G. NMR Spectroscopy of Sialic Acids

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I. Introduction

High-resolution NMR spectroscopy has become an invaluable technique in the study of biopolymers and of their constituents. Using ^{1}H or ^{13}C nuclei as probes information can be obtained about primary structures, conformations and intermolecular interactions of biomolecules in solution. In particular, the possibility to record spectra of underivatized compounds in aqueous solutions allows to afford further insight into the way of action of biomolecules under physiological conditions. For general reviews of high-resolution NMR spectroscopy in the study of biological systems the reader is referred to the recent books of BERLINER and REUBEN (1978, 1980), JARDETZKY and ROBERTS (1981), and SHULMAN (1979).

In 1977 we introduced the application of high-resolution ^{1}H-NMR spectroscopy as a new method for structure elucidation of carbohydrate chains present in glycoproteins. These studies have shown that sialylated carbohydrate chains can completely be characterized with regard to the sialic acid residues. In this chapter relevant ^{1}H- and ^{13}C-NMR parameters of free and glycosidically bound sialic acids will be discussed.

II. ^{1}H-NMR Spectroscopy

1. N-Acetyl- and N-Glycolylneuraminic Acid

As early as 1968 60 MHz ^{1}H-NMR spectra were published of Neu5Ac in ^2H_2O (CHAPMAN *et al.* 1968, KIMURA and TSURUMI 1968, BLIX and JEANLOZ 1969). Such a spectrum is given in Fig. 1. It shows a singlet belonging to the acetamido methyl protons at δ 2.05. Two broad signals are found at δ 3.7 and δ 3.9 comprising most of the skeleton protons. Although the investigators realized that this technique could be very promising for biochemical purposes, hardly any structural information can be obtained from this low-resolution NMR spectrum.

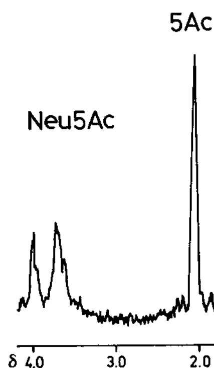


Fig. 1. 60 MHz ^{1}H-NMR spectrum of Neu5Ac dissolved in ^2H_2O, recorded at ambient temperature (taken from KIMURA and TSURUMI 1968).

The recent development of NMR spectrometers operating at higher magnetic fields (up to 11.7 Tesla, equivalent to a frequency of 500 MHz for protons) together with the advances in computer capabilities have led to an enormous improvement in spectral resolution and in sensitivity. This progress is excellently reflected in the 500 MHz ^{1}H-NMR spectrum of Neu5Ac in ^2H_2O, as presented in Fig. 2. The spectrum consists of two subspectra belonging to the α- and β-anomer of Neu5Ac. These anomers occur in a molar ratio of 7 : 93, respectively (JAQUES *et al.* 1977, HAVERKAMP *et al.* 1978, DABROWSKI *et al.* 1979, BEAU *et al.* 1980, FRIEBOLIN *et al.* 1980 a, b, HAVERKAMP *et al.* 1982). The 500 MHz ^{1}H-NMR spectrum given in Fig. 2 allows the assignment of several of the signals for the α-anomer of Neu5Ac. The spectrum of the β-anomer could completely be interpreted (BROWN *et al.* 1975, BEAU *et al.* 1980, HAVERKAMP *et al.* 1982). Chemical shifts and coupling constants are summarized in Table 1. It should be noted that the chemical shifts of Neu5Ac protons are pH-dependent. For the β-anomer the signals shift upfield upon increasing the pH from 2 to 7, e.g. for H3eq from δ 2.313 to δ 2.208 and for H3ax from δ 1.880 to δ 1.827 (see Table 1) (HAVERKAMP *et al.* 1982).

Interestingly, in the pH range 6.5-9.0, H3ax can be exchanged. This could be observed in alkaline ${}^2H_2O$ solution of Neu5Ac ($p^2H = 9.0$), wherein a complete replacement of H3ax by ${}^2H$ occurred (Dorland *et al.* 1982). In the ${}^1H$-NMR spectrum this exchange leads to the disappearance of the H3ax signal and of its

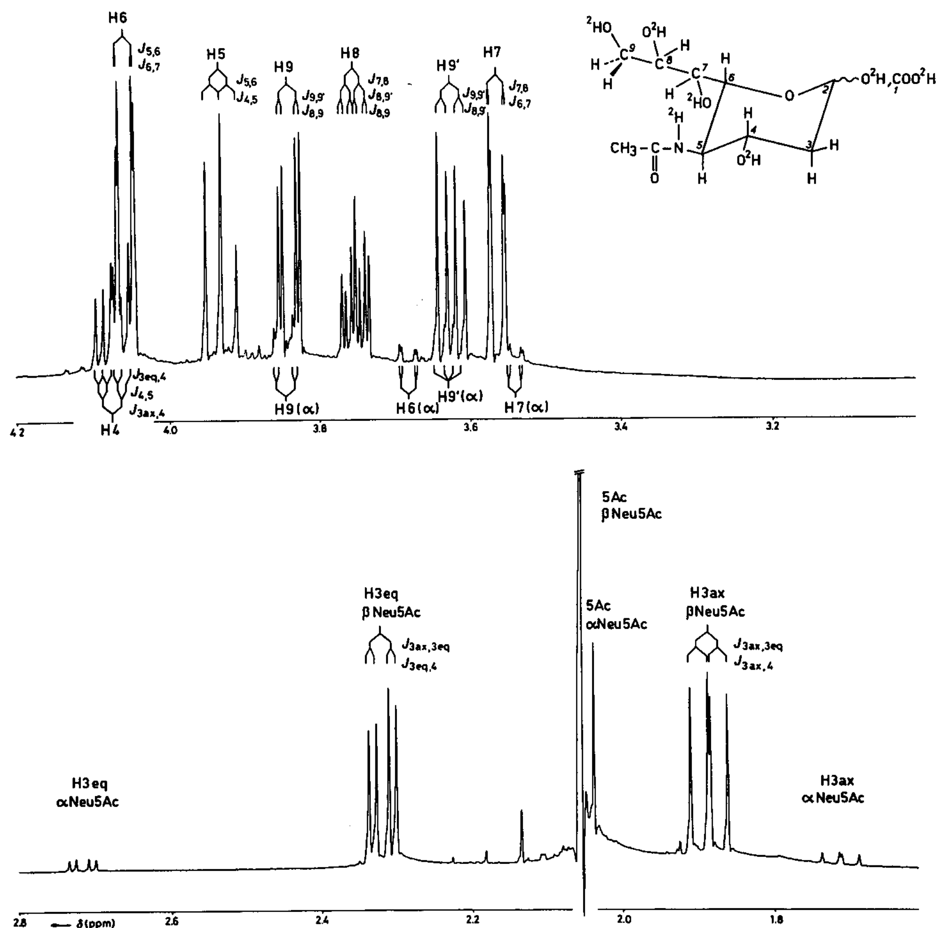


Fig. 2. Resolution-enhanced 500 MHz ${}^1H$-NMR spectrum of Neu5Ac dissolved in ${}^2H_2O$, recorded at p^2H 1.4 and 27 °C. The intensity ratio of the signals from corresponding protons reflects the molar ratio of the α- and β-anomer under the applied measuring conditions to be 7 : 93. Detailed splitting patterns are indicated for all resonances from the β-, and for the greater part of those from the α-anomer.

coupling with H3eq and H4. This phenomenon can excellently be utilized for the preparation of specifically labelled (${}^2H$ or ${}^3H$) cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac), thereby allowing the enzymic introduction of labelled Neu5Ac in glycoconjugates (Dorland *et al.* 1982). Further increase of the p^2H to 12.4 causes also exchange of H3eq (Friebolin *et al.* 1981 b). The

Table 1. *^{1}H-NMR data for Neu5Ac and Neu5Gc*

Chemical shift values are given in ppm downfield from DSS for solutions in $^{2}H_2O$ at 25 °C and at the indicated p^{2}H values. Coupling constants (J) are given in Hz. n.d., value could not be determined

Compound	p^{2}H	Chemical shift											References
		H3ax	H3eq	H4	H5	H6	H7	H8	H9	H9′	5Ac	5Gc	
βNeu5Ac	1.4	1.880	2.313	4.067	3.931	4.056	3.556	3.750	3.841	3.619	2.053	—	HAVERKAMP *et al.* 1982
αNeu5Ac	1.4	1.705	2.718	n.d.	3.85	3.684	3.53	3.75	3.85	3.62	2.036	—	HAVERKAMP *et al.* 1982
βNeu5Ac	7.0	1.827	2.208	4.024	3.899	3.984	3.514	3.753	3.835	3.608	2.050	—	HAVERKAMP *et al.* 1982
αNeu5Ac	7.0	1.621	2.730	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.030	—	HAVERKAMP *et al.* 1982
βNeu5Gc	7.0	1.840	2.243	4.127	4.002	4.106	3.549	3.777	3.821	3.613	—	4.143	HAVERKAMP *et al.* 1982
αNeu5Gc	7.0	1.644	2.749	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	4.12	HAVERKAMP *et al.* 1982

Compound	p^{2}H	Coupling constant										References
		$^{2}J_{3ax,3eq}$	$^{3}J_{3ax,4}$	$^{3}J_{3eq,4}$	$^{3}J_{4,5}$	$^{3}J_{5,6}$	$^{3}J_{6,7}$	$^{3}J_{7,8}$	$^{3}J_{8,9}$	$^{3}J_{8,9'}$	$^{2}J_{9,9'}$	
βNeu5Ac	1.4	—13.2	11.8	5.0	10.4	10.7	1.2	9.4	2.8	6.4	—12.4	BEAU *et al.* 1980
αNeu5Ac	1.4	—13.0	11.5	4.5	n.d.	10.5	1.5	9.0	n.d.	6.5	—12.5	—
βNeu5Gc	7.0	—12.6	11.4	4.6	10.2	10.2	1.0	9.0	2.8	6.2	—11.4	JAQUES *et al.* 1980a

exchange reaction can also be traced in the ^1H-NMR spectrum of Neu5Gc in ^2H_2O ($p^2H \sim 7$) as published by JAQUES *et al.* (1980a); the reduced intensity of the H3ax signal has to be ascribed to partial replacement by ^2H rather than to saturation effects as proposed by the authors. The ^1H-NMR data of Neu5Gc are also compiled in Table 1. It is worthy to note that, except for H9, the chemical shift values for the protons of Neu5Gc are found at lower field than the corresponding protons of Neu5Ac if both measured in neutral solution. These chemical shift differences may result from changes in the microenvironment of the various protons. In this respect it would be interesting to carry out studies on the occurrence of hydrogen bonds of Neu5Gc in a similar way as has been performed

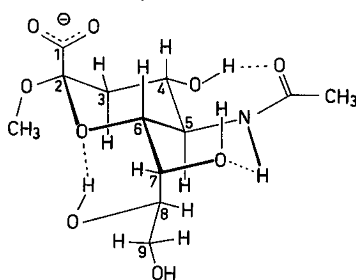


Fig. 3. Conformational model of the anion of αNeu5Ac2Me showing the proposed hydrogen bonds and preferred conformation of the glycerol side chain (taken from CZARNIECKI and THORNTON 1976). Although shown here only for the α-anomer, this conformation is independent of the anomeric configuration.

for Neu5Ac derivatives by CZARNIECKI and THORNTON (1976, 1977a). These authors have studied the spatial structure of the α-methyl glycoside of Neu5Ac in ^2H_2O solution on the basis of the proton-proton coupling constants (see Table 1) in combination with the ^{13}C spin-lattice relaxation times (T_1). The T_1 values provide information about the internal mobility of the molecules in solution. The similarity of the values for C7, C8 and the ring carbon atoms point to an isotropic motion of these atoms. From this observation the authors postulated that the amido-NH is hydrogen-bonded to the oxygen at C7 and that the OH at C8 is hydrogen-bonded to the ring-oxygen. A third hydrogen bond between the acetamido-carbonyl and the OH at C4 was suggested on the basis of model building. The proposed structure is given in Fig. 3. Also REUTER *et al.* (unpublished results) found that the T_1 values for ring and glycerol side chain carbon atoms of derivatives of Neu5Ac are similar. In the latter study the inversion-recovery method was used in combination with T_1 calculation, following the method of SASS and ZIESSOW (1977). This approach gives more accurate T_1 determinations than the fast inversion-recovery method, used by CZARNIECKI and THORNTON (1976, 1977a). Apparently, the anomeric centre is not involved in any hydrogen bond, leading to the same conformation for α- and β-anomers of Neu5Ac.

2. *O-Acetylated Neuraminic Acid Derivatives*

For neuraminic acids bearing O-acetyl groups, it has been demonstrated that high-resolution ^{1}H-NMR spectroscopy can efficiently be employed to determine the number and position of such substituents (HAVERKAMP *et al.* 1982). The number of acetyl groups can be determined on the basis of number and relative intensities of the acetyl signals. O-acetylation causes some specific downfield shifts

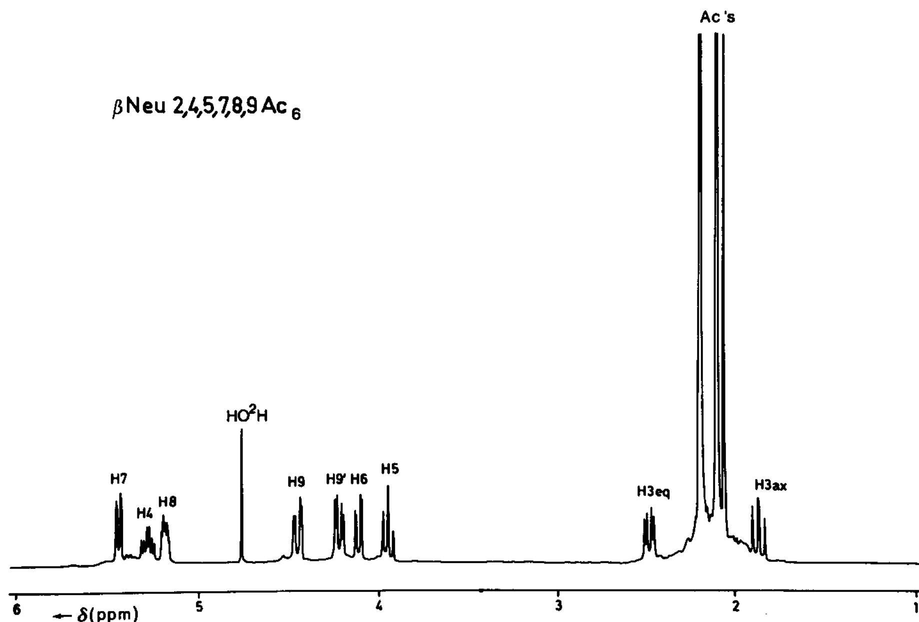


Fig. 4. 360 MHz ^{1}H-NMR spectrum of βNeu2,4,5,7,8,9Ac$_6$ dissolved in ^2H_2O, recorded at p^{2}H ~ 7 and 25 °C.

which are that characteristic that they can be applied for the assignment of the positions of the acetyl groups. In case when a secondary carbon atom is O-acetylated, the proton attached to this carbon undergoes a downfield shift which varies from 1-1.5 ppm. This so-called α-effect amounts to 1.2 ppm for H4 and 1.5 ppm for H7. Upon introduction of an acetyl substituent at the primary hydroxyl group of C9, the effect is shared over the protons H9 and H9′, each being about 0.5-0.6 ppm.

