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Isolation and structural characterization of sialic acid-containing storage material from mucopolipidosis I (sialidosis) fibroblasts

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Sialic acid-containing storage material was isolated from cultured human mucopolipidosis I (sialidosis) fibroblasts by gel permeation chromatography on Bio-Gel P-6 followed by medium-pressure anion-exchange chromatography on Mono Q. The structure determination of the isolated sialyloligosaccharides was carried out by 500-MHz ¹H-NMR spectroscopy in conjunction with sugar analysis and analytical HPLC. The storage material showed completely sialylated mono-, di- and triantennary *N*-glycosidic *N*-acetylglucosamine oligosaccharides having the Man β 1 \rightarrow 4GlcNAc sequence at the reducing end in common. Heterogeneity occurred with respect to the linkages between terminal sialic acid and the penultimate galactose residues (α 2 \rightarrow 3/ α 2 \rightarrow 6). It turned out that all the identified carbohydrate chains are consistent with the neuraminidase deficiency.

Introduction

Mucopolipidosis I is a rare inherited metabolic disease which leads to severe clinical symptoms, such as psychomotor retardation and neurodegeneration [1]. The defect in the catabolism of glycoproteins is caused by an α -neuraminidase deficiency (sialidase) leading to an accumulation of sialic acid-containing material in tissues, leucocytes and fibroblasts [2] and to an excessive excretion of glycopeptides [3] and sialyloligosaccharides

in urine. The structures of the urinary sialyloligosaccharides are rather well documented [4–9]. Much less is known about the nature of the accumulated sialic acid-containing storage material in cultured fibroblasts, although these cells are frequently used for the diagnosis of genetic diseases [10]. In fibroblasts from mucopolipidosis I patients a 3–7-fold increase of the amount of bound sialic acid, in comparison to normal cell lines, has been observed [11–14]. Therefore, fibroblasts seem to be a representative cell type for studying the effect of the neuraminidase deficiency on lysosomal catabolism. In the case of human α -mannosidosis [15] and of β -mannosidosis in goats [16], the accumulation of oligosaccharides in fibroblasts has been demonstrated by thin-layer chromatography. To gain insight into the structural features of the storage material from mucopolipidosis I fibroblasts, sialic acid-containing glycoconjugates have been isolated and analyzed by 500-MHz ¹H-NMR

Abbreviations: HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; Man, mannose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid.

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spectroscopy combined with sugar analysis and analytical HPLC. Here, we report on the identification of the primary structures of accumulated sialyloligosaccharides.

Materials and Methods

Fibroblasts

Skin fibroblasts of a dysmorphic mucopolidosis I patient (E.V.) with infantile onset were cultured in Ham's F10 medium supplemented with 10% fetal calf serum. In the fibroblasts, α -neuraminidase activity was greatly diminished, but all other enzymes tested, including β -galactosidase, showed normal activities [17].

Cells from eight 75 cm² Falcon flasks were harvested by trypsinization after 3 weeks of confluency, washed three times with phosphate-buffered saline and resuspended in 4 ml distilled water. The cells were disrupted by sonicating twice for 20 s at 0 °C. After centrifugation for 10 min at 10 000 \times g, the supernatant was collected and the pellet was extracted once more with 3.5 ml distilled water. The combined supernatants were used for further investigations.

Colorimetric methods

The sialic acid content was measured by the periodic acid–thiobarbituric acid method of Warren [18], before and after hydrolysis with 0.05 M H₂SO₄ (1 h, 80 °C). For the determination of the amount of free and total sialic acid, 100 and 50 μ l aliquots of the supernatant were used, respectively. Free NeuAc was used as a standard.

The protein concentration in the supernatant was determined according to Lowry et al. [19], using 10 and 20 μ l aliquots of the supernatant. Bovine serum albumin was applied as a standard.

Gel permeation chromatography

Part of the supernatant (7.25 ml) was fractionated on a Bio-Gel P-6 (200–400 mesh, Bio-Rad) column (120 \times 2.4 cm) at 4 °C with a 0.1 M ammonia-acetic acid buffer (pH 5.4) as eluent (22 ml/h, 4.8 ml fractions). The elution profile was obtained by determination of the hexose content in the various fractions with the phenol-sulfuric acid assay [20]. In addition the ultraviolet absorption at 280 nm was measured.

