue. As mentioned above, the NAc signal of this GlcNAc in the asialo branch is found at 2.051 ppm, while in the (2→6)-sialylated branch it undergoes a

Table 5. *Structures of the acidic oligosaccharides isolated from meconium. The numbering of monosaccharide units in all compounds corresponds to that given for A-4*



shift increment to 2.069 ppm. However, only the resonance position of H-1 of Man-4 or Man-4′ can be used for determination of the location of the α(2→6)-linked sialic acid residue [19]. Oligosaccharide A-3a is identical to compound V of the series of urinary sialidosis oligosaccharides [19].

A-4

The carbohydrate composition of A-4 (Table 1) shows the presence of an additional NeuAc residue when compared with that of A-3. Methylation studies (Table 3) give strong indication that this NeuAc residue is attached to position C-6 of the terminal Gal in A-3. The 360-MHz ^{1}H-NMR spectral data of A-4 (Table 4) prove it to by a completely sialylated biantennary oligosaccharide having GlcNAc-2 in terminal position (Table 5). Both sialic acid residues are α-(2→6)-linked as can be concluded from the observed long distance effects as well as from the positions of the axial and equatorial H-3 atoms of the NeuAc residues. A-4 was found to be identical to compound IX in the sialidosis series [19].

DISCUSSION

In the present paper the characterization of the major oligosaccharides accumulating in new-born meconium is described. Meconium can be relatively easily collected; it is well suited as a starting material for the isolation of large amounts of carbohydrate components. From the about a hundred carbohydrate constituents 15 major oligosaccharides were isolated and purified by various chromatographic techniques. Application of methylation analysis and 360-MHz ^{1}H-NMR spectroscopy led to the elucidation of the complete structures of these oligosaccharides.

It is tempting to suggest that oligosaccharides N-1, N-2, N-3, N-5 and N-6 stem from the catabolism of *O*-glycosidic-type glycans. This hypothesis implies that endo-*N*-acetyl-α-D-galactosaminidase, endo-*N*-acetyl-β-D-glucosaminidase and endo-β-D-galactosidase are involved in human catabolism pathway of mucin-type glycoproteins. Oligosaccharides N-4 and N-7 are also present in human milk [21].

The neutral oligosaccharide N-8 and the acidic oligosaccharides A-1 to A-4 originate from the catabolism of *N*-

glycosidically linked glycans. Their occurrence is indicative of the action of an endo-*N*-acetyl-*β*-D-glucosaminidase on sialylated glycoproteins or glycopeptides. Though this enzyme activity has never been characterized in human tissue, its existence has been postulated previously with regard to the nature of sialyl oligosaccharides excreted in the urine of patients with a genetic neuraminidase deficiency [19, 22, 23].

It can be expected that a detailed study of the other oligosaccharides present in meconium will furnish structural information leading to further insight in the metabolism of glycoconjugates at the end of embryonal development.

This investigation was supported in part by the *Centre National de la Recherche Scientifique* (L. A. no. 217: *Biologie physico-chimique et Moléculaire des Glucides Libres et Conjugués*, Dir.: Prof. J. Montreuil, and RCP no. 529: *Glucides et Glycoconjugués*), by the *Institut National de la Santé et de la Recherche Médicale* (U 124: *Recherches Ultrastructurales et Biochimiques sur les Cellules Normales et Cancéreuses*, Dir.: Prof. G. Biserte), by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO) and by the Netherlands Foundation for Cancer Research (KWF). The authors are indebted to Prof. M. Delecour (*Maternité R. Salengro*, Lille) for important help with collecting meconium, and thank M. G. Ricart for recording the mass spectra and Mrs A. Lemaire-Poitau for technical assistance.