There are also effects on protons at carbon atoms adjacent to the O-acetylated carbon. These β-effects are in the cases of O-acetylation of C4, C7, and C9 about 0.2 ppm. The geminal protons H3eq and H3ax form an exception since they undergo shifts of about 0.04 and 0.14 ppm, respectively. The β-effects on H3eq and H3ax are unequal due to the rigidity of the C3-C4 part of the molecule. The γ- and δ-effects on protons, attached to more remote carbon atoms are small and irregular in direction.

In di-O-acetylated neuraminic acids the effects of O-acetylation on the chemical shifts of skeleton protons are composed of the individual contributions of each O-acetyl group. Apparently, the induced shifts are additive. This is a general phenomenon, which can be illustrated for the peracetylated neuraminic acid. The ^{1}H-NMR spectrum of this compound is shown in Fig. 4. The resonance positions of H5, H6, H7, H8, H9, and H9′ can be estimated on the basis of the presumed α- and β-effects of O-acetylation at position C4, C7, C8, and C9.

In principle, the chemical shifts of the acetyl protons contain information on the position of these substituents. The protons of the acetyl group at C9 are found in the range δ 2.10–2.13, at C4 near δ 2.06 and at C8 near δ 2.09. Furthermore, the occurrence of an acetyl group at C4 or C7 gives rise to a significant upfield shift of the N-acetyl signal. In case when the substituents at C4 and C7 are both present, their effects are additive (HAVERKAMP *et al.* 1982). ^{1}H-NMR data of a series of O-acetylated sialic acids and of some reference compounds are presented in Table 2.

An elegant illustration of the suitability of the above mentioned approach for the determination of the number and position of O-acetyl groups in sialic acids has been given for a trisaccharide from echidna milk (HAVERKAMP *et al.* 1982, KAMERLING *et al.* 1981, 1982). It was found that the trisaccharide is identical to Neu5Acα(2–3)Galβ(1–4)Glc having one O-acetyl substituent at the Neu5Ac residue. The H3, H4, and H5 signals of Neu5Ac have undergone shifts (see Table 2), typical for O-acetylation of C4. The 500 MHz ^{1}H-NMR spectrum of this trisaccharide is presented in Fig. 5.

It may be clear that this NMR method can generally be applied for the determination of the number and positions of O-acetyl groups in glycosidically linked sialic acids, occurring in oligosaccharides or glycopeptides derived from glycoconjugates.

Obviously the isolation procedure for the carbohydrates should be that mild that O-deacetylation is suppressed as far as possible and that migration of O-acetyl groups does not occur. In principle, the method can be extended to other O-acyl substituents, e.g. O-lactyl, provided that adequate reference compounds are available.

On the basis of the shifts which occur upon etherification of sialic acid, e.g. O-methylation, ^{1}H-NMR spectroscopy may also afford valuable information about the positions of these substituents (BEAU *et al.* 1980).

3. *Analysis of Sialic Acid Linkage Types in Glycoconjugates*

High-resolution ^{1}H-NMR spectroscopy has proved to be a suitable method for the determination of the type of linkage of sialic acid residues to carbohydrate units of glycoconjugates (DORLAND *et al.* 1978, SCHUT *et al.* 1978, LEGER *et al.* 1978, DORLAND 1979, VLIEGENTHART 1980, VAN HALBEEK *et al.* 1980, 1981 a, b, NOMOTO *et al.* 1981, INOUE *et al.* 1981, SPIK *et al.* 1982, VLIEGENTHART *et al.* 1981, 1982). First of all, the resonance positions of the sialic acid structural reporter group signals H3eq and H3ax are indicative of the configuration and position of the glycosidic linkage. These chemical shifts are influenced to some extent by the total structure of the carbohydrate chain to which sialic acid is attached. Secondly, several reporter group signals in the carbohydrate chain are shifted in a very

Table 2. [1]*H-NMR chemical shift data for O-acetylated sialic*
Chemical shift values are given for solutions in ^2H_2O at 25 °C and at the indicated p^2H
could not be determined at all, due to complication of the spectra of the corresponding β-

Compound	p^2H	Chemical shift					
		H3ax	H3eq	H4	H5	H6	H7
βNeu4,5Ac_2	7	1.951	2.249	5.274	4.15	4.15	3.570
βNeu5,7Ac_2	4	1.905	2.236	3.950	3.767	4.246	5.045
αNeu5,7Ac_2	4	1.649	2.757	n.d.	n.d.	n.d.	n.d.
βNeu5,9Ac_2	7	1.833	2.221	4.024	3.913	3.991	3.571
αNeu5,9Ac_2	7	1.624	2.720	n.d.	n.d.	n.d.	n.d.
βNeu9Ac5Gc	7	1.842	2.234	4.14	4.006	4.109	3.570
αNeu9Ac5Gc	7	1.649	2.751	n.d.	n.d.	n.d.	n.d.
βNeu5,7,9Ac_3	2	1.924	2.303	3.978	3.775	4.293	5.162
αNeu5,7,9Ac_3	2	1.686	2.751	n.d.	n.d.	n.d.	n.d.
βNeu5,8,9Ac_3	7	1.838	2.189	3.978	3.903	3.780	3.838
βNeu5,8,9Ac_3	2	1.862	2.250	4.006	3.912	3.830	3.866
βNeu2,4,5,7,8,9Ac_6	7	1.862	2.467	5.276	3.934	4.105	5.438
βNeu5Ac1Me	7	1.913	2.315	4.067	3.916	4.067	3.552
αNeu5Ac1Me	7	1.732	2.723	n.d.	n.d.	n.d.	n.d.
βNeu5Ac2Me	7	1.645	2.337	4.009	3.88	3.785	3.532
αNeu5Ac2Me	7	1.626	2.718	3.675	3.803	3.689	3.586
βNeu5Ac1,2Me_2	7	1.784	2.392	4.044	3.917	3.87	3.581
αNeu5Ac1,2Me_2	7	1.798	2.676	3.756	3.86	3.83	3.558
βNeu4,5$Ac_2$1,2Me_2	7	1.927	2.442	5.260	4.157	4.035	3.623
βNeu5,9$Ac_2$1,2Me_2	7	1.788	2.395	4.059	3.938	3.910	3.637
βNeu4,5,9$Ac_3$1,2Me_2	7	1.921	2.445	5.299	4.188	4.069	3.686
αNeu4,5,7,8,9$Ac_5$2Me	7	1.701	2.637	4.858	3.834	4.498	5.365
αNeu4,5,7,8,9$Ac_5$1,2Me_2	7	1.930	2.718	4.924	3.909	4.273	5.390
Neu5Acα(2-3)Galβ(1-4)Glc	6	1.799	2.757	3.688	3.825	n.d.	n.d.
Neu5Gcα(2-3)Galβ(1-4)Glc	7	1.816	2.777	n.d.	n.d.	n.d.	n.d.
Neu5Acα(2-6)Galβ(1-4)Glc	6	1.739	2.715	3.658	3.836	n.d.	n.d.
Neu4,5Ac_2α(2-3)Galβ(1-4)Glc	5	1.926	2.768	4.955	4.088	n.d.	n.d.

[a] Values may be interchanged.
[b] Also including the 2Ac signal.
[c] Individual acetyl signals could not be assigned.

characteristic way upon extension with sialic acid. This information was derived from comparative ^{1}H-NMR studies on a large series of sialo-oligosaccharides and sialo-glycopeptides (DORLAND *et al.* 1978, VLIEGENTHART 1980, VLIEGENTHART *et al.* 1981, 1982, VAN HALBEEK *et al.* 1980, 1981 a, b).

a) The Anomeric Configuration of Sialic Acid

The anomeric configuration of Neu5Ac or Neu5Gc can usually be inferred from the chemical shifts of the H3eq and H4 resonances. Analysis of a series of model

acids and some reference compounds (HAVERKAMP *et al.* 1982)
values. n.d., value could not be determined. Values for αNeu4,5Ac$_2$ and αNeu5,8,9Ac$_3$
anomers as a result of the presence of small non-carbohydrate contaminants in the samples.

Chemical shift										
H8	H9	H9′	5Ac	4Ac	7Ac	8Ac	9Ac	5Gc	1Me	2Me
3.775	3.844	3.619	1.992	2.065	—	—	—	—	—	—
3.911	3.629	3.444	1.976[a]	—	2.144[a]	—	—	—	—	—
n.d.	n.d.	n.d.	1.947	—	2.128	—	—	—	—	—
3.977	4.365	4.187	2.057	—	—	—	2.119	—	—	—
n.d.	n.d.	n.d.	n.d.	—	—	—	n.d.	—	—	—
3.970	4.365	4.183	—	—	—	—	2.115	4.144	—	—
n.d.	n.d.	n.d.	—	—	—	—	n.d.	4.123	—	—
4.140	4.106	4.106	1.981[a]	—	2.134[a]	—	2.106	—	—	—
n.d.	n.d.	n.d.	1.956	—	n.d.	—	n.d.	—	—	—
5.114	4.528	4.287	2.057	—	—	2.089	2.105	—	—	—
5.115	4.545	4.287	2.059	—	—	2.091	2.107	—	—	—
5.182	4.446	4.212	1.940	2.047;2.081;2.089;2.171;2.179[b,c]				—	—	—
3.731	3.834	3.619	2.051	—	—	—	—	—	3.838	—
n.d.	n.d.	n.d.	2.036	—	—	—	—	—	3.838	—
3.88	3.85	3.662	2.047	—	—	—	—	—	—	3.200
3.886	3.869	3.641	2.033	—	—	—	—	—	—	3.341
3.87	3.841	3.667	2.050	—	—	—	—	—	3.868	3.274
3.84-3.88	3.84-3.88	3.654	2.034	—	—	—	—	—	3.880	3.383
3.87	3.846	3.667	1.990	2.059	—	—	—	—	3.868	3.304
4.087	4.433	4.201	2.057	—	—	—	2.128	—	3.878	3.278
4.109	4.443	4.214	1.999	2.059	—	—	2.125	—	3.885	3.302
5.422	4.382	4.265	1.910	2.028; 2.089; 2.162; 2.162[c]				—	—	3.305
5.428	4.356	4.243	1.922	2.041; 2.093; 2.167; 2.211[c]				—	3.887	3.356
n.d.	n.d.	n.d.	2.030	—	—	—	—	—	—	—
n.d.	n.d.	n.d.	—	—	—	—	—	4.119	—	—
n.d.	n.d.	n.d.	2.030	—	—	—	—	—	—	—
n.d.	n.d.	n.d.	1.963	2.070	—	—	—	—	—	—

substances has shown that for α-anomers the chemical shift of H3eq varies between δ 2.6 and δ 2.8 and that of H4 between δ 3.6 and δ 3.8. For β-anomers these ranges are δ 2.1–δ 2.5 and δ 3.9–δ 4.2, respectively (HAVERKAMP *et al.* 1978, 1982). Furthermore, the signal of the N-acyl group protons furnishes additional evidence for the anomeric configuration (VAN HALBEEK *et al.* 1981 b, HAVERKAMP *et al.* 1982). The signals, which are useful for the determination of the anomeric configuration are compiled in Table 3.

Although O-substitution, e.g. acylation or etherification, may influence the

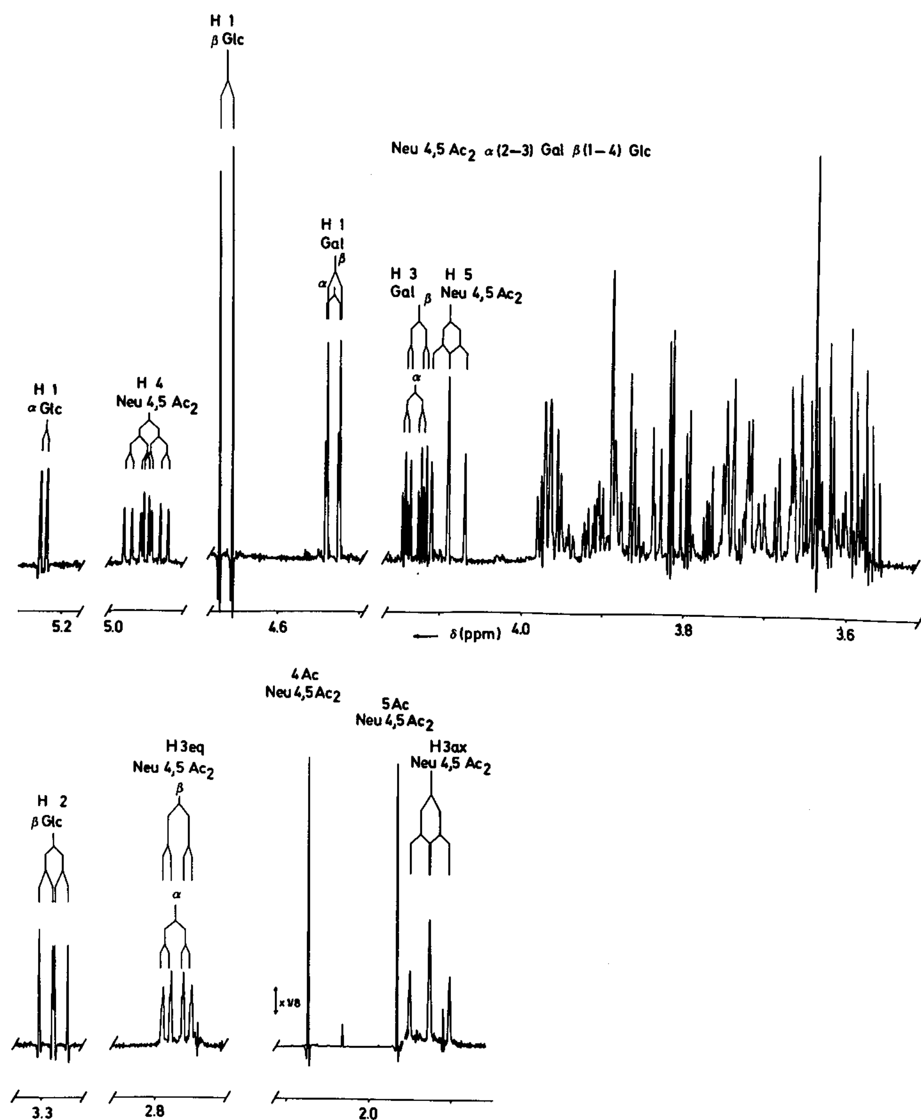


Fig. 5. Resolution-enhanced 500 MHz ^{1}H-NMR spectrum of Neu4,5Ac$_2$α(2-3)Galβ(1-4)Glc dissolved in ^2H_2O, recorded at p^{2}H 7 and 27 °C. Signals of corresponding protons of the two anomers of the trisaccharide (molar ratio α : β = 7 : 10) coincide, unless indicated otherwise. (The relative intensity scale of the acetyl methyl proton signals differs from that of the other parts of the spectrum.)

