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Migration of *O*-acetyl groups in *N*,*O*-acetylneuraminic acids

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Highly purified *N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac_2), *N*-acetyl-7-*O*-acetylneuraminic acid (Neu5,7Ac_2) and *N*-acetyl-7,9-di-*O*-acetylneuraminic acid (Neu5,7,9Ac_3) were used to study spontaneous migrations of acetyl groups between hydroxyl groups. The techniques applied involved thin-layer chromatography, gas-liquid chromatography/mass spectrometry, high-performance liquid chromatography and 360-MHz ^{1}H-NMR spectroscopy. It was found that at pH values at which no significant de-*O*-acetylation is observed: (a) Neu5,7Ac_2 can easily be transformed into Neu5,9Ac_2, (b) Neu5,7,9Ac_3 yields an equilibrium of Neu5,7,9Ac_3 and Neu5,8,9Ac_3 in a molar ratio of approximately 1:1, and (c) Neu4,5Ac_2 does not give rise to *O*-acetyl migrations. The importance of these findings is discussed in terms of the biosynthesis of *O*-acetylated sialic acids.

Sialic acids are known to form part of many complex carbohydrates. They can bear *O*-acetyl groups at different positions [1]. Especially, bovine submandibular gland glycoprotein has been shown to be a rich source of many members of the sialic acid family, i. e. mono-, di-, and tri-*O*-acetylated *N*-acetyl- and *N*-glycolylneuraminic acids [2]. With regard to the biological role of *O*-acetyl groups in sialic acids, information is accumulating about specific functions of these sialic acids. Typical examples are the decrease of the rate of the sialidase-initiated degradation of sialocarbohydrate chains [3], the involvement in the tumor antigenic properties of human melanoma cells [4], the role in environmental adaptation [5] and binding of influenza C viruses [6, 7].

Prior to chemical characterization of the sialic acids, they are in most cases released by acid or enzymic hydrolysis followed by various isolation methods [8, 9]. Among the *O*-acetylated species, obtained in this way, Neu5,9Ac_2 is the most common neuraminic acid derivative [1, 2]. In erythrocytes from various mammals, glycosidically bound Neu5,9Ac_2 is the predominant *O*-acetylated sialic acid species, as determined on intact cells or isolated membranes by treatment with periodate followed by acetylacetone [10, 11]. Neu5,9Ac_2 often appears to be accompanied by the 7-*O*-acetyl analogue in variable amounts. The observation that Neu5,7Ac_2 decreases frequently in favour of Neu5,9Ac_2 during storage indicates *O*-acetyl migration. Spontaneous migration of acetyl groups between hydroxyl groups has been observed in various carbohydrates and is believed to proceed intramolecularly through orthoester intermediates [12 – 15].

To obtain more insight into the origin of the *O*-acetyl substitution patterns for sialic acids, in relation to enzymic acetylation [3] and non-enzymic migration, a migration study using different analytical approaches was undertaken using highly purified Neu4,5Ac_2, Neu5,7Ac_2 and Neu5,7,9Ac_3. A preliminary communication dealing with Neu5,7Ac_2 has appeared [16].

MATERIALS AND METHODS

Isolation of sialic acids

Neu5,7Ac_2, Neu5,9Ac_2 and Neu5,7,9Ac_3 were released from fresh bovine submandibular gland glycoprotein by mild acid hydrolysis and purified via anion-exchange and cellulose chromatography, essentially as described [2]. The anion-exchange resin Dowex 2 × 8 (100 – 200 mesh) was replaced by Dowex 1 × 2 (minus 400 mesh). Column eluates were monitored for sialic acids by the orcinol/Fe^{3+}/HCl assay [17], as well as by TLC and HPLC (see below).

Neu4,5Ac_2 was available from equine submandibular gland glycoprotein [18].

HPLC of sialic acids

Qualitative and quantitative HPLC analysis of sialic acids was performed on a Spectra-Physics SP-8000 apparatus [9]. Sialic acid samples (0.01 – 1 μg), dissolved in 20 μl distilled water, 50 mM Tris/HCl buffers (pH 7 – 9), or 50 mM sodium phosphate buffers (pH 3 – 5), were analysed on stainless steel columns (40 × 4.6 mm) filled with Aminex A-28 or A-29 (Bio-Rad, München). The elutions were carried out isocratically by using 0.75 mM sodium sulfate at a flow rate of 0.5 ml/min at 1.5 MPa. The eluate was monitored at 200 nm.

