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A Detailed Study of the Periodate Oxidation of Sialic Acids in Glycoproteins

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Periodate oxidation of terminal *N*-acetyl- and *N*-glycoloylneuraminic acid residues in the mucins from edible bird nest substance and pig submandibular gland, respectively, can be carried out under conditions which exclusively give rise to the formation of the C-7 analogues of these sialic acids. In contrast, the C-8 compounds can be obtained in a maximum yield of about 40%. Under identical conditions, *N*-glycoloylneuraminic acid is oxidized about 1.5 times faster than the *N*-acetylated derivative. After release of the sialic acids by acid hydrolysis, the characterization of the oxidation products was carried out by TLC, by GLC and GLC-MS of the corresponding pertrimethylsilyl derivatives, and by 500-MHz ^{1}H-NMR spectroscopy. In addition, molar response factors for GLC analysis and extinction coefficients in the orcinol/Fe^{3+}/HCl assay were determined.

Conversion of *N*-acetylneuraminic acid into the C-7/C-8 analogues can be performed by mild oxidation with periodate followed by reduction with sodium borohydride or -tritide [1]. This modification is an important tool to establish the presence of sialic acids in biological materials by a sensitive method and to label sialoglycoconjugates specifically[2, 3] facilitating their identification, e.g. by SDS-PAGE [4].

A detailed investigation about the chemistry and analytical features of the periodate oxidation of sialic acids, the products formed in this reaction, and the accessibility of different sialic acids to oxidation is still missing. Therefore, the oxidation conditions for *N*-

Abbreviations: EI, electron-impact; Me_3Si or $SiMe_3$, trimethylsilyl; Neu5Ac, *N*-acetylneuraminic acid; C-7 Neu5Ac, 5-acetamido-3,5-dideoxy-L-*arabino*-heptulosnic acid; C-8 Neu5Ac, 5-acetamido-3,5-dideoxy-D-*galacto*-octulosonic acid; Neu5Gc, *N*-glycoloylneuraminic acid; C-7 Neu5Gc, 5-glycoloylamido-3,5-dideoxy-L-*arabino*-heptulosonic acid; C-8 Neu5Gc, 5-glycoloylamido-3,5-dideoxy-D-*galacto*-octulosonic acid; PSM, pig submandibular gland mucin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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acetyl- and *N*-glycoloylneuraminic acids in glycoproteins were studied, and the periodate-oxidized analogues of *N*-acetyl- and *N*-glycoloylneuraminic acid analyzed by packed and capillary GLC-MS as pertrimethylsilyl derivatives, together with a further analytical characterization of these compounds by TLC, 500-MHz ^{1}H-NMR spectroscopy, and determination of the molar extinction coefficients in the orcinol/Fe^{3+}/HCl assay and the molar detector response factors in GLC.

Materials and Methods

Oxidation and Isolation of Sialic Acids

Mucins from edible bird nests and pig submandibular glands were kindly provided by Dr. G. Phannschmidt, University of Kiel. 60-100 mg of the mucins were dissolved in 50 ml portions of 0.1 M sodium acetate buffer, pH 5.5. Different amounts of sodium metaperiodate in the same buffer were added to give a molar ratio of sialic acid (as estimated by the orcinol/Fe^{3+}/HCl assay [5, 6]) to periodate ranging from 1:1 to 1:10. The individual oxidation mixtures were kept at 4°C in the dark for periods varying from 20 min to 2 h. After this time glycerol was added in a threefold molar excess, as compared to periodate, and the mixtures were dialyzed for 16 h against 2 l 0.1 M sodium acetate buffer, pH 5.5, with one intermediate change. In each case the solution in the dialysis bag was adjusted to pH 9 with 0.2 M NaOH, and a ten-fold molar amount of sodium borohydride, as compared to periodate, was added. After 1 h at room temperature, the solution was adjusted to pH 4-5 with 50% aqueous acetic acid. Finally, the reaction mixture was dialyzed for 16 h against distilled water (3 x 1 l).