Table 3. ^{1}*H-NMR chemical shift data, useful for discrimination between α- and β-glycosidically linked sialic acids* (HAVERKAMP *et al.* 1978, 1982)
Chemical shift values are given for solutions in ^2H_2O at 25 °C and at $p^2H \simeq 7$. Values have to be modified (see Table 2) if C4 and/or C7 of the sialic acid bear a substituent (e.g., an O-acetyl group)

Configuration of glycosidic linkage of sialic acid	Chemical shift range			
	H3eq	H4	5Ac	5Gc
α	2.6-2.8	3.6-3.8	2.025-2.035	4.11-4.13
β	2.1-2.5	3.9-4.2	2.045-2.055	4.13-4.15

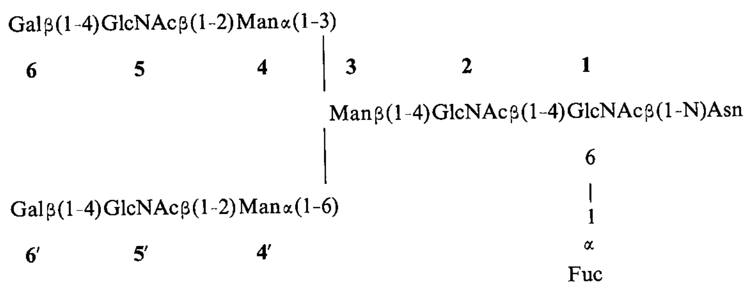
Table 4. ^{1}*H-NMR chemical shift data discriminative for α(2-3)- or α(2-6)-linkage of sialic acid to galactose* (DORLAND *et al.* 1978)
Chemical shift values are given for solutions in ^2H_2O at 25 °C and at $p^2H \simeq 7$

Structural element	Chemical shift	
	H3ax	H3eq
Neu5Acα(2-3)Galβ(1-·)	1.80	2.76
Neu5Acα(2-6)Galβ(1-·)	1.72	2.67

chemical shifts of some of the reporters which are employed for the determination of the anomeric configuration of the glycosidic linkage, they are still useful for this purpose (BEAU *et al.* 1980, HAVERKAMP *et al.* 1982, KAMERLING *et al.* 1982).

b) Sialic Acid in Carbohydrate Chains of the N-Acetyllactosamine Type

The diantennary glycan structure



and sialylated extensions thereof which frequently occur in glycoproteins, were extensively studied by ^{1}H-NMR spectroscopy (DORLAND *et al.* 1978, SCHUT *et al.* 1978, LEGER *et al.* 1978, VLIEGENTHART *et al.* 1981, 1982). The N-acetyllactosamine

type branches can be terminated by Neu5Ac residues in α(2-6)- or α(2-3)-linkages to the Gal residues. Additional Neu5Ac-N-acetyllactosamine branches can be present at C4 of Man **4** and/or C6 of Man **4′**. In the ^{1}H-NMR spectrum the set of chemical shifts of H3eq and H3ax contains information concerning the type of linkage of the sialic acid. It can generally be stated that the sets of chemical shifts for H3eq and H3ax, given in Table 4, are discriminative for a Neu5Ac residue in α(2-6)- or α(2-3)-linkage to Gal.

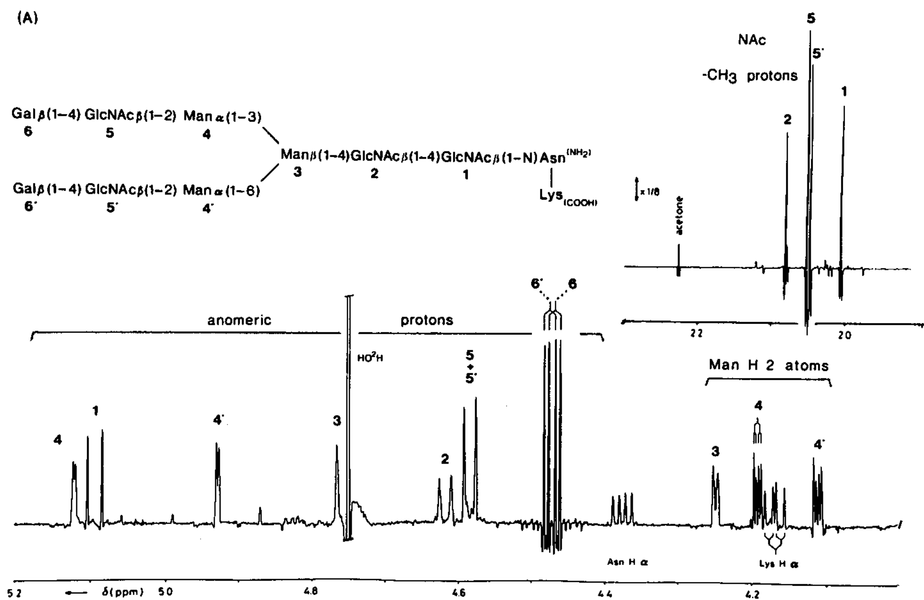


Fig. 6A

Fig. 6. Structural-reporter-group regions of the resolution-enhanced 500 MHz ^{1}H-NMR spectra of an asialo diantennary glycopeptide (*A*) and its (2-6)-sialylated analogue (*B*) (see next page) (^2H_2O; p[2]H 7; 27 °C). The bold numbers in the spectra refer to the corresponding residues in the structures. The relative-intensity scale of the acetyl methyl proton regions differs from that of the other parts of the spectra, as indicated.
The effects of extension of the N-acetyllactosamine branches with Neu5Ac in α(2-6)-linkage to galactose can be traced from comparison of spectra (*A*) and (*B*). Comparison is facilitated by the fact that sample (*B*) contains a small amount of compound (*A*), as is clearly inferred from the signals marked by asterisks.

To indicate the effects of extension of the N-acetyllactosamine branches with sialic acid, relevant data of the asialo-diantenna are used as a reference for mono- and disialo-compounds. The chemical shift alterations of structural-reporter-group signals in the diantenna due to attachment of two Neu5Ac residues in α(2-6)-linkage to Gal, can be traced from Fig. 6. Downfield shifts are observed for the anomeric proton signals of Man **4** and **4′** and for the anomeric proton signals of GlcNAc **5** and **5′**. The anomeric proton signals of Gal **6** and **6′** shift upfield. For monosialo-diantennae the changes in chemical shift are restricted to the branch which bears the Neu5Ac residue. The sialic acid bearing branch can easily be

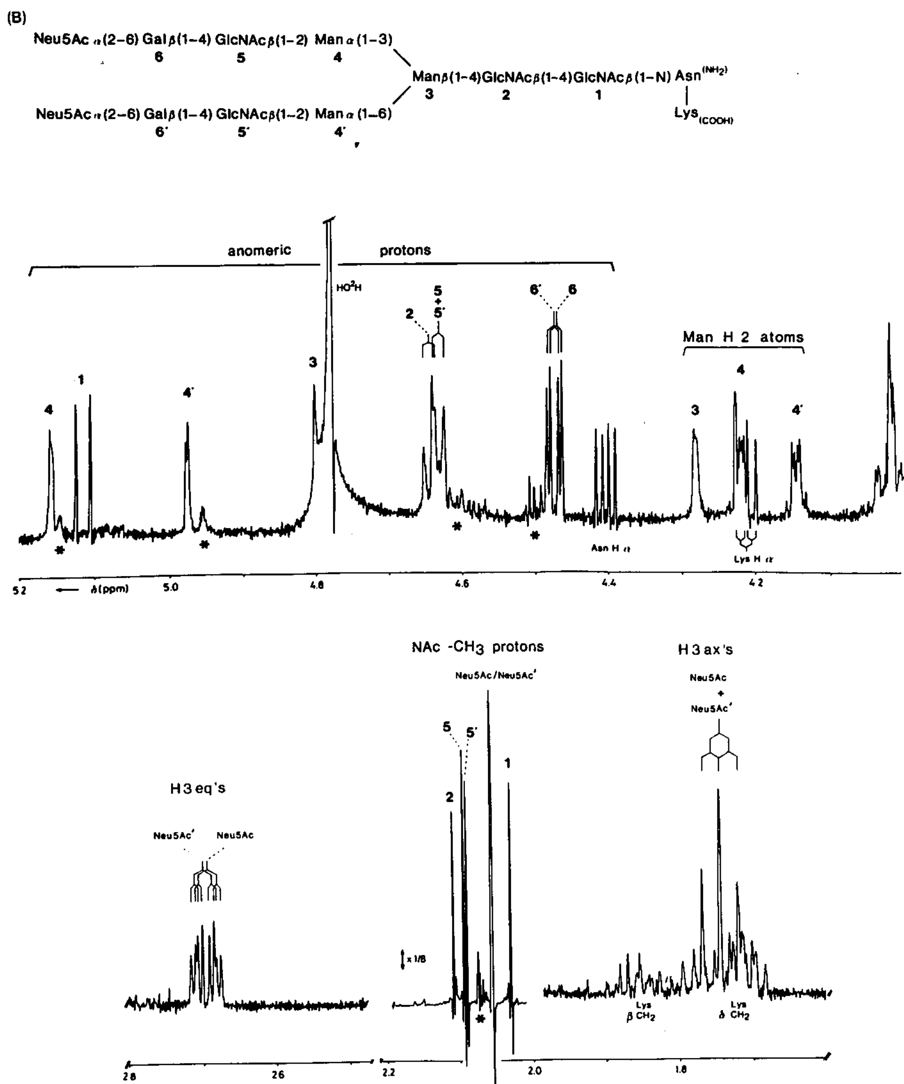


Fig. 6B

identified on the basis of the chemical shifts of H1 of the α-linked mannose residues (Man **4** and **4′**). With regard to monoantennary partial structures it should be noted that the chemical shifts of their structural reporter groups are virtually identical to those of the corresponding branch in the diantennary structure.

In contrast to the α(2-6)-linked sialic acid the α(2-3)-linked analogue influences only pronouncedly the chemical shifts of protons of the sugar residue to which it is attached. In case of a Neu5Ac α(2-3)Gal-linkage, the H1 of Gal shifts from δ 4.47 to δ 4.54 while the H3 of Gal shifts from δ 3.67 to δ 4.11. By consequence,

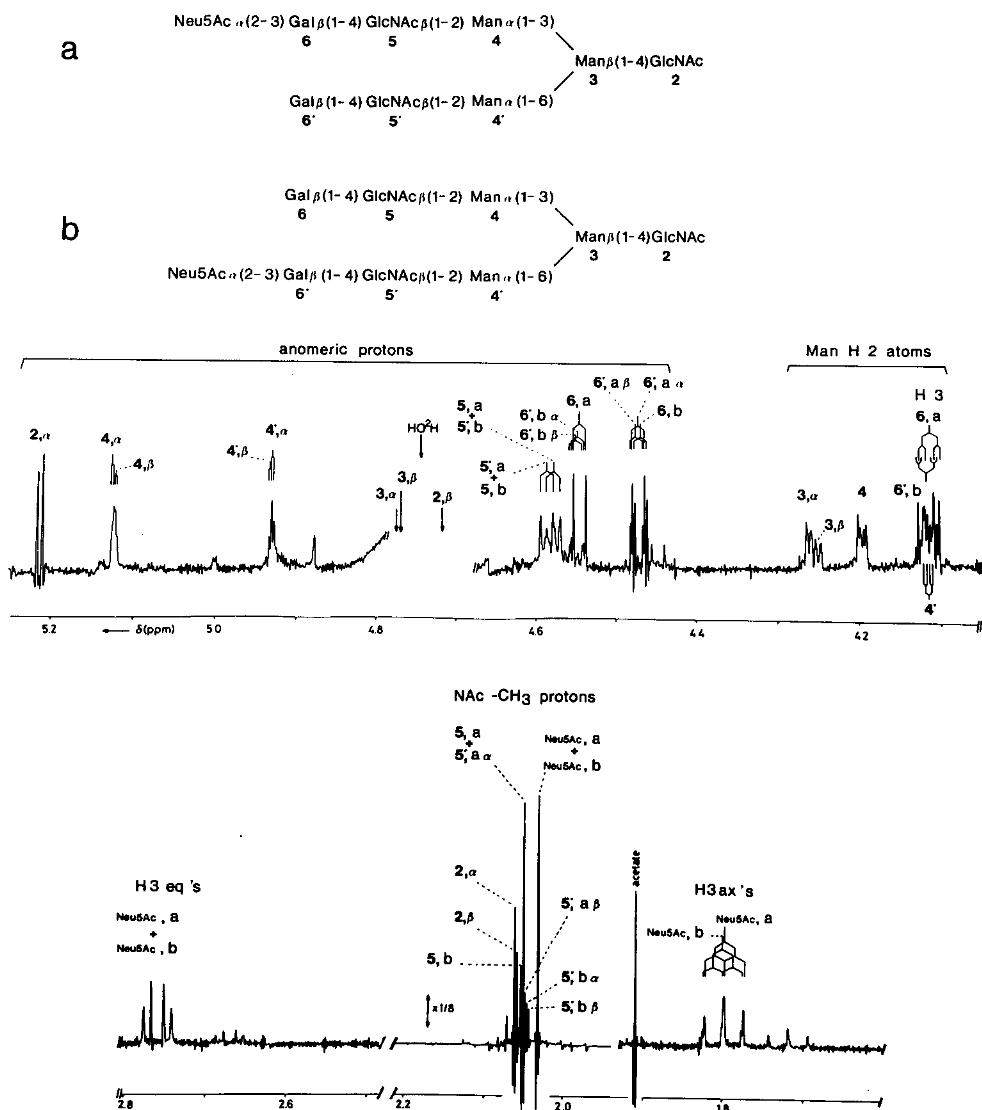


Fig. 7. Structural-reporter-group regions of the resolution-enhanced 500 MHz ^{1}H-NMR spectrum of a mixture containing monosialo diantennary oligosaccharides *a* and *b* in the ratio of 3 : 1 (^2H_2O; p^{2}H 7; 27 °C; anomeric ratio for both *a* and *b*, $\alpha:\beta = 2:1$). The bold numbers in the spectrum refer to the corresponding residues in the structures, the letters *a* and *b* to the compounds in the mixture. (E.g., anomeric proton signal designated as **6'**,*a*β means: H1 of Gal **6'** in the β-anomer of compound *a*.) Signals of corresponding protons in *a* and *b* coincide, unless indicated otherwise. The relative-intensity scale of the acetyl methyl proton region differs from that of the other parts of the spectrum. The HO^{2}H-resonance has been omitted; its position is indicated by an arrow.

in monosialo-diantennary compounds it is complicated to determine the branch to which the α(2-3)-linked Neu5Ac residue is attached. This problem can be solved in cases when the signals of the H1's of Gal **6** and **6'** can be assigned unambiguously (see Table 5). This depends on the quality of the sample and the resolution of the ^{1}H-NMR spectrum. Furthermore, as a result of Neu5Ac in α(2-3)-linkage to Gal, a slight effect occurs on the N-acetyl signal of the N-acetyllactosamine unit bearing the Neu5Ac residue. These small effects are useful

Table 5. *^{1}H-NMR chemical shift data, useful for the localization of α(2-6)- and α(2-3)-linked sialic acid in diantennary glycopeptides of the N-glycosidic N-acetyllactosamine type* (VLIEGENTHART *et al.* 1981, 1982)
For numbering of residues, see Figs. 6 and 7. n.d., value could not be determined

Reporter group	Chemical shift in		
	asialo branch	α(2-6)-sialylated branch	α(2-3)-sialylated branch
H1 of Gal **6**	4.467	4.442	4.544
H1 of Gal **6'**	4.473	4.447	4.548
H3 of Gal **6**	3.67	n.d.	4.113
H3 of Gal **6'**	3.67	n.d.	4.115
H1 of GlcNAc **5/5'**	4.582	4.603	4.578
NAc of GlcNAc **5**	2.050	2.069	2.048
NAc of GlcNAc **5'**	2.046	2.064	2.043
H1 of Man **4**	5.121	5.135	5.120
H1 of Man **4'**	4.928	4.946	4.926

in case when a mixture of monosialo-compounds has to be analysed. An example is given in Fig. 7, where the spectrum of a mixture of two isomeric monosialo-diantennary oligosaccharides is presented. The reducing character of the compounds implicates that protons in certain positions of the chain i.e. the H1's of GlcNAc **2**, Man **3**, **4**, and **4'**, Gal **6'** and the N-acetyl protons of GlcNAc **2** and **5'**, each show a double set of resonances. Despite of this complication the position of sialic acid can be derived from the H1 signals of the Gal residues and from the N-acetyl signals of the GlcNAc residues **5** and **5'**. The chemical shifts for the key resonances in various asialo-, mono- and disialo-structures are compiled in Table 5.

