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NATURAL OCCURRENCE AND PREPARATION OF *O*-ACYLATED 2,3-UNSATURATED SIALIC ACIDS

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ABSTRACT

Three *O*-acylated, unsaturated sialic acids, *N*-acetyl-9-*O*-acetyl-, *N*-acetyl-9-*O*-lactoyl-, and 2-deoxy-*N*-glycoloyl-9-*O*-lactoyl-2,3-didehydroneuraminic acid (5-acetamido-9-*O*-acetyl-, 5-acetamido-9-*O*-lactoyl-, and 2,6-anhydro-3,5-dideoxy-5-glycoloylamido-9-*O*-lactoyl-D-*glycero*-D-*galacto*-non-2-enonic acid) were isolated from urine or submandibular glands of rat, pig, and cow. Mass spectrometric evidence for the existence of 2,3-unsaturated 9-*O*-acetyl-*N*-glycoloylneuraminic acid in porcine urine was also obtained. The sialic acids were purified by dialysis, gel- and ion-exchange chromatography, and preparative thin-layer chromatography. They were analyzed by thin-layer chromatography, high-pressure liquid chromatography, and capillary gas–liquid chromatography–mass spectrometry. For comparison, *O*-acetylated unsaturated sialic acids were synthesized.

INTRODUCTION

Sialic acids are known to occur in saturated or unsaturated (double bond between C-2 and C-3) forms in Nature[1]. Besides, of more than 20 saturated sialic acids, up to three natural unsaturated derivates of sialic acids are presently known, namely, *N*-acetyl-2-deoxy-2,3-didehydroneuraminic acid (5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu5Ac2en)[2], 2-deoxy-*N*-glycoloyl-2,3-didehydroneuraminic acid (2,6-anhydro-3,5-dideoxy-5-glycoloylamido-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu2en5Gc)[3], and 2-deoxy-*N*-glycoloyl-8-*O*-methyl-2,3-didehydroneuraminic acid (2,6-anhydro-3,5-dideoxy-5-glycoloylamido-8-*O*-methyl-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu2en5Gc8Me)[1,4]. With regard to the biological origin of unsaturated sialic acids, two hypotheses have been forwarded. It has been shown that Neu5Ac2en and Neu2en5Gc can be formed in a nonenzymic elimination reaction from the corresponding CMP-sialic acids. As this reaction can also occur under physiological conditions, the formation

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of natural, unsaturated sialic acids from tissue CMP-sialic acids has been suggested[3,5]. Furthermore, it has been postulated, on the basis of experiments with mammalian brain, that Neu5Ac2en might be produced enzymically from sialoglycoconjugates[6]. As *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) has been reported to occur in tissues and body fluids in free form[7,8], and linked to CMP[7,9] or to various glycoconjugates[9,10], the natural existence of *O*-acylated unsaturated sialic acids was expected.

EXPERIMENTAL

Chemicals. — *N*-Acetylneuraminic acid (Neu5Ac) and *N*-glycoloylneuraminic acid (Neu5Gc) were isolated either from edible birds' nest substance or porcine submandibular-gland glycoproteins[11]. Neu5Ac2en was purchased from Boehringer (Mannheim). Neu5Gc2en was synthesized according to Nöhle *et al.*[3]. Ion-exchange resins, polyacrylamide gel (Bio-Gel P-2), and Aminex A-29 for l.c. were purchased from Bio-Rad (Munich), and Sephadex G-25 was from Pharmacia (Freiburg). Cellulose thin-layer (0.1 mm) plates, solvents, and chemicals of analytical grade were products of Merck (Darmstadt) and the cellulose powder MN 2100 ff and the per(trimethylsilyl)ating agent, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were obtained from Macherey–Nagel & Co (Düren).

Thin-layer chromatography. — Screening of sialic acid fractions isolated from biological materials and control of synthesis of *O*-acetylated unsaturated derivatives of neuraminic acid were monitored by t.l.c. on 0.1-mm cellulose. Cellulose plates were prerun in 0.1M HCl and dried before application of the sample. After development in 1:2:1 (v/v) butanol–propanol–0.1M HCl, sialic acid spots were detected with the orcinol–Fe^{3+}–HCl spray reagent[11].

