Eur. J. Biochem. 56, 253–258 (1975)

Isolation and Identification of 2-Deoxy-2,3-dehydro-*N*-Acetylneuraminic Acid from the Urine of a Patient with Sialuria

Johannis P. KAMERLING and Johannes F. G. VLIEGENTHART

Organisch Chemisch Laboratorium der Rijksuniversiteit, Utrecht

Roland SCHAUER

Institut für Physiologische Chemie, Arbeitsgruppe für Zellchemie, Ruhr-Universität Bochum

Gérard STRECKER and Jean MONTREUIL

Laboratoire de Chimie Biologique de la Faculté des Sciences de Lille et Laboratoire associé au Centre National de la Recherche Scientifique

(Received March 7/April 28, 1975)

N-Acetylneuraminic acid preparations from the urine of a patient with sialuria contain 1–2% of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid. This new human sialic acid was isolated by ion-exchange and partition chromatography. The structure has been elucidated by mass spectrometry and confirmed by comparison with the synthetic compound. The properties of this unsaturated sialic acid in the orcinol/Fe^{3+}/HCl and the periodic acid/thiobarbituric acid tests as well as in thin-layer and gas-liquid chromatography are described. It does not react with acylneuraminate pyruvate-lyase. The origin of this new human sialic acid is discussed.

The excretion of 11 to 36 g of *N*-acetylneuraminic acid (NeuNAc) per l urine of a mentally retarded boy, who is now ten-years old, has been reported earlier [1,2]. Only one case of this disease, which has been called sialuria, is known. In this paper it will be described that the urine of the sialuria patient also contains small amounts of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (desH^{2,3}-NeuNAc) which was not known to occur in nature before.

MATERIALS AND METHODS

Isolation Procedure for Sialic Acids

NeuNAc from fresh urine maintained at + 4 °C during 24 h was routinely isolated and crystallized as described previously [1].

To isolate the new sialic acid, the urine was frozen (– 18 °C) immediately after urination and transported to the laboratory. The sialic acids were purified at + 2 °C by direct rinsing the urine through Dowex 50 (H^+ form) and adsorption of the sialic acids on Dowex 2-X8 (HCO_2^- form) [3]. The resin was washed with 2 bed-volumes of water and the sialic acids were eluted with a gradient of 0–2 M HCOOH. The eluate containing the sialic acids was freeze-dried.

500 mg of lyophilized sialic acids were dissolved in 3 ml of a mixture of *n*-butanol–*n*-propanol–water (1:2:1, v/v/v) and fractionated on a cellulose 2100 ff (Macherey, Nagel & Co.) column (1.50 m × 3 cm) using the same solvent for elution [3]. The 10-ml fractions containing the unknown compound were lyophilized and rechromatographed on a 1.50 m × 1-cm column in the same system.

In order to further purify the new sialic acid eluting from cellulose, preparative thin-layer chromatography was carried out. Trace amounts of radioactive NeuNAc were added to the unknown sample as a marker. The radioactive NeuNAc was localized by radioscanning (thin-layer radioscanner No. LB 2723, Dr Berthold, Wildbad). The unknown compound was localized on the basis of its migration rate relative to NeuNAc and isolated by water extraction of the silica gel.

Abbreviations. NeuNAc, *N*-acetylneuraminic acid; desH^{2,3}-NeuNAc, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid.

Enzymes. Sialic acid aldolase or acylneuraminate pyruvate-lyase (EC 4.1.3.3); lactate dehydrogenase (EC 1.1.1.27); neuraminidase or acylneuraminyl hydrolase (EC 3.2.1.18).

Colorimetry

Sialic acids were analyzed quantitatively by the orcinol/Fe^{3+}/HCl reagent [4] and the periodic acid/thiobarbituric acid reagent [5]. Crystalline NeuNAc served as reference substance.

Identification of Acyl Substituents

The presence of *O*-acyl groups was tested with the hydroxylamine-Fe^{3+} reagent [6] or by employing two-dimensional thin-layer chromatography with intermediary ammonia treatment of the sialic acids [7]. To establish the nature of the *N*-acyl group, the sialic acid was methanolyzed. The methyl ester of the acyl substituent that was formed was characterized as its hydroxamate using thin-layer chromatography [7]. Glycoloylhydroxamate and acetylhydroxamate served as reference substances.