Anion-exchange chromatography

Medium-pressure anion-exchange chromatography was carried out on a Mono Q HR 5/5 column utilizing a Fast Protein Liquid Chromatography apparatus, equipped with a Liquid Chromatography Controller LCC-500 and two P-500 pumps (Pharmacia) [21]. For the separation of sialyloligosaccharides a linear gradient of 0–100 mM NaCl in 10 ml water (Lichrosolv, Merck) at a flow rate of 2.0 ml/min and a pressure of 2.4 MPa was applied.

The eluate was monitored at 214 nm using a Pharmacia UV-1/214 detector, operating at 1.0 AU sensitivity, connected with a dual chart recorder. The injection volumes were 0.5 ml. Specific fractions were desalted on a Bio-Gel P-2 (100–200 mesh, Bio-Rad) column (18 \times 1 cm) with distilled water as eluent and subsequently lyophilized.

Sugar analysis

Sugar analysis was carried out by gas-liquid chromatography on a capillary CPSil5 WCOT fused silica column (25 m \times 0.32 mm i.d., Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis (1.0 M methanolic HCl, 24 h, 85 °C), *N*-reacetylation and trimethylsilylation [22].

500-MHz ¹H-NMR spectroscopy

Sialyloligosaccharides were repeatedly exchanged in ²H₂O (99.96 atom% ²H, Aldrich) with intermediate lyophilization. ¹H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier transform mode at a probe temperature of 27 °C. Resolution-enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [23]. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225 ppm in ²H₂O at 27 °C) with an accuracy of 0.002 ppm [24].

HPLC

Analytical HPLC was carried out on a Kratos

liquid chromatograph consisting of two Spectroflow-400 Solvent Delivery Systems, a Spectroflow 450 Solvent Programmer and a Rheodyne injection valve module. For the separation a 10 μm Lichrosorb-NH₂ column (250 \times 4.6 mm, Chrom-pack) was used.

The elution of the monosialylated compounds was performed isocratically with a mixture of acetonitrile/30 mM KH₂PO₄, pH 4.7 (66:34, v/v) [25]. The di- and trisialylated compounds were eluted isocratically with a mixture of acetonitrile/30 mM KH₂PO₄-K₂HPO₄ buffer, pH 7.0 (62.5:37.5, v/v). In both cases the flow rate was 2.0 ml/min. The eluate was monitored by a Spectroflow 783 Programmable Absorbance Detector at 205 nm and a sensitivity of 0.01 AU. Peak areas were calculated by a Spectra Physics SP 4290 Integrator. The HPLC column was calibrated using well-defined sialyloligosaccharides isolated from urine of the same sialidosis patient (unpublished data).

Results

Determination of the protein content in the water-phase (7.5 ml) after disruption of the mucopolipidosis I fibroblasts yielded 2.25 mg protein/ml. The amounts of free and total sialic acid were 7 and 72 nmol/mg protein, respectively. The quantity of bound sialic acid, 65 nmol/mg protein, is strongly enhanced as compared to control values (5–10 nmol/mg protein) [10–13].

The elution pattern of the supernatant on Bio-Gel P-6 is presented in Fig. 1. As is evident from the figure, the supernatant contained mainly high-molecular-mass material. The phenol-sulfuric acid assay turned out to be too insensitive to trace the presence of smaller hexose-containing carbohydrate chains. Because medium-size carbohydrate chains had to be expected, the fractions were pooled as indicated and subjected to sugar analysis.