To illustrate the effect of sialic acid in higher-branched compounds of the N-acetyllactosamine type the asialo-triantenna is used as a reference. In the triantenna bearing three Neu5Ac residues, α(2-6)-linked to Gal, the effects of sialylation of Gal **6** and **6'** are similar to those observed in the corresponding diantenna. Compared with the spectrum of the diantenna the extra branch is

Table 6. *Pertinent* ^{1}*H-NMR chemical shift data for asialo and sialo triantennary structures of the N-glycosidic N-acetyllactosamine type* (VLIEGENTHART *et al.* 1981, 1982)

Reporter group	Chemical shift in				
	asialo glycopeptide **8**-**7** \\ **6**-**5**-**4** \\ **3**-**2**-**1**-Asn / | **6′**-**5′**-**4′** Lys	sialylated glycopeptide N*6-**8**-**7** \\ N6-**6**-**5**-**4** \\ **3**-**2**-**1**-Asn / | N′6-**6′**-**5′**-**4′** Lys	anomer of oligo-saccharide	asialo oligo-saccharide **8**-**7** \\ **6**-**5**-**4** \\ **3**-**2** / **6′**-**5′**-**4′**	sialylated oligo-saccharide N*3-**8**-**7** \\ N6-**6**-**5**-**4** \\ **3**-**2** / N′6-**6′**-**5′**-**4′**
H1 of Gal **6**	4.468	4.440	α, β	4.468	4.443
H1 of Gal **6′**	4.473	4.448	α	4.471	4.443
			β	4.473	4.447
H1 of Gal **8**	4.462	4.439	α, β	4.463	4.545
NAc of GlcNAc **5**	2.048	2.069	α, β	2.050	2.067
NAc of GlcNAc **5′**	2.045	2.065	α	2.048	2.067
			β	2.046	2.065
NAc of GlcNAc **7**	2.075	2.101	α, β	2.078	2.074
H3ax of NeuAc	—	1.717	α, β	—	1.720
H3ax of NeuAc′	—	1.717	α, β	—	1.717
H3ax of NeuAc*	—	1.706	α, β	—	1.801
H3eq of NeuAc	—	2.670	α, β	—	2.670
H3eq of NeuAc′	—	2.674	α, β	—	2.672
H3eq of NeuAc*	—	2.670	α, β	—	2.757

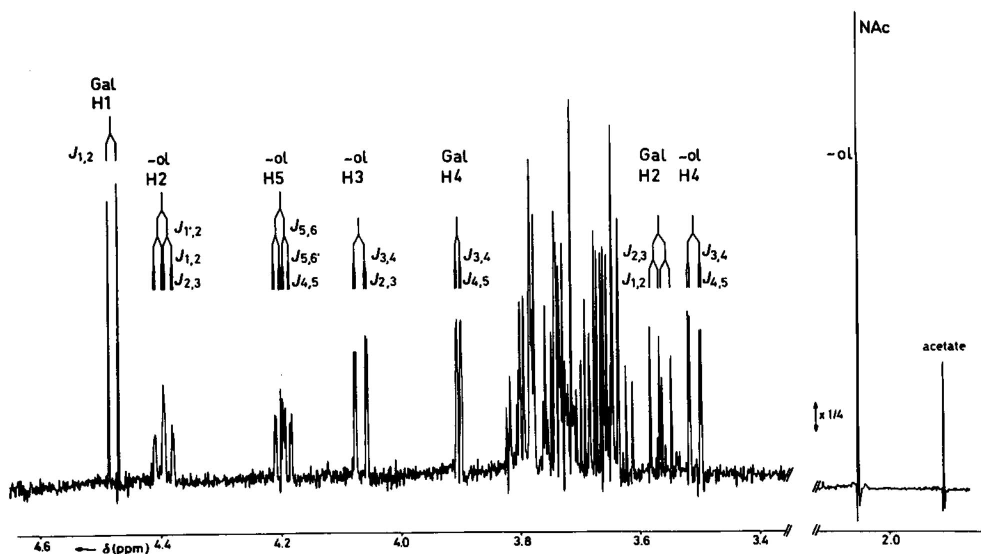


Fig. 8. Resolution-enhanced 500 MHz ^{1}H-NMR spectra of Galβ(1-3)GalNAc-ol (*A*) and Neu5Acα(2-3)Galβ(1-3)GalNAc-ol (*B*), dissolved in ^2H_2O, recorded at 27 °C and p^{2}H 7. Detailed splitting patterns are indicated for structural-reporter-group signals from residues other than Neu5Ac. The relative-intensity scale of the acetyl proton regions differs from that of the other parts of the spectrum, as indicated.

Table 7. [1]*H-NMR chemical shift data for Neu5Ac-containing oligosaccharide-alditols of the* VLIEGENTHART

Reporter group	Residue	Chemical shift in Galβ(1-3)-GalNAc-ol	Neu5Acα(2-3)-Galβ(1-3)-GalNAc-ol*	Neu5Acα(2-6)-GalNAc-ol*	Galβ(1-3)-[Neu5Acα(2-6)]-GalNAc-ol*
H3ax	Neu5Acα(2-3)	—	1.800	—	—
H3eq		—	2.774	—	—
5Ac		—	2.034	—	—
H3ax	Neu5Acα(2-6)	—	—	1.700	1.693
H3eq		—	—	2.728	2.729
5Ac		—	—	2.033	2.034
H1	Gal	4.478	4.547	—	4.475
H3		3.671	4.122	—	3.67
H2	GalNAc-ol	4.395	4.390	4.245	4.378
H3		4.065	4.074	3.842	4.058
H4		3.507	3.498	3.413	3.538
H5		4.196	4.187	4.024	4.245
H6		3.69	3.68	3.837	3.85
H6′		3.628	3.65	3.528	3.492
NAc		2.050	2.046	2.056	2.048

* For the analogues of these compounds containing Neu5Gc instead of Neu5Ac, see Table 8.

characterized by the structural-reporter-group signals of the constituting monosaccharides, in particular the H3 signals of Neu5Ac*, the H1 signal of Gal **8** and the N-acetyl signals of GlcNAc **7** and Neu5Ac*, as indicated in Table 6. For the trisialo-triantennary structure possessing a Neu5Ac residue in α(2-3)-linkage to Gal in the **7**-**8** branch, a downfield shift of H1 of Gal **8** is observed, in comparison with the asialo-analogue (see Table 6). So far, no NMR data have been described for Neu5Ac in other types of glycosidic linkages to N-acetyllactosamine structures.

c) Sialic Acid in O-Glycosidically Linked Carbohydrate Chains

Several types of O-glycosidically linked carbohydrate chains occur in glycoproteins. These carbohydrate chains are usually analysed in the form of oligosaccharide-alditols, prepared by alkaline borohydride reductive cleavage (CARLSON 1966). The obtained oligosaccharide-alditols have frequently in common:

Galβ(1-3)GalNAc-ol.

O-glycosidic mucin type and some reference substances (VAN HALBEEK *et al.* 1980, 1981 a, *et al.* 1981)

Chemical shift in		
Neu5Acα(2-3)-Galβ(1-3)[Neu5Acα(2-6)]-GalNAc-ol	Galβ(1-3)-[Galβ(1-4)GlcNAcβ(1-6)]-GalNAc-ol	Neu5Acα(2-3)Galβ(1-3)-[Galβ(1-4)GlcNAcβ(1-6)]-GalNAc-ol
1.800	—	1.801
2.774	—	2.774
2.032	—	2.033
1.692	—	—
2.723	—	—
2.032	—	—
4.541	4.465	4.534
	4.470	4.470
4.117	3.900	3.922
	3.925	3.931
4.378	4.394	4.390
4.067	4.060	4.072
3.524	3.465	3.456
4.240	4.282	4.272
3.84	3.931	3.927
3.475	3.7	3.7
2.042	2.067	2.066

This structural element can be extended with sialic acid in different ways:

α) in α(2-6)-linkage to GalNAc-ol
β) in α(2-3)-linkage to Gal
γ) in α(2-8)-linkage to the sialic acid residue mentioned under α)
δ) in α(2-3)-linkage to GalNAc, which is β(1-3)-linked to Galβ(1-3)GalNAc-ol.

The NMR parameters of a few of these compounds will be discussed. The 500 MHz ^{1}H-NMR spectrum of Galβ(1-3)GalNAc-ol, as shown in Fig. 8 *A*, could almost completely be interpreted. The H2, H3, H4, and H5 of GalNAc-ol, which resonate outside of the bulk, are suitable structural reporter groups in case of further substitution of this core structure. Extension with Neu5Ac in an α(2-3)-linkage to Gal gives the trisaccharide-alditol, the ^{1}H-NMR spectrum of which is shown in Fig. 8 *B*. Comparison of this spectrum with that of the disaccharide-alditol reveals downfield shifts for H1 and H3 of Gal (VAN HALBEEK *et al.* 1980) and a small but significant upfield shift for the N-acetyl protons of GalNAc-ol. Similar effects have been observed for Neu5Acα(2-3)Galβ(1-3)[Galβ(1-4)GlcNAcβ(1-6)]GalNAc-ol

when compared to its asialo-analogue (VAN HALBEEK *et al.* 1981 a). The Neu5Ac residue itself is characterized by the set of chemical shifts of H3ax, H3eq, and 5Ac (see Table 7).

Further extension of the aforementioned trisaccharide with a Neu5Ac residue α(2-6)-linked to GalNAc-ol affords the following tetrasaccharide-alditol:

Neu5Acα(2-3)Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol.

The additional Neu5Ac residue affects the chemical shifts of H4, H5, and H6′ of GalNAc-ol (see Table 7). The signal of H6′ of GalNAc-ol is now situated apart from the bulk as a result of the substitution at C6 of GalNAc-ol. Furthermore, attachment of Neu5Acα(2-6) to GalNAc-ol introduces a specific shift of the N-acetyl protons of GalNAc-ol, when compared with the trisaccharide-alditol Neu5Acα(2-3)Galβ(1-3)GalNAc-ol. The Neu5Ac residue in α(2-6)-linkage to GalNAc-ol is characterized by the additional set of chemical shifts of its H3 protons (see Table 7).

Fig. 9 shows the ^{1}H-NMR spectrum of a pentasaccharide-alditol containing the bloodgroup A determinant. The NMR parameters of this compound and of its partial structures are presented in Table 8 (VAN HALBEEK *et al.* 1981 b). These compounds were derived from glycoproteins with A^+, H^+ or A^-H^- immunological properties. No structural differences could be found between the oligosaccharide-alditols of the corresponding A^+, H^+, and A^-H^- series, except for the pentasaccharide-alditol which was only present in the A^+ series. The set of chemical shifts for H3eq (δ 2.745) and H3ax (δ 1.721) of Neu5Gc, is indicative of the α(2-6)-linkage of Neu5Gc to GalNAc-ol, bearing no other substituents. This type of attachment of sialic acid to GalNAc-ol is further reflected by oppositely directed shifts for H6 and H6′ of GalNAc-ol and the change in geminal coupling constant $J_{6,6'}$ from −11.7 Hz to −9.8 Hz. Attachment of Gal in β(1-3)-linkage to GalNAc-ol introduces a characteristic upfield shift for H3ax (Δδ −0.008). A similar feature can be observed for the Neu5Ac analogues, as is evident from Table 7 (*cf.* Neu5Acα(2-6)GalNAc-ol, Galβ(1-3)[Neu5Acα(2-6)]GalNAc-ol). The presence of the Fuc and GalNAc residues does not affect the structural-reporter-group signals of Neu5Gc and GalNAc-ol.

A type of sialo-oligosaccharide-alditols, obtained from trout eggs and having in common the following pentasaccharide structure:

GalNAcβ(1-4)Galβ(1-4)GalNAcβ(1-3)Galβ(1-3)GalNAc-ol

were investigated by INOUE *et al.* (1981) and NOMOTO *et al.* (1981) using 270 MHz ^{1}H-NMR spectroscopy. Neu5Gc can be attached to GalNAc-ol in α(2-6)- and/or in α(2-3)-linkage to the internal GalNAc. Furthermore, the former Neu5Gc can be elongated with one or more Neu5Gc residues each in α(2-8)-linkage. The characteristic ^{1}H-NMR data of this class of compounds are compiled in Table 9.

d) CMP-N-acetylneuraminic Acid and Degradation Products

A key intermediate in the biosynthesis of glycoconjugates is the sugar nucleotide cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac). To check its

identity ^{1}H-NMR spectroscopy can effectively be used (HAVERKAMP *et al.* 1979 a). The 360 MHz ^{1}H-NMR spectrum of this compound is given in Fig. 10 and the NMR parameters are compiled in Table 10. The chemical shifts for H3eq, H4 and the N-acetyl protons of the Neu5Ac residue point to β-configuration of the glycosidic linkage (see Tables 3 and 10). Another proof for the configuration of the glycosidic bond is found in the heteronuclear long-range coupling constants $^4J_{H3ax,P}$ (6.1 Hz) and $^4J_{H3eq,P}$ (0 Hz) indicating a planar "W"-shape of the bonds in

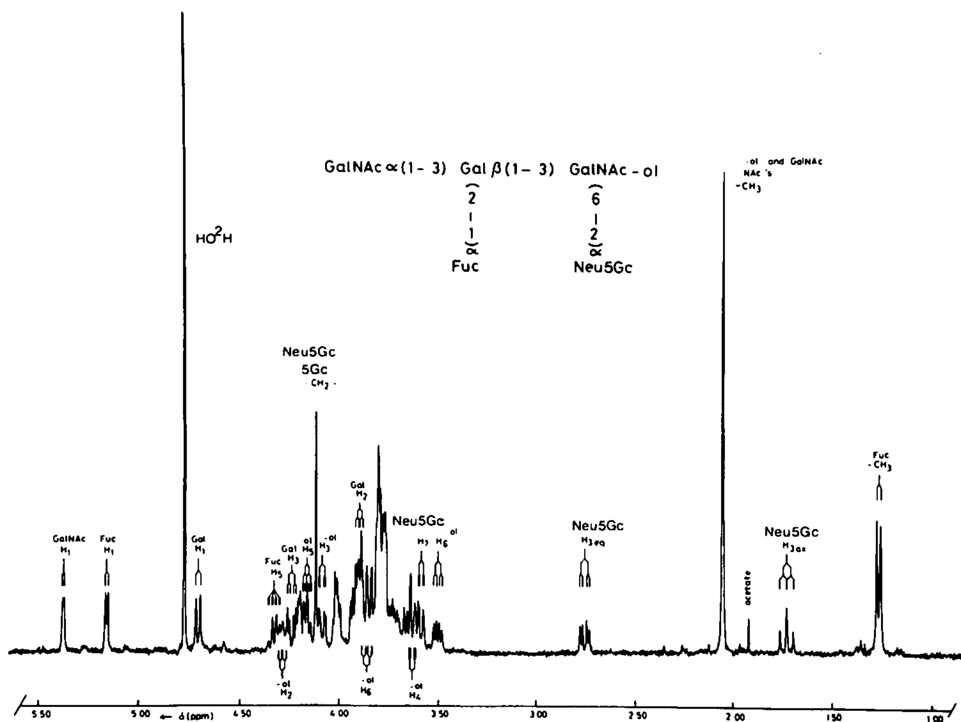


Fig. 9. 360 MHz ^{1}H-NMR spectrum of a mucin-type acidic pentasaccharide-alditol containing the bloodgroup-A determinant, dissolved in ^2H_2O, recorded at p^{2}H 7 and 25 °C.

the H3ax-C3-C2-O-P system ("all trans") (DAVIES and DANYLUK 1974, 1975, LEE and SARMA 1976). These long-range coupling constants provide information on the spatial structure of the compound in aqueous solution. Another approach for the determination of the β-configuration of the glycosidic linkage, based on the heteronuclear vicinal coupling constants $^3J_{C1,H3ax}$ and $^3J_{C1,H3eq}$, will be discussed in section G.III.5.