For the release of modified sialic acids, the residue in the dialysis bag was adjusted to pH 2 with concentrated HCl and hydrolyzed for 30 min at 80°C, followed by neutralization with 1 M NaOH and dialysis through an Amicon UM 20 membrane. The diffusate was lyophilized and passed through a column (2.5 x 60 cm) packed with Sephadex G-10 (Pharmacia, Freiburg, W. Germany) using water as eluent.

Elution was monitored with the orcinol/Fe^{3+}/HCl assay. The sialic acid-containing fractions were pooled, applied to a column (3 x 20 cm) of Dowex 50W-X8 (20-50 mesh, H^+-form) and eluted with 100 ml water. After lyophilization of the total eluate, the residue was dissolved in a small amount of water, applied on a column (2 x 30 cm) of Dowex 1-X8 (200-400 mesh, $HCOO^-$-form). The resin was washed with 100 ml water and the sialic acids eluted by 500 ml of a linear gradient of 0.2 to 1.2 M formic acid. The fractions containing sialic acids were pooled and lyophilized.

For the isolation of individual sialic acids, these fractions were further purified by liquid chromatography on cellulose MN 2100ff (Macherey - Nagel, Düren, W. Germany) using a column of 1 x 100 cm and *n*-butanol/*n*-propanol/water, 1/2/1 by vol, as eluent [5, 6], and by preparative TLC in the system described below for analytical TLC. Chromatography of individual fractions was repeated until of the modified sialic acids were homogeneous.

The oxidation of free Neu5Ac and Neu5Gc was carried out at 4°C with a molar ratio of periodate to sialic acid of about 40:1. After different times, aliquots were withdrawn from the reaction mixtures and further analyzed in the periodic acid/thiobarbituric acid assay [5, 6].

Colorimetry

Quantitative determination of sialic acids by colorimetry [5, 6] was performed using the orcinol/Fe^{3+}/HCl reagent, in some cases followed by measuring the spectra of the color complexes on a Hitachi 220 scanning photometer in the range 400-700 nm.

Thin Layer Chromatography

TLC was carried out on plastic sheets coated with 0.2 mm cellulose (Merck, Darmstadt, W. Germany), prerun in 0.1 M HCl, with *n*-butanol/*n*-propanol/0.1 M HCl, 1/2/1 by vol, as solvent system [5, 6]. Sialic acid-containing spots were visualized by spraying with the orcinol/Fe^{3+}/HCl spray reagent [5, 6].

Gas Liquid Chromatography

GLC was carried out on a Packard 429 gas chromatograph with either a glass column (0.4 x 200 cm) packed with 3.8% SE-30 on Chromosorb W (system I) or a capillary CP-Sil 5 WCOT fused silica column (25 m x 0.32 mm, system II), both from Chrompack, Müllheim, W. Germany. For system I the oven temperature was kept at 160°C for 5 min, then raised at 2°C/min to 220°C and held at 220°C for 15 min. The flow rate of the nitrogen carrier gas was 30 ml/min. In system II, the oven temperature was kept at 140°C for 5 min, then programmed at 2°C/min to 220°C and held at 220°C for 15 min. The nitrogen flow was 1 ml/min, to which make-up gas was added to give a total flow of 30 ml/min. The split ratio was adjusted to 1:20. In both systems a flame-ionization detection was used, and the temperatures of the detector and injector were both 250°C.

The compounds were analyzed as trimethylsilyl ester, per-*O*-trimethylsilyl ether derivatives, as obtained by treatment with pyridine/hexamethyldisilazane/trimethylchlorosilane, 5/1/1 by vol, for 60 min at room temperature. For determination of the relative molar detector responses, the derivatized sialic acids were analyzed on system II with Neu5Ac as internal standard.

Mass Spectrometry

Mass spectrometry was performed on a Varian 3700 gas-chromatograph/Varian MAT 44S mass spectrometer/Varian SpectroSpin 200 data system combination operating at 70 eV, an ion source temperature of 220°C and an ionization current of 300 μA. The GLC-conditions for both columns were the same as described above.