Liquid chromatography. — L.c. analysis of sialic acids isolated from natural sources and determination of the reaction products from chemical *O*-acetylation of Neu5Ac2en and Neu2en5Gc were performed with a Spectra-Physics SP 8000 apparatus operated at 0.5–3 MPa and equipped with a photometer (Spectroflow monitor SF 770, Schoeffel Instruments Corp.) an integrator (Spectra-Physics SP 4000), and a column (40 × 4.6 mm i.d.) filled with Aminex A-29, isocratically eluted with 0.75mM Na_2SO_4, and monitored at 200 nm for the detection of sialic acids, as described[12].

Isolation of unsaturated sialic acids. — Free sialic acids were isolated either from submandibular glands of cow, horse, and pig (200 g each) or from urine of rat, pig, cow, and horse (1 L each). The techniques applied (homogenization of tissues, dialysis of free sialic acids, and purification by different chromatographic steps on columns and cellulose thin-layers) are exactly as described in ref. 3.

Synthesis of O-acetylated unsaturated sialic acids. — This synthesis was carried out similarly to the procedure described by Haverkamp *et al.*[13]. Dried Neu5Ac2en or Neu2en5Gc (10 mg each) was dissolved in dry pyridine (1 mL). After addition of *N*-acetylimidazole (4 mg) dissolved in dry pyridine (1 mL), the

mixture was kept at room temperature for 12 h or longer. The formation of *O*-acetyl derivatives of the unsaturated sialic acids was monitored by t.l.c. and l.c. by withdrawing samples (10 μL) at regular intervals. The samples were lyophilized before per(trimethylsilyl)ation and analysis of the sialic acids by g.l.c.–m.s.

Capillary gas–liquid chromatography. — A sample of freeze-dried sialic acid (~100 μg) was dissolved in dry pyridine (30 μL). After the addition of MSTFA (50 μL), the solution was thoroughly mixed and kept at room temperature for 60 min to achieve complete trimethylsilylation[14] and give the trimethylsilyl ester of per(trimethylsilyl) ethers. In the case of very pure sialic acids, the *N*-(trimethylsilyl) derivatives were also formed. G.l.c. was carried out on a Packard Becker gas chromatograph, model 428, equipped with a flame-ionization detector and an all-glass inlet system. Sialic acids were separated on a 25-m, fused-silica OV-101 capillary column (0.4 mm i.d., Macherey–Nagel & Co., Düren), the oven temperature being programmed from 150 to 280° at a rate of 2°/min. Injector and detector unit were heated to 250°. The N_2 flow-rate through the capillary column was adjusted to 1 mL/min at a split ratio of 1:20. Make-up gas was added to a total flow rate of 20 mL/min; the speed of H_2 and air was 30 mL and 250 mL/min, respectively. Peak areas and retention times were calculated by a Spectra-Physics 4100 integrator.

Gas–liquid chromatography–mass spectrometry. — Separation of the derivatized sialic acids was achieved by means of a Varian gas chromatograph, model 3700, equipped with a 25-m, fused-silica OV-101 capillary column (0.4 mm i.d.). The oven temperature was programmed from 150 to 280° at a rate of 2°/min, while He gas was used as carrier gas (flow rate 1 mL/min, split ratio 1:10). The gas chromatograph was lined *via* an open-split coupling (line and separator kept at 220° each) to a quadrupole, mass spectrometer (Varian MAT 44S), which was connected