To study the relation between *O*-acetyl migration and pH, samples of Neu5,7Ac_2 were incubated for several hours at 37 °C in 50 mM Tris/HCl buffers (5 μg/100 μl) with pH values

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Abbreviations. Neu5Ac, *N*-acetylneuraminic acid; Neu4,5Ac_2, *N*-acetyl-4-*O*-acetylneuraminic acid; Neu5,7Ac_2, *N*-acetyl-7-*O*-acetylneuraminic acid; Neu5,8Ac_2, *N*-acetyl-8-*O*-acetylneuraminic acid; Neu5,9Ac_2, *N*-acetyl-9-*O*-acetylneuraminic acid; Neu5,7,9Ac_3, *N*-acetyl-7,9-di-*O*-acetylneuraminic acid; Neu5,8,9Ac_3, *N*-acetyl-8,9-di-*O*-acetylneuraminic acid; Neu5,7,8,9Ac_4, *N*-acetyl-7,8,9-tri-*O*-acetylneuraminic acid; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography.

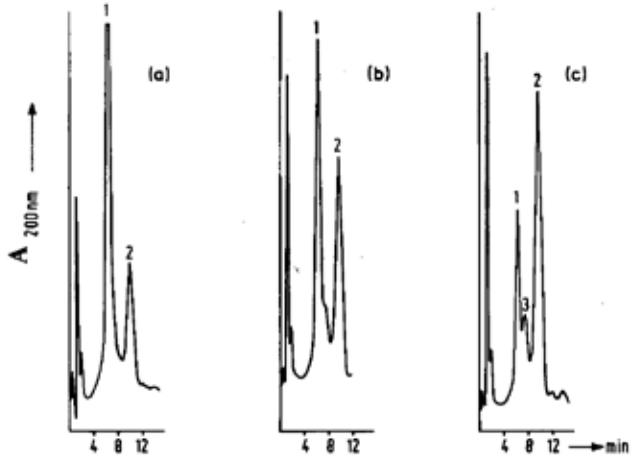


Fig. 1. *HPLC analysis on Aminex A-29 of the conversion of Neu5,7Ac₂ into Neu5,9Ac₂.* Neu5,7Ac₂, contaminated already by a low amount of Neu5,9Ac₂, was kept in 50 mM Tris/HCl buffer pH 7.0 at 37°C for the time periods: (a) $t = 0$, (b) $t = 7$ h, (c) $t = 16$ h. The elution was carried out isocratically with 0.75 mM sodium sulfate at a flow rate of 0.5 ml/min. Assignment of the peaks: 1, Neu5,7Ac₂; 2, Neu5,9Ac₂; 3, Neu5Ac

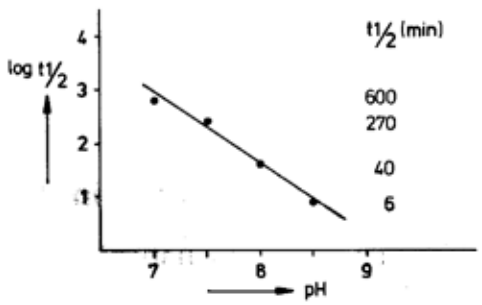


Fig.2. *pH dependency of the conversion of Neu5,7Ac₂ into Neu5,9Ac₂ as determined by HPLC.* The $t_{1/2}$ values were determined for Neu5,7Ac₂ samples (5 µg/100 µl) incubated with 50 mM Tris/HCl buffers of pH 7.0, 7.5, 8.0, 8.5 and 9.0 at 37°C

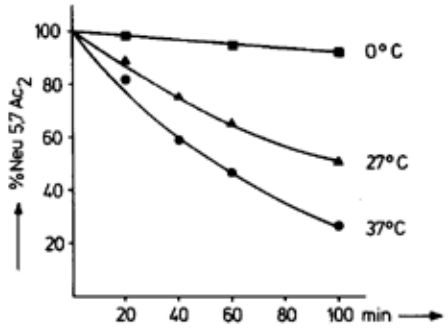


Fig. 3. *Temperature dependency of the conversion of Neu5,7Ac₂ into Neu5,9Ac₂, as determined by HPLC.* The plot was constructed for Neu5,7Ac₂ samples (5 µg/100 µl) incubated with 50 mM Tris/HCl buffer pH 8.0 at 0°C, 27°C and 37°C

of 7.0, 7.5, 8.0, 8.5 and 9.0, respectively, and in 50 mM sodium phosphate buffers at pH 3.0, 4.0 and 5.0, respectively. At regular time intervals 20-µl aliquots were taken for HPLC analysis. Neu4,5Ac₂ was studied in 50 mM Tris/HCl buffer pH 8.0, only.