Fraction I contained mainly glucose and trace

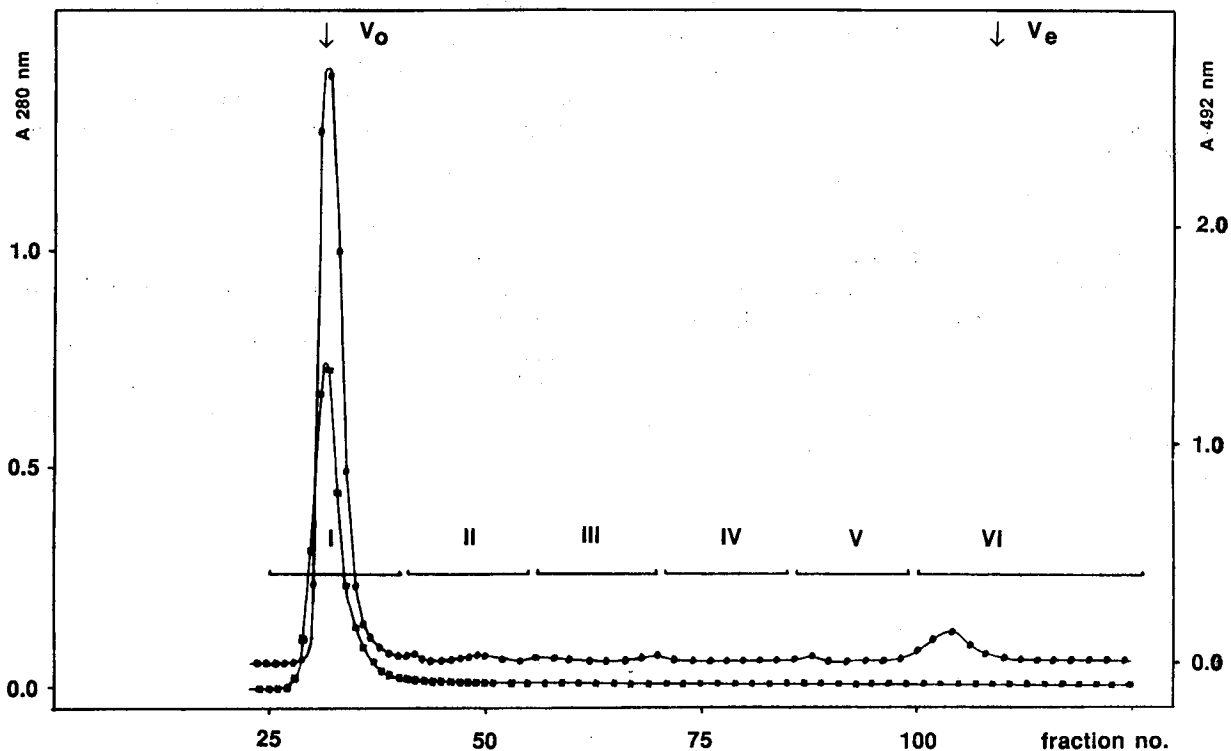


Fig. 1. Bio-Gel P-6 elution pattern of the supernatant of mucopolipidosis I fibroblasts, using a 0.1 M ammonia-acetic acid buffer, pH 5.4, as eluent. The ultraviolet 280 nm profile is indicated by (■—■) and the results of the phenol-sulfuric acid determinations (492 nm), using 0.2 ml of the fractions, by (●—●). Fractions I–VI were pooled as indicated.