500-MHz ^{1}H-NMR Spectroscopy

Sialic acids were repeatedly exchanged in ^2H_2O (99.6 atom % ^{2}H, Aldrich, Milwaukee, WI, USA) with intermediate lyophilization. Resolution-enhanced 500-MHz ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) at 27°C and p^{2}H ~6. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ = 2.225 ppm) [7].

Table 1. Analytical data of Neu5Ac, Neu5Gc and their C-7/C-8 analogues.

Sialic acid	TLC	Color-imetry	GLC			MS						
			Relative detector response	R_{Neu5Ac}[d]		m/z-Values of fragment ions[e]						
	R_f[a]	ε[b]		system I	system II	A	B	C	D	E	F	G
C-7 Neu5Ac	0.64	6500	1.2	0.50	0.68	522	420	-	-	375	-	173
C-8 Neu5Ac	0.59	9600	1.1	0.72	0.81	624	522	536	356	375	"103"	173
Neu5Ac	0.55	5700	1.0	1.00	1.00	726	624	536	356	375	205	173
C-7 Neu5Gc	0.54	6300	1.4	0.81	0.87	610	508	-	-	375	-	261
C-8 Neu5Gc	0.49	9300	1.3	0.96	0.98	712	610	624	444	375	"103"	261
Neu5Gc	0.47	6500	1.1	1.25	1.06	814	712	624	444	375	205	261

[a] R_f Value for TLC on cellulose, using *n*-butanol/*n*-propanol/0.1M HCl, 1/2/1 by vol, as solvent system and the orcinol/Fe^{3+}/HCl spray reagent for detection.
[b] Extinction coefficients of chromophores in the orcinol/Fe^{3+} /HCl assay at 572 nm.
[c] Relative molar GLC detector response determined for Me_3Si esters, per-*O*-$SiMe_3$ ethers in system II.
[d] GLC as Me_3Si-esters, per-*O*-$SiMe_3$ ethers; for further details see the Materials and Methods section.
[e] The fragment ions A - G are highly characteristic for the determination of sialic acid structures [8-10].

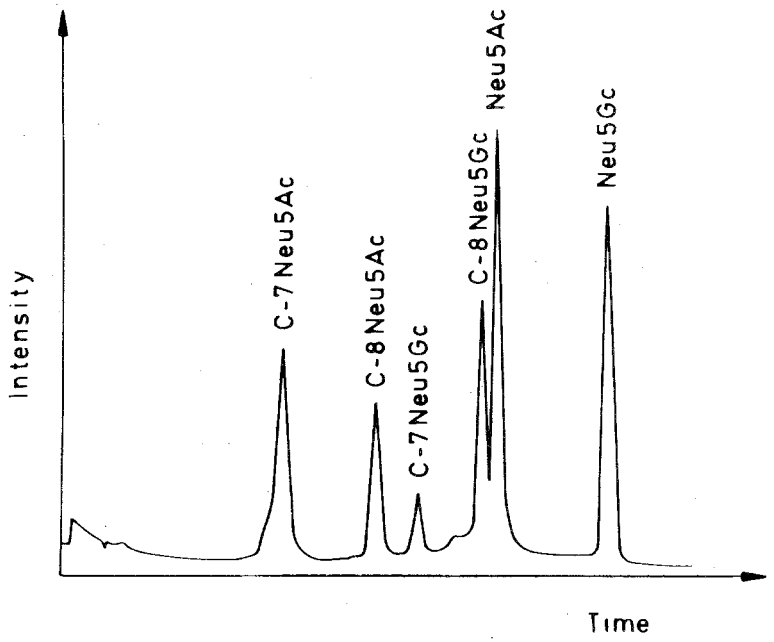

Figure 1. Total ion current of an artificial mixture of Neu5Ac, Neu5Gc and their C-7/C-8 analogues obtained by GLC-MS in system I. For details see the Materials and Methods section.