Reaction with Acylneuraminate Pyruvate-Lyase

The susceptibility of the sialic acid to the action of acylneuraminate pyruvate-lyase (Sigma Chemical Company) in the presence of lactate dehydrogenase (from porcine muscle; Boehringer Mannheim GmbH) and NADH was tested photometrically (Hitachi photometer, model 101) [8].

Preparation of Methyl Esters, Trimethylsilyl Ethers of Sialic Acids

To 400 μg of sialic acid in 1 ml of methanol, diazomethane in ether was added until a faint yellow colour was obtained, immediately followed by evaporation of the solution. The residue was dissolved in 1 ml of pyridine. Subsequently 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane were added. After 2 h at room temperature 2 ml of chloroform and 2 ml of water were added to the turbid mixture. The chloroform layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* [9].

Preparation of Trimethylsilyl Esters, Ethers of Sialic Acids

To 100 μg of sialic acid 25 μl of *N*-trimethylsilylimidazole was added. This solution was heated for 5 min at 60 °C under nitrogen [10].

Thin-Layer Chromatography

Thin-layer chromatography was performed on 0.2-mm plates of silica gel H nach Stahl (Merck AG) and cellulose MN 300 (Macherey, Nagel & Co.) for analytical purposes and on 0.5-mm plates of silica gel H nach Stahl for preparative purposes. For silica gel *n*-propanol—water (7:3, v/v) [11] was used and for cellulose *n*-butanol—*n*-propanol—0.1 M HCl (1:2:1, v/v/v) [12] or *n*-butanol—acetic acid—water (4:1:5, v/v/v) [13]. The sialic acid spots were stained with the orcinol/Fe^{3+}/HCl or the periodic acid/thiobarbituric acid spray reagent [7]. Unsaturated compounds chromatographed on silica gel were stained by exposure to iodine vapour at room temperature.

Gas-Liquid Chromatography

Gas-liquid chromatography of methyl esters, trimethylsilyl ethers of sialic acids was carried out on a Pye 104 gaschromatograph, equipped with flame ionization detector and glass columns (1.60 m × 4.0 mm) on 3.8% SE-30 on Chromosorb W-AW DMCS, HP, 80—100 mesh. Column-oven temperature: 206 °C; gas flow rate for N_2 30 ml/min. The retention times (R_{NeuNAc}) are given relative to the pertrimethylsilyl derivative of the methyl ester of NeuNAc.

Gas-liquid chromatography of pertrimethylsilyl sialic acids was carried out on a Hewlett-Packard apparatus model 402, equipped with flame ionization detector and a metal column (1.60 m × 2.0 mm) packed with 3% OV-17 on Gas Chrom Q, 80—100 mesh. Column-oven temperature: 210 °C; gas flow rate for N_2 50 ml/min. The retention times (R'_{NeuNAc}) are given relative to the pertrimethylsilyl derivative of NeuNAc.

Mass Spectrometry

75-eV mass spectra were recorded on a Jeol JGC-1100/JMS-07 combination (column material 3% SE-30 on Chromosorb W-AW DMCS, 80—100 mesh; oven temperature 200 °C; ion source temperature 250 °C; accelerating voltage 1.5 kV; ionizing current 300 μA) and on an AEI MS-902 apparatus (ion source temperature 100 °C; accelerating voltage 8 kV; ionizing current 500 μA). High-resolution mass measurements were performed with a dynamic resolving power of 10000 and a scan speed of 16 s per mass decade by using an AEI MS-902 mass spectrometer connected on-line with a Ferranti Argus 500 computer. The exact masses measured were converted into element lists as described by Van't Klooster *et al.* [14].