TABLE I

OCCURRENCE OF UNSATURATED SIALIC ACIDS IN FLUIDS AND TISSUES OF VARIOUS ANIMAL SPECIES AND MAN

Species	*Source*	*Unsaturated sialic acids*		
Man[a]	Saliva	Neu5Ac2en		
	Urine	Neu5Ac2en		
	Serum	Neu5Ac2en		
Cow	Submandibular gland	Neu5Ac2en		Neu5,9$Ac_2$2en
	Urine	Neu5Ac2en		Neu5,9$Ac_2$2en
Horse	Submandibular gland	Neu5Ac2en	Neu2en5Gc	
	Urine	Neu5Ac2en	Neu2en5Gc	
Pig	Submandibular gland	Neu5Ac2en	Neu2en5Gc	Neu5Ac2en9Lt, Neu2en5Gc9Lt
	Urine	Neu5Ac2en	Neu2en5Gc	Neu9Ac5Gc2en
Rat	Urine	Neu5Ac2en	Neu2en5Gc	Neu5,9$Ac_2$2en
Hen[b]	Erythrocytes	Neu5Ac2en		
Starfish[c]	Whole animal		Neu2en5Gc	Neu2en5Gc8Me

[a]Ref. 2 and 8. [b]Ref. 1. [c]Ref. 1 and 4.

to a Spectro Spin 55 200 data system. Mass spectra were obtained by electron-impact ionization at 70 eV (ion-source temperature 220°, ionization current 0.5 mA).

RESULTS

By application of the preparation procedure described earlier[3], it was possible to purify the fractions of free sialic acids from submandibular glands and urine to such an extent that the nature of individual sialic acids occurring in even very small amounts in these fractions could be identified with the aid of the improved analytical methods described herein. Thus, unsaturated sialic acids were detected in all biological materials investigated. The amounts of these neuraminic acid derivatives listed in Table I were low, ranging from about 0.1 to 5% of the total, nonglycosidically bound sialic acids. Besides Neu5Ac2en and Neu2en5Gc, four other *O*-acylated unsaturated sialic acids were identified, namely, *N*-acetyl-9-*O*-acetyl-2-deoxy-2,3-dehydroneuraminic acid (5-acetamido-9-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, $Neu5,9Ac_2 2en$), found in bovine and rat urine and in bovine submandibular glands; *N*-acetyl-2-deoxy-9-*O*-lactoyl-2,3-dehydroneuraminic acid (5-acetamido-9-*O*-lactoyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu5Ac2en9Lt) and 2-deoxy-*N*-glycoloyl-9-*O*-lactoyl-2,3-dehydroneuraminic acid (2,6-anhydro-3,5-dideoxy-5-glycoloyl-