Results and Discussion

Analytical Data of C-7/C-8 Analogues of Neu5Ac and Neu5Gc

The oxidation of sialic acids was carried out similar to the conditions used earlier for modification of Neu5Ac-containing gangliosides [1], followed by purification procedures generally applied in sialic aicd analysis [5, 6]. Table 1 surveys a series of analytical characteristics of the C-7/C-8 analogues of *N*-acetyl- and *N*-glycolylneuraminic acid together with those of the parent sugars.

Neu5Ac, Neu5Gc, and the periodate-oxidized compounds were analyzed as Me_3Si ester, per-*O*-$SiMe_3$ ether derivatives by GLC and GLC-MS. The retention times relative to Neu5Ac in system I are similar to those published earlier for the methyl ester, per-*O*-$SiMe_3$ ether derivatives [1, 3, 8]. A comparison of the total ion current chromatogram of all six sialic acids investigated (Fig. 1; Me_3Si esters) with the selected ion chromatogram in [3] (methyl esters) indicates that the separation between C-8 Neu5Gc and Neu5Ac is better for the Me_3Si esters under the conditions used here. Analysis of the same derivatives on a capillary system instead (system II) revealed an improved separation.

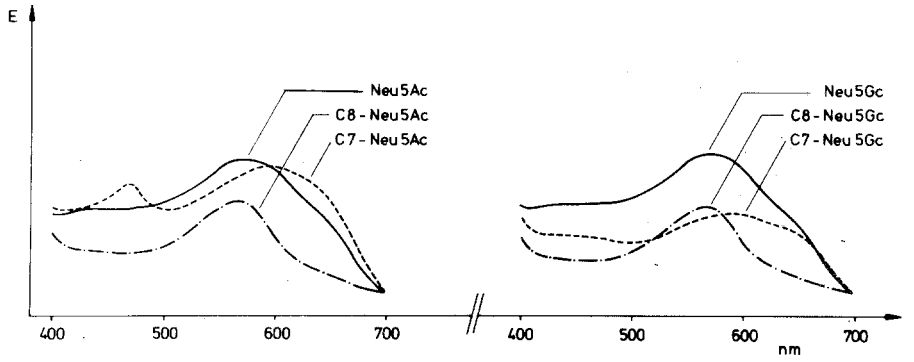

Figure 2. Absorption curves of the chromophores obtained from Neu5Ac or Neu5Gc and their C-7/C-8 analogues in the orcinol/Fe3+/HCl assay between 400 and 700 nm.

The relative molar detector responses of the Me_3Si derivatives were determined in GLC system II using Neu5Ac as internal standard. From the data in Table 1 it is evident that these values are similar to each other, ranging from 1.1 for Neu5Gc and C-8 Neu5Ac to 1.4 for C-7 Neu5Gc. The EI mass spectra of C-7 Neu5Ac, C-8 Neu5Ac, C-7 Neu5Gc, and C-8 Neu5Gc were obtained for the pertrimethylsilylated compounds. They are identified by the set of the highly characteristic fragment ions A-G, reported earlier for the identification of *N,O*-acylneuraminic acids [8, 9]. As has been observed earlier for these derivatives [10], fragment A is less intense relative to fragment B than in the case of the methyl esters. Fragment E is always accompanied by a peak corresponding to E - Me_3SiOH at m/z 285, which has a higher relative intensity than fragment E itself [10]. The other peaks correspond well to those observed for the methyl ester derivatives of these compounds [8], except of the shift of +58 mass units for all fragments retaining the C-1 part of the molecule. It has to be noted that the m/z values for the fragments A of the C-7 analogues are the same as for the fragments B of the C-8 analogues; similarly, the m/z values of the fragments A of the latter compounds correspond to the fragments B of the parent sialic acids.