RESULTS

Isolation and Identification of the New Human Sialic Acid

In Fig. 1 the cellulose thin-layer chromatogram of the urinary sialic acid mixture (before cellulose column chromatography) in the solvent system *n*-butanol—*n*-propanol—0.1 M HCl (1:2:1, v/v/v) is given. The

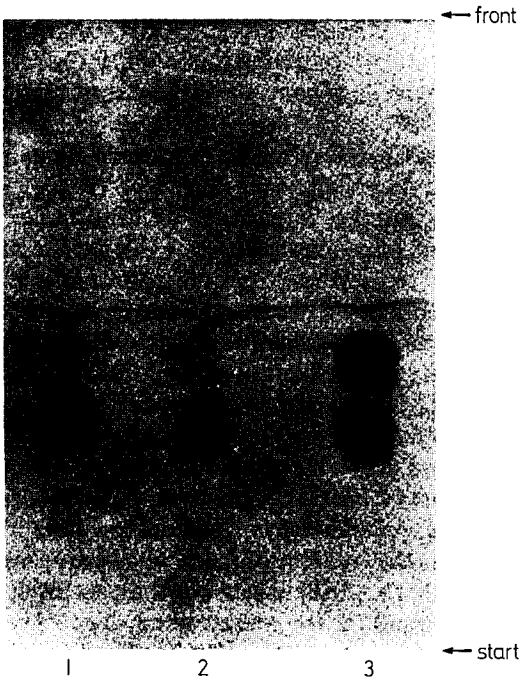

Fig. 1. *Thin-layer chromatography of urinary sialic acids of sialuria (1) on cellulose.* Solvent: *n*-butanol–*n*-propanol–0.1 M HCl; 1:2:1, v/v/v. Reference substances: (2), synthetic NeuNAc; (3), synthetic desH^{2,3}-NeuNAc co-chromatographed with synthetic NeuNAc

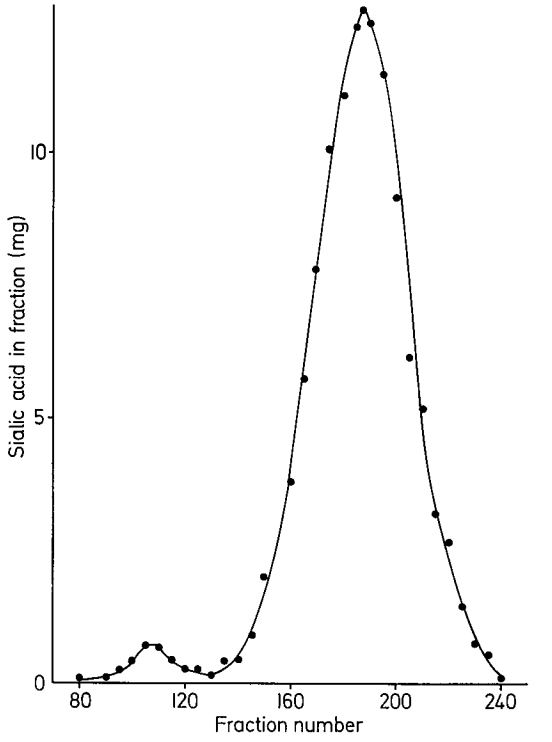

Fig. 2. *Fractionation of sialic acids (500 mg) from sialuria urine on cellulose.* Column size: 1.50 m × 3 cm; solvent: *n*-butanol–*n*-propanol–water (1:2:1, v/v/v). Fraction volume: 10 ml. Fraction 90–130, mixture of desH^{2,3}-NeuNAc (maximum of its relative amount in the peak region), NeuNAc and its propyl and butyl esters; Fraction 131–240, Neu NAc

Table 1. *R_F-values of NeuNAc and the new sialic acid (natural and synthetic desH^{2,3}-NeuNAc) obtained by thin-layer chromatography on silica gel and cellulose in several solvent systems*
Solvent A: *n*-propanol–water (7:3, v/v); solvent B: *n*-butanol–*n*-propanol–0.1 M HCl (1:2:1, v/v/v); solvent C: *n*-butanol–acetic acid–water (4:1:5, v/v/v)

Compound	R_F value on		
	silica gel in solvent A	cellulose in solvent	
		B	C
NeuNAc	0.51	0.60	0.57
desH^{2,3}-NeuNAc	0.73	0.71	0.69

R_F values are presented in Table 1. The new sialic acid is less hydrophilic than NeuNAc. With the orcinol/Fe^{3+}/HCl spray reagent NeuNAc as well as the unknown compound give rise to a lilac colour. On cellulose thin-layer chromatograms both spots stain red by spraying with the periodic acid/thiobarbituric acid reagent.

By gas-liquid chromatography of the petrimethylsilylated native urinary sialic acid mixture on 3% OV-17, a R'_{NeuNAc} value of 1.79 is found for the new human sialic acid.

