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Neuraminic Acid Derivatives Newly Discovered in Humans: *N*-Acetyl-9-*O*-L-lactoylneuraminic Acid, *N*,9-*O*-Diacetylneuraminic Acid and *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic Acid*

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Summary: The free and glycosidically bound acylneuraminic acids from human serum and saliva and the free acylneuraminic acids from human urine have been characterized by thin-layer chromatography and gas-liquid chromatography/mass spectrometry. Acylneuraminic acid mixtures obtained from serum and saliva contain mainly *N*-acetylneuraminic acid and *N*-acetyl-9-*O*-L-lactoylneuraminic acid, whereas small amounts of *N*,9-*O*-diacetylneuraminic acid are also present. No free *N*,*O*-diacylneuraminic acids could be detected in the urine samples. None of the investigated fluids contained *N*-glycoloylneuraminic acid.

The unsaturated *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid is usually a component of the free acylneuraminic acid fractions of serum, saliva and urine.

The body fluids of a patient with sialuria contain the same *O*-acylated and unsaturated *N*-acetylneuraminic acid derivatives as mentioned above, but the total amounts of free acylneuraminic acids in these materials are significantly higher than found for normal persons.

Neue Neuraminsäurederivate des Menschen: N-Acetyl-9-O-L-lactoylneuraminsäure, N,9-O-Diacetylneuraminsäure und N-Acetyl-2,3-dehydro-2-desoxyneuraminsäure

Zusammenfassung: Im Blutserum, Speichel und Urin des Menschen vorkommende freie und glykosidisch gebundene Acylneuraminsäuren wurden durch Dünnschichtchromatographie bzw. Gaschromatographie/Massenspektrometrie charakterisiert. Dabei konnten die Strukturen von vier Neuraminsäurederivaten ermittelt werden. Das Vorkommen von drei dieser Substanzen beim gesunden Menschen war bisher nicht bekannt gewesen.

Das aus Serum und Speichel isolierte Acylneuraminsäuregemisch besteht aus *N*-Acetylneuraminsäure, *N*-Acetyl-9-*O*-L-lactoylneuraminsäure (maximal 25%), *N*,9-*O*-Diacetylneuraminsäure (maximal 5%) und *N*-Acetyl-2,3-dehydro-2-desoxyneuraminsäure. Das ungesättigte Neuraminsäurederivat kommt nur in der Fraktion freier Acylneuraminsäuren vor; die relativen Anteile betragen 1 - 80%. Im Urin wurden keine freien *N*,*O*-Diacyl-

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Enzymes:
D-Lactate dehydrogenase, D-lactate:NAD$^{\oplus}$ oxidoreductase (EC 1.1.1.28);
L-Lactate dehydrogenase, L-lactate:NAD$^{\oplus}$ oxidoreductase (EC 1.1.1.27).

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neuraminsäuren gefunden, sondern nur *N*-Acetylneuraminsäure und *N*-Acetyl-2,3-dehydro-2-desoxyneuraminsäure. Die untersuchten Flüssigkeiten enthielten keine *N*-Glykoloylneuraminsäure.

Die beschriebenen Acylneuraminsäuren wurden sowohl bei gesunden Individuen als auch in den entsprechenden Körperflüssigkeiten eines Patienten mit Sialurie entdeckt. Die Konzentrationen freier Acylneuraminsäuren sind in den untersuchten Flüssigkeiten des Sialurie-Patienten wesentlich höher als bei normalen Personen.

Key words: *N*-Acetylneuraminic acid, *N*-acetyl-9-*O*-L-lactoylneuraminic acid, *N*,9-*O*-diacetylneuraminic acid, *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid, gas-liquid chromatography/mass spectrometry.

Neuraminic acid is a common constituent of animal glycoconjugates. The compound occurs as the *N*-acetyl or the *N*-glycoloyl derivative; the hydroxyl functions at positions 4, 7 and/or 9 can be esterified[1-7]. Up to now *O*-acetyl[4,5], *O*-L-lactoyl[6] and *O*-glycoloyl[4,7] substituents have been found.