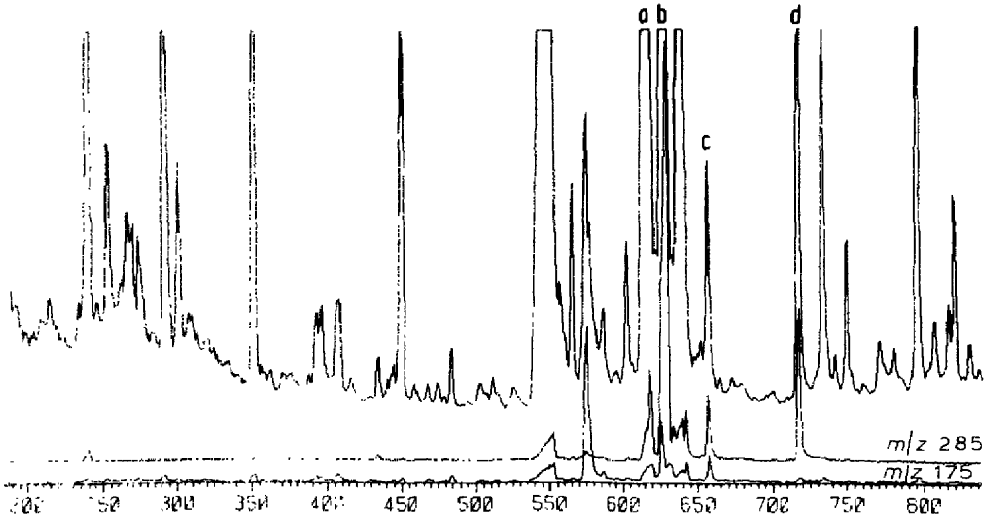


Fig. 1. Capillary g.l.c. combined with m.s. of sialic acid preparations obtained from rat urine after preparative g.l.c.[3]. Sialic acids were analyzed as Me_3Si esters, per$SiMe_3$ ethers on a 25-m fused, silica OV-101 capillary column, 150–280 °, 2°/min. Upper trace: Total ion current. Two lower traces: Selected-ion monitoring at *m/z* values useful as marker ions for unsaturated or 9-*O*-acetylated sialic acids: (a) Neu5Ac, (b) Neu5Ac2en and a microamount of $Neu5,9Ac_2$ (*m/z* 175), (c) $Neu5,9Ac_2$2en, and (d) Neu5Gc.

amido-9-*O*-lactoyl-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu2en5Ac9Lt), detected in porcine submandibular glands. Additionally, the results of mass spectrometry strongly suggested the existence of 9-*O*-acetyl-2-deoxy-*N*-glycoloyl-2,3-didehydroneuraminic acid (9-*O*-acetyl-2,6-anhydro-3,5-dideoxy-5-glycoloylamido-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu9Ac2en5Gc) in porcine urine.

Figure 1 illustrates the capillary gas–liquid chromatogram obtained from the sialic acid fraction of rat urine. Although different purification steps were applied, this chromatogram shows, besides various sialic acid peaks, several substances other than sialic acids. These interfering components may represent anionic substances of low-molecular weight, which could not be separated from sialic acids. However, the individual components of the complex mixture of neuraminic acid derivatives could be identified after derivatization with MSTFA, and the efficient separation on a capillary column by selected-ion monitoring, followed by mass spectrometry.

For comparison with the natural compounds, *O*-acetylated 5-acetamido- (or 5-glycoloylamido)-2,5-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acids were synthesized with *N*-acetylimidazole as acetylating reagent. This method was originally used for the preparation of *O*-acetylated *N*-acetylneuraminic acid methyl ester methyl β-glycosides[13]. The degree of *O*-acetylation both of Neu5Ac2en and Neu2en5Gc was monitored by l.c. (Table II) and t.l.c. (Fig. 2, and Table II). Substitution of the free hydroxyl groups of Neu5Ac2en is dependent on time and the amount of reagent[13]. During the reaction (24 h), *O*-acetylation mainly took place at O-9 (Figs. 2 and 3), followed by O-7. Thus, Neu5Ac2en derivatives with either one *O*-acetyl group at O-9 (Neu5,9$Ac_2$2en) or with two *O*-acetyl groups, at O-7

TABLE II

M.S., G.L.C., L.C., AND T.L.C. DATA OF *N,O*-ACYL-2-DEOXY-2,3-DIDEHYDRONEURAMINIC ACIDS[a]

Compound[b]	*Fragment*						R_T		R_F *(t.l.c.)*[e]
	A	*C*	*D*	*E*	*F*	*H*	*G.l.c.*[c]	*L.c.*[d]	
Neu5Ac2en (n, s)	636	446	356	285	205	368	1.02	1.0	0.45
Neu5,9$Ac_2$2en (n, s)	606	446	356	285	175	368	1.08	1.2	0.66
Neu5,7,9$Ac_3$2en (s)	576			285	175		1.10	*f*	0.80
Neu5Ac2en9Lt (n)	708	446	356	285	277		1.26	*f*	*f*
Neu2en5Gc (n, s)	724	534	444	285	205	456	1.17	1.3	0.35
Neu9Ac5Gc2en (n, s)	694		444	285	175		1.22	1.6	0.48
Neu2en5Gc9Lt (n)	796	534	444	285	277	456	1.45	*f*	*f*
Neu2en5GcAc (s)	694	504	414	285	205	426	1.19	*f*	*f*

[a]For mass-spectrometric analysis, the unsaturated sialic acids were converted into trimethylsilyl esters, per(trimethyl) ethers. Fragment nomenclature follows the known fragmentation pattern[17,19]; details are described in the text. Fragments *B* and *G* (ref. 17, 19) are not observed for unsaturated sialic acids. [b]Natural (n); synthetic (s). [c]Capillary g.l.c. on OV-101. Values relative to the trimethylsilyl ester, per(trimethylsilyl) ether of Neu5Ac. [d]Reference substance, Neu5Ac2en. [e]See also Fig. 2. [f]Not determined.