Column chromatography on cellulose of the sialic acids from the sialuria urine results in a small peak eluting before NeuNAc (Fig. 2). Because this peak contained besides the new sialic acid still small amounts of NeuNAc, rechromatography on cellulose was applied. By this method, from 500 mg of urine sialic acids finally 1 mg of the unknown sample was obtained in a purity grade of 95% (as shown by gas-liquid chromatography on OV-17). This corresponds to 0.2% of the total sialic acids.

The new sialic acid does not contain *O*-acyl groups, because the hydroxamate-Fe^{3+} reaction was negative and no de-*O*-acylated product was detectable after two-dimensional thin-layer chromatography with intermediate NH_3 treatment. Identification of the acylhydroxamate after methanolysis of the sialic acid indicated the presence of an *N*-acetyl group.

The new human sialic acid is resistant to the action of the aldolase. No pyruvic acid was formed in the enzyme assay, as indicated by the constancy of the NADH concentration in the presence of lactate dehydrogenase, and no acylmannosamine was found as reaction product. In contrast, NeuNAc was readily

split by the enzyme, leading to pyruvic acid and *N*-acetylmannosamine as cleavage products.

For combined gas-liquid chromatography/mass spectrometry fraction 105, as indicated in Fig. 2, was used. In Fig. 3 the gas chromatogram on 3.8% SE-30 of the esterified and trimethylsilylated sample is given. Four peaks are present in the chromatogram with retention times of R_{NeuNAc} 1.00, 1.10, 1.42 and 1.77 respectively. From these peaks mass spectra were recorded.

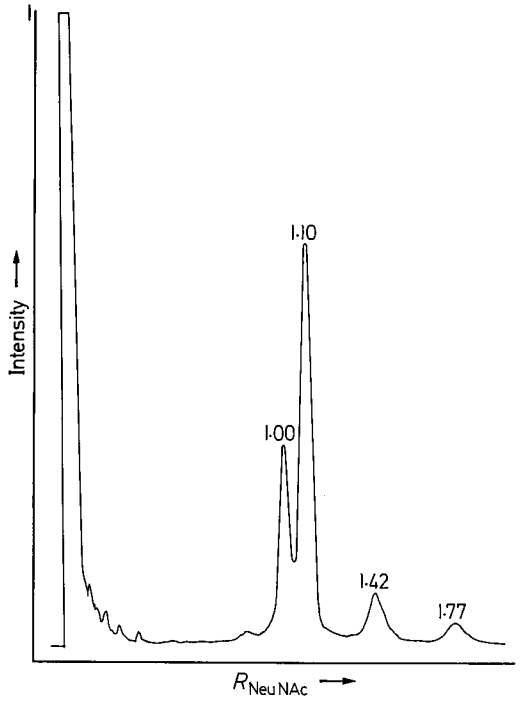

Fig. 3. *Gas-liquid chromatography on 3.8% SE-30 of a sialic acid mixture enriched in desH^{2,3}-NeuNAc eluting from cellulose (fraction 105 of Fig. 2).* Peaks are related to the methyl ester of NeuNAc (R_{NeuNAc} 1.00) and identified by mass spectrometry. R_{NeuNAc} 1.10, methyl ester of desH^{2,3}-NeuNAc; R_{NeuNAc} 1.42, propyl ester of NeuNAc; R_{NeuNAc} 1.77, butyl ester of NeuNAc