For investigation of the dependence of *O*-acetyl migration on temperature, samples of Neu5,7Ac₂ were incubated at 0°C, 27°C and 37°C in 50 mM Tris/HCl buffer pH 8.0 (5 µg/100 µl). At regular time intervals 20-µl aliquots were taken for HPLC analysis.

TLC of sialic acids

TLC of sialic acids was carried out on 0.2-mm cellulose plastic sheets (E. Merck, Darmstadt) using the solvent system *n*-butanol/*n*-propanol/0.1 M HCl (1:2:1, v/v). Before application of the sialic acids (10–20 µg) the cellulose plates were prerun in 0.1 M HCl. Sialic acid spots were visualized by using the orcinol/Fe^{3+}/HCl spray reagent [17].

For *O*-acetyl migration studies, samples of Neu5,7Ac₂ were incubated for several hours at 37°C in 10 mM pyridinium acetate buffers (2 µg/µl) with pH values of 5.0 and 7.5. The rate of isomerisation was followed by TLC.

GLC-MS of sialic acids

GLC-MS of trimethylsilylated methyl esters of sialic acids was carried out as reported [19].

^{1}H-NMR spectroscopy of sialic acids

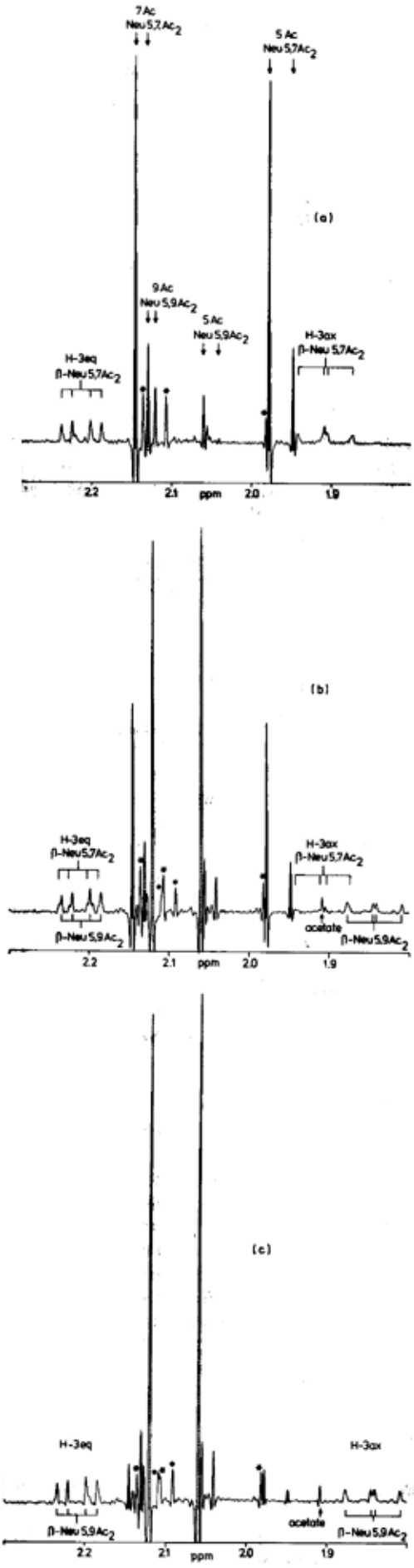
For ^{1}H-NMR spectroscopy, purified Neu5,7Ac₂ (1.5 mg) and Neu5,7,9Ac₃ (1.0 mg) samples were exchanged three times in ^2H_2O (99.96 atom % ^{2}H, Aldrich) at p^{2}H ≈ 4, with intermediate lyophilisation. Subsequently, the sialic acids were dissolved in 0.3 ml 0.1 M $Na^2H_2PO_4/Na_2{}^2HPO_4$ in ^2H_2O (p^{2}H 7.7) and transferred to 5-mm NMR tubes. The progress of *O*-acetyl migration in the Neu5,7Ac₂ (final p^{2}H 7.2) and Neu5,7,9Ac₃ (final p^{2}H 7.5) samples was followed directly on a Bruker HX-360 spectrometer (SON hf-NMR-facility, Department of Physical Chemistry, State University of Groningen, The Netherlands) operating at 360 MHz in the Fourier-transform mode at a probe temperature of 37°C. At regular time intervals 64 scans were accumulated. Resolution enhancement of the spectra was achieved by Lorentzian to Gaussian transformation, according to Ernst [20]. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone in ^2H_2O (δ = 2.225 ppm).