In studies on the chemical stability of CMP-Neu5Ac in dependence on the pH two interesting decomposition products were observed, *viz.* β-N-acetylneuraminic acid-2-phosphate (Neu5Ac2P) and 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en) (BEAU *et al.*, unpublished results). Their ^{1}H-NMR spectra are given in Fig. 11 and 12, respectively. The presence of a phosphate group in Neu5Ac2P

Table 8. *Relevant ^{1}H-NMR chemical shift data for Neu5Gc-containing oligosaccharide-*

Reporter group	Residue	Chemical shift in			
		GalNAc-ol	Neu5Gcα(2-6)-GalNAc-ol	Galβ(1-3)-GalNAc-ol	Neu5Gcα(2-3)-Galβ(1-3)-GalNAc-ol
H3ax	Neu5Gcα(2-3)	—	—	—	1.817
H3eq		—	—	—	2.787
5Gc		—	—	—	4.122
H3ax	Neu5Gcα(2-6)	—	1.721	—	—
H3eq		—	2.746	—	—
5Gc		—	4.124	—	—
H1	Gal	—	—	4.478	4.547
H3		—	—	3.671	4.132
H2	GalNAc-ol	4.255	4.253	4.395	4.389
H3		3.847	3.842	4.065	4.073
H4		3.385	3.416	3.507	3.495
H5		3.928	4.025	4.196	4.188
H6		3.67	3.842	3.69	3.68
H6′		3.64	3.536	3.628	3.65
NAc		2.055	2.057	2.050	2.045

comes to expression in an additional splitting of the H3ax signal as a result of long-range coupling between P and H3ax. The criteria used for the determination of the β-glycosidic linkage as described for CMP-Neu5Ac (i.e. δ H3eq, H4 and N-acetyl; $^4J_{H3,P}$) also hold for this compound (see Table 10).

The ^{1}H-NMR data of Neu5Ac2en are compiled in Table 10. The chemical shifts depend strongly on the pH. The double bond formed in the decomposition reaction of CMP-Neu5Ac, is characterized by the doublet near δ 5.7 which is ascribed to H3. The small value observed for $^3J_{3,4}$ is an indication for an almost coplanar orientation of the ring-O-C2-C3-C4 part of the six-membered ring. The other proton-proton coupling constants are similar to those observed in Neu5Ac (Table 1), in CMP-Neu5Ac and in Neu5Ac2P (Table 10). This leads to the conclusion that the conformation of the glycerol side chain, including the hydrogen bridges, is virtually unaltered with respect to the aforementioned compounds. In view of its property to act as an inhibitor of sialidase (MEINDL *et al.* 1974, KAMERLING *et al.* 1975, HAVERKAMP *et al.* 1976; KAMERLING *et al.* 1979) it is relevant to have methods available for the accurate determination of the concentration of Neu5Ac2en in biological samples. High-resolution ^{1}H-NMR

alditols of the O-glycosidic mucin type and for some reference compounds (VAN HALBEEK *et al.* 1981 b)

Chemical shift in				
Galβ(1-3)-[Neu5Gcα(2-6)]-GalNAc-ol	Fucα(1-2)-Galβ(1-3)-GalNAc-ol	Fucα(1-2)-Galβ(1-3)-[Neu5Gcα(2-6)]-GalNAc-ol	GalNAcα(1-3)-[Fucα(1-2)]-Galβ(1-3)-GalNAc-ol	GalNAcα(1-3)-[Fucα(1-2)]-Galβ(1-3)-[Neu5Gcα(2-6)]-GalNAc-ol
—	—	—	—	—
—	—	—	—	—
—	—	—	—	—
1.711	—	1.714	—	1.711
2.746	—	2.747	—	2.750
4.123	—	4.122	—	4.123
4.477	4.584	4.586	4.720	4.719
3.669	3.880	3.870	4.254	4.248
4.380	4.398	4.386	4.302	4.291
4.061	4.089	4.087	4.100	4.089
3.541	3.520	3.545	3.606	3.624
4.249	4.162	4.226	4.125	4.185
3.860	3.68	3.84	3.68	3.862
3.497	3.63	3.484	3.64	3.493
2.049	2.046	2.043	2.048	2.046

spectroscopy allows the detection of Neu5Ac2en down to concentrations of about 2% in mixtures of sialic acids. As marker signals for this determination the N-acetyl singlet at δ 2.068 and the doublet of H3 at δ 5.690 are used.

4. *Enzymic and Chemical Conversions*

^{1}H-NMR spectroscopy is suitable for following the course of reactions either directly in the NMR-tube or by analysis of isolated reaction products. Simultaneous measurements of substrate and product concentrations allows under proper conditions the deduction of kinetic reaction parameters (FRIEBOLIN *et al.* 1981 c). Alternatively, the formed products can be isolated and analysed separately by NMR spectroscopy. For example in the saponification of sialic acid methyl esters under mild alkaline conditions both types of approaches are useful (HAVERKAMP *et al.* 1982). The potency of NMR spectroscopy as a real-time analytical probe is in particular evident in the study of enzyme reactions.

^{1}H-NMR evidence for the release of αNeu5Ac as the primary product of sialidase action on various natural and synthetic sialoglycoconjugates was obtained by FRIEBOLIN *et al.* (1980 b, 1981 d). This initial formation of the α-

Table 9. *Some pertinent* [1]*H-NMR chemical shift data for polysialosyl oligosaccharide-alditols of the O-glycosidic mucin type, and for some reference substances* (NOMOTO *et al.* 1981)

Compound	Chemical shift		
	H3ax	H3eq	5Gc
Galβ(1-3)[Neu5Gcα(2-6)]GalNAc-ol*	1.769	2.737	4.125
Galβ(1-3)[Neu5Gcα(2-6)]GalNAc-ol	1.660	2.659	n.d.
8 | 2 α Neu5Gc	1.743	2.779	n.d.
Galβ(1-3)[Neu5Gcα(2-6)]GalNAc-ol	1.668	2.659	n.d.
8 | 2 α Neu5Gc	1.711	2.709	n.d.
8 | 2 α Neu5Gc	1.756	2.779	n.d.
GalNAcβ(1-4)Galβ(1-4)GalNAcβ(1-3)Galβ(1-3)GalNAc-ol			
3 | 2 α Neu5Gc	1.855	2.554	4.120
GalNAcβ(1-4)Galβ(1-4)GalNAcβ(1-3)Galβ(1-3)[Neu5Gcα(2-6)]GalNAc-ol	1.669	2.659	n.d.
8 | 2 α Neu5Gc	1.743	2.780	n.d.
3 | 2 α Neu5Gc	1.865	2.559	n.d.

Table 9 (Continued)

Compound	Chemical shift		
	H3ax	H3eq	5Gc
GalNAcβ(1-4)Galβ(1-4)GalNAcβ(1-3)Galβ(1-3)[Neu5Gcα(2-6)]GalNAc-ol	1.672	2.660	n.d.
3 (of Galβ(1-4)); 8 (of Neu5Gcα(2-6)) — 2 — α Neu5Gc	1.714	2.709	n.d.
8 — 2 — α Neu5Gc	1.757	2.780	n.d.
2 — α Neu5Gc (attached to 3 of Galβ(1-4))	1.856	2.560	n.d.

* For unclarified reasons, data for this compound are not in accordance with those in Table 8 (VAN HALBEEK *et al.* 1981b).

n.d. = value not given in quoted reference (NOMOTO *et al.* 1981).

Fig. 10. 360 MHz ^{1}H-NMR spectrum of CMP-Neu5Ac (as triethylammonium-salt) dissolved in ^2H_2O, recorded at p^{2}H 8 and 25 °C. Multiplets at δ 1.3 and 3.2 represent ethyl groups of triethyl ammonium counter ions.

Table 10. *Relevant* ^{1}H*-NMR data for CMP-Neu5Ac* (Haverkamp *et al.* 1979a), *Neu5Ac2P and Neu5Ac2en*
Chemical shift values are given in ppm relative to DSS for solutions in $^{2}H_2O$ at 25 °C and at the indicated p^2H values. Coupling constants (J) are given in Hz

Compound	p^2H	Chemical shift									
		H3ax	H3eq	H4	H5	H6	H7	H8	H9	H9′	5Ac
CMP-Neu5Ac	8	1.639	2.484	4.066	3.92	4.141	3.456	3.92	3.90	3.622	2.054
Neu5Ac2P	7.5	1.548	2.403	4.093	3.888	4.239	3.386	4.028	3.883	3.581	2.045
Neu5Ac2en*	6	—	5.690	4.470	4.051	4.213	3.601	3.936	3.885	3.646	2.068

Compound	p^2H	Coupling constant											
		$^{2}J_{3ax,3eq}$	$^{4}J_{3ax,P}$	$^{4}J_{3eq,P}$	$^{3}J_{3ax,4}$	$^{3}J_{3eq,4}$	$^{3}J_{4,5}$	$^{3}J_{5,6}$	$^{3}J_{6,7}$	$^{3}J_{7,8}$	$^{3}J_{8,9}$	$^{3}J_{8,9'}$	$^{2}J_{9,9'}$
CMP-Neu5Ac	8	—13.4	6.1	< 1.0	11.6	5.0	10.4	10.5	1.2	9.7	2.8	6.2	—11.7
Neu5Ac2P	7.5	—12.8	4.2	< 1.0	11.2	4.8	9.1	10.5	< 1.0	9.6	2.6	6.9	—11.7
Neu5Ac2en*	6	—	—	—	—	2.3	8.8	10.9	1.0	9.3	2.8	6.3	—11.9

* For comparison with the data for Neu5Ac2en1Me, see Kumar *et al.* 1981.

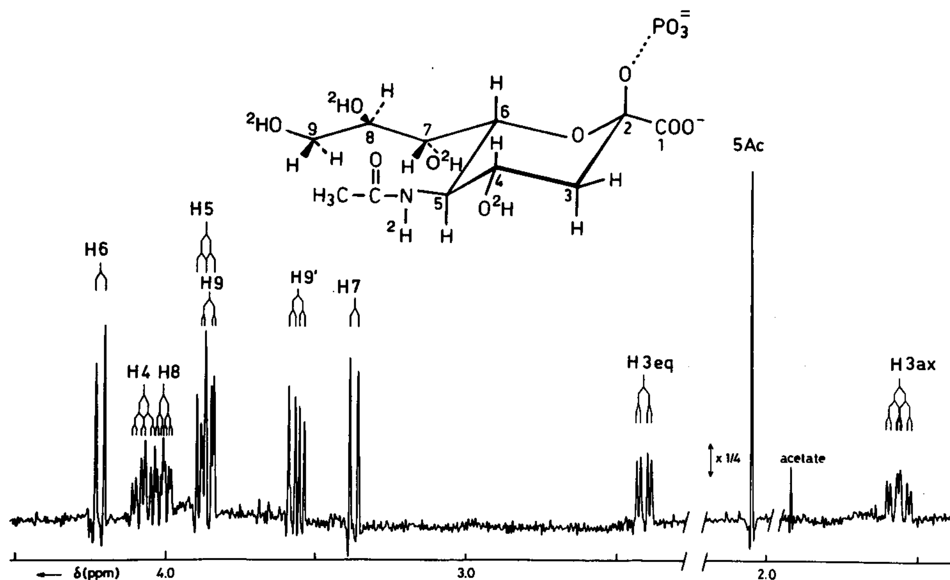


Fig. 11. Resolution-enhanced 360 MHz ^{1}H-NMR spectrum of β-N-acetylneuraminic acid-2-phosphate (βNeu5Ac2P) dissolved in $^{2}H_2O$, recorded at p^{2}H 7.5 and 25 °C. The relative-intensity scale of the 5Ac methyl proton signal differs from that of the other parts of the spectrum, as indicated.

Fig. 12. Resolution-enhanced 360 MHz ^{1}H-NMR spectrum of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en) dissolved in $^{2}H_2O$, recorded at p^{2}H 6 and 25 °C. The relative-intensity scale of the 5Ac methyl proton signal differs from that of the other parts of the spectrum, as indicated.

anomer offers the possibility to study the kinetics of the mutarotation by means of ^{1}H-NMR spectroscopy. FRIEBOLIN *et al.* (1980a, 1981a) also studied the anomerisation in dependence on the pH.

The positional specificity of sialidases can be monitored by the action of the enzymes on mixtures of isomeric sialyllactoses (FRIEBOLIN *et al.* 1981c). In the same study also kinetic parameters of these enzyme reactions were determined. For sialidase isolated from Newcastle disease virus PAULSON *et al.* (1982) demonstrated by 500 MHz ^{1}H-NMR spectroscopy that this enzyme hydrolyzes preferentially sialic acid residues α(2-3)-linked to α_1-acid glycoprotein.

The specificity of a colostrum sialyltransferase was investigated by VAN DEN EIJNDEN *et al.* (1980) using glycopeptides derived from asialo-α_1-acid glycoprotein as substrates. The positional specificity as well as the branch specificity of the enzyme were studied.