The most characteristic mass values of the spectrum of the unknown sialic acid derivative (R_{NeuNAc} 1.10) together with the intensities, brutoformulae and explanations are summarized in Table 2. The atomic formulae were deduced from high-resolution mass measurements of the esterified and trimethylsilylated sample by using the direct inlet system of the mass spectrometer. In the high-mass range of the spectrum with R_{NeuNAc} 1.10 a number of peaks were observed 90 daltons lower than the corresponding values in the spectrum of the pertrimethylsilyl derivative of the methyl ester of NeuNAc, pointing to a difference of trimethylsilanol. Therefore the unknown sialic acid contains one hydroxyl group and one hydrogen atom less than NeuNAc. This leads to the conclusion that this compound is an unsaturated NeuNAc. The occurrence of the fragment ions *m/e* 205, *i.e.* $CH_2OSi(CH_3)_3-CH=\overset{+}{O}Si(CH_3)_3$, and *m/e* 307, *i.e.* $CH_2OSi(CH_3)_3-CHOSi(CH_3)_3-CH=\overset{+}{O}Si(CH_3)_3$, shows that the side chain C-7–C-9 is intact. To the intensity of the peak at *m/e* 186, generally occurring in *N*-acetylneuraminic acids, two fragments can contribute, namely: $CH_3CO-\overset{+}{N}H=CH-C[OSi(CH_3)_3]=CH_2$ (C-3–C-5) and $CH_3CO-\overset{+}{N}H=CH-CH=CHOSi(CH_3)_3$ (C-5–C-7). The presence of *m/e* 186 in the spectrum eliminates the possibility of a double bond between C-4 and C-5. The mass spectrum does not show an intense peak at *m/e* 534 (M^+ minus $\cdot COOCH_3$); this type of fragment ion is always present in saturated sialic acids [15,9]. The hindrance of the

Table 2. *Interpretation of some important fragment ions, present in the mass spectrum of the pertrimethylsilyl derivative of the methyl ester of the new human sialic acid (desH^{2,3}-NeuNAc) with R_{NeuNAc} 1.10 on 3.8% SE-30*
The figures in brackets are the intensities of the ions, relative to that of *m/e* 227

m/e	Atomic formula	Fragment
593 (2)	$C_{24}H_{51}NO_8Si_4$	$M^{+\cdot}$
578 (5)	$C_{23}H_{48}NO_8Si_4$	$M^{+\cdot}$ minus $\cdot CH_3$
388 (3)	$C_{16}H_{30}NO_6Si_2$	$M^{+\cdot}$ minus $\cdot CHOSi(CH_3)_3-CH_2OSi(CH_3)_3$
341 (18)	$C_{15}H_{25}O_5Si_2$	$M^{+\cdot}$ minus $HOSi(CH_3)_3$ minus $\cdot CH_2OSi(CH_3)_3$ minus NH_2COCH_3
307 (3)	$C_{12}H_{31}O_3Si_3$	$CH_2OSi(CH_3)_3-CHOSi(CH_3)_3-CH=\overset{+}{O}Si(CH_3)_3$
298 (6)	$C_{13}H_{20}NO_5Si$	$M^{+\cdot}$ minus $\cdot CHOSi(CH_3)_3-CH_2OSi(CH_3)_3$ minus $HOSi(CH_3)_3$
227 (100)	$C_{10}H_{15}O_4Si$	$M^{+\cdot}$ minus $\cdot CHOSi(CH_3)_3-CHOSi(CH_3)_3-CH_2OSi(CH_3)_3$ minus NH_2COCH_3
217 (7)	$C_9H_{21}O_2Si_2$	$CH_2=COSi(CH_3)_3-CH=\overset{+}{O}Si(CH_3)_3$
205 (7)	$C_8H_{21}O_2Si_2$	$CH_2OSi(CH_3)_3-CH=\overset{+}{O}Si(CH_3)_3$
186 (37)	$C_8H_{16}NO_2Si$	$CH_3CO-\overset{+}{N}H=CH-CH=CHOSi(CH_3)_3$ $CH_3CO-\overset{+}{N}H=CH-COSi(CH_3)_3=CH_2$

elimination of $COOCH_3$ can adequately be explained by the occurrence of a double bond between C-2 and C-3. Therefore, the structure of the new human sialic acid can be formulated as 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (desH^{2,3}-NeuNAc). It has to be noted that the peak at *m/e* 173, *i.e.* $CH_3CO-\overset{+}{N}H=\dot{C}H-CHOSi(CH_3)_3$, is absent. This suggests that also the formation of the latter fragment ion is prohibited by the double bond.

The mass spectrum of the compound with R_{NeuNAc} 1.00 is identical to that of the pertrimethylsilyl derivative of the methyl ester of NeuNAc [15, 9]. The spectra of the compounds with R_{NeuNAc} 1.42 and 1.77 originate from the pertrimethylsilyl derivatives of the propyl and butyl esters of NeuNAc respectively. These two minor compounds have to be considered as artefacts, formed by reaction of NeuNAc with the solvent system used during the isolation procedure. Some relevant fragment ions are given in Table 3 [9].