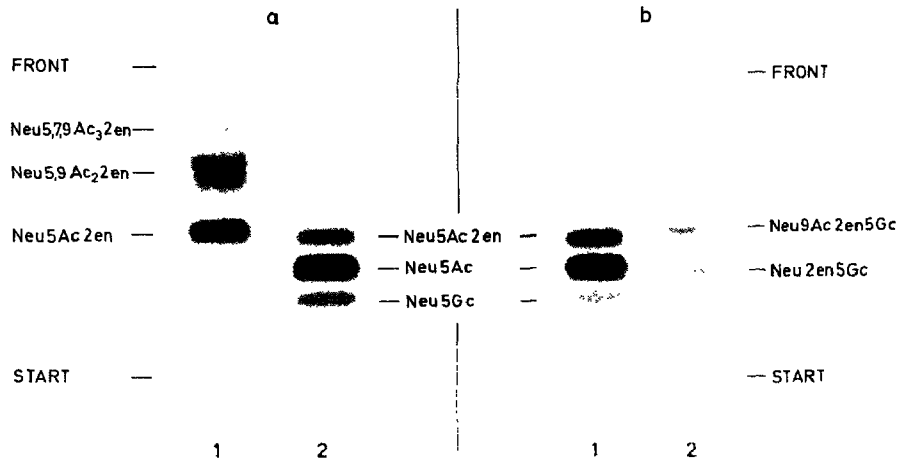


Fig. 2. T.l.c. of *O*-acetylated unsaturated sialic acids on 0.1-mm cellulose plates in 1:2:1 (v/v) butanol–propanol–0.1M HCl[11]. (a) Lane 1: synthetic *O*-acetylated derivatives of Neu5Ac2en; lane 2: reference sialic acids. (b) Lane 1: reference sialic acids; lane 2: synthetic *O*-acetylated derivatives of Neu2en5Gc.

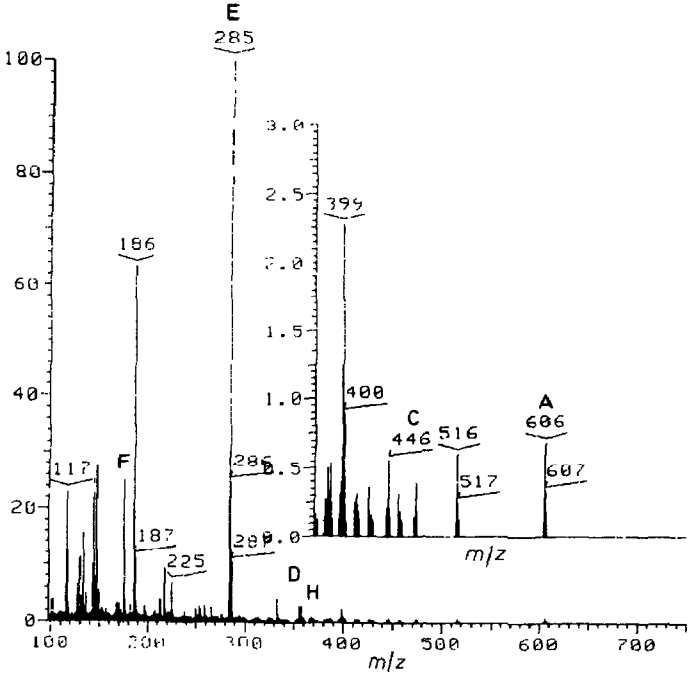


Fig. 3. E.i. mass spectrum of *N*-acetyl-9-*O*-acetyl-2-deoxy-2,3-didehydroneuraminic acid, analyzed as Me_3Si ester, per$SiMe_3$ ether.