5. *Complexes of Sialic Acids with Ca^{2+}*

^{1}H-NMR studies on the interaction of Neu5Ac and Neu5Gc with Ca^{2+} ions have been carried out by JAQUES *et al.* (1977, 1980a). Induced proton shifts are observed in the NMR spectra of sialic acids upon increasing the concentration of $CaCl_2$ (the Ca^{2+}/sialic acid ratios varied from 0 to 6.0). The direction of all of the Ca^{2+}-induced chemical shifts is the same for Neu5Ac and Neu5Gc, indicating that the geometries in the two complexes are similar. The signs of the shifts roughly determine the position of the calcium ion relative to the ring and side chain protons. The downfield shifts of H3eq, H4, and H6 and the upfield shift of H3ax indicate that the ion is above the ring. The relatively large downfield Ca^{2+}-induced shifts of H7 and H8 suggest that these protons are closest to the calcium ion but with their C-H bond directions pointing away from it. The major difference in the Ca^{2+}-Neu5Gc complex, when compared to the Ca^{2+}-Neu5Ac complex, is that H7 is somewhat less deshielded. The extra binding of Ca^{2+} to the hydroxyl group of the glycolyl chain as was postulated on the basis of molecular (CPK) model building studies could not clearly be inferred from ^{1}H- and ^{13}C-NMR chemical shift and coupling data. The coupling constants $^{3}J_{7,8}$, $^{3}J_{8,9}$, and $^{3}J_{8,9'}$ have undergone significant changes upon complexation with Ca^{2+}. This indicates a change in the conformation of the glycerol side chain by which the interaction with the ion is enhanced. The coupling constants of the pyranose ring protons remain unaltered, which demonstrates that the $^{2}C_5$ conformation of the ring does not change upon addition of Ca^{2+}. The approximate position of the Ca^{2+} ion in the Ca^{2+}-sialic acid complex was determined from the Ca^{2+}-induced shifts by using Buckingham's electric-field-shift theory (BUCKINGHAM 1960). For the Ca^{2+}-Neu5Ac complex this theory leads to a model, which is presented in Fig. 18 (section G.III.6). The model is corroborated by ^{13}C-NMR spectroscopy (JAQUES *et al.* 1977, 1980a). As pointed out by BEHR and LEHN (1973), the glycerol side chain is in a position to effectively "wrap around the Ca^{2+}".

III. ^{13}C-NMR Spectroscopy

1. *N-Acetyl- and N-Glycolylneuraminic Acid*

^{13}C-NMR spectroscopy is another frequently used technique for structure analysis of biomolecules, however, requiring at least 10-100 times more substance.

Carbon spectra provide direct information about the molecular skeleton. Furthermore, ^{13}C resonances are stronger influenced than ^{1}H resonances by neighbouring dipoles and/or electric charges. Hence the ^{13}C signals are observed in a broad range of frequencies and are sensitive to changes in the substitution pattern, molecular conformation and interaction with other compounds. With regard to structural studies of sialic acids, an advantage of ^{13}C-NMR over ^{1}H-NMR is that C1 and C2 which do not bear protons can directly be observed. The

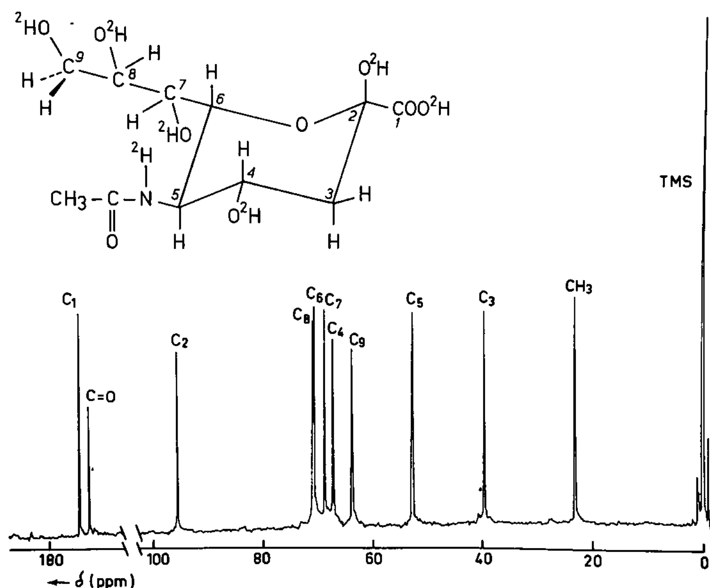


Fig. 13. Proton-noise-decoupled 25 MHz ^{13}C-NMR spectrum of Neu5Ac dissolved in $^{2}H_2O$, recorded at p^2H ~ 2 and 25 °C. Only the signals stemming from the β-anomer are clearly observable.

^{13}C-NMR spectrum of Neu5Ac is shown in Fig. 13. This proton noise-decoupled spectrum shows 11 intense singlets. Assignment of the resonances was made by comparison with spectral data of carbon atoms in similar chemical environments of model compounds and by various proton decoupling techniques (Bhattacharjee *et al.* 1975). The relatively low intensities for the carbonyl carbons and for C2 are due to the restricted nuclear Overhauser enhancement as a result of the absence of proton substituents at these carbon atoms. In addition, the spectrum shows several small resonances (see Table 11) belonging to the αNeu5Ac anomer which is present for ~ 9% in the equilibrium mixture (Jaques *et al.* 1977).

It has to be noted that the spectrum of Fig. 13 has been recorded with TMS as an external standard. Addition of an internal standard, e.g. the sodium salt of 2,2,3,3-tetradeutero-3-(trimethyl)propionic acid as applied by Eschenfelder *et al.* (1975) in a study of a series of monomeric αNeu5Ac and βNeu5Ac derivatives (glycosides and esters), can seriously affect several of the carbon resonances if the

Table 11. ^{13}C-NMR chemical shift data
Chemical shifts are given in ppm downfield from external TMS for

Compound	p²H	T	Chemical shift					
			C1	C2	C3	C4	C5	C6
βNeu5Ac	1.6	25 °C	176.06	96.46	40.00	67.88	53.26	71.34
βNeu5Ac	7	25 °C	177.87	97.61	40.63	68.51	53.50	71.45
αNeu5Ac	7	25 °C	n.d.	98.42	41.94	69.38	53.07	72.75
βNeu5Ac	7	37 °C	176.0	97.6	40.6	68.5	53.5	71.5*
βNeu5Ac	n.d.	37 °C	176.0	96.4	39.9	67.8	53.2	71.3*
βNeu5Gc	7	25 °C	178.08	97.65	40.61	68.18	53.18	71.20
βNeu5Gc	n.d.	37 °C	176.8	96.4	40.0	67.6	52.9	71.5
Neu5Ac2en	n.d.	28 °C	169.7	148.1	107.8	67.7	50.1	75.5

* Assignments may be interchanged.

addition of internal standard leads to ionization of the carboxyl group. In comparison to the spectrum of the protonated Neu5Ac (pH ~ 2) the signal of C1 shifts downfield (Δδ 1.8; see Table 11). Downfield shifts for carboxyl group carbons are generally observed when going from the free acid to the corresponding carboxylate anion.

Furthermore, significant chemical shift differences between α- and β-anomers are observed for C4 and C6 as a result of 1,3-diaxial interactions between the protons in these positions and the anomeric centre (BHATTACHARJEE *et al.* 1975). The resonance positions of C2, C3, C5, C7, and C9 show only minor differences between α- and β-anomers, whereas C1 and C8 occupy intermediate positions in this respect. The ^{13}C resonances for βNeu5Ac and βNeu5Gc in aqueous solutions are compiled in Table 11. Hydroxylation of the CH_3 group of the N-acetyl substituent results in two alterations in the ^{13}C-NMR spectrum: a strong downfield shift for the resonance of the involved carbon and a small downfield shift for the neighbouring carbonyl carbon.

2. *O-Acetylated Neuraminic Acid Derivatives*

The effect of O-acetylation at various positions of the sialic acid skeleton has been studied for aqueous solutions (BHATTACHARJEE *et al.* 1975). The main effects induced by O-acetylation at C9 of Neu5Ac are the downfield shift of 3.1 ppm for C9 and the upfield shift of -3.1 ppm for C8. In methanol as solvent the directions and magnitudes of these shifts are similar (HAVERKAMP *et al.* 1975); for βNeu4,5,9Ac$_3$1,2Me$_2$ O-acetylation at C4 causes a downfield shift of 2.9 ppm for C4 and upfield shifts of -3.3 and -3.4 ppm for C3 and C5, respectively. These

for Neu5Ac, Neu5Gc, and Neu5Ac2en
solutions in ^2H_2O at various temperature and p^2H values

Chemical shift						References
C7	C8	C9	5Ac (Me)	5Gc (CH_2)	5Ac/Gc (C=O)	
69.43	71.62	64.37	23.27	—	174.49	JAQUES *et al.* 1977
69.82	71.59	64.55	23.34	—	175.98	JAQUES *et al.* 1977, 1980 a
69.38	73.77	64.10	n.d.	—	n.d.	JAQUES *et al.* 1977
69.6	71.7*	64.5	23.3	—	176.0	JENNINGS *et al.* 1977 b
69.4	71.5*	64.3	23.2	—	174.3	BHATTACHARJEE *et al.* 1975
69.63	71.62	64.47	—	62.25	176.85	JAQUES *et al.* 1980 a
69.3	71.5	64.3	—	62.2	174.3	BHATTACHARJEE *et al.* 1975
68.4	70.0	63.3	22.4	—	174.9	CZARNIECKI and THORNTON 1977 a

observations from methanolic solutions may be helpful to predict the magnitude and direction of the acetylation shifts for directly bound and neighbouring carbon atoms in aqueous solutions.

3. *Polysaccharides*

^{13}C-NMR spectra are especially useful for studying the structure of sialic acid polymers because ^{1}H-NMR spectra of such polymers have rather bad resolution (*cf.* EGAN *et al.* 1977). The capsular polysaccharides of *Neisseria meningitidis* serogroups B, C, W-135, Y, BO and the *Escherichia coli* capsular polysaccharides K1 and K92 (strains Bos 12 (O16K92NM), N67 and MT411) were investigated by BHATTACHARJEE *et al.* (1975), EGAN *et al.* (1977), JENNINGS *et al.* (1977 a, b) and LIU *et al.* (1977). The aim of these studies was to establish relations between structure and effectiveness of the polysaccharide antigens as vaccines. The meningococcal serogroups B and C and the *E. coli* K92 polysaccharides are polymers of Neu5Ac with α(2-8)-, α(2-9)- and alternating α(2-8)-, α(2-9)-linkages, respectively. Those of groups W-135 and Y are composed of α(2-6)-linked disaccharide repeating units Galα(1-4)Neu5Ac and Glcα(1-4)Neu5Ac, respectively. The serogroup C, W-135 and Y polysaccharides can partially be O-acetylated to a degree depending on the strain and growth conditions.

Signal assignment in the proton noise-decoupled spectra of the various polysaccharides was made on the basis of comparison with each other and with appropriate sialic acids and hexose derivatives as model compounds. ^{13}C-NMR spectral data of the various polymers and relevant model compounds are compiled in Table 12. The meningococcal serogroup B polysaccharide shows a simple spectrum (see Fig. 14 *a*) consisting of 11 singlets (BHATTACHARJEE *et al.* 1975). This

Table 12. ^{13}*C-NMR chemical shift data for*
Chemical shifts are given in ppm downfield from external

Compound	p^2H	T	Linkage type	Chemical shift			
				C1	C2	C3	C4
Neisseria meningitidis Serogroup B polysaccharide	7	37 °C	α(2-8)	174.4	102.1	40.9	69.4*
Escherichia coli K1 capsular polysaccharide	7	37 °C	α(2-8)	174.4	102.2	41.0	69.4*
Neisseria meningitidis Serogroup C polysaccharide [a]	<7	37 °C	α(2-9)	172.5	100.1	40.6	68.7
Neisseria meningitidis Serogroup C polysaccharide [a]	7	37 °C	α(2-9)	174.9	101.4	41.2	69.5
Escherichia coli K92 capsular polysaccharide	7	35 °C	α(2-9)	174.5	101.9	40.9	69.8*
	7	35 °C	α(2-8)	174.5	102.0	41.1	69.8
αNeu5Ac2Me	<7	37 °C	—	172.3	100.3	40.0	68.5*
αNeu5Ac2Me	7	37 °C	—	174.6	101.9	41.3	69.5
βNeu5Ac2Me	<7	37 °C	—	174.1	100.6	40.6	67.8
βNeu5Ac2Me	7	37 °C	—	175.9	101.7	41.1	68.4
Neu5Acα(2-9)Neu5Ac	7	37 °C	nr.	174.9	101.6	41.0	69.1*
			r.	175.8*	97.5	40.3	68.3

* Values may be interchanged.
[a] O-deacetylated.
nr. = non reducing unit; r. = reducing unit.

indicates a linear chain with one type of glycosidic linkage. The α(2-8)-linkages introduce characteristic downfield shifts for C2 (1.8 ppm) and C8 (6.8 ppm) with respect to the corresponding resonances in αNeu5Ac2Me. The upfield shift of the C9 resonance (—1.9 ppm) indicates its position vicinal to the glycosidic linkage. Shift effects for the other carbons are less pronounced but fit with the presence of α(2-8)-linkages between the Neu5Ac residues. The α-configuration of the glycosidic linkages was deduced from the chemical shifts of C1, C4, and C6 (see Table 12). The ^{13}C-NMR data for the *E. coli* K1 capsular polysaccharide (colominic acid) match exactly with those described above for the *N. meningitidis* serogroup B capsular polysaccharide indicating identical structures (BHATTACHARJEE *et al.* 1975, JENNINGS *et al.* 1977 a, b, LIU *et al.* 1977). However,

Neu5Ac-polymers and for some model compounds
TMS for solutions in ^2H_2O at the indicated p^{2}H values

Chemical shift								References
C5	C6	C7	C8	C9	5Ac (Me)	5Ac (C=O)	2Me	
53.6	74.3	70.4*	78.8	62.4	23.6	176.1	—	BHATTACHARJEE *et al.* 1975
53.7	74.4	70.6*	78.6	62.5	23.7	176.1	—	BHATTACHARJEE *et al.* 1975
53.1	73.9	69.3	70.9	66.6	23.4	176.1	—	JENNINGS *et al.* 1977b
53.0	73.6	69.5	71.4	66.3	23.3	176.1	—	BHATTACHARJEE *et al.* 1975
52.8	73.5	69.0*	71.5	62.4	23.3	175.9	—	EGAN *et al.* 1977
53.5	74.5	70.4	78.8	66.2	23.5	176.0	—	
52.9	74.0	69.5*	72.0	64.3	23.2	176.1	52.7	BHATTACHARJEE *et al.* 1975
53.2	73.8	69.5	72.9	63.9	23.3	176.3	52.8	JENNINGS *et al.* 1977b
53.1	71.2*	69.4	71.8*	64.7	23.3	176.1	52.1	BHATTACHARJEE *et al.* 1975, JENNINGS *et al.* 1977b
53.3	71.4	69.7	71.4	64.8	23.4	176.0	51.8	JENNINGS *et al.* 1977b
53.0	73.6	69.4*	72.8	63.8	23.1	176.1	—	JENNINGS *et al.* 1977b
53.3	71.3	69.4	70.2	66.7	23.1	176.1*	—	

both polymers may differ in average chain length. The ^{13}C-NMR spectral data of the serogroup C polysaccharide (see Fig. 14*b*, Table 12) clearly demonstrate the α(2-9) type of glycosidic linkage in this polymer. The downfield shift (2.0 ppm) for the resonance of the primary C9 on formation of the glycosidic linkage is only small when compared to the shifts observed for secondary carbon atoms in glycosidic linkage. The chemical shifts for this polysaccharide together with those of the disaccharide Neu5Acα(2-9)Neu5Ac prepared by mild acid hydrolysis of the polymer (JENNINGS *et al.* 1977b) are given in Table 12. Assignment of the anomeric configuration of the monomeric units was made on the basis of the resonance positions of the carboxylate carbon (C1), C4 and C6.