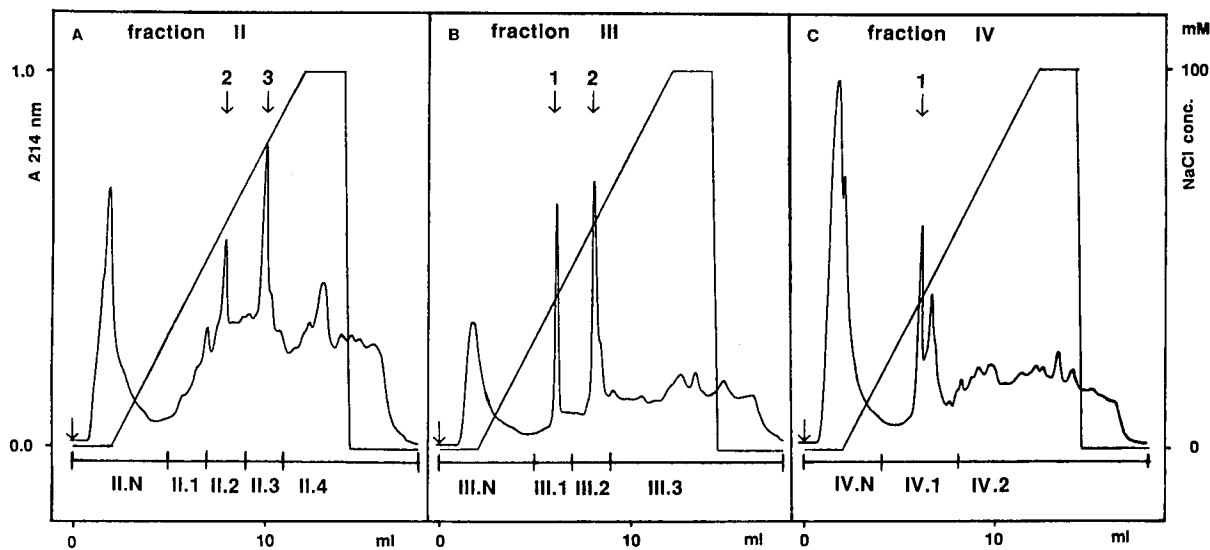


Fig. 2. Mono Q elution profiles of fractions II, III and IV at 214 nm, using a linear gradient of 0–100 mM NaCl in 10 ml water. The subfractions were pooled as indicated. The numerals above the arrows correspond to the number of sialic acid residues bound in reference compounds isolated from sialidosis urine [21].

amounts of mannose, galactose, GlcNAc and NeuAc, suggesting the occurrence of glycoproteins and glycogen fragments. Fractions II, III and IV showed the presence of mannose, galactose, GlcNAc and NeuAc. Fraction V contained no detectable amounts of sugar and fraction VI only glucose.

The sialic acid-containing fractions II, III and IV were further fractionated by anion-exchange chromatography on Mono Q (Fig. 2). Comparison of the retention times of the various peaks moni-

TABLE I
MOLAR CARBOHYDRATE COMPOSITION OF THE RELEVANT MONO Q SUBFRACTIONS OF THE SUPERNATANT OF MUCOLIPIDOSIS I FIBROBLASTS

Monosaccharide	II.2	II.3	III.1	III.2	IV.1
Man ^a	3.0	3.0	2.0	3.0	2.0
Gal	2.4	2.8	1.0	2.2	0.8
GlcNAc	3.6	3.9	1.5	3.1	1.4
NeuAc	1.6	2.9	0.6	2.0	0.8
Amount ^b	10	22	42	30	20

^a Mannose taken as 3 or 2 as indicated.

^b Estimated amount (nmol) of accumulated oligosaccharide.

tored at 214 nm with those of reference compounds isolated from sialidosis urine strongly suggested the presence of tri- and disialylated compounds in fraction II, di- and monosialylated compounds in fraction III and monosialylated compounds in fraction IV [21]. Pooled subfractions as indicated in Fig. 2A–C were subjected to sugar analysis. In Table I a summary of the sugar analysis data of the relevant Mono Q subfractions, together with the estimated amount of material, is given. The various results point to the presence of *N*-glycosidic type of carbohydrate chains with different types of branching and numbers of sialic acid residues. The additional fractions not included in Table I did not contain detectable amounts of sugar, except for the neutral fractions (N) which showed the presence of small amounts of glucose.

The fractions II.2, II.3, III.1, III.2 and IV.1 were further investigated by 500-MHz ¹H-NMR spectroscopy. The structural-reporter-group ¹H-NMR data of these fractions, together with those of reference compounds [5,24], have been compiled in Table II.

In all fractions, heterogeneities in the type of NeuAc linkages ($\alpha 2 \rightarrow 3/\alpha 2 \rightarrow 6$) were observed

H-2	Man	3 α	4.244	4.245	4.240	4.266	4.263	4.256	4.254	4.228	4.223	4.223	
		3 β	4.233	4.234	4.240	4.255	4.251	4.244	4.254	4.254	4.217	4.223	4.223
		4 α	4.198	4.197	4.197	4.199	4.197	4.192	4.201	4.201	4.221	4.223	4.223
		4 β	4.196	-	-	-	4.119	4.117	4.119	4.119	4.119	4.121	4.121
		4'	-	-	-	4.121	4.119	4.117	4.119	4.119	4.119	4.121	4.121
H-3a	NeuAc	2 \rightarrow 6 ^b	1.716	-	1.717	1.721	1.719	-	1.712	1.712	1.720	1.713	1.713
		NeuAc' 2 \rightarrow 6	-	-	-	1.719	-	-	-	1.712	1.717	1.713	-
		NeuAc 2 \rightarrow 3	-	1.799	-	-	-	1.797	-	-	-	-	-
		NeuAc' 2 \rightarrow 3	-	-	-	-	1.800	1.799	-	-	-	-	-
		NeuAc* 2 \rightarrow 3	-	-	-	-	-	-	1.799	1.797	-	-	1.799
H-3e	NeuAc	2 \rightarrow 6 ^b	2.670	-	2.670	2.669	2.668	-	2.664	2.664	2.670	2.666	2.666
		NeuAc' 2 \rightarrow 6	-	-	-	2.672	-	-	-	2.664	2.672	2.666	-
		NeuAc 2 \rightarrow 3	-	2.758	-	-	-	2.758	-	-	-	-	-
		NeuAc' 2 \rightarrow 3	-	-	-	-	2.756	2.758	-	-	-	-	-
		NeuAc* 2 \rightarrow 3	-	-	-	-	-	-	2.750	-	-	-	2.754
Nac	GlcNAc	2 α	2.043	2.043	2.042	2.063	2.061	2.061	2.059	2.062	2.060	2.060	
		2 β	2.041	2.041	2.042	2.060	2.057	2.058	2.059	2.059	2.060	2.060	
		5	2.069	2.049	2.045	2.071	2.069	2.049	2.067	2.067	2.067	2.068	2.068
		5' α	-	-	-	2.069	2.045	2.046	2.067	2.067	2.067	2.068	2.047
		5' β	-	-	-	2.066	2.044	2.043	2.067	2.042	2.065	2.068	2.047
NeuAc	7	-	2.030	2.029	2.030	2.030 ^c	2.030 ^c	2.032 ^c	2.030 ^c	2.030 ^d	2.075	2.075	
		-	-	-	-	-	-	-	-	-	2.074	2.075	
		-	-	-	-	-	-	-	-	-	2.030 ^d	2.031 ^d	