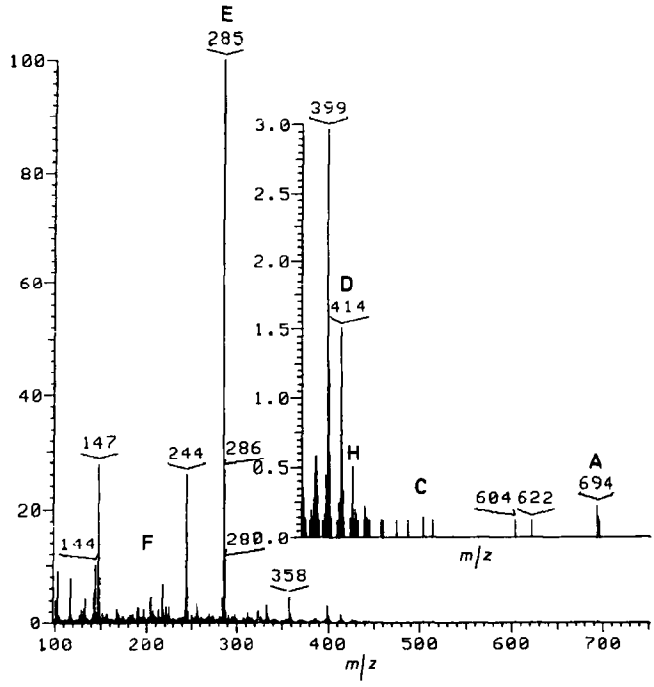
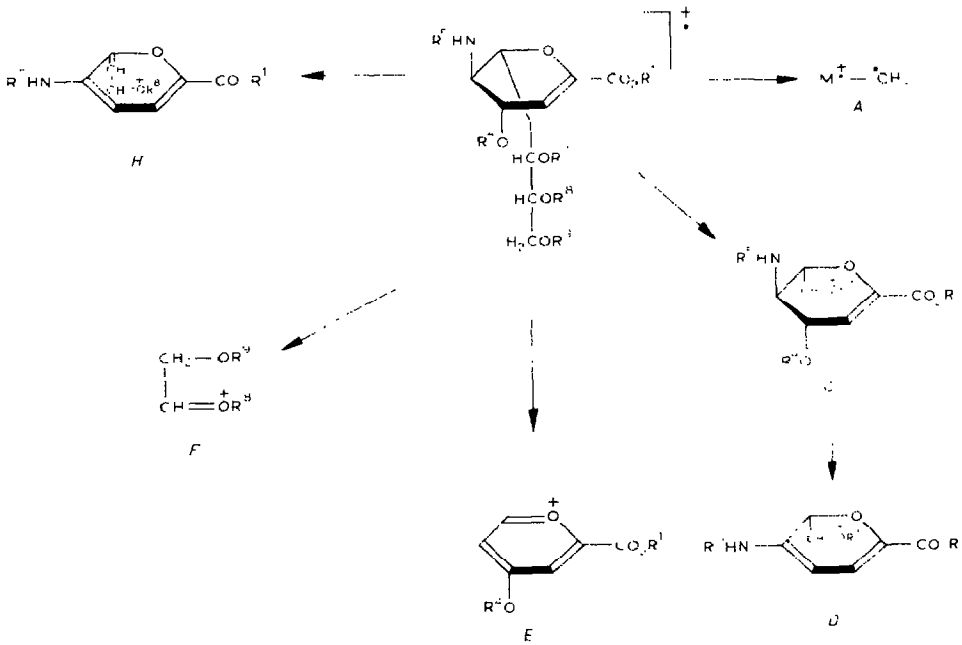


Fig. 4. E.i. mass spectrum of *N*-acetoxyacetyl-2-deoxy-2,3-didehydroneuraminic acid, analyzed as Me_3Si ester, per$SiMe_3$ ether.