About the presence of *N*,*O*-acylneuraminic acids in man little has been reported[2]. The occurrence of such components, free or glycosidically bound, has been described for normal human urine[9] and for glycoproteins from human bile[10], colon[11,12], saliva[13] and serum[14]. Evidence for the presence of *N*,*O*-diacetylneuraminic acid in sialyllactose from human milk and colostrum has also been obtained[15]. However, in none of these cases have the number, nature and position of the ester groups been identified unequivocally.

In this paper the isolation and identification of human *N*-acetylneuraminic acid derivatives with *O*-acetyl or *O*-L-lactoyl groups at C-9 will be described. In addition, *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid, which has been found earlier as a natural compound in the urine of a patient with sialuria[16], has now been discovered to be a regular constituent of urine, serum and saliva from normal human individuals. A comparison concerning these new (in humans) acylneuraminic acids is made between normal individuals and the patient with sialuria.

Materials and Methods

Collection of human materials

Urine from normal individuals (30 *l*) and from a patient with sialuria (1 *l*) was collected and immediately frozen. The diffusate (10 *l*) from 10 *l* of blood plasma from normal persons was obtained in frozen state from the Blutspendezentrale in D-5800 Hagen. Serum from normal individuals (20 m*l*) and from the sialuria patient (30 m*l*) were prepared and lyophilized. Saliva from normal persons (100 m*l*) and from the sialuria patient (10 m*l*) was collected without pharmacological stimulation and immediately freeze-dried. During collection of the saliva samples, bacterial growth was prevented by addition of chloroform.

Isolation of acylneuraminic acids

All purification procedures of acylneuraminic acids were carried out at 2 °C. Free acylneuraminic acids from normal urine, filtered after thawing, were adsorbed on Dowex 1 × 4, $HCOO^{\ominus}$-form and eluted with 0.2M acetic acid, brought to pH 6 with pyridine. The eluate containing acylneuraminic acids was freeze-dried and the residue was fractionated on a column (1 × 150 cm) of cellulose MN 2100 ff (Macherey, Nagel & Co., Düren) using the solvent system n-butanol/n-propanol/water (1 : 2 : 1, v/v/v)[8]. Free acylneuraminic acids from sialuria urine were isolated as described in an earlier paper[16]. Free acylneuraminic acids from the serum and saliva samples were isolated by dialysis of the materials for 24 h against 10 vol. water with 3 changes. The diffusates were freed from lipid materials by three extractions with 0.1 vol. of ether. Acylneuraminic acids in the diffusates were further purified by rinsing the solution through a column (2.5 × 40 cm) of Dowex 50, $H^{\oplus}$-form, 50 - 100 mesh, after which the acids were adsorbed on a column (2.5 × 40 cm) of Dowex 2 × 8, $HCOO^{\ominus}$-form, 50 - 100 mesh. The column was washed with 1 *l* of water, the acylneuraminic acids were eluted with 0.5 *l* of 1M formic acid and the eluate was freeze-dried[8,17].

The acylneuraminic acids in the diffusate from 10 *l* of blood plasma were isolated in a similar way using a column (7 × 80 cm) of Dowex 50, $H^{\oplus}$-form and a column (4 × 60 cm) of Dowex 1 × 8, $HCOO^{\ominus}$-form. The latter column was eluted with 4 *l* of a gradient from 0 - 2M formic acid. The eluate containing the acylneuraminic acids was lyophilized and the residue was fractionated on cellulose as described above.

Table 1. Thin-layer and gas-liquid chromatographic data of reference acylneuraminic acids.
R_F values on cellulose (system A) and on silica gel (system B); R_{NeuNAc} values of the compounds as their methyl ester trimethylsilyl ethers on 3.8% SE-30.