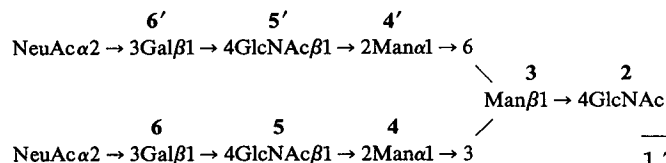
^a n.d. = not determined.

^b NeuAc denotes the sialic acid linked to Gal-6, NeuAc' the one linked to Gal-6' and NeuAc* the one linked to Gal-8.

^c Protons stemming from two NAc groups.

^d Protons stemming from three NAc groups.

ical shifts of the H-1 signals of GlcNAc-5' and Gal-6' show both NeuAc α 2 \rightarrow 3 and α 2 \rightarrow 6 attachment. Analytical HPLC of III.2 confirmed the presence of both compounds in a ratio of 7:5. Furthermore, a small peak was observed with the same retention time as the reference diantennary compound with two NeuAc residues in α 2 \rightarrow 3 linkage (see Scheme I):



The $^1\text{H-NMR}$ spectrum of subfraction II.2 demonstrates the presence of disialylated diantennary structures, having heterogeneity with respect

to the NeuAc linkages in both antennae. The chemical shift values of the H-1 signals of Man-4 and Man-4' (α 2 \rightarrow 6: H-1 Man-4, δ 5.142/H-1 Man-4', δ 4.95; α 2 \rightarrow 3: H-1 Man-4, δ 5.125/H-1 Man-4', δ 4.92) in combination with the NeuAc H-3e and H-3a signals (α 2 \rightarrow 6: H-3e, δ 2.670/H-3a, δ 1.717; α 2 \rightarrow 3: H-3e, δ 2.754/H-3a, δ

1.799) indicate a heterogeneity of the NeuAc linkage type in both branches. This is confirmed by the H-1 signals and the NAc singlets of the GlcNAc-5/5' residues (α 2 \rightarrow 6: H-1, δ 4.60/NAc, δ 2.071; α 2 \rightarrow 3: H-1, δ 4.58/NAc-5, δ