The RF-values in the standard TLC system used for sialic acid analysis consisting of cellulose plates and *n*-butanol/*n*-propanol/0.1M HCl, 1/2/1 by vol, as solvent system and detection by the orcinol/Fe^{3+}/HCl spray reagent [5, 6] are listed in Table 1. They are in agreement with the literature data [1, 11].

The extinction coefficients obtained in this study are close to the data available from the literature for the parent sialic acids and the oxidation products of Neu5Ac [1]; the corresponding values for C-7 and C-8 Neu5Gc have not yet been published. The absorption spectra in the range of 400-700 nm of all six natural or modified sialic acids investigated are shown in Fig. 2. The parent sialic acids and the C-8 analogues behave similar with maxima at 556 and 572 nm, respectively. The extinction maxima for both C-7 analogues are shifted to higher wavelengths, i.e. 596 nm for C-7 Neu5Ac and 593 nm for C-7 Neu5Gc. When the extinction coefficients are determined at these wavelengths, they increase to 7200 for the Neu5Ac and to 6900 for the Neu5Gc derivative.

Table 2. Chemical shifts of protons of C-7/C-8 analogues of Neu5Ac and Neu5Gc, at p^2H~6, relative to internal acetone (δ = 2.225 ppm) and coupling constants *J*., as obtained by 500-MHz ^1H-NMR spectroscopy.

	Chemical shifts (ppm)			
	C-7 β-Neu5Ac	C-7 β-Neu5Gc	C-8 β-Neu5Ac	C-8 β-Neu5Gc
H-3ax	1.821	1.840	1.814	1.832
H-3eq	2.238	2.253	2.203	2.218
H-4	3.992	4.096	3.998	4.097
H-5	3.710	3.798	3.892	3.976
H-6	3.800	3.906	3.772	3.887
H-7	3.671	3.674	3.722	3.709
H-7′	3.634	3.632	-	-
H-8	-	-	3.655	3.657
H-8′	-	-	3.586	3.589
NAc	2.044	-	2.055	-
NGc	-	4.133	-	4.144
	Coupling constants (Hz)			
*J*3ax, 3eq	- 13.0	-13.0	-12.9	-12.9
*J*3ax,4	11.5	11.7	11.6	11.5
*J*3eq,4	5.1	5.0	4.9	4.9
*J*4,5	10.0	9.9	10.1	9.8
*J*5,6	10.5	10.5	10.2	10.4
*J*6,7	2.6	2.5	1.0	1.2
*J*6,7′	4.9	4.9	-	-
*J*7, 7′	- 12.5	-12.5	-	-
*J*7,8	-	-	7.2	7.3
*J*7,8′	-	-	5.7	5.5
*J*8,8′	-	-	- 11.3	-11.2
	Chemical shifts (ppm)			
	C-7 α-Neu5Ac	C-7 α-Neu5Gc	C-8 α-Neu5Ac	C-8 α-Neu5Gc
H-3ax	1.599	N.D.[a]	N.D.[a]	1.622
H-3eq	2.630	2.650	N.D.	2.665
NAc	2.026	-	2.038	-
NGc	-	4.116	-	4.129

[a] N.D. = not determined.

Although the chemical nature of the chromophore of sialic acids in the orcinol/Fe^{3+}/HCl assay is not known, the absorption spectra presented here strongly suggest that C-8 of sialic acids is part of the purple chromophore, whereas C-9 and the substituent at the nitrogen may not be important for color formation.

The ^1H-NMR parameters of the modified sialic acids, acquired at 500 MHz and p_2H~6, are presented in Table 2. Each of the four modified sialic acids occurs mainly in the β-anomeric form (>92%). The chemical shift values of H-3ax and H-3eq of the modified and parent β-sialic acids are observed in the same δ–range (H-3ax, δ = 1.81-1.84 ppm; H-3eq, δ = 2.20-2.25 ppm; for the chemical shift values of Neu5Ac and Neu5Gc see [12]). The NAc or NGc