Compound	R_F value in		R_{NeuNAc}
	System A	System B	
N-Acetylneuraminic acid	0.57	0.39	1.00
N-Glycoloylneuraminic acid	0.48	0.39	1.81
N,4-*O*-Diacetylneuraminic acid	0.76	0.61	1.18
N,7-*O*-Diacetylneuraminic acid	0.76	0.61	1.04
N,9-*O*-Diacetylneuraminic acid	0.76	0.61	1.13
N,7,9-*O*-Triacetylneuraminic acid	0.83	0.73	1.14
N-Acetyl-9-*O*-L-lactoylneuraminic acid	0.70	0.61	2.55
N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid	0.67	0.70	1.09

Glycosidically bound acylneuraminic acids from dialysed serum were isolated by hydrolysis in aqueous formic acid, pH 2, at 70 °C for 1 h, performed three times with intermediate dialysis[8]. Acylneuraminic acids from saliva samples were released in the same way, without previous dialysis. The acylneuraminic acids in the diffusates of the hydrolysis mixtures were purified as described for the free acylneuraminic acids.

Identification of acylneuraminic acids

Acylneuraminic acids were analysed with the orcinol/$Fe^{3\oplus}$/HCl reagent[18] and with the periodic acid/thiobarbituric acid reagent[19], using *N*-acetylneuraminic acid as a standard.

Thin-layer chromatography of acylneuraminic acids was carried out on cellulose MN 300 (Macherey, Nagel & Co., Düren) in the solvent system n-butanol/n-propanol/0.1M HCl (1 : 2 : 1, v/v/v; system A) and on silica gel H (Merck AG, Darmstadt) in the solvent system n-propanol/water (7 : 3, v/v; system B)[8]. Gas-liquid chromatography of the methyl ester trimethylsilyl ethers of acylneuraminic acids was performed on 3.8% SE-30 at 210 °C[5,6,16]. Table 1 shows the thin-layer and gas chromatographic retention values, relative to *N*-acetylneuraminic acid, of a number of reference acylneuraminic acids, obtained from bovine[4,6] and equine submandibular glands[4]. *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic acid was synthesized according to Meindl[20] and purified by chromatography on cellulose[16].

The *N*-acyl groups of *N*,*O*-diacylneuraminic acids were determined by two-dimensional thin-layer chromatography in system A with intermediate ammonia treatment[4]. *O*-Acyl groups were identified by thin-layer chromatography of their hydroxamates on cellulose in the solvent system n-propanol/10% aqueous ammonium carbonate/5M ammonium hydroxide (6 : 2 : 1, v/v/v)[4,6].

The molar ester contents of the acylneuraminic acid mixtures were determined with the alkaline hydroxylamine/$Fe^{3\oplus}$ reagent[21] using ethyl acetate as a standard. The configuration and the relative amounts of lactate were estimated with L-lactate dehydrogenase (EC 1.1.1.27, from porcine muscle, Boehringer Mannheim GmbH) and D-lactate dehydrogenase (EC 1.1.1.28, from *Lactobacillus leichmannii*, Boehringer Mannheim GmbH) after release with 0.05M NaOH from the acylneuraminic acids[6]. L-Lactate and D-lactate were used as standards.

Combined gas-liquid chromatography/mass spectrometry of the methyl ester trimethylsilyl ethers of the various acylneuraminic acids was used for determination of the molecular weight, the type of the *N*-acyl substituents and the number, type and position of the *O*-acyl substituents. Full details of this technique have been published recently[5,6,16].

Results and Discussion

The structures of the main acylneuraminic acids occurring free or glycosidically bound in blood serum, saliva and urine of normal individuals and the sialuria patient were determined as described in the experimental part. A survey of the results is given in Table 2. The *N*,*O*-diacylneuraminic acids present in the mixtures were identified as *N*,9-*O*-diacetylneuraminic acid and *N*-acetyl-9-*O*-L-lactoylneuraminic acid. The lactoyl group has the L-configuration, as was determined enzymically. Both *N*,9-*O*-diacetylneuraminic acid and *N*-acetyl-9-*O*-L-lactoylneuraminic acid turned out to be identical with the *N*,*O*-diacylneuraminic acids known to occur in bovine submandibular gland glycoproteins[5,6].

Table 2. Types and amounts of acylneuraminic acids present in serum, saliva and urine from normal persons and from a patient with sialuria.