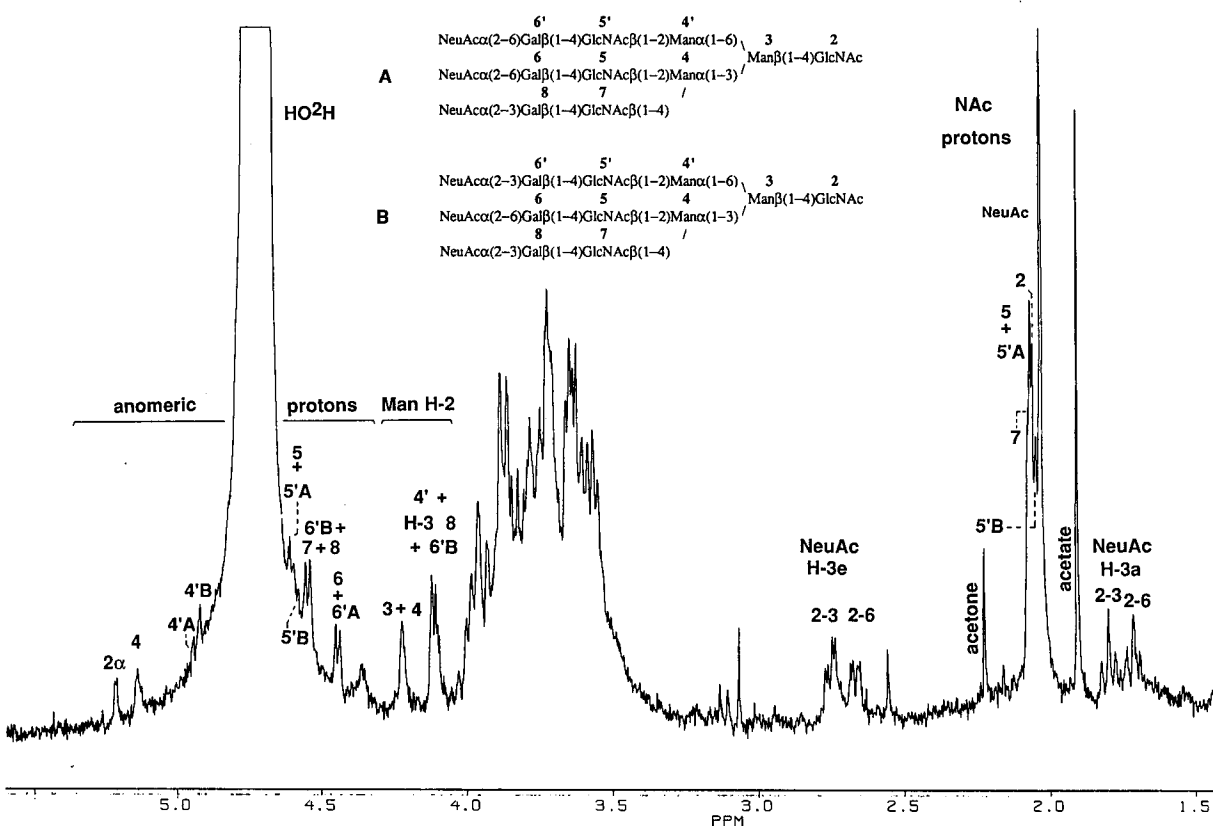
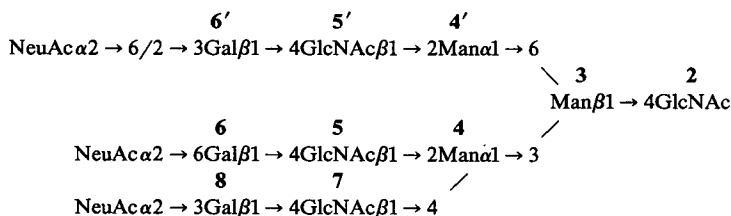


Fig. 3. 500-MHz $^1\text{H-NMR}$ spectrum (3600 scans) of fraction III.2 in $^2\text{H}_2\text{O}$ at 27°C , obtained from mucopolipidosis I fibroblasts after anion-exchange chromatography on Mono Q.

2.051/NAc-5', δ 2.045) and the H-1 signals of the Gal-6/6' residues ($\alpha 2 \rightarrow 6$: δ 4.445; $\alpha 2 \rightarrow 3$: δ 4.546). The $^1\text{H-NMR}$ data (not included in Table II) are similar to those of a mixture of reference compounds **29**, **39** and **38** [5,24]. The analytical HPLC profile showed the presence of the same three structures as mentioned above for III.2, but now in ratio of 2 : 1 : 1 (Scheme I).