The identity of the unsaturated compound was confirmed by comparison with synthetic 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid [16]. Thin-layer co-chromatography (Fig. 1 and Table 1) showed that both compounds have the same R_F values; similar colours are obtained with the two sialic acid spray reagents. Both the natural and the synthetic desH^{2,3}-NeuNAc were stainable on silica gel in iodine vapour at room temperature in accordance with the presence of the double bond. Also in gas-liquid co-chromatographic experiments, identical R_{NeuNAc} and R'_{NeuNAc} values were found. Furthermore, the mass spectra of both compounds are the same. It has to be noted that the resistance of the synthetic desH^{2,3}-NeuNAc towards the aldolase has been described earlier [8].

Quantitative Analysis

For the colorimetric analyses experiments, desH^{2,3}-NeuNAc of 95% purity grade obtained after cellulose column chromatography, was further purified by preparative thin-layer chromatography. In the orcinol/Fe^{3+}/HCl test the molar absorption coefficient of the natural desH^{2,3}-NeuNAc corresponded exactly to that of the authentic one [16].

Therefore, the quantitative analyses of the isolated desH^{2,3}-NeuNAc were based on this test. Preparative thin-layer chromatography of the native urinary sialic acid mixture and quantitative estimation of the sialic acid spots using the orcinol/Fe^{3+}/HCl reagent has shown that this mixture contains 1–2% of desH^{2,3}-NeuNAc.

The human desH^{2,3}-NeuNAc produced only 8.7% of colour in the periodic acid/thiobarbituric acid test when compared with NeuNAc. This value is in good agreement with that of the authentic sample: in our hands, it was not completely negative in this test as has been reported [16], but exhibited a molar absorption coefficient of 5.3% when compared with NeuNAc.

Table 3. *Some important fragment ions in the mass spectra of the pertrimethylsilyl derivatives of the methyl, propyl and butyl esters of NeuNAc (R_{NeuNAc} 1.00, 1.42 and 1.77 respectively on 3.8% SE-30) [9]*

A: $M^{+\cdot}$ minus CH_3; B: $M^{+\cdot}$ minus COOR (R = CH_3, C_3H_7 or C_4H_9); C: $M^{+\cdot}$ minus $CHOSi(CH_3)_3-CH_2OSi(CH_3)_3$; D: $M^{+\cdot}$ minus $CHOSi[CH_3)_3-CH_2OSi(CH_3)_3$ minus $HOSi(CH_3)_3$ minus $HOSi(CH_3)_3$; E: $M^{+\cdot}$ minus $CHOSi(CH_3)_3-CHOSi(CH_3)_3-CH_2OSi(CH_3)_3$ minus NH_2COCH_3; F: $CH_2OSi(CH_3)_3-\overset{+}{C}H=\overset{+}{O}Si(CH_3)_3$; G: $CH_3CO-\overset{+}{N}H=CH-CHOSi(CH_3)_3$

R_{NeuNAc}	A	B	C	D	E	F	G
1.00	668	624	478	298	317	205	173
1.42	696	624	506	326	345	205	173
1.77	710	624	520	340	359	205	173

DISCUSSION

The results show that in the urine of a patient with sialuria desH^{2,3}-NeuNAc is also present. The excretion rate of this compound is 1–2% of the total sialic acids. So far, desH^{2,3}-NeuNAc has not been found in biological material. The origin of this new sialic acid is unknown. We suggest that desH^{2,3}-NeuNAc is a metabolic product because it is also present in blood serum and in saliva of the sialuria patient (unpublished results). Furthermore, there are no indications that it is an artificial degradation product of NeuNAc. The compound occurs in similar amounts in 4-year-old sialic acid preparations from urine as in urine collected a few days before isolation of the sialic acids. Storage of frozen urine for months or incubation of urine for 48 h at 37 °C do not significantly alter the content of desH^{2,3}-NeuNAc. The compound is not formed from synthetic NeuNAc subjected to the isolation procedure applied to urine. Storage of crystalline NeuNAc for longer times at −18 °C does not give rise to detectable amounts of desH^{2,3}-NeuNAc.

It is conceivable that in the organism desH^{2,3}-NeuNAc is formed either in an elimination reaction between a glycosidic hydroxyl group and a proton at C-3 during the enzymic condensation of *N*-acetylmannosamine with phospho*enol*pyruvate [17] or by elimination of CMP from CMP-NeuNAc.

