¹H-NMR study on ganglioside amide protons: evidence that the deuterium exchange kinetics are affected by the preparation of samples

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The kinetics of $H/^2H$ chemical exchange of the amide proton has been suggested as one of the tools available for investigating hydrogenbond stabilizing interactions in gangliosides.

The amide proton/deuterium ($NH/^2H$) exchange rates in GM2 ganglioside were studied by ¹H-NMR spectroscopy on 12 samples prepared following different procedures. In samples passed through a sodium salt Chelex-100 cation exchange resin column prior to being analysed the N-acetylneuraminic acid NH exchange occurred in less than 10 min and that of ceramide NH in 30 min. The N-acetylgalactosamine acetamido NH exchange was slower, the half-life of the signal ranging from 15 min to 3.5 h. Contact of the Chelex-treated GM2 samples with water, through a dialysis process, modified the $NH/^2H$ exchange rate values, the N-acetylgalactosamine acetamido NH exchange becoming faster than that of ceramide NH and similar to that of N-acetylneuraminic acid NH. Our results indicate that the deuterium/proton exchange rate strongly depends on sample preparation (ion content and minor contaminants present in water). The three-dimensional model involving the N-acetylgalactosamine acetamido NH and the N-acetylneuraminic acid carboxyl group hydrogen-bonding, which is supported by experimental evidence, cannot be confirmed by NH-exchange measurement.

Keywords: gangliosides; ¹H-NMR; amide protons; deuterium exchange; interaction processes; calcium ion

Introduction

The conformational properties of the ganglio-series gangliosides‡, characterized by the neutral oligosaccharide chain β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc- have been studied. The 'a' sub-series of these gangliosides have only one sialic acid unit linked at position 3 of the inner galactose [1]. It now seems established that the three-dimensional structure of the oligosaccharide chain mainly derives from the conformational properties of the -β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal- portion. This portion shows a rather rigid conformation, which is only slightly affected by the addition of other saccharides and is stabilized by a number of through-space interactions, such as those between the N-acetylneuraminic acid lateral chain and the N-acetylgalactosamine unit [2–5]. It has been suggested that the conformation is favourable for a hydrogen bond between the N-acetylgalactosamine amide proton and the N-acetylneuraminic acid carboxylic group [6, 7], but direct experimental proof is not available. Levery [7] proposed different proton-NMR approaches pointing out the inter-residue amide-carboxyl hydrogen bond and the role of this interaction in the stabilization of a preferred ganglioside conformation. The authors examined chemical shifts, temperature shift coefficients and $^3J_{2,NH}$ arguments together with $H/^2H$ amide proton exchange measurements. In particular, the very low N-acetylgalactosamine amide proton exchange rate seemed to be consistent with the proposed inter-residue hydrogen bonding network.

This paper shows that the amide proton/deuterium ($NH/^2H$) exchange in GM2, β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-4)-Cer, strongly depends on the sample preparation.

Materials and methods

Materials

Water was doubly purified by ion exchange chromatography followed by passage through a Milli-Q Water Purification System (Millipore, USA). Deuterated dimethylsulfoxide

(I)

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Chelex  ──  + Ca²⁺  ──  dialysis  ──  dialysis  ──  dialysis  ──  Chelex
                         H₂O 30L       EDTA        H₂O 30L
          a b c                 d                        e              f
            g                   h                        i
```

(II)

```
Chelex  ──  dialysis
            H₂O 30L
       j              k
```

(III)