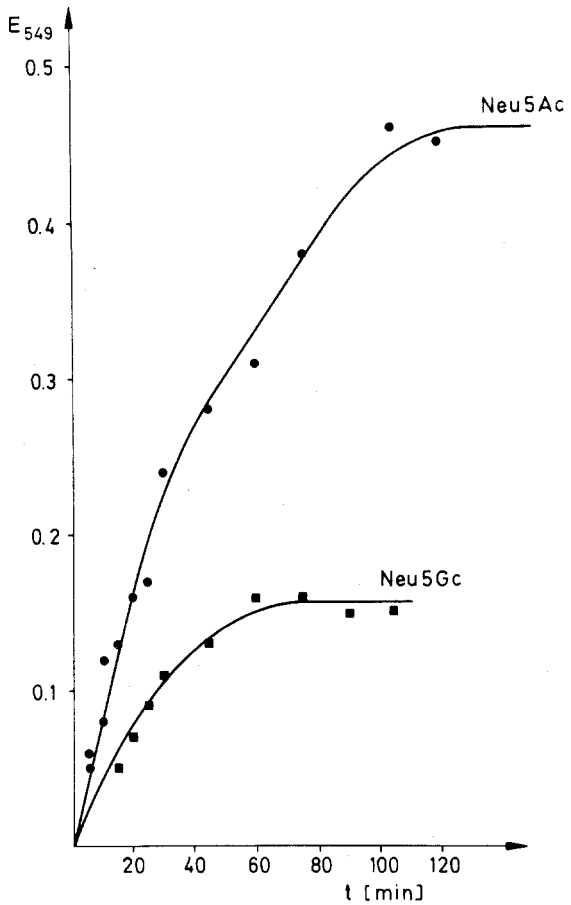

Figure 3. Time course of the periodate oxidation of free Neu5Ac and Neu5Gc in the periodic acid/thiobarbituric acid assay. For details see the text.

singlets of the four α-forms resonate in each case at a higher field than those of the four β-forms ($\Delta\delta$ = -0.015 to -0.018 ppm), comparable with the findings for Neu5Ac and Neu5Gc ($\Delta\delta$ = -0.020 and -0.023 ppm, respectively). It is interesting to note that the replacement of a *N*-acetyl group by a *N*-glycoloyl group at C-5 causes similar shift increments for H-3ax, H-3eq, H-4, H-5, and H-6, when the pairs of C-7, C-8 and parent sialic acids are compared. The largest influences are observed for H-4 ($\Delta\delta$ = +0.099 to +0.104 ppm), H-5 ($\Delta\delta$ = +0.084 to +0.103 ppm), and H-6 ($\Delta\delta$ = +0.106 to +0.122 ppm).

Optimization of Oxidation Conditions

As model substances to study the optimal oxidation conditions of Neu5Ac- and Neu5Gc-containing glycoproteins, collocalia mucin and pig submandibular gland mucin were taken. The bird mucin is known to contain only Neu5Ac, whereas the pig material has about 80% Neu5Gc and 20% Neu5Ac. In both cases, *O*-acetylated sialic acids are absent [13]. These relatively homogeneous starting materials should allow discrimination between the oxidation of Neu5Ac and Neu5Gc residues, if differences were to be observed.

Collocalia mucin was first oxidized with a molar ratio of periodate:sialic acid of 1.5:1 for different times from 20 min to 2 h. TLC analysis of the products obtained after oxidation, reduction, mild acid hydrolysis, dialysis, and purification over ion-exchange resins showed that after 30 min about 10% C-7 and 40% C-8 Neu5Ac were formed, the remainder being Neu5Ac; after 1 h this ratio was 30%: 40% and remained constant up to 2 h.

In the case of PSM the same experiment gave 40%, each, of C-7 and C-8 Neu5Gc after 20 min. After 40 min no Neu5Gc was left. C-7 Neu5Ac which is formed by oxidation of the Neu5Ac residues in PSM was already present after 20 min, increased in intensity up to 2 h, whereas only a trace amount of C-8 Neu5Ac was found at 20 min.