The spectrum of the capsular polysaccharide K92 of *E. coli* is given in Fig. 14*c*

(EGAN et al. 1977). The set of 20 resonances forms almost a superposition of the spectra given in Figs. 14a and 14b. The proposal that this polymer is a linear Neu5Ac chain with alternating α(2-8)- and α(2-9)-linkages was corroborated by ^{13}C-NMR spectroscopy of the periodate-oxidized polymer. The possibility that the

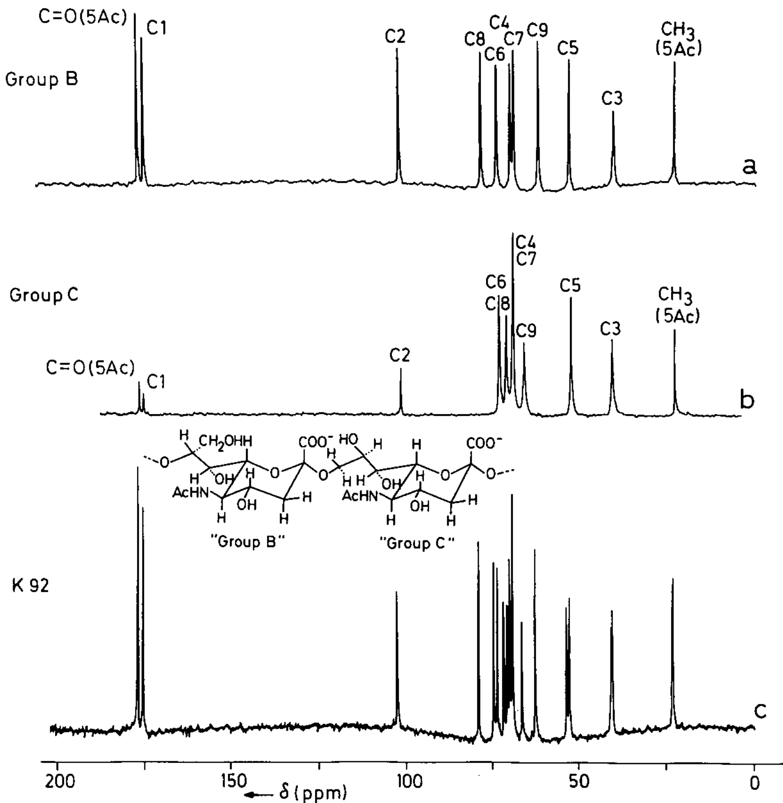


Fig. 14. *a* Proton noise-decoupled 68 MHz ^{13}C-NMR spectrum of meningococcal serogroup B polysaccharide, being an α(2-8)-linked Neu5Ac-homopolymer, dissolved in ^2H_2O, recorded at p^2H 7 and 35 °C (taken from EGAN et al. 1977). *b* Proton noise-decoupled 25 MHz ^{13}C-NMR spectrum of O-deacetylated meningococcal serogroup C polysaccharide, being an α(2-9)-linked Neu5Ac-homopolymer, dissolved in ^2H_2O, recorded at p^2H 7 and 37 °C (taken from BHATTACHARJEE et al. 1975). *c* Proton noise-decoupled 68 MHz ^{13}C-NMR spectrum of the capsular polysaccharide K92 from *E. coli*, a Neu5Ac-homopolymer with alternating α(2-8)- and α(2-9)-linkages, dissolved in ^2H_2O, recorded at p^2H 7 and 35 °C (taken from EGAN et al. 1977).

K92 preparation consists of a mixture of α(2-8)-homopolymer and α(2-9)-homopolymer chains was excluded since no precipitation was observed with anti-group B serum.

The spectra of the capsular polysaccharides of meningococcal serogroups W-135 and Y can easily be interpreted on the basis of sialic acid and hexose reference data (BHATTACHARJEE et al. 1976). The spectra are given in Fig. 15 and contain two

singlets in the anomeric region, which is consistent with the presence of disaccharide repeating units.

The signal assignments are given in Table 13 and are supported by comparison with the ^{13}C-NMR data of the Hexα(1-4)Neu5Ac disaccharide fragments prepared by mild acid hydrolysis. The glycosylation at position 4 of Neu5Ac is reflected by the downfield shifts for C4 and the upfield shifts for C3 and C5 in

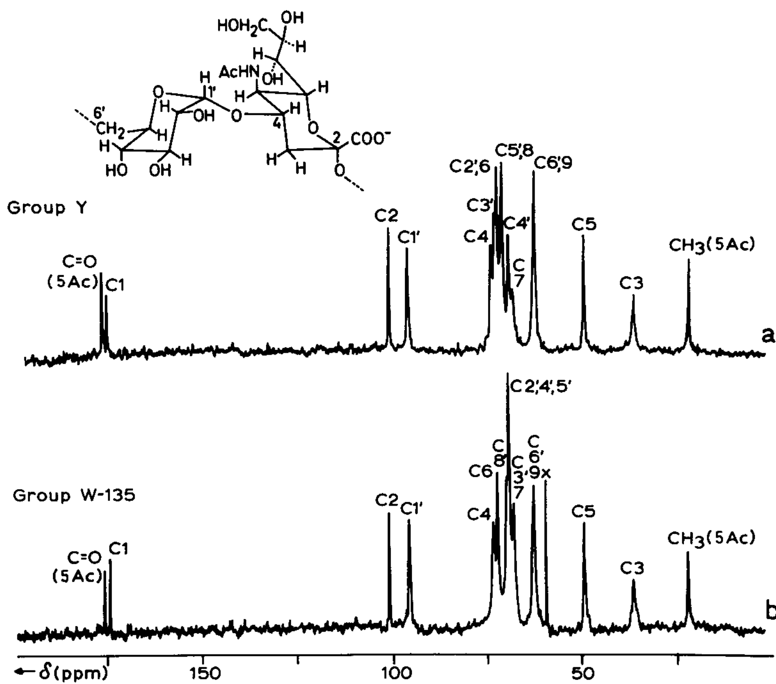


Fig. 15. *a* Proton noise-decoupled 25 MHz ^{13}C-NMR spectrum of O-deacetylated meningococcal serogroup Y polysaccharide, being a {-6)Glcα(1-4)Neu5Acα(2-} polymer, dissolved in ^2H_2O, recorded at p^{2}H 7 and 37 °C (taken from Bhattacharjee *et al.* 1976). *b* Proton noise-decoupled 25 MHz ^{13}C-NMR spectrum of meningococcal serogroup W-135 polysaccharide, being a {-6)Galα(1-4)Neu5Acα(2-} polymer, dissolved in ^2H_2O, recorded at p^{2}H 7 and 37 °C (taken from Bhattacharjee *et al.* 1976).

disaccharides as well as polysaccharides with respect to αNeu5Ac2Me as a reference (see Table 13). The downfield shifts of the hexose-C6 resonances in the polymers (1.5-2.2 ppm) with respect to the methyl hexoside model compounds are indicative of the involvement of this carbon atom in the glycosidic linkage to Neu5Ac. The α-configuration of the monomeric units is evident from the chemical shifts of hexose C1-C5 and of Neu5Ac C1 and C6.

In a study concerning the immunospecificity of the capsular polysaccharides of *Streptococcus* group B, type III, Jennings *et al.* (1981) applied ^{13}C-NMR spectroscopy as a main tool for structure analysis. The native capsular polysaccharide consists of a backbone of -6)GlcNAcβ(1-3)Galβ(1-4)Glcβ(1-

Table 13. ^{13}*C-NMR chemical shift data for Neu5Ac-hexose*
Chemical shifts are given in ppm downfield from external

Compound	Structure	Chemical shift in		
		Neu5Ac		
		C1	C2	C3
Neisseria meningitidis				
Serogroup Y polysaccharide[a]	{-6)Glcα(1-4)Neu5Acα(2-}$_n$	174.2	101.3	38.0
Serogroup Y disaccharide	Glcα(1-4)Neu5Ac	174.4	95.9	36.6
αGlc*p*		—	—	—
αNeu5Ac2Me[b]		174.6	101.9	41.3
Neisseria meningitidis				
Serogroup W-135 polysaccharide	{-6)Galα(1-4)Neu5Acα(2-}$_n$	174.3	101.4	37.8
Serogroup W-135 disaccharide	Galα(1-4)Neu5Ac	174.4	96.0	36.6
αGal*p*		—	—	—

* Values may be interchanged.
[a] O-deacetylated.
[b] JENNINGS *et al.* 1977b (p^{2}H = 7).

repeating units with side chains of Neu5Acα(2-6)Galβ(1- attached to C4 of the core GlcNAc residues. Sialic acid in this (N-acetyllactosamine-type) structural element is characterized by a signal at δ 101.1 for the anomeric C2. The carbon atoms involved in the various glycosidic linkages resonate at δ 78-104. Comparison of these signals in the spectra of the native polysaccharide, the asialo-analogue and the (linear) asialo-agalacto-backbone polysaccharide shows significant shifts in the resonance positions of carbon atoms in corresponding positions in the three polymeric structures. This indicates that distinct interglycose conformational changes, i.e. changes in the torsion angles of the glycosidic bonds occur on specific removal of sialic acid and side chain galactose residues. Serological studies on the native polymer and the derived structures reveal that the sialic acid residues—although not an integral part of the main determinant—influence its specificity by controlling the torsion angles of the glycosidic bond between the penultimate Gal residue and the GlcNAc residue in the polymer backbone. It was postulated that this conformational control could involve specific hydrogen bonds between sialic acid carboxylate groups and the polysaccharide backbone of the native antigen. This suggestion is corroborated by the fact that the carboxylate-reduced polymer behaves both serologically and ^{13}C-NMR spectroscopically like the asialo-polysaccharide. This work clearly demonstrates the power of ^{13}C-NMR spectroscopy as a tool in immunochemical investigations on polysaccharides in general and more in particular for the study of the function of sialic acid residues.

polymers and for some model compounds (BHATTACHARJEE *et al.* 1976)
TMS for solutions in ^2H_2O at 37 °C and unspecified p^{2}H values

Chemical shift in													
Neu5Ac								hexose					
C4	C5	C6	C7	C8	C9	5Ac (Me)	5Ac (C=O)	C1′	C2′	C3′	C4′	C5′	C6′
74.8*	50.8	73.3*	69.4	72.1	63.7	23.4	175.5	96.4	73.3*	74.1	70.2	72.1	63.7
73.3*	50.7	71.3	69.3	71.3	64.3	23.3	175.4	96.3	72.6*	73.9	70.3	72.1*	61.5
—	—	—	—	—	—	—	—	93.1	72.5	73.8	70.7	72.5	61.7
69.5	53.2	73.8	69.5	72.9	63.9	23.3	176.3	—	—	—	—	—	—
74.3*	50.7	73.3*	69.2	70.9	63.8	23.6	175.6	96.1	70.2	69.3	70.2	70.2	63.8
72.5*	50.8	71.5	69.1	71.5	64.4	23.4	175.6	96.6	70.3	69.4	70.7	72.3*	62.0
—	—	—	—	—	—	—	—	93.4	70.3	69.4	70.4	71.6	62.3

4. *Esterified Polysaccharides*

The ^{13}C-NMR spectra of polysaccharides can in principle provide information about the presence of O-acetyl substituents with respect to molar content and localization. The introduction of an O-acetyl group gives rise to characteristic shifts for the O-acetylated carbon atom and for adjacent carbon atoms (see section G.III.2). The degree of O-acetylation can be determined by comparison of the intensities of the characteristic methyl signals of the O-acetyl and N-acetyl groups. For the native meningococcal serogroup C polysaccharide this yields an O-acetyl content of 1.16 mol OAc/Neu5Ac unit (BHATTACHARJEE *et al.* 1975). Comparison of the spectra of the native and O-deacetylated polysaccharide did not allow a precise assignment of the positions of the O-acetyl substituents. This is due to the high complexity of the spectrum of the native polysaccharide as a result of a non-uniform distribution of the O-acetyl groups over the polymer. Using reference chemical shift data of the model compound Neu4,5,7,8,9Ac$_5$ the presence of an O-acetyl group at C4 of the serogroup C polysaccharide could be excluded. Hence, O-acetylation is restricted to C7 and/or C8.

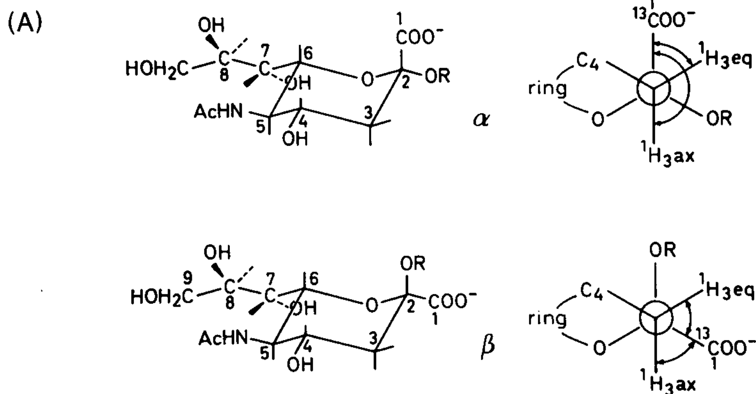
In a similar study of the meningococcal serogroup Y polysaccharide (1.3 mol OAc/Neu5Ac unit) tracing of the positions of the O-acetyl groups was even more difficult since the substituents can be present at C3 or C4 of the Glc units and/or at C7 of the Neu5Ac units (BHATTACHARJEE *et al.* 1976).

