Acid and enzymatic hydrolysis of the internal sialic acid residue in native and chemically modified ganglioside GM1

Pablo E. A. Rodriguez,[1] Bruno Maggio, and Federico A. Cumar[2]

Departamento de Química Biológica-CIQUIBIC, Facultad de Ciencias Químicas-CONICET, Universidad Nacional de Córdoba, Casilla de Correo 61, 5016 Córdoba, Argentina

Abstract The sialic acid of gangliosides not containing GalNAc (i.e., GM3, GD3) is readily hydrolyzed either enzymatically by sialidases or chemically in acid conditions. On the other hand, in gangliosides having the sialic acid on the internal galactose residue linked to GalNAc (i.e., GM1, GM2) the Neu5Ac is largely resistant to acid or enzymatic hydrolysis. In the present work GM1(NH_4^+), GM1(H^+), and several de-acetylated derivatives in the sialic acid and in both sialic acid and N-acetylgalactosamine moieties were prepared. Studies by counterion exchange with DEAE-Sephadex A-25 and Dowex 50WX8, acid-base titration, and acid or enzymatic hydrolysis with sialidases were performed on these derivatives. Our results provide cumulative evidence supporting that a hydrogen bonding interaction between the hydrogen atom of un-ionized carboxyl group in Neu5Ac and the oxygen atom of the carbonyl group in GalNAc reduces the dissociation of the Neu5Ac carboxyl group and impairs its enzymatic and acid hydrolysis. In addition, our results suggest that the enzymatic hydrolysis of the ionized form of sialic acid in GM1(Na^+) and GM1(NH_4^+) is impaired by a second hydrogen bonding interaction between the proton of the acetamide group in GalNAc and the carbonyl moiety of the carboxyl group of the Neu5Ac.—**Rodriguez, P. E. A., B. Maggio, and F. A. Cumar.** Acid and enzymatic hydrolysis of the internal sialic acid residue in native and chemically modified ganglioside GM1. *J. Lipid Res.* 1996. **37:** 382–390.

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In gangliosides having the sialic acid on the internal galactose residue linked to GalNAc (i.e., GM1, GM2) the Neu5Ac is largely resistant to acid or enzymatic hydrolysis. The generally accepted mechanism for the acid hydrolysis of sialic acid involves protonation of the ketosidic oxygen atom followed by a rate-determining decomposition reaction to an alcohol and a resonance-stabilized carbonium-oxonium ion (1). This ion reacts subsequently with water to form the free sugar. The protonation of the glycosidic oxygen, facilitated by the proximity of the ionized carboxyl group, stabilizes the carbonium ion of the postulated intermediate in the hydrolytic reaction. Thus, hydrolysis of the ketosidic linkage in the sialic acid depends on a specific catalysis by the ionized carboxyl group (1).

Conceptual speculations regarding the explanation of an impaired hydrolysis of the sialic acid in gangliosides with a core oligosaccharide moiety such as GalNAc(β,1 → 4)[Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′) have considered the existence of structural rigidity of the inner sialic acid; among other effects this rigidity was proposed to be mediated by hydrogen bonding between

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Abbreviations: GM3: Neu5Ac(α,2 → 3)Gal(β,1 → 4)Glc(β,1 → 1′)Cer; GM2: GalNAc(β,1 → 4)[Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Cer; GM1: Gal(β,1 → 3)GalNAc(β,1 → 4)[Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Cer; GM1(H^+), GM1 H^+ form; GM1(NH_4^+), GM1 NH_4^+ form; GM1(Na^+), GM1 Na^+ form; GM1-CH_3, N-methylamide derivative, at the carboxyl group of N-acetylneuraminic acid, of ganglioside GM1; NA, neuraminic acid, $GalNH_2$, galactosamine; GM1(NA): Gal(β,1 → 3)GalNAc(β,1 → 4)[NA(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Cer; GM1(NA)-CH_3, N-methylamide derivative, at the carboxyl group of neuraminic acid, of GM1(NA); GM1(NA/$GalNH_2$): Gal(β,1 → 3)$GalNH_2$(β,1 → 4)[NA(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Cer; GM1(NA/$GalNH_2$)-CH_3, N-methylamide derivative, at the carboxyl group of neuraminic acid, of GM1(NA/$GalNH_2$); lysoGM1(NA): Gal(β,1 → 3)GalNAc(β,1 → 4)[NA(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Sph; lysoGM1(NA/$GalNH_2$): Gal(β,1 → 3)$GalNH_2$(β,1 → 4)[NA(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Sph; lysoGM1(NA/$GalNH_2$)-CH_3, N-methylamide derivative, at the carboxyl group of neuraminic acid, of lysoGM1(NA/$GalNH_2$); GD3: Neu5Ac(α,2 → 8)Neu5Ac(α,2 → 3)Gal(β,1 → 4)Glc (β,1 → 1′)Cer; GD1a: Neu5Ac(α,2 → 3)Gal(β,1 → 3)GalNAc(β,1 → 4)[Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc (β,1 → 1′)Cer; GD1b: Gal(β,1 → 3)GalNAc(β,1 → 4)[Neu5Ac(α,2 → 8)Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Cer; GT1b: Neu5Ac(α,2 → 3)Gal(β,1 → 3)GalNAc(β,1 → 4)[Neu5Ac(α,2 → 8)Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Cer; GQ1b: Neu5Ac(α,2 → 8)Neu5Ac(α,2 → 3)Gal(β,1 → 3)GalNAc(β,1 → 4)[Neu5Ac(α,2 → 8)Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 → 1′)Cer; TLC, thin-layer chromatography.