```
Chelex  ──  dialysis  ──  H₂O 10mL
            H₂O 10mL
       l         m              n
```
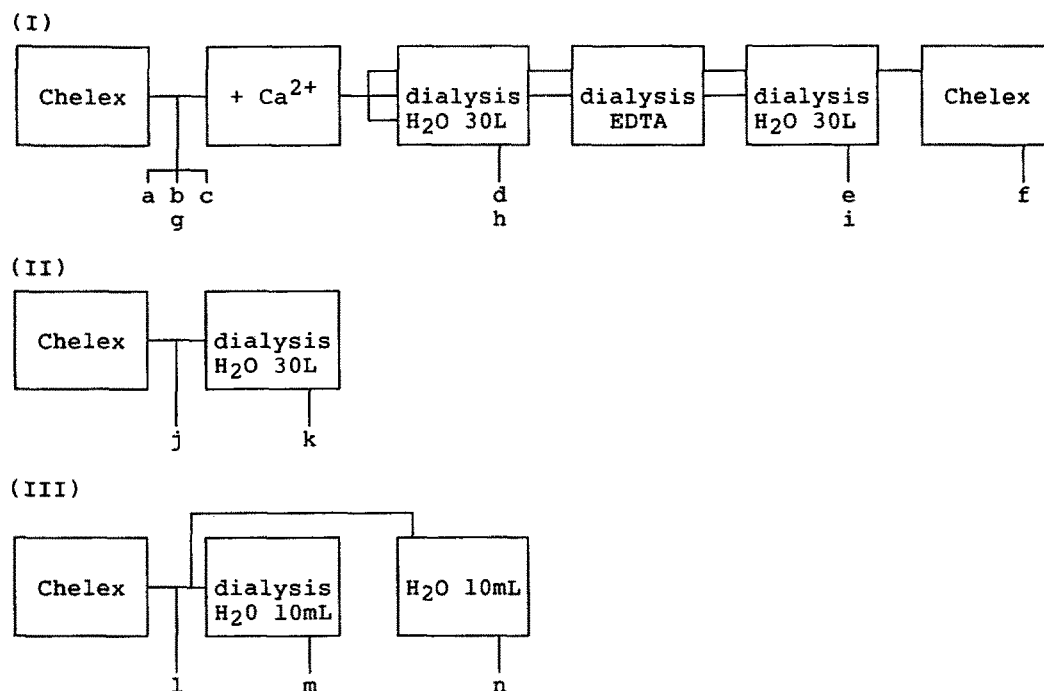
Figure 1. Schemes I, II and III of the procedures followed for sample preparation.

(DMSO-d_6) isotopically pure ($>99.98\%$), calcium chloride and tetrahydrated Suprapur^R calcium chloride were purchased from Merck (Germany); ethylenediaminotetraacetic acid, sodium salt, from Riedel-De Haen (Germany); Chelex 100 (100–200 mesh, sodium form) from Bio-Rad (USA); and N-acetylneuraminic acid from Sigma (USA).

Ganglioside GM2 was extracted from calf brain [8] and purified to above 99% by silica gel, ion exchange and molecular sieve column chromatographies, dialysis and precipitation from acetone [9]. Its structural and homogeneity characterization was performed as described elsewhere [9].

Preparation of samples for ^1H-NMR analyses

Samples containing about 1 mg of GM2 were prepared following Schemes I, II and III shown in Fig. 1. After each step, samples coded a to n (see Table 1) were taken for NH/^{2}H exchange NMR analyses.

Scheme I. A water solution of ganglioside (about 10 mg ml^{-1}) was passed through a Chelex 100 cation exchange resin column (pH 6, 0.5 × 4 cm). The column was washed three times with 3 ml of water. Three identical aliquots of the eluate were taken as samples a, b and c. CaCl$_2$ was added to the remaining eluate to reach a final concentration of 50 mM. After 24 h of continuous stirring, this solution was divided into three aliquots which were dialysed in the same tank for 3 days with 10 l of water changed every day. One of these is sample d. The remaining two samples were dialysed with a solution of EDTA (50 mM) and then with water. One is sample e. The remaining sample f was finally passed through a Chelex 100 column. Another 3 mg of GM2

was treated following Scheme I, but using Suprapure CaCl$_2$ (samples g, h, and i).

Scheme II. GM2 (2 mg) was treated with Chelex 100 as above. Half of the eluate is sample j and the remaining sample k was dialysed for 3 days with water (3 × 10 l).

Scheme III. GM2 (3 mg) was treated with Chelex 100 as above. The eluate was divided into three samples. One is sample l. Sample n was left in solution with 10 ml water for 4 days and sample m was kept in a dialysis tube floating in a minimum amount of water (about 10 ml) for 4 days.

All samples from a to n were lyophilized, maintained under vacuum and transferred to a dry-box until they were dissolved in DMSO-d_6.

Deuterium exchange kinetics of amide protons