RESULTS

7-O-Acetyl migration in Neu5,7Ac₂

Introductory experiments by TLC demonstrated that Neu5,7Ac₂, dissolved in 10 mM pyridinium acetate buffer pH 7.5 and kept at 37°C, was almost completely converted into Neu5,9Ac₂ after 30 h. During the incubation about 10% of the *O*-acetylated sialic acids were de-*O*-acetylated, yielding Neu5Ac. The relative amounts of the various sialic acids were determined by GLC-MS using the trimethylsilylated methyl ester derivatives. For the electron impact mass spectra of the (*O*-acetylated) sialic acid derivatives, see [19]. When the incubation experiment was carried out at pH 5.0, Neu5,7Ac₂ was found to be relatively stable.

More detailed investigations at various pH and temperature values were performed by HPLC on Aminex A-28 and A-29 [9]. To illustrate the *O*-acetyl migration, in Fig. 1 the increase of Neu5,9Ac₂ and the concomitant decrease of Neu5,7Ac₂ are depicted for a Neu5,7Ac₂ sample, contaminated already at $t = 0$ by 27% of Neu5,9Ac₂, after incubation with 50 mM Tris/HCl buffer pH 7.0 at 37°C for



various times. In addition to the conversion of $Neu5,7Ac_2$ into $Neu5,9Ac_2$, the appearance of a small amount of Neu5Ac due to de-*O*-acetylation is visible. Longer incubation times lead to a nearly complete migration of the *O*-acetyl group from C7 to C9 (see also ^{1}H-NMR studies). The rate of the intramolecular rearrangement strongly depends on the pH of the incubation medium. As is evident from Fig. 2, a linear plot is obtained for log $t_{1/2}$ of $Neu5,7Ac_2$ against pH, showing differences in $t_{1/2}$ values by two orders of magnitude, comparing pH 7.0 ($t_{1/2}$ = 600 min) and pH 8.5 ($t_{1/2}$ = 6 min) at 37°C. At pH 9.0 a very fast isomerisation is observed ($t_{1/2}$ < 1 min). Incubation of $Neu5,7Ac_2$ with 50 mM sodium phosphate buffer pH 3.0, 4.0 and 5.0 at 37°C does not lead to significant *O*-acetyl migration within 24 h. However, at pH 3.0 the sialic acid was de-*O*-acetylated by 20%. As was shown earlier [21] for $Neu5,9Ac_2$, no significant de-*O*-acetylation occurs in the range of pH 2–11 within 10 min at 58°C. At pH > 11 and < 2, however, a rapid de-*O*-acetylation was observed at this temperature. The *O*-acetyl migration reaction is also influenced by the temperature, as shown in Fig. 3.

The *O*-acetyl migration from C7 to C9 was also followed by 360-MHz ^{1}H-NMR spectroscopy. For the interpretation of the 360-MHz ^{1}H-NMR spectra advantage was taken of the NMR data of a number of naturally occurring *O*-acetylated sialic acids [22]. From these data it was anticipated that the ^{1}H-chemical shifts and the relative intensities of the different *N*- and *O*-acetyl signals should be excellent structural parameters to monitor the *O*-acetyl migration. ^{1}H-NMR spectra of a $Neu5,7Ac_2$ sample (90% purity as determined by HPLC), dissolved in 0.1 M sodium phosphate buffer (final p^{2}H 7.2), were recorded at 37°C at different time intervals up to 1250 min, keeping the NMR tube continuously in the instrument. A compilation of the ^{1}H chemical shifts of the β-anomers of $Neu5,7Ac_2$ and $Neu5,9Ac_2$ is presented in Table 1. It has to be noted that the data reported earlier for these substances [22] were obtained under deviating experimental conditions (temperature, pH, solvent), explaining the small differences observed in chemical shift for some signals. The sialic acid NMR parameters are relatively sensitive to these conditions. Chemical shift values of relevant reporter groups of the concerned sialic acids in the range δ = 1.8–2.3 ppm are compiled in Table 2. For the interpretation of the spectra it has to be taken into account that each sialic acid occurs as a mixture of two anomeric forms [22]. For $Neu5,7Ac_2$ the equilibrium ratio under the applied conditions of solvent, pH and temperature amounts to $\alpha:\beta$ = 23:77, and for $Neu5,9Ac_2$ = 9:91 (see also [2]). Therefore, as a result of the migration, an anomerisation process will also occur. Compared to the migration, this process proceeds very fast. In Fig. 4 the *N*,*O*-acetyl/H-3*eq* (β)/H-3*ax* (β) regions of the ^{1}H-NMR spectra (δ = 1.8–2.3 ppm) obtained at t = 12 min (start), t = 160 min and t = 1250 min (finish) are presented.