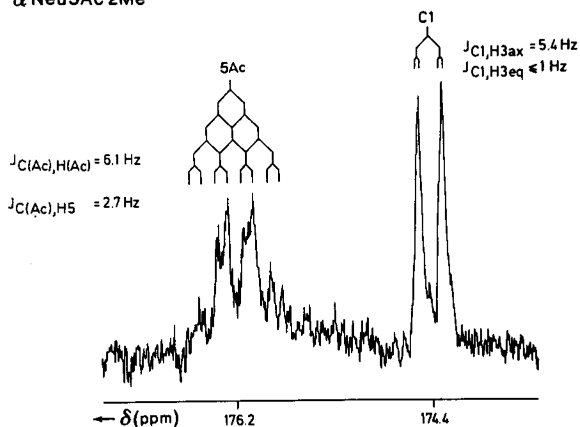
An interesting phenomenon observed for the α(2-8)- and α(2-9)-linked sialo-polysaccharides upon storage in slightly acidic solutions (pH 3-6) is the formation

Table 14. ^{13}*C-NMR data for Neu5Ac carbonyl carbons in CMP-Neu5Ac and some reference compounds* (Haverkamp *et al.* 1979 b)
Chemical shifts (δ) are given in ppm downfield from external TMS, for solutions in $^{2}H_2O$ at the indicated temperatures and p²H values. Coupling constants (J) are given in Hz. n.d., value could not be determined

Compound	p²H	T	C1				5Ac (C=O)		
			δ	$^{3}J_{C1,H3ax}$	$^{3}J_{C1,H3eq}$	$^{3}J_{C1,H\text{-}ester}$	δ	$^{2}J_{CAc,HAc}$	$^{3}J_{CAc,H5}$
βNeu5Ac*	7	25 °C	177.7	≤ 1 [a]	≤ 1 [a]	—	175.8	6.1	2.8
βNeu5Ac1,2Me$_2$	7	25 °C	171.6	≤ 1 [a]	≤ 1 [a]	3.7	175.6	5.9	1.8
αNeu5Ac1,2Me$_2$	7	25 °C	171.1	5.9	≤ 1 [a]	3.4	175.9	6.2	2.2
αNeu5Ac2Me**	7	25 °C	174.4	5.4	≤ 1 [a]	—	176.2	6.2	2.4
CMP-Neu5Ac	8	15 °C	175.3	≤ 1 [a]	≤ 1 [a]	—	175.7	n.d.	n.d.

[a] Estimated from the line width of the unresolved (double) doublet (see Figs. 16 and 17).
* Compare with Table 11.
** Compare with Tables 12 and 13.





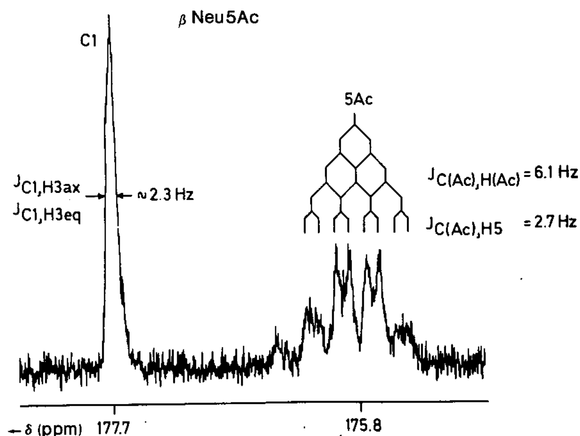


Fig. 16. *A* Structure of Neu5Ac α- and β-anomers and the torsion angles between C1 and the H3 atoms (R = H or aglycon). *B* Carbonyl carbon resonances in the undecoupled 25 MHz ^{13}C-NMR spectrum of αNeu5Ac2Me, dissolved in $^{2}H_2O$, recorded at p^{2}H 7 and 25 °C. *C* Carbonyl carbon resonances in the undecoupled 25 MHz ^{13}C-NMR spectrum of Neu5Ac, dissolved in $^{2}H_2O$, recorded at p^{2}H 7 and 25 °C. Only the signals from the β-anomer are observable.

of internal ester linkages (LIFELY *et al.* 1981). ^{13}C-NMR investigation of these products provided evidence for the occurrence of inter-residue ester bridges. In the α(2-8)-linked polymers these bridges involve the carboxyl group of one residue and the hydroxyl group at C9 of the adjoining residue as becomes evident from the upfield shift (Δδ-7.0) for C1 and the downfield shift (Δδ 5.6) for C9. Although an upfield shift of ~ -3 ppm can be expected for C8 as a result of a neighbouring ester substituent, the much larger upfield shift for C8 (Δδ-7.2) and the upfield shift for C2 (Δδ-4.3) can be explained by assuming a strain in the newly formed 6-membered ring structure between neighbouring Neu5Ac units. ^{13}C-NMR spectroscopy points out that a similar inter-residue ester formation takes place in α(2-9)-linked sialopolymers under mild acidic conditions involving C1 and the hydroxyl group at C8.

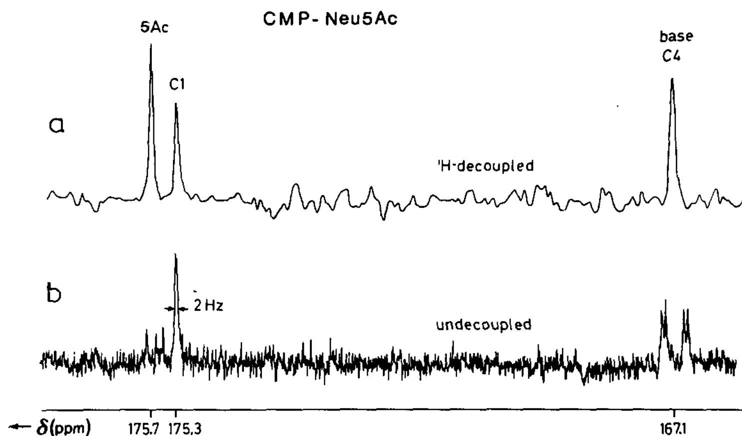


Fig. 17. Partial proton noise-decoupled (*a*) and undecoupled (*b*) 25 MHz ^{13}C-NMR spectrum of CMP-Neu5Ac, dissolved in ^2H_2O, recorded at p^{2}H 8 and 15 °C. The 5Ac carbonyl multiplet in spectrum (*b*) is not well developed. The $^3J_{C1,H3ax}$ value (< 1 Hz) indicates the β-configuration of Neu5Ac in this sugar nucleotide.

5. *CMP-N-acetylneuraminic Acid and 2-Deoxy-2,3-dehydro-N-acetylneuraminic Acid*

Another example of structure investigation of a neuraminic acid derivative by ^{13}C-NMR spectroscopy is the elucidation of the anomeric configuration of the Neu5Ac residue in CMP-Neu5Ac (HAVERKAMP *et al.* 1979 b). This study concerns in particular the heteronuclear vicinal coupling constants $^3J_{C1,H3ax}$ and $^3J_{C1,H3eq}$. The magnitudes of these coupling constants depend according to a Karplus-type relation on the torsion angle between C1 and the protons at position 3. The values observed for CMP-Neu5Ac together with those for some reference compounds are compiled in Table 14. Antiperiplanar orientation of C1 and H3ax, as present in the α-anomers (see Fig. 16) gives rise to a relatively large value for $^3J_{C1,H3ax}$ (5.4-5.9 Hz), whereas a synclinal orientation leads to a small value for this vicinal coupling constant (~ 1 Hz). From the measured value for $^3J_{C1,H3ax}$, as presented in Fig. 17, it was concluded that the Neu5Ac unit in CMP-Neu5Ac has β-

configuration. This result is in agreement with the information obtained via the resonance positions of H6 in the ^{1}H-NMR study of the sugar nucleotide (see section G.II.3.d). By consequence, CMP-Neu5Ac belongs to the class of sugar nucleotides having an axially oriented glycosidic linkage for the activated sugar residue.

The ^{13}C-NMR spectrum of Neu5Ac2en together with its complete interpretation are described by ESCHENFELDER *et al.* (1975) and CZARNIECKI and THORNTON (1977a). The chemical shift values are summarized in Table 11.

6. *Complexes of Sialic Acids with Ca^{2+}*

The property of sialic acids to form complexes with alkaline earth ions has also been investigated by ^{13}C-NMR spectroscopy. This complexation seems to play an important role in the erythrocyte cell membrane where sialic acid units are present as terminal sugar residues at the carbohydrate chains of glycolipids (gangliosides) and glycoproteins. Model studies by ^{13}C-NMR spectroscopy on the interaction of Neu5Ac and Neu5Gc with Ca^{2+} ions have been described in several reports (BEHR and LEHN 1973, CZARNIECKI and THORNTON 1977b, JAQUES *et al.* 1977, JAQUES *et al.* 1980a). The chemical shifts of the sialic acid skeleton carbons are very sensitive to complex formation. The downfield shift in the resonance position of C8 on titration with Ca^{2+} results from a direct involvement of the hydroxyl group at C8 in the Neu5Ac-Ca^{2+} complex formation. However, for the shifts of other carbon resonances CZARNIECKI and THORNTON (1977b) and JAQUES *et al.* (1977) offer different explanations leading to different spatial models for the

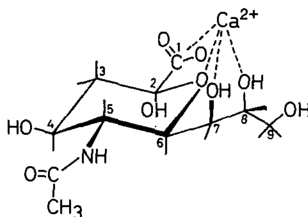


Fig. 18. Conformational model proposed for the complex of αNeu5Ac with Ca^{2+}, based on Ca^{2+}-induced ^{1}H- and ^{13}C-chemical shifts (taken from JAQUES *et al.* 1977).

Neu5Ac-Ca^{2+} complex. The model postulated by the latter authors is given in Fig. 18 and is supported by ^{1}H-NMR studies (see G.II.5). Similar studies with Neu5Gc (JAQUES *et al.* 1980a) reveal that, although the extra hydroxyl function of the glycolyl group could participate in the Ca^{2+} binding, a close resemblance exists between the spatial structures of the Neu5Ac-Ca^{2+} and the Neu5Gc-Ca^{2+} complexes. CZARNIECKI and THORNTON (1977b) propose another model for the Neu5Ac-Ca^{2+} complex in which the anomeric O, the ring-O and the O at C8 are the main binding sites ("oxygen cage") whereas the carboxylate group is not involved in the complexation.

In the complex of αNeu5Ac with Ca^{2+} the set of oxygen atoms involved in the binding differs from that in the βNeu5Ac-Ca^{2+} complex. This leads to a less strong complex for the α-anomer. In view of this observation the rather strong binding of gangliosides with Ca^{2+} cannot exclusively be ascribed to the binding of the αNeu5Ac units of these glycolipids. As also the sequence Neu5Acα(2-3)Galβ(1-4)Glc (and its α(2-6)-analogue) as free oligosaccharides do not show a pronounced complexation behaviour (Jaques *et al.* 1980 b) this trisaccharide will probably not be the active unit in the complexation of gangliosides with Ca^{2+} provided this structural element will have the same conformation in gangliosides and in the free form. Probably, a more complex interaction of the cation with the carbohydrate chain is involved (Behr and Lehn 1973, Czarniecki and Thornton 1977 c). The relatively weak complexation of colominic acid with Ca^{2+} (Jaques *et al.* 1977) may be interpreted in the same way as for monomeric α-anomers (Czarniecki and Thornton 1977 b).

7. *^{13}C-Relaxation Studies on Sialic Acids and Sialyllactoses*

Studies on the spatial structure of Neu5Ac derivatives by using ^{13}C spin-lattice relaxation times have already been discussed in conjunction with ^{1}H-NMR parameters in section G.II.1. The measurement of T_1-values has also been applied to obtain information on internal molecular dynamics of sialo-oligosaccharides. Czarniecki and Thornton (1977 c) and Jaques *et al.* (1980 b) investigated isomers of sialyllactose as model functional units of gangliosides and glycoproteins. Comparison of the relative T_1-values for protonated carbons in the spectra of both Neu5Acα(2-3)Galβ(1-4)Glc and the corresponding α(2-6)-isomer (from bovine colostrum) demonstrates a segmental anisotropic motion of the sugar residues along the main axis of both of the molecules, with the Neu5Ac residue being the least mobile and the Glc residue being the most mobile. Probably the solvated, negatively charged carboxylate group stabilizes the oligosaccharide chain conformation and it is this stabile conformation that is recognized as an immunological determinant.

IV. Analytical Procedures

1. *Instrumentation*

The most advanced ^{1}H-NMR spectra of carbohydrate chains reported so far, were obtained at 500 MHz on a Bruker WM 500 spectrometer operating in the pulsed Fourier transform mode. These spectra were taken up in 16 k memory with an acquisition time of 3.3 s and a spectral width of 2.5 kHz. Adequate resolution enhancement was achieved by Lorentzian to Gaussian transformation from quadrature phase detection followed by employment of a 32 k point complex Fourier transformation. The probe temperature was stabilized at 300 ± 0.1 K. At this temperature the HO^{2}H-resonance in neutral aqueous solution is found at δ 4.76. For the detection of proton resonances in this range of the spectrum the HO^{2}H line can be shifted by slightly changing the temperature (*cf.* Vliegenthart *et al.* 1981). Chemical shifts are measured by reference to internal acetone (δ 2.225 ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS)) with an accuracy of 0.001 ppm.

500 MHz ^1H-NMR instrumentation as specified above has several advantages over instruments working at lower field strengths. The very strong magnetic field together with the computer resolution enhancement facility allows a precise determination of chemical shifts of structural-reporter-groups and a highly detailed information on splitting patterns of proton multiplets. Furthermore, marked increase in the sensitivity is obtained; samples as small as 25 nmoles of carbohydrate can be adequately analysed with sufficient signal-to-noise ratio. This gain in spectral resolution as well as in sensitivity makes possible the assignment of many structural-reporter-groups and enables the structure elucidation of minor constituents in complex samples or of microheterogeneity in carbohydrate chains.

For instruments operating at lower magnetic fields there is obviously a loss in sensitivity and spectral resolution with respect to the instrument described above. Consequently, the type of problems which can be solved with such less advanced instruments is restricted. Verification of the identity of unknown carbohydrate chains on the basis of comparison with standard reference data sets is possible with 200–360 MHz ^1H-NMR instruments.

Usually, ^{13}C-NMR spectra of glycoconjugates and polysaccharides are obtained at 25–50 MHz (corresponding with 100–200 MHz for ^1H) instruments operating in the Fourier transform mode. Frequently, spectra are recorded at about 310 K. Chemical shifts are expressed relative to external tetramethyl silane (TMS). Again instruments operating at higher fields will give improvements with regard to spectral resolution and sensitivity. The low natural abundance (1.1%) and the relatively low intrinsic sensitivity of ^{13}C make that sample amounts have to be considerably larger than for ^1H-NMR spectroscopy; about 10–100 μmoles of substance is required. For details about special NMR techniques such as specific heteronuclear decoupling, 2D-NMR, relaxation measurements, etc. the reader is referred to the books of BERLINER and REUBEN (1978, 1980), JARDETZKY and ROBERTS (1981), SHULMAN (1979), and BREITMAIER and VOELTER (1974).

2. *Solutions*

Solutions in ^2H_2O of the compounds to be analysed were in general adjusted to p^{2}H 7 and ^2H-exchanged by 5-fold lyophilization of the solution finally using $\geq$ 99.96 atom % ^2H. For ^1H-NMR spectroscopy in general 0.06–3 mM solutions of the compounds in 0.4 ml ^2H_2O were used; for ^{13}C-NMR spectroscopy typical sample concentrations lay in the range of 10–100 mM. Amount of material and solubility limit the maximum concentration which can be applied. Too high viscosity of the solution, especially in ^1H-NMR spectroscopic measurements, will reduce the spectral resolution. Contamination of the samples with organic or inorganic compounds such as salts and buffer materials has to be avoided, since they affect negatively the spectral quality. Also the presence of paramagnetic materials such as transition metal ions which may be retained or acquired during isolation and purification procedures can disturb spectral resolution. The same holds for contaminants that give rise to sample inhomogeneities like colloidal or particulate matter.

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