The mean values of free (A) or glycosidically bound (B) acylneuraminic acids are recorded as μg/ml fluid for numbers of determinations given in parentheses. For saliva, analytical data of glycosidically bound acylneuraminic acids are not given explicitly because the free acylneuraminic acids cannot be completely removed from the viscous materials within a relatively short dialysis time, which is necessary for preservation of the *O*-acyl groups. These samples are therefore denominated as "A + B". The data for normal saliva "A + B" were obtained from samples which contain only traces of *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid. The approximate relative amounts (mean values from at least two analyses) of the constitutive acylneuraminic acids are given in % of the total amount; "traces" means 0.5 - 2%. The variable amounts of *N*-acetylneuraminic acid (NeuNAc) and *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid of normal saliva "A" are explained in the text.

Sample		Acylneuraminic acids [μg/ml]	NeuNAc [%]	2,3-Dehydro-2-deoxy-NeuNAc [%]	9-*O*-Ac-NeuNAc [%]	9-*O*-Lactoyl-NeuNAc [%]
Normal blood serum	A	1 (10)	80	traces	traces	20
	B	360 (10)	100	–	–	–
Sialuria serum	A	69 (2)	75	traces	–	25
	B	374 (2)	100	–	–	–
Normal saliva	A	8 (6)	variable	variable	traces	–
	A + B	44 (2)	70	traces	5	25
Sialuria saliva	A	120 (2)	95	traces	5	–
	A + B	1770 (2)	90	traces	traces	10
Normal urine	A	0.2 (3)	100	traces	–	–
Sialuria urine	A	14000 (5)	100	traces	–	–

Some indications for the presence of *N*,7-*O*-diacetylneuraminic acid and *N*,7,9-*O*-triacetylneuraminic acid in saliva were obtained by mass spectrometry. However, for a definite assessment more material is needed. The presence of *N*,4-*O*-diacetylneuraminic acid, *N*-glycoloylneuraminic acid and *O*-acylated *N*-glycoloylneuraminic acid derivatives in the mixtures could be excluded.

In most cases *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid was found in relatively small amounts. Estimation of these small quantities, in addition to *N*-acetylneuraminic acid, was carried out by gas-liquid chromatography/mass spectrometry with reference to the relative intensity of the peak at *m/e* 227, which is the base peak in the region $> m/e$ 100 of the mass spectrum of *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid. This relative intensity was measured as a function of the retention time in the range from R_{NeuNAc} 1.0 - 1.1. The identification of *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid from serum and urine was facilitated by partial separation of the compound from *N*-acetylneuraminic acid using cellulose column chromatography[16].

A typical thin-layer chromatogram of the various free acylneuraminic acids isolated from serum and urine of the sialuria patient is shown in Fig. 1. Gas-liquid chromatographic separations of acylneuraminic acids from saliva of two normal persons are represented in Fig. 2.

The quantitative data given in Table 2 do not refer to a statistical evaluation of the average relative amounts of the different acylneuraminic acids present as free or glycosidically bound components in the materials. Moreover, such a study was not intended in view of the lability of the ester linkages, which makes it almost impossible to obtain accurate values for the *N*,*O*-diacylneuraminic acid contents.

In nearly all cases the acylneuraminic acid mixture contains predominantly *N*-acetylneuraminic acid, as can be seen from Table 2. There are significant differences between the mixtures with respect to the relative amounts of *N*,*O*-diacylneur-

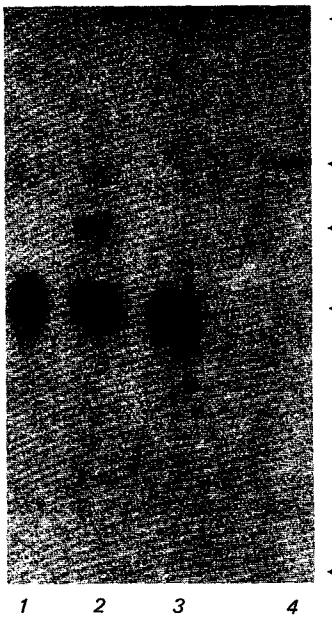

Fig. 1. Thin-layer chromatography on silica gel (system B) of free acylneuraminic acids from urine (1) and serum (2) of the sialuria patient.