REFERENCES

1. Côté, R. H. (1970) in *Blood and Tissue Antigens* (Aminoff, D., ed.) pp. 249–264, Academic Press, London.
2. Fraser, D. & Clamp, J. R. (1975) *Clin. Chim. Acta*, *59*, 301–307.
3. Karlson, K. A. & Larson, G. (1978) *FEBS Lett.* *87*, 283–287.
4. Karlson, K. A. & Larson, G. (1979) *J. Biol. Chem.* *254*, 9311–9316.
5. Strecker, G. & Lemaire-Poitau, A. (1977) *J. Chromatogr. Biomed. Appl.* *143*, 553–569.
6. Partridge, S. M. (1950) *Biochem. Soc. Symp.* *3*, 52–61.
7. Zanetta, J. P., Beckenridge, W. C. & Vincendon, G. (1972) *J. Chromatogr.* *69*, 291–301.
8. Hakomori, S. I. (1964) *J. Biochem. (Tokyo)* *55*, 205–208.
9. Fournet, B. & Montreuil, J. (1973) *J. Chromatogr.* *75*, 29–40.
10. Fournet, B., Strecker, G., Leroy, Y. & Montreuil, J. (1981) *Anal. Biochem.* in the press.
11. Fournet, B., Dhalluin, J. M., Strecker, G. & Montreuil, J. (1980) *Anal. Biochem.* *108*, 35–56.
12. Strecker, G., Fournet, B., Montreuil, J., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G. & Dubesset, D. (1978) *Biochimie (Paris)* *60*, 725–734.
13. Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Binette, J.-P. & Schmid, K. (1978) *Biochemistry*, *17*, 5206–5214.
14. Vliegenthart, J. F. G., van Halbeek, H. & Dorland, L. (1980) in *IUPAC 27th International Congress of Pure and Applied Chemistry* (Varmavuori, A., ed.) pp. 253–262, Pergamon Press, Oxford.
15. Strecker, G., Herlant-Peers, M.-C., Fournet, B., Montreuil, J., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G. & Farriaux, J.-P. (1977) *Eur. J. Biochem.* *81*, 165–171.
16. Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Spik, G., Fournet, B. & Montreuil, J. (1979) *Eur. J. Biochem.* *100*, 569–574.
17. Montreuil, J. (1975) *Pure Appl. Chem.* *42*, 431–477.
18. Kornfeld, R. & Kornfeld, S. (1976) *Annu. Rev. Biochem.* *45*, 217–237.
19. Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Strecker, G., Michalski, J.-C., Fournet, B., Spik, G. & Montreuil, J. (1978) *Eur J. Biochem.* *87*, 323–329.
20. Dorland, L., Haverkamp, J., Schut, B. L., Vliegenthart, J. F. G., Spik, G., Strecker, G., Fournet, B. & Montreuil, J. (1977) *FEBS Lett.* *77*, 15–20.
21. Montreuil, J. (1960) *Bull. Soc. Chim. Biol.* *42*, 1399–1447.
22. Strecker, G., Fournet, B., Hondi-Assah, T., Spik, G., Montreuil, J., Maroteaux, P., Durand, P. & Farriaux, J.-P. (1976) *C. R. Acad. Sci. (Paris)* *282*, 671–674.
23. Strecker, G., Peers, M.-C., Michalski, J.-C., Hondi-Assah, T., Fournet, B., Spik, G., Montreuil, J., Farriaux, J. P., Maroteaux, P. & Durand, P. (1977) *Eur. J. Biochem.* *75*, 391–403.

G. Strecker and J. Montreuil, Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille I, Boîte postale 36, F-59650 Villeneuve d'Ascq, France

M.-C. Herlant Peers, Institut de Recherches sur le Cancer, Cité Hospitalière, 2 Place de Verdun, F-59020 Lille-Cedex, France

L. Dorland, H. van Halbeek, G. A. Veldink, and J. F. G. Vliegenthart, Organisch-Chemisch Laboratorium, Rijksuniversiteit te Utrecht, Croesestraat 79, NL-3522-AD Utrecht, The Netherlands