and O-9 (Neu5,7,9$Ac_3$2en), were formed. The analytical data of these substances are listed in Table II. *O*-Acetylation of Neu2en5Gc also led to the 9-*O*-acetyl derivative. A Neu5Gc derivative, which has the *N*-glycoloyl group acetylated, was found as a minor product. In this way, a sialic acid containing an *N*-acetoxyacetyl group was synthesized for the first time (5-acetoxyacetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid, Neu2en5GcAc, Fig. 4 and Table II). The possible existence of this sialic acid in the hematoside from equine erythrocytes was discussed earlier[15], but this was later identified as 4-*O*-acetyl-*N*-glycoloylneuraminic acid[16,17].

The behaviors of the Neu5,9$Ac_2$2en preparations, either synthesized or isolated from natural sources, were identical in g.l.c.–m.s. analysis. Based on the mass spectrum of this sialic acid derivative and on that of *N*-acetyl-9-*O*-lactoylneuraminic acid isolated from bovine submandibular gland[18], both Neu5Ac2en9Lt and Neu2en5Gc9Lt (Fig. 5), isolated from porcine submandibular glands, could be identified by their mass-fragmentation patterns. As shown in Figs. 3–5 and Table II, mass fragmentation of the unsaturated sialic acids follows the known fragmentation scheme of sialic acids[17,19] (Scheme 1).



Scheme 1. E.i. mass-spectrometric fragmentation pattern of 2,3-unsaturated sialic acids (for *m/z* values, see also Table II).

$R^1 = R^4 = R^8 = SiMe_3$; R^5 = Ac, $COCH_2OSiMe_3$ or $COCH_2OAc$; R^7 = $SiMe_3$ or Ac; R^9 = $SiMe_3$, Ac or lactoyl.

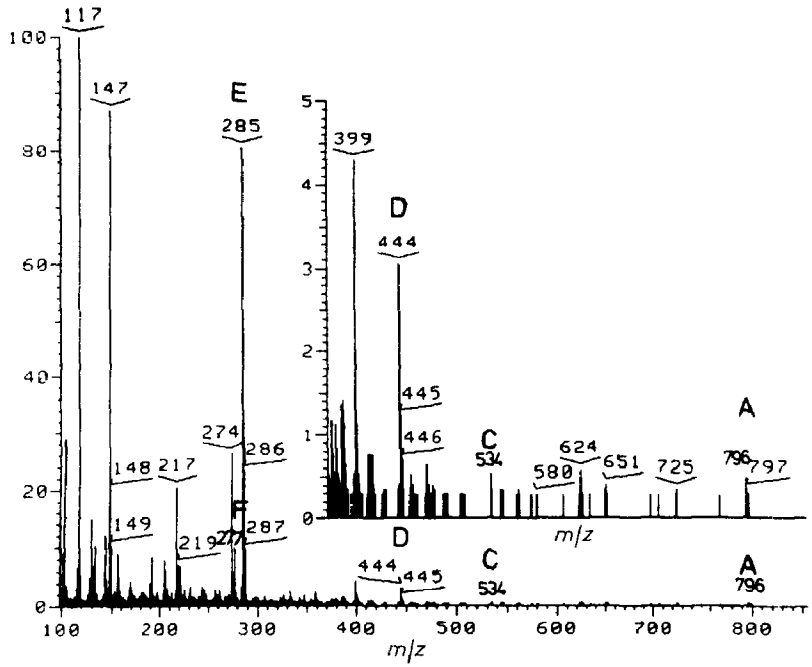


Fig. 5. E.i. mass spectrum of 2-deoxy-*N*-glycoloyl-9-*O*-lactoyl-2,3-didehydroneuraminic acid, analyzed as Me_3Si ester, per$SiMe_3$ ether.