Samples in 0.5 ml of DMSO-d_6 were equilibrated at 305 ± 0.02 K. Spectra were run at 500 MHz using a Bruker AM500 spectrometer equipped with an ASPECT 3000 computer, a process controller and an array processor; 160 transients were collected per spectrum. Proton chemical shifts were assigned by one- and two-dimensional TOCSY experiments [10–12]. The proton/deuterium exchange was analysed for GM2 N-acetylneuraminic acid, ceramide and N-acetylgalactosamine amides at 7.95, 7.44 and 7.36 ppm respectively. The amide proton exchange was initiated by addition of 10 μl of ^{2}H$_2$O followed by gentle shaking. The first spectrum was recorded 5 min after adding the ^{2}H$_2$O, necessary for re-shimming, and the following were recorded once every 15 min for the first 2 h and once every 30 min for the next 12–20 h. The extent of NH/^{2}H amide proton exchange was determined by analysing the decay of the

Table 1. Half-life (h), for deuterium exchange of N-acetylneuraminic acid (Neu5Ac), N-acetylgalactosamine (GalNAc) and ceramide amide (Cer) protons of GM2 samples prepared following sequential procedural steps as reported in the Methods section.

Sample	Neu5Ac	GalNAc	Cer
Levery [7]	14.5	368	78.9
Scheme I			
a Chelex	≈ 0.1	1.6	≈ 0.1
b Chelex	≈ 0.1	≈ 0.2	≈ 0.1
c Chelex	≈ 0.1	≈ 0.5	≈ 0.1
d Chelex + Ca^{2+}	1.7	3.5	12.2
e Chelex + Ca^{2+} + EDTA	3.0	1.1	9.4
f Chelex + Ca^{2+} + EDTA + Chelex	≈ 0.1	0.9	0.7
g Chelex	≈ 0.1	3.3	≈ 0.2
h ^{a}Chelex + Ca^{2+}	9.2	3.4	33.2
i ^{a}Chelex + Ca^{2+} + EDTA	4.2	broad signal	19.8
Scheme II			
j Chelex	≈ 0.1	≈ 0.5	≈ 0.2
k Chelex + dialysis	4.4	3.3	18.5
Scheme III			
l Chelex	≈ 0.1	≈ 0.5	≈ 0.2
m Chelex + 10 ml water	≈ 0.2	4.5	1.0
n Chelex + 10 ml dialysis	≈ 0.2	6.5	1.3

^{a} Suprapur^R calcium chloride tetrahydrated was used.
$\approx$ approximate value. For other data, errors range between 3% and 10%.

NH-resonance peak areas. A X32 Bruker satellite station equipped with standard Bruker UXNMR software was used for data processing and integral evaluations. The NH-resonance half-lives were determined by linear fit in a logarithmic scale of the measured integrals versus time. The resonance of the unexchangeable vinylic proton of the long chain base (Sph5) at 5.53 ppm was used as an internal reference for integral calibration. The standard deviation of the distribution of Sph5-resonance integral values was estimated in order to define the errors or 'weights' needed as a normalization factor for the weighted least squared procedure used to perform the fit. For fast decaying signals very few observations were collected and only approximate half-lives are given. For the other data, errors range between 3% and 10%.

Quantitative assays

The ganglioside content was determined as bound N-acetylneuraminic acid by the resorcinol-HCl method [13–14], pure N-acetylneuraminic acid being used as the reference standard.

Ion content was determined by qualitative and quantitative analyses of the emission spectrum using a Jobin Yvon Inducted Coupled Plasma (ICP) System. Calcium was determined at all the three emissions at 315.887, 317.933 and 393.366 nm, and sodium at 330.237 nm. Calibration was done on the carbon emission at 193.031 nm.