Reference substances: *N*-acetylneuraminic acid (3) and *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (4).

aminic acids. In both serum and saliva, *N*-acetyl-9-*O*-L-lactoylneuraminic acid is the main *N,O*-diacylneuraminic acid, representing from 0 - 25% of the acylneuraminic acids in the various fractions. *N*,9-*O*-diacetylneuraminic acid was found in the same fluids in lower amounts, ranging from 0 - 5%. The differences in the *N*-acetyl-9-*O*-L-lactoylneuraminic acid and *N*,9-*O*-diacetylneuraminic acid contents between the various acylneuraminic acid fractions from serum and saliva may arise partly from individual or functional differences in the tissues producing these acylneuraminic acids. In addition, appreciable de-*O*-acylation is known to occur during the isolation and purification procedures[4]. Hydrolysis of the ester groups during passage through the kidney tubuli could explain the absence of free *N,O*-diacylneuraminic acids in urine.

Nothing is known about the biosynthesis of the human *N,O*-diacylneuraminic acids. Enzyme systems leading to acetylation and L-lactoylation of position 9 of *N*-acetylneuraminic acid are assumed to be present in man. Analogous systems, introducing acetyl groups at position 7 and/or 9 of *N*-acetylneuraminic acid and *N*-glycoloylneuraminic acid in bovine submandibular glands and at position 4 of *N*-acetylneuraminic acid and *N*-glycoloylneuraminic acid in equine submandibular glands have been reported[7,22].

N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid was found in all samples containing free acylneuraminic acids from serum, saliva and urine. The compound, which cannot occur in glycosidic linkage to glycoconjugates, was absent in dialysed serum samples. In serum and urine, it represents about 1% of the total free acylneuraminic acids. Appreciable variance was found in the relative amount of the compound in the different samples of free saliva acylneuraminic acids. The samples from two normal persons and the sialuria patient contained only traces, whereas samples obtained from two other normal persons were found to contain about 60 and 80% *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid, respectively (see Fig. 2b). The total amounts of free acylneuraminic acids in the four different saliva samples from normal individuals did not differ significantly.

The biosynthesis as well as the biological significance of *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid in the human body is unknown. The compound may be formed from CMP-*N*-acetylneuraminic acid by an enzymic or spontaneous reaction, as was suggested previously[16]. The metabolic origin of *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid may also be assumed on basis of its frequent occurrence in human fluids. The reasons for the large differences in the amount of the compound in the saliva of normal persons are unknown. They may reflect differences in functional states of the salivary glands. It is also possible that the amount present in the saliva samples is connected with the activity of bacterial enzymes in the oral cavity. The compound, known to be a strong inhibitor of some bacterial and viral neuraminidases[23,24], occurs in the saliva of some of our subjects at concentrations ($\sim 2 \times 10^{-5}$ M) which are in the range of the inhibitor constants measured for these enzymes. Therefore, it may be speculated that *N*-acetyl-2,3-