DISCUSSION

The recent developments in sialic acid analysis, especially l.c. and g.l.c.–m.s., allow the determination of sialic acids in the nanogram range, which is a prerequisite for the discovery of the labile, *O*-acylated unsaturated sialic acids in urine and submandibular glands. L.c. analysis of sialic acids again proved to be very useful for the screening of sialic acids during the isolation procedure or also in the course of *O*-acetylation reactions with *N*-acetylimidazole.

The four new unsaturated sialic acids found in biological materials could be identified by g.l.c.–m.s. on the basis of their fragmentation patterns (Table II, Figs. 3–5). These findings increase the number of known natural *N*,*O*-substituted unsaturated sialic acids to seven, including 2-deoxy-*N*-glycoloyl-8-*O*-methyl-2,3-didehydroneuraminic acid (Neu2en5Gc8Me) of starfish[1,4] (see Table I).

In the mass-spectrometric analysis of the trimethylsilyl ethers, the base fragment for all unsaturated sialic acids is at *m/z* 285, which corresponds to the fragment at *m/z* 227 for methyl ester derivatives[19], is considered as fragment *E* of the known fragmentation pattern[17,19]. The high relative-abundance shows that it is formed easily. In agreement with the fragmentation of saturated sialic acids, the molecular weight can be deduced from fragment *A* (M − 15). Generally, the fragmentation corresponded well to the literature data[19]. In all cases, the expected shifts for the corresponding fragments were observed (Table II, Figs. 3–5). The fragment at *m/z* 186 for *N*-acetylated sialic acids[19] is of interest, too, as it gave additional information about the substituent at the amino group. Thus, it was shifted to *m/z* 274 for *N*-glycoloyl derivatives. In the case of the *N*-acetoxyacetyl group of Neu2en5GcAc obtained by synthesis (Table II, Fig. 4), this fragment had an *m/z* value of 244. To confirm the structure of the 9-*O*-acetylated, unsaturated sialic acid isolated from natural materials, the synthesis of *O*-acetylated, unsaturated sialic acids was of great value, as both mass spectra were identical (Fig. 3, Table II).

In the studies described herein, *O*-acetylation of unsaturated sialic acids with *N*-acetylimidazole did not lead to 4-*O*-acetylated derivatives, in contrast to the methyl ester, methyl β-glycoside of Neu5Ac reported earlier[13]. This difference may be due to the altered conformation of the neuraminic acid derivatives used for the acetylation reaction. In the case of Neu2en5Gc, the presence of *N*-acetylimidazole also led to *O*-acetylation of the *N*-glycoloyl substituent (Fig. 4, Table II), which could have been anticipated, as this is a primary alcohol group, too.

The excretion of Neu5,9Ac$_2$2en by cow and rat supports the assumption that unsaturated sialic acids can be formed from glycosidically bound sialic acids under *in vivo* conditions[5,7]. Glycoconjugates of both cow and rat contain a relatively large proportion of *O*-acetylated sialic acids[9,10], which may mirror a high activity of a specific *O*-acetyltransferase[9,20,21]. The origin of Neu5,9Ac$_2$2en and Neu2en5Gc from the CMP-glycosides of Neu5,9Ac$_2$ and Neu5Gc can be taken into consideration, as these activated sialic acids have been isolated from bovine[7] and porcine[22] submandibular glands, respectively. If this is the correct route for the formation of

natural 2,3-unsaturated sialic acids, the absence of *N*-acetyl-4-*O*-acetyl-2-deoxy-2,3-didehydroneuraminic acid in horse submandibular gland and urine may indicate that in horse, which is a rich source of 4-*O*-acetylated sialic acids and contains high activity of the corresponding acetyltransferase[9,20,23], *O*-acetylation takes place only on glycosidically linked but not on free sialic acids. Consequently, CMP-Neu4,5Ac_2 is not expected to be an intermediate in horse tissues. This pathway was further supported by the absence of free Neu4,5Ac_2, either in equine urine or in equine submandibular gland. Thus, the discovery of different, *N*,*O*-substituted 2,3-unsaturated sialic acids may give a better insight into the biosynthetic route of *N*,*O*-acylated sialic acids as components of glycoconjugates.

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