Fig. 4. *Conversion of $Neu5,7Ac_2$ into $Neu5,9Ac_2$ by* O*-acetyl migration as monitored by 360-MHz ^{1}H-NMR spectroscopy.* Partial 360-MHz ^{1}H-NMR spectra of the *N*,*O*-acetyl/H-3*eq*(β)/H-3*ax*(β) region at (a) t = 12 min (start), (b) t = 160 min, and (c) t = 1250 min (finish) of a $Neu5,7Ac_2$ sample (1.5 mg/300 µl) (90% purity as determined by HPLC), dissolved in 0.1 M sodium phosphate buffer (final p^{2}H 7.2) at 37°C. The black signals belong to the *N*,*O*-acetyl groups of $Neu5,7Ac_2$ and the dotted ones to those of $Neu5,9Ac_2$. The signals assigned by ★ and ● belong to the contaminants $Neu5,7,9Ac_3$ and $Neu5,8,9Ac_3$, respectively

Table 1. *^{1}H-NMR chemical shift data and coupling constants for neuraminic acid derivatives*
Chemical shifts (δ) are given for 0.1 M sodium phosphate solutions in ^2H_2O at 37 °C at the indicated p^{2}H values, using internal acetone (δ = 2.225 ppm) and acetate (δ = 1.907 ppm)

Atom	Chemical shift values (δ) in				
	β-Neu5Ac p^{2}H 7.2 (500 MHz)	β-Neu5,7Ac$_2$ p^{2}H 7.2 (360 MHz)	β-Neu5,9Ac$_2$ p^{2}H 7.2 (360 MHz)	β-Neu5,7,9Ac$_3$ p^{2}H 7.5 (360 MHz)	β-Neu5,8,9Ac$_3$ p^{2}H 7.5 (360 MHz)
	ppm				
H-3*eq*	2.207	2.211	2.208	2.209	2.169
H-3*ax*	1.838	1.906	1.842	1.914	1.853
H-4	4.023	3.942	4.029	3.943	3.982
H-5	3.899	3.759	3.906	3.751	3.890
H-6	3.979	4.233	3.986	4.241	3.766
H-7	3.515	5.041	3.568	5.137	3.825
H-8	3.754	3.915	3.964	4.144	5.109
H-9	3.832	3.629	4.362	4.113	4.522
H-9′	3.612	3.447	4.189	4.070	4.291
5Ac	2.050	1.976[a]	2.058	1.980[b]	2.059
7Ac	–	2.144[a]	–	2.134[b]	–
8Ac	–	–	–	–	2.090
9Ac	–	–	2.119	2.106	2.107
Coupling constant					
	Hz				
$J_{3eq,3ax}$	−13.0	−13.1	−13.0	−13.1	−12.8
$J_{3eq,4}$	4.9	4.9	4.9	4.9	4.7
$J_{3ax,4}$	11.5	11.4	11.4	11.5	11.4
$J_{4,5}$	9.9	10.0	9.7	10.0	10.0
$J_{5,6}$	10.4	10.5	10.2	10.5	10.0
$J_{6,7}$	1.3	2.0	1.2	2.0	1.2
$J_{7,8}$	9.0	8.4	9.3	8.8	8.4
$J_{8,9}$	2.8	3.3	2.7	3.0	2.6
$J_{8,9'}$	6.4	6.5	5.6	4.8	5.5
$J_{9,9'}$	−11.8	−12.0	−11.7	−11.9	−12.4

[a] Values may be interchanged.
[b] Values may be interchanged.

Table 2. *^{1}H-NMR chemical shifts of* N,O-*acetyl structural-reporter groups for neuraminic acid derivatives involved in the* O-*acetyl migration studies (see Figs 4–7)*
Chemical shifts (δ) are given for 0.1 M sodium phosphate solutions in ^2H_2O at 37 °C, using internal acetone (δ = 2.225 ppm) and acetate (δ = 1.907 ppm). n. d. = not determined

Compound	δ for				p^{2}H
	5Ac	7Ac	8Ac	9Ac	
	ppm				
β-Neu5Ac	2.050	–	–	–	7.2
α-Neu5Ac	2.033	–	–	–	7.2
β-Neu5,7Ac$_2$	1.976	2.144	–	–	7.2
α-Neu5,7Ac$_2$	1.946	2.128	–	–	7.2
β-Neu5,9Ac$_2$	2.058	–	–	2.119	7.2
α-Neu5,9Ac$_2$	2.039	–	–	2.129	7.2
β-Neu5,7,9Ac$_3$	1.980	2.134	–	2.106	7.5
α-Neu5,7,9Ac$_3$	1.948	2.119[a]	–	2.122[a]	7.5
β-Neu5,8,9Ac$_3$	2.059	–	2.090	2.107	7.5
α-Neu5,8,9Ac$_3$	n. d.	–	n. d.	n. d.	7.5

[a] Values may be interchanged.