The absence of EDTA in the EDTA-treated samples was confirmed by NMR.

Results and discussion

The Chelex 100 chelating-exchange column allows removal of traces of divalent cations from the ganglioside solution [15–17], which may prevent high resolution NMR spectra.

It was experimentally observed that although gangliosides are solubilized in DMSO, a solvent in which complex amphiphilic lipids do not aggregate, a few OH and NH ^{1}H-NMR signals may appear extremely broadened. The improvements derived from Chelex treatment are shown in Fig. 2, where ^{1}H-NMR spectra of Chelex-treated and untreated GM2 ganglioside are compared. Spectrum 2a shows the Neu5Ac-OH8 and GalNAc-NH signals at 6.10 and 7.35 ppm respectively which were almost completely lost in the Chelex-untreated sample (Fig. 2b).

Studying the NH/^{2}H exchange in samples of different Chelex-treated gangliosides we soon noticed a large discrepancy with previously published results. In particular, the exchange process of amide protons was hundreds of times faster than quoted [7]. We then engaged in a more systematic study of these processes in gangliosides. We chose GM2 and analysed 12 samples prepared as in Fig. 1.

In samples a, b, c, g, j, l (those only passed through a Chelex-100 column) the exchange rate of Neu5Ac-NH was
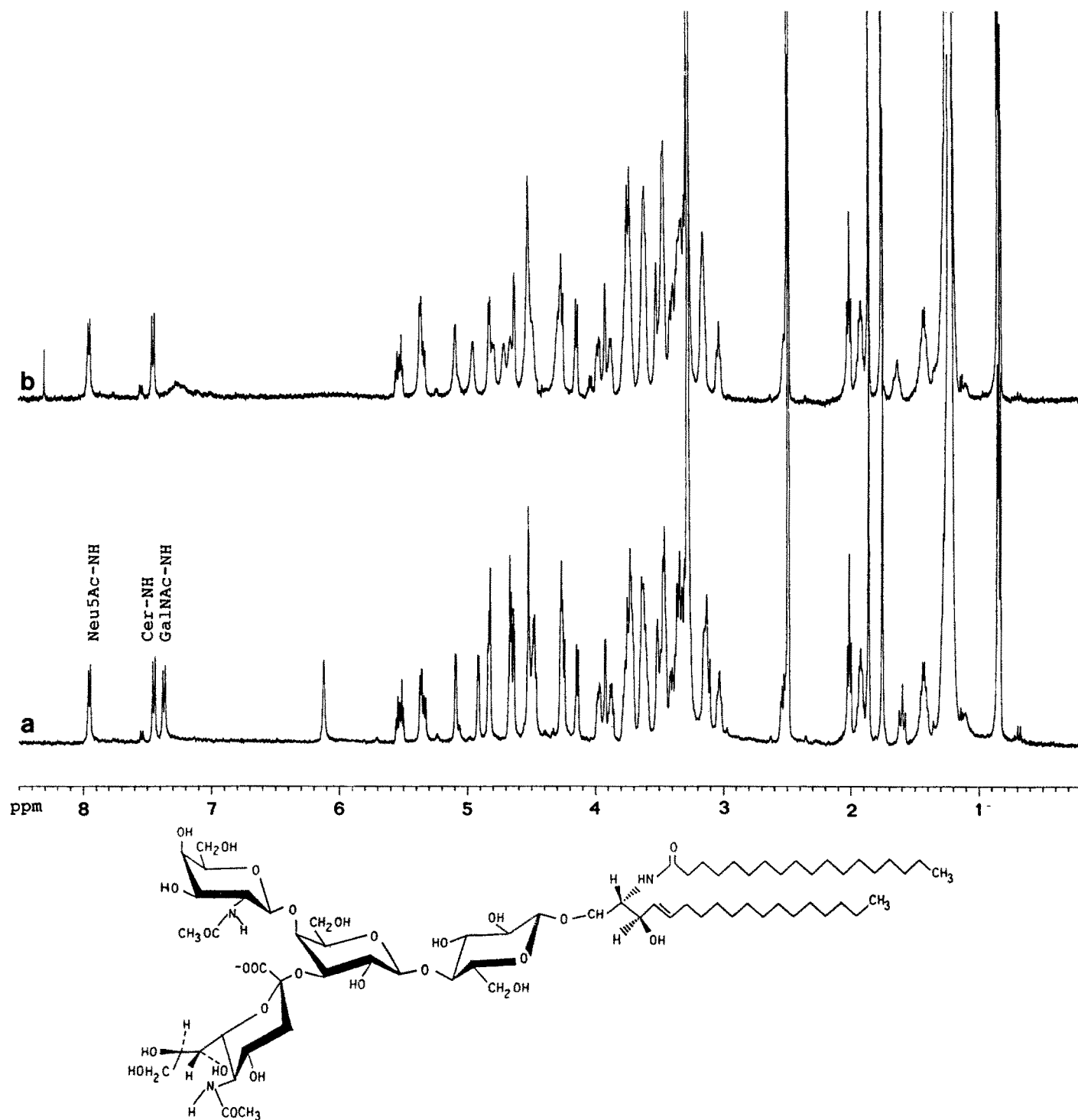
Figure 2. ^{1}H-NMR GM2 spectra. Chelex-treated (a) and Chelex-untreated (b) samples are compared.

so fast that 10 min after adding ^{2}H$_2$O no signal was observed. The Cer-NH disappeared in 30 min, while the half-life of the GalNAc-NH signal ranged from 15 min to 3.5 h (see Table 1). The discrepancy between these results and those reported [7] is the more significant since the NH/^{2}H exchange process is claimed to be related to conformational properties.

Excluding any influence of organic contamination on the observed differences in the rates of exchange, we tentatively associated these differences to interaction processes involving cations. In our samples, only sodium was expected to be present, the gangliosides being prepared as the sodium salt [18] passed through a sodium salt Chelex 100 column. Accordingly we followed Scheme I in Fig. 1, analysing if,

Table 2. Molar ratio between ganglioside GM1, sodium and calcium in samples prepared following Scheme I of Fig. 1.

	GM1	Na	Ca
Chelex	1	0.97	0.00
Chelex + Ca^{2+}	1	0.41	0.35
Chelex + Ca^{2+} + EDTA	1	a	0.06
Chelex + Ca^{2+} + EDTA + Chelex	1	0.94	0.02

a not determined.

Table 3. Chemical shift (δ) (ppm from tetramethylsilane), $^3J_{2,NH}$ (Hz) coupling constants and temperature shift coefficients[a] (ppb deg^{-1}) of GM2 amide protons in DMSO-d_6 at 305 K.