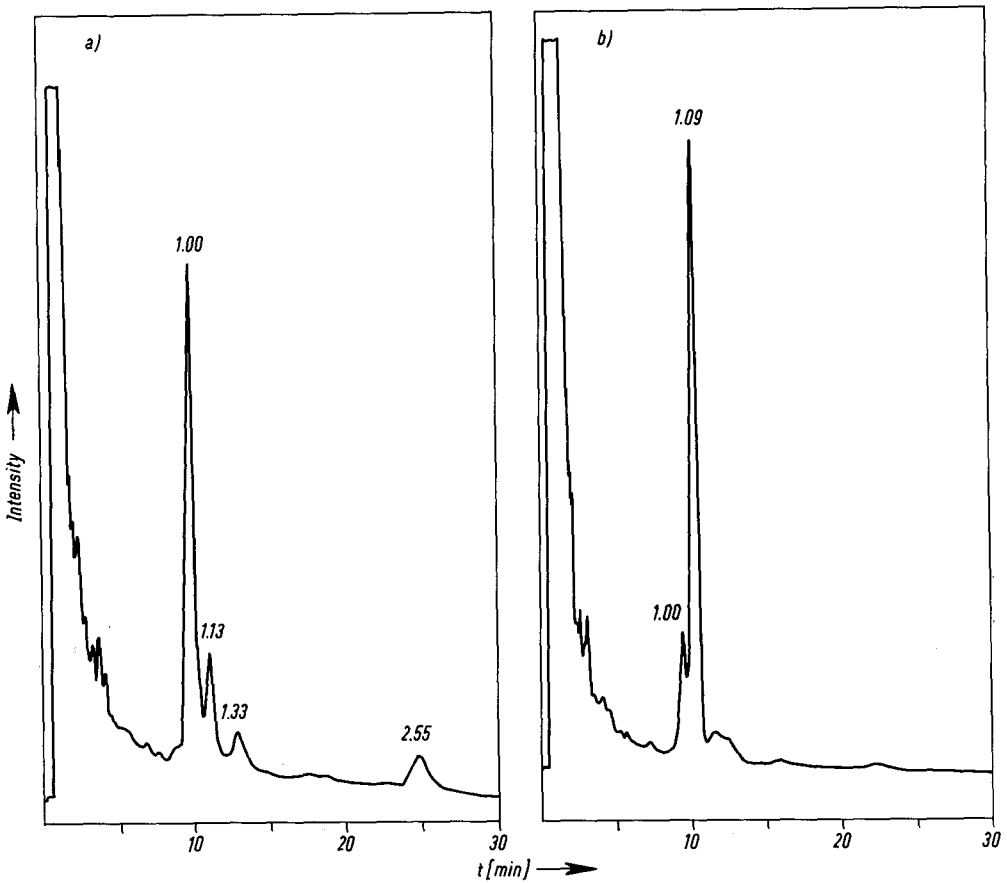

Fig. 2. Gas-liquid chromatography on 3.8% SE-30 of mixtures of acylneuraminic acids obtained from saliva of two normal individuals.

a) Free and glycosidically bound acylneuraminic acids from one person, b) free acylneuraminic acids from another person secreting relatively large quantities of *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid.

Components are identified as their methyl ester trimethylsilyl ethers by combined gas-liquid chromatography/mass spectrometry: R_{NeuNAc} 1.00, *N*-acetylneuraminic acid; R_{NeuNAc} 1.09, *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (not visible as a distinct peak in chromatogram a); R_{NeuNAc} 1.13, *N*,9-*O*-diacetylneuraminic acid; R_{NeuNAc} 1.33, undersilylation product of *N*-acetylneuraminic acid; R_{NeuNAc} 2.55, *N*-acetyl-9-*O*-L-lactoylneuraminic acid.

dehydro-2-deoxyneuraminic acid inhibits neuraminidases produced by bacteria present in the oral cavity[25,26]. It seems worthwhile to consider the influence of this natural inhibitor on caries and periodontitis.

The occurrence of *N*,9-*O*-diacetylneuraminic acid, *N*-acetyl-9-*O*-L-lactoylneuraminic acid and *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid in addition to *N*-acetylneuraminic acid in the fluids of both normal individuals and the sialuria patient suggests that sialuria, which is believed to be an error in acylneuraminic acid metabolism[27], is not connected with a significant alteration in *O*-acylation or dehydratation of *N*-acetylneuraminic acid. The only differences in acylneuraminic acid values between normal individuals and the sialuria patient are the appreciably higher concentrations of free acylneuraminic acids ob-

served in sialuria urine[16,27], serum[27] and saliva (more than 10^4-fold for urine, Table 2). In contrast to the free acylneuraminic acids, the concentrations of glycosidically bound acylneuraminic acids in serum glycoconjugates from normal individuals and the sialuria patient are only slightly different (see also ref.[27]).

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