Comparison of these three spectra shows the increase of the *N*- and *O*-acetyl signals characteristic of Neu5,9Ac$_2$ and the concomitant decrease of the corresponding signals belonging to Neu5,7Ac$_2$. The progress of the migration is also clear from the related H-3*eq*(β) and H-3*ax*(β) signals. In Fig. 5 the conversion of Neu5,7Ac$_2$ into Neu5,9Ac$_2$ is plotted against the incubation time. For the calculation of the percentages of both compounds, the peak intensities of the *N*-acetyl signals (5Ac) of α,β-Neu5,7Ac$_2$ and α,β-Neu5,9Ac$_2$ have been used. From the different ^{1}H-NMR spectra it can be concluded (see Fig. 5) that an almost complete migration of the 7-*O*-acetyl group (7Ac) to position C9 (9Ac) occurs, without significant de-*O*-acetylation. The various spectra show some additional, relatively low signals which partly arise from acetyl groups of contaminating sialic acids such as Neu5,7,9Ac$_3$ and Neu5,8,9Ac$_3$ (see Tables 1 and 2) and from acetate. No clear indications were obtained for the presence of Neu5,8Ac$_2$ in the incubation mixture at any time. It should be noted that typical ^{1}H-NMR parameters of Neu5,8Ac$_2$ are not known. The latter sialic acid has been found up to now only in trace amounts in the sialic acid mixture isolated from bovine submandibular gland glycoprotein as determined by GLC-MS [2]. Efforts to trace this sialic acid by GLC-MS in the incubation

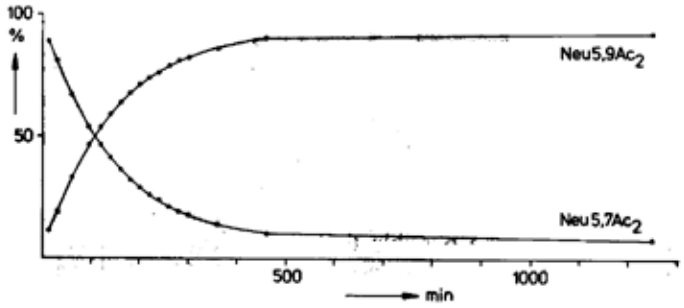


Fig. 5. *Schematic presentation of the decrease of Neu5,7Ac$_2$ and the increase of Neu5,9Ac$_2$ as a function of the time of incubation.* For experimental details, see text and Fig. 4. The used intensity of the β5Ac signal of Neu5,9Ac$_2$ has not been corrected for the small contribution of the β5Ac signal of the contaminant Neu5,8,9Ac$_3$

mixture used for ^{1}H-NMR at $t = t_{1/2}$ for Neu5,7Ac$_2$ failed. It should be noted that the $t_{1/2}$ value of 110 min, deduced from the curve in Fig. 5, and that calculated from the plot in Fig. 2 ($t_{1/2}$ = 447 min) differ from each other, indicating the influence of the concentration.

7-O-Acetyl migration in Neu5,7,9Ac$_3$

When Neu5,7,9Ac$_3$ was incubated under similar conditions as reported for *O*-acetyl migration in Neu5,7Ac$_2$, no significant alterations were observed by TLC and HPLC. It has to be noted that Neu5,7,9Ac$_3$ and Neu5,8,9Ac$_3$ cannot be sufficiently separated in the TLC and HPLC systems routinely applied. However, migration of the 7-*O*-acetyl group to C8 was indicated by ^{1}H-NMR spectroscopy.

A Neu5,7,9Ac$_3$ sample (75–80% purity as determined by HPLC) was dissolved in 0.1 M sodium phosphate buffer (final p^{2}H 7.5), and *O*-acetyl migration was followed by 360-MHz ^{1}H-NMR spectroscopy at 37°C. The ^{1}H chemical shifts of the β-anomers of Neu5,7,9Ac$_3$ and Neu5,8,9Ac$_3$ are presented in Table 1. Spectra were recorded at different time intervals up to 725 min. Chemical shift values of the relevant reporter groups, being the *N*- and *O*-acetyl signals, are included in Table 2. For Neu5,7,9Ac$_3$ the anomeric ratio amounts to $\alpha:\beta$ = 22:78. For Neu5,8,9Ac$_3$ the acetyl signals of the α-anomer could not be traced. In Fig. 6 the *N*,*O*-acetyl/H-3*eq*(β)/H-3*ax*(β) regions of the spectra obtained at t = 12 min (start), t = 95 min and t = 725 min (finish) are depicted. The 9-*O*-acetyl signals of the two β-sialic acids (β9Ac) differ only slightly in their δ values, as can be seen from the insets in the figure. Comparison of the three spectra illustrates that Neu5,7,9Ac$_3$ is transformed into a nearly equimolar mixture of Neu5,7,9Ac$_3$ and Neu5,8,9Ac$_3$. De-*O*-acetylation does occur to a small degree, as is evident from the increase in intensity of the free acetate signal at δ = 1.907 ppm. In connexion with this, it is not evident which partially de-*O*-acetylated sialic acids are formed. The course