	Neu5Ac	GalNAc	Cer
δ	7.94	7.36	7.44
$^3J_{2,NH}$	8.1	9.2	8.8
dδ/dT	−4.75	+0.44	−5.33

a seven points were collected from 300–323 K.

and to what extent, a cation content alteration could influence the rate of NH/^{2}H exchange processes. Calcium was chosen for these experiments keeping in mind the special interest in calcium-ganglioside interaction processes [19, 20].

The measured half-lives for the NH/^{2}H exchange are reported in Table 1.

All treatment following the passage through the Chelex column slowed down the exchange process, although we never attained half-life values close to those previously published [7]. We found a quite large variability in the data, but from Tables 1 and 2 it seems that the addition of calcium is not the only reason for the modification in the exchange rates. In fact, treating the Ca-ganglioside solution with a strong calcium chelating agent such as EDTA is ineffective in restoring the initial values of half-lives. The capability of EDTA to remove Ca^{2+} from the ganglioside solutions was confirmed by ICP analyses (see Table 2). These analyses are destructive and were performed on the less-costly GM1 ganglioside, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-Cer, treated exactly as GM2. Preliminary experiments on the NH/^{2}H exchange rate for GM1 indicated behaviour similar to that for GM2. Addition of calcium chloride transformed half of the sodium salt ganglioside into the calcium salt and calcium was largely removed by EDTA treatment.

Only a final passage through a Chelex column restored the starting NH/^{2}H exchange rate (sample f), thus supporting the hypothesis that whatever contaminants are introduced after a first Chelex treatment can always be removed by the Chelex itself. On the other hand, the not exclusive implication of calcium in modulating the NH/^{2}H exchange rate is confirmed by the results for sample k prepared by direct dialysis of Chelex-treated GM2 (see Scheme II of Fig. 1). The dialysis process itself slows down the exchange rate by many hours.

In order to exclude any direct influence from the dialysis tube and to investigate the relevance of the amount of water in contact with GM2 in determining the exchange rates, we prepared samples following Scheme III of Fig. 1 (samples l, m and n). Samples of GM2 dialysed with 10 ml water or solubilized in 10 ml water showed similar behaviour. Cer-NH and Neu5Ac-NH exchanged almost as fast as after

Chelex treatment only, whereas the half-lives of GalNAc-NH were the highest measured for this amide. Following these results, we conclude that the contact of gangliosides with a large amount of water, as in the dialysis procedure, is a reason for the long half-lives of N-acetylneuraminic acid and ceramide amide protons while the exchange rate of GalNAc-NH is strongly influenced by contact with water, no matter what amount.