It remains to be investigated if this compound occurs only in the sialuria patient and if it has any influence on the sialic acid metabolism. It has been shown that synthetic desH^{2,3}-NeuNAc inhibits competitively the action of neuraminidase [18], but it

does not affect the acylneuraminate pyruvate-lyase [8]. Further studies are necessary to elucidate if desH^{2,3}-NeuNAc inhibits enzymes involved in the biosynthesis of sialoglycoproteins and thus may lead to the striking urinary excretion of NeuNAc.

We thank Mrs Margret Wember and Mr G. J. Gerwig for their technical assistance, Mr C. Versluis (Laboratory of Analytical Chemistry, State University, Utrecht) for recording the mass spectra, Dr P. Meindl (Ernst Boehringer-Institut für Arzneimittelforschung, Bender & Co. Ges.m.b.H., Vienna) for the gift of synthetic 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, Dr J.-P. Farriaux (Service de Pédiatrie de la Cité Hospitalière de Lille) for supplying the urine samples and Dr J. Haverkamp for helpful discussions. This investigation was supported by the *Deutsche Forschungsgemeinschaft* (grant Scha 202/3), the *Fonds der Chemischen Industrie*, the *Erwin Riesch-Stiftung*, the *Centre National de la Recherche Scientifique* and the *Fondation pour la Recherche Médicale Française*.

REFERENCES

1. Montreuil, J., Biserte, G., Strecker, G., Spik, G., Fontaine, G. & Farriaux, J.-P. (1968) *Clin. Chim. Acta, 21*, 61–69.
2. Fontaine, G., Biserte, G., Montreuil, J., Dupont, A., Farriaux, J.-P., Strecker, G., Spik, G., Puvion, E., Puvion-Dutilleul, F., Sezille, G. & Picqué, M. T. (1968) *Helv. Paediatr. Acta, 23, Suppl. XVII*, Fasc. 3.
3. Schauer, R. & Faillard, H. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349*, 961–968.
4. Böhm, P., Dauber, S. & Baumeister, L. (1954) *Klin. Wochenschr. 32*, 289–292.
5. Aminoff, D. (1961) *Biochem. J. 81*, 384–392.
6. Hestrin, S. (1949) *J. Biol. Chem. 180*, 249–261.
7. Buscher, H.-P., Casals-Stenzel, J. & Schauer, R. (1974) *Eur. J. Biochem. 50*, 71–82.
8. Schauer, R., Wember, M., Wirtz-Peitz, F. & Ferreira do Amaral, C. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1073–1080.
9. Kamerling, J. P., Vliegenthart, J. F. G., Versluis, C. & Schauer, R. (1975) *Carbohyd. Res. 41*, 7–17
10. Casals-Stenzel, J., Buscher, H.-P. & Schauer, R. (1975) *Anal. Biochem. 65*, 507–524
11. Granzer, E. (1962) *Hoppe-Seyler's Z. Physiol. Chem. 328*, 277–279.
12. Svennerholm, E. & Svennerholm, L. (1958) *Nature (Lond.) 181*, 1154–1155.
13. Crumpton, M. J. (1959) *Biochem. J. 72*, 479–486.
14. Van 't Klooster, H. A., Vaarkamp-Lijnse, J. S. & Dijkstra, G. (1974) *Org. Mass Spectrom. 8*, 303–316.
15. Kamerling, J. P., Vliegenthart, J. F. G. & Vink, J. (1974) *Carbohyd. Res. 33*, 297–306.
16. Meindl, P. & Tuppy, H. (1969) *Monatsh. Chem. 100*, 1295–1306.
17. Warren, L. & Felsenfeld, H. (1961) *Biochem. Biophys. Res. Commun. 4*, 232–235.
18. Meindl, P. & Tuppy, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350*, 1088–1092.

J. P. Kamerling and J. F. G. Vliegenthart, Organisch Chemisch Laboratorium der Rijksuniversiteit, Croesestraat 79, Utrecht, The Netherlands

R. Schauer, Institut für Physiologische Chemie der Ruhr-Universität Bochum, D-4630 Bochum-Querenburg, Postfach 2148, Buscheystraße, Federal Republic of Germany

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