In another experiment collocalia mucin and PSM were treated with a molar ratio of periodate to sialic acids ranging from 1:1 to 10:1 keeping the incubation time at 60 min. With this reaction time a two-fold molar excess of periodate is necessary for the formation of appreciable amounts of C-7 Neu5Ac in collocalia mucin. Even then, about 30% of the C-8 compound is left. Under these conditions the complete conversion of Neu5Ac to C-7 Neu5Ac requires a 10-fold molar excess of periodate compared to sialic acid.

Under the same conditions, PSM is already completely oxidized with a lower periodate concentration. A three-fold excess is sufficient to give C-7 Neu5Gc as the only derivative of *N*-glycoloyl sialic aicd.

The thiobarbituric acid/periodic acid assay of Warren [14] is often carried out for sialic acid determination [5, 6]. Periodate-oxidized (30 min, room temperature) sialic acids undergo a sequence of reactions to give β-formyl pyruvic acid, which yields the chromophore with thiobarbituric acid. Only free sialic acids can be assayed in this test. To follow the periodate oxidation in this test, Neu5Ac and Neu5Gc were treated at 4°C with a 40-fold molar excess of periodate over sialic acids. After different times, aliquots were withdrawn from the reaction mixture and further processed as in the usual thiobarbituric acid/periodic acid assay. The time curve obtained is shown in Fig. 3. The different extinction values reached after full oxidation are due to different amounts of the two sialic acids used for the test. It can be recognized that the oxidation of Neu5Gc is complete after about 60 min whereas Neu5Ac is fully oxidized only after 110 min. A comparison of the relative oxidation rated reveales that Neu5Gc is oxidized about 1.5 times faster than Neu5Ac.

The above results demonstrate that the speed of the reaction is dependent on the type of sialic acid. The results from this colorimetric assay of free sialic acids are similar to those obtained after periodate oxidation of the mucins, where Neu5Gc residues were oxidized at milder conditions than Neu5Ac residues. Thus, the faster oxidation of Neu5Gc is probably a general phenomenon and not dependent on the type of substrate.

In order to prepare pure sialic acid analogues, oxidation conditions were chosen for an optimum yield of the different compounds, i.e. a molar ratio of periodate/sialic acid of 10:1 and 4:1 for C-7 Neu5Ac and C-7 Neu5Gc, respectively, and 2:1 and 1:1 for C-8 Neu5Ac and C-8 Neu5Gc, respectively, each with a reaction time of 60 min.

Because only a maximum yield of about 40% of the C-8 sialic acids could be obtained, separation of these compounds from the C-7 analogues and not oxidized sialic acids was

necessary after purification of the sialic acid mixtures by gel chromatography on Sephadex G-10 and cation- and anion-exchange chromatographies. The anion-exchange chromatography step led already to a partial purification of the modified sialic acids. In the case of C-8 Neu5Gc the isolation became especially difficult because this compound had to be separated not only from C-7 Neu5Gc and Neu5Gc but also from Neu5Ac, which is originally present in PSM, and its oxidation products. For this purpose chromatography on cellulose was carried out. Preparative TLC on cellulose also used for analytical investigations was more efficient than column chromatography on cellulose, and finally gave homogeneous preparations. For C-8 Neu5Ac the same purification procedure was used. The C-7 analogues which were almost pure after anion exchange chromatography, were finally purified by column chromatography on cellulose.

The sensitivity of the sialic acid moiety in the total sialoglycoconjugate towards periodate oxidation can be recognized when the data obtained are compared to those published for the oxidation of glycolipid-bound Neu5Ac [1]. Even after 2 h with a ten-fold molar excess of periodate, about 25% Neu5Ac in G_{M1}-ganglioside are not oxidized. Thus, periodate oxidation followed by sodium borotritide reduction can hardly be used alone as a reliable method for an estimation of the amount of sialic acids in sialoglycoconjugates without detailed knowledge about the oxidation behaviour of the compound under investigation. On the other hand, the conditions might be varied considerably if surface labeling is needed.

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