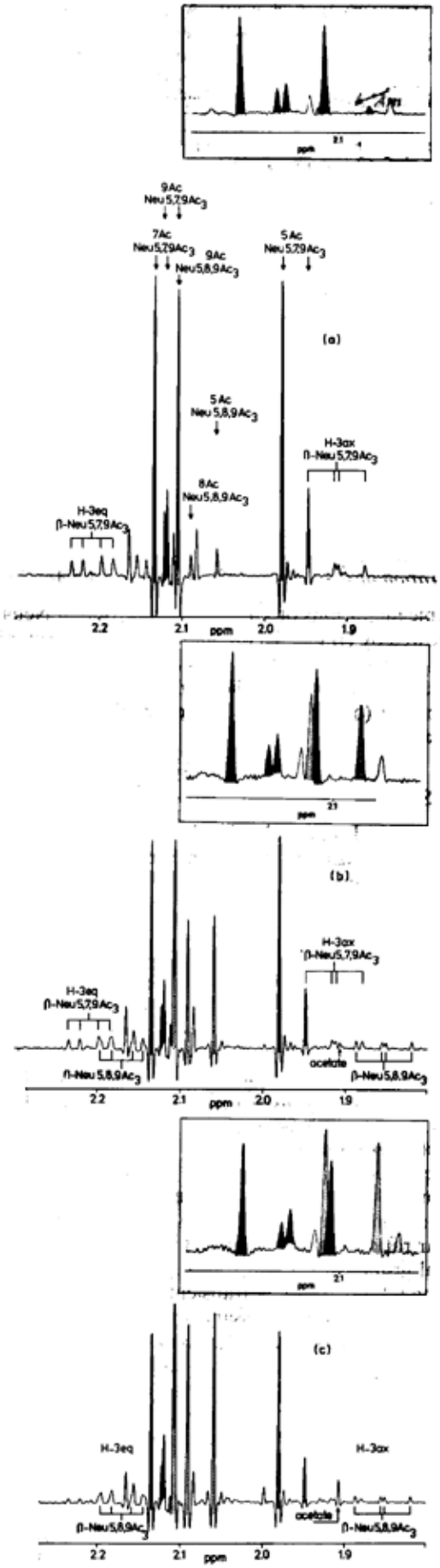


Fig. 6. *Partial conversion of Neu5,7,9Ac$_3$ into Neu5,8,9Ac$_3$ by O-acetyl migration as monitored by 360-MHz ^{1}H-NMR spectroscopy.* Partial 360-MHz ^{1}H-NMR spectra of the *N*,*O*-acetyl/H-3*eq*(β)/H-3*ax*(β) region at (a) t = 12 min (start), (b) t = 95 min, and (c) t = 725 min (finish) of a Neu5,7,9Ac$_3$ sample (1.0 mg/300 µl) (75–80% purity as determined by HPLC), dissolved in 0.1 M sodium phosphate buffer (final p^{2}H 7.5) at 37°C. The black signals belong to the *N*,*O*-acetyl groups of Neu5,7,9Ac$_3$ and the dotted ones to those of Neu5,8,9Ac$_3$. The insets show the areas around 2.1 ppm at more detail

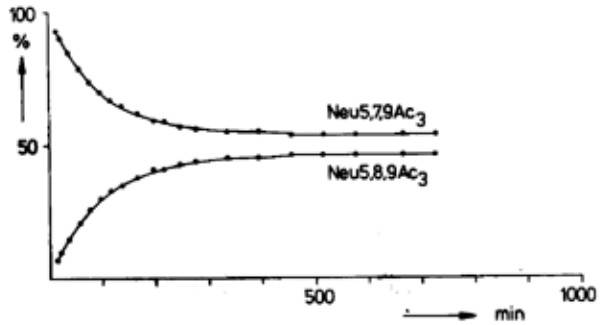


Fig. 7. *Schematic presentation of the decrease of Neu5,7,9Ac$_3$ and the increase of Neu5,8,9Ac$_3$ as a function of the time of incubation.* For experimental details, see text and Fig. 6. The used intensity of the α5Ac signal of Neu5,7,9Ac$_3$ has not been corrected for the small contribution of one of the four signals of H-3*ax* of β-Neu5,7,9Ac$_3$

of the *O*-acetyl migration as a function of the time is presented in Fig. 7. For the calculation of the percentages of the two compounds, the peak intensities of the *N*-acetyl signals (5Ac) of α,β-Neu5,7,9Ac$_3$ and β-Neu5,8,9Ac$_3$ were used.