The $^1\text{H-NMR}$ spectrum of subfraction II.3 is given in Fig. 3 and shows the presence of the following trisialylated triantennary structures:



The chemical shifts of the NeuAc H-3a and H-3e signals show both $\alpha 2 \rightarrow 6$ and $\alpha 2 \rightarrow 3$ attachments ($\alpha 2 \rightarrow 6$: H-3e, δ 2.666/H-3a, δ 1.713; $\alpha 2 \rightarrow 3$: H-3e, δ 2.754/H-3a, δ 1.799). The NeuAc residue bound to Gal-6' is heterogeneous with respect to the linkage type, being $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$. This is reflected in the chemical shift values of the H-1 signals of Man-4' ($\alpha 2 \rightarrow 6$: δ 4.939; $\alpha 2 \rightarrow 3$: δ 4.918) and the NAc signals of GlcNAc-5' ($\alpha 2 \rightarrow 6$: δ 2.068; $\alpha 2 \rightarrow 3$: δ 2.047). The Gal-6' and GlcNAc-5' H-1 signals confirm the NeuAc heterogeneity ($\alpha 2 \rightarrow 6$: δ 4.445/ δ 4.601; $\alpha 2 \rightarrow 3$: δ 4.545/ δ 4.584, respectively). The Gal-6 is mainly $\alpha 2 \rightarrow 6$ -sialylated, as is deduced from the H-1 signal of Man-4 (δ 5.134), in combination with the H-1 signals of Gal-6 and GlcNAc-5 (δ 4.445 and 4.601, respectively). Gal-8 is exclusively $\alpha 2 \rightarrow 3$ -sialylated as can be concluded from the only and characteristic NAc signal of GlcNAc-7 (δ 2.075) [24]. The chemical shift values of structure A from Fig. 3 are in accordance with those of reference compound **41** [24]. Structure B differs from structure A by one NeuAc, being bound $\alpha 2 \rightarrow 3$ to Gal-6' instead of $\alpha 2 \rightarrow 6$. The HPLC profile of II.3 revealed that the trisialylated triantennary structures occur in a ratio of 2 : 3. In addition, a peak with the same retention time as the triantennary reference structure with three $\alpha 2 \rightarrow 3$ -linked NeuAc residues was observed (see Scheme I).

Discussion

The increase of bound sialic acid in neuraminidase-deficient fibroblasts, as reported by several groups [10–13], is caused by the storage of completely sialylated oligosaccharides, derived from *N*-glycoproteins. The isolated structures are of the mono-, di- and triantennary *N*-acetylglucosamine type of oligosaccharides having GlcNAc-2 at the reducing terminus. The observed hetero-

geneity with respect to the NeuAc linkages is consistent with the neuraminidase deficiency for both $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ linkages. The same types of structures have been found in sialidosis urine, but also partially sialylated structures have been isolated from this source [4,5,9]. The latter structures were not found in fibroblasts, which could be due to the limited amount of material available. On the other hand, it cannot be excluded that the urinary, partially sialylated structures have been formed during storage or working-up procedures. Scarce amounts of material prompted us to perform $^1\text{H-NMR}$ spectroscopy before HPLC separations were carried out. However, the combination of NMR spectroscopy with analytical HPLC turns out to be a sensitive and accurate method for the structural analysis of small amounts of heterogeneous mixtures of sialyloligosaccharides. It has to be noted that the insensitivity of the carbohydrate-detection method resulted in an inaccurate pooling of the Bio-Gel P-6 fractions. This accounts for the occurrence of monoantennary structures in fractions III and IV and of diantennary structures in fractions II and III.

The finding of GlcNAc-2 at the reducing end indicates a very efficient removal of the peptide part and of the GlcNAc-1 residue in the lysosomes of the fibroblasts. Previously an endoglucosaminidase splitting the *N,N'*-diacetylchitobiose

unit was believed to be responsible for this phenomenon [26], but recently indications have been found for another pathway. After the removal of the peptide moiety the asparagine residue is split off from the carbohydrate chain and then the GlcNAc-1 residue is cleft from the oligosaccharide [27,28].

The accumulated sialyloligosaccharides are most likely derived from intracellular metabolism of endogenous glycoproteins. The nature of the isolated structures is in agreement with naturally occurring human sialylated carbohydrate chains of glycoproteins. Moreover, a recent report shows the storage of sialic acid-containing material in mucopolipidosis II fibroblasts. The latter material was formed after incubation with a radioactive precursor and was found in the trichloroacetic acid-soluble fraction [29]. Strikingly, part of the trisialylated triantennary compounds resembles the carbohydrate chains of fetuin [30–32], which was added to the culture medium. Therefore it cannot completely be excluded that part of the storage material is derived from fetuin or other glycoproteins present in the medium, which are digested by the lysosomes after endocytosis and of which the carbohydrate chains, due to the neuraminidase deficiency, cannot be broken down.

Acknowledgements

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