[1]To whom correspondence should be addressed.

[2]Dr. Federico A. Cumar passed away on March 16, 1994. This paper concludes work initiated by him and represents our posthumous homage to his tireless efforts in this Center, to his incomparable qualities as a person and as a scientist, and to an unforgettable teacher and friend.

the protonated carboxyl group of the inner sialic acid residue and the carbonyl group of the GalNAc residue (2).

Considerable interest has focused on the interactions taking place among carbohydrate residues of the glycolipid oligosaccharide chain, as revealed by NMR, following the pioneering studies of R. K. Yu and collaborators (3) that first assigned the carbohydrate resonance frequencies for several glycolipids. Of particular importance for our work are the interactions described between the Neu5Ac and GalNAc residues. Koerner et al. (4) considered a number of possible secondary structural elements, consistent with the observed chemical shift data for a whole series of monosialo- and asialo-ganglioside structures: a dipole-charge interaction between the GalNAc acetamide and Neu5Ac carboxylate, hydrophobic interactions involving the GalNAc amide methyl and Neu5Ac C_3 methylene groups, hydrogen bonding interactions between GalNAc amide and Neu5Ac C_4 hydroxyl groups, and counterion effects. Two extensive studies of the three-dimensional structure of GM1 ganglioside have been reported (5, 6). Scarsdale, Prestegard, and Yu (5) suggested that the allowed conformation for the GalNAc(β,1 → 4)[Neu5Ac(α,2 → 3)]Gal(β,1 → 1′) trisaccharide in GM1 is favorable for the formation of a hydrogen bond between the GalNAc-NH and the Neu5Ac-COO^- groups. Acquotti et al. (6) proposed a similar type of H-bond to stabilize this structure. On the basis of comparative analysis of ^{1}H-NMR of the exchangeable amide protons of GM2, GA2, and GM3, Levery (7) suggested that such a hydrogen bonding interaction exists and could contribute to the stabilization of a single conformer only slightly different from that proposed by Scarsdale et al. (5) and Acquotti et al. (6).

In this work we provide evidence supporting that a hydrogen bonding interaction between the hydrogen atom of an un-ionized carboxyl group in the Neu5Ac and the oxygen atom of the carbonyl group in GalNAc favors the stability of the undissociated forms of Neu5Ac and impairs its acid and enzymatic hydrolysis. In addition, our results suggest that the enzymatic hydrolysis of the ionized form of sialic acid in GM1(Na^+) and GM1(NH_4^+) is impaired by a second hydrogen bonding interaction between the proton of the acetamide group in GalNAc and the carbonyl moiety of the carboxyl group of the Neu5Ac.

MATERIALS AND METHODS

Chemicals

Solvents and chemicals were of the highest purity available. Sialidase (type V) from *Cl. perfringens* was purchased from Sigma Chemical Co. (St. Louis, MO), sialidase from *V. cholerae* and from *A. ureafaciens* were purchased from Boehringer Mannheim Gmbh (Germany). Sephadex and DEAE-Sephadex A-25 (Cl^- form) and Dowex-50WX8 (H^+ form) were purchased from Sigma Chemical Co. Iatrobeads 6RS8060 silica gel was obtained from Iatron Lab. (Tokyo, Japan). Sep-Pak C_{18} cartridges were obtained from Waters (Milford, MA). Pre-coated TLC plates (silica gel 60) were from Merck (Darmstaad, Germany).

Total bovine brain gangliosides were prepared according to Folch, Lees, and Sloane Stanley (8) and purified by successive column chromatography on DEAE-Sephadex and Iatrobeads 6RS8060 columns (9). The content of major gangliosides in the eluted mixture, as determined by scanning the TLC plates in a CS 930 Shimadzu UV/VIS scanner, was GM1 20%, GD1a 40%, GD1b 16%, and GT1b 19%. GM1 ganglioside was prepared from the purified total ganglioside mixture by treatment with *Cl. perfringens* sialidase according to Wenger and Wardell (10) and purified by successive chromatography on Sephadex G-25 (11) and Iatrobeads 6RS8060 (9) columns. The preparation showed a single spot after TLC [resorcinol reagent (12)] running as standard GM1.

GM1 and GM3 derivatives

GM1 or 4-OAc-Neu5Gc-GM3, H^+ or NH_4^+ forms, (see abbreviations for nomenclature) were prepared in batch from solutions of GM1 or 4-OAc-Neu5Gc-GM3, Na^+ form, (0.1% w/v) in distilled water. The solutions were treated for 2 h, at room temperature, under continuous stirring, with Dowex 50WX8 (H^+ or NH_4^+), respectively, at a ratio of Neu5Ac/Dowex 1/10. The suspensions were centrifuged at 1,500 rpm; the supernatant was lyophilyzed and stored at -20°C until use. Alternatively, GM1 or 4-OAc-Neu5Gc-GM3, H^+ form, were prepared as follows: GM1(NH_4^+) or 4-OAc-Neu5Gc-GM3(NH_4^+) were incubated during 30 min at room temperature in acid conditions (5.6 mM formic acid, pH 2.8 or 50 mM sodium acetate buffer, pH 5.3). The mixtures were applied to