Non-migration of the 4-O-acetyl group in Neu4,5Ac$_2$

Possible migrations of the 4-*O*-acetyl group in Neu4,5Ac$_2$ were followed by HPLC. To this end a Neu4,5Ac$_2$ sample was dissolved in 50 mM Tris/HCl buffer pH 8.0 at 37°C. It was found that migration of the *O*-acetyl group at C4 to position C7, C8 or C9 (or C2) does not occur; de-*O*-acetylation is insignificant.

DISCUSSION

The reported data clearly demonstrate that isolated Neu5,7Ac$_2$ can easily be transformed into Neu5,9Ac$_2$ at pH values at which no significant de-*O*-acetylation is observed. *O*-Acetyl migration is even possible under physiological pH. The rate of reaction is dependent on the pH and the temperature (see also [16]). In the migration process Neu5,8Ac$_2$ could not be traced as an intermediate. For this feature two different explanations are possible: (a) Neu5,8Ac$_2$ is not involved as an intermediate in the migration mechanism, leading to a direct intramolecular migration of the *O*-acetyl group from C7 (secondary ester group) to C9 (more stable primary ester group); (b) when Neu5,8Ac$_2$ is involved, the rate of migration from C8 to C9 must be at least an order of magnitude larger than the rate of migration from C7 to C8.

In the case of Neu5,7,9Ac$_3$ the *O*-acetyl migration proceeds from C7 (secondary ester group) to C8 (secondary ester group), until an equilibrium of Neu5,7,9Ac$_3$ and Neu5,8,9Ac$_3$ in a molar ratio of approximately 1:1 has been reached. The functional groups at C7 and C8 in sialic acids show a *trans*-orientation (cf. [22–24]). A transient distortion of this *trans*-orientation seems to be necessary to explain the migration of the acetyl group between the two secondary hydroxyl groups.

The data presented on the *O*-acetyl migration in *N*,*O*-acetylneuraminic acids have several consequences for the routine isolation procedures of sialic acids, when aimed at the localization of the *O*-acetyl substituents. It is evident that in fact pH values below 4 and over 6 should be avoided to prevent migration of the *O*-acetyl group at C7 and hydrolysis of *O*-acetyl groups as much as possible (see also [25]). Although this has not yet been studied, it is assumed that migration of *O*-acetyl groups also occurs in *N*-glycolylneuraminic acid and in glycosidically bound *N*-acetyl- or *N*-glycolylneuraminic acids.

With regard to the biosynthesis of *O*-acetylated sialic acids, the easy non-enzymic migration of the *O*-acetyl groups from C7 in the glycerol side chain may represent one of the steps. In bovine submandibular glands an *O*-acetyltransferase was identified that, when incubated with Neu5Ac, led to the formation of the 7-*O*-, 9-*O*- and 7,9-di-*O*-acetylated species [3, 26]. It may be possible that the enzyme introduces the *O*-acetyl group into the glycerol side chain at C7, followed by a chemical migration of this group from C7 to C9. When Neu5,9Ac$_2$ is formed, an additional *O*-acetyl group can be transferred to C7 leading to the formation of Neu5,7,9Ac$_3$ and making it possible to obtain Neu5,8,9Ac$_3$ also via migration. Finally, all OH groups in the glycerol side chain can be *O*-acetylated in this way. For this reason, only the *O*-acetylation of C4 needs a separate *O*-acetyltransferase, the existence of which was shown in equine submandibular gland [3, 26].

In summary, we propose that for *O*-acetylation of *N*-acylneuraminic acids presented above, nature uses only two *O*-acetyltransferases, namely a 4-*O*-acetyltransferase (acetyl-CoA:*N*-acyl-neuraminate 4-*O*-acetyltransferase, EC 2.3.1.44) and a 7-*O*-acetyltransferase (acetyl-CoA:*N*-acylneuraminate 7-*O*-acetyltransferase, EC 2.3.1.45). This concept will be the subject of further studies.

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