In order to compare samples directly analysed after Chelex treatment with those submitted to Ca^{2+} addition and dialysis, chemical shifts, temperature shift coefficients and $^3J_{2,NH}$ coupling constants of amide protons were examined. No difference was observed. Data are reported in Table 3.

Conclusions

Our results clearly indicate that when gangliosides, prior to being dissolved in dimethylsulfoxide for NMR analysis are passed through a Chelex-100 cation exchange resin the general exchange process of the amide protons is quite rapid. At the same time it is clear that the processes involving Neu5Ac-NH, GalNAc-NH and Cer-NH occur at different rates, GalNAc-NH being slowest. The contact of GM2 with water strongly reduces the exchange rates. GalNAc-NH exchange is then faster than that of Cer-NH and similar to that of Neu5Ac-NH.

Our results do not explain how the exchange process is modulated but clearly indicate that the amide-proton exchange rates can be influenced by a number of factors that, at present, remain unknown. Very minor contaminants in water are suspected. Dialysis of gangliosides is usually performed with large amounts of water, so that even part per billion contaminants may become important for interaction processes involving the acetamide groups of gangliosides and so interfere with the NH/^{2}H exchange.

Furthermore, it appears that Chelex treatment is capable of restoring rapid exchange for N-acetylneuraminic acid and ceramide amide protons whereas the N-acetylgalactosamine amide proton seems to behave in a more variable way. The lower, 15 min, half-life value measured for GalNAc-NH is close to those obtained for Neu5Ac-NH, while the 3.3 h half-life in sample g is similar to half-lives

obtained for GalNAc-NH itself after adding calcium, followed by dialysis (samples d, h, k). Thus, we argue that it is possible to obtain a very high exchange rate for the N-acetylgalactosamine amide proton too, but it is also easy to introduce contaminants which are hard to remove.

Evidence of hydrogen-bonding is known for the ceramide amide which acts as acceptor and donor in the interaction with Sph-OH and glycosidic oxygen, respectively [21], and for N-acetylgalactosamine amide interacting with the carboxylic group of N-acetylneuraminic acid (see above). Amide proton exchange rate measurements fail to confirm this. In fact, as it appears from our data, a direct comparison of the half-lives of different amide protons of the same ganglioside sample cannot be readily indicative of the hydrogen-bond implication of some of them.

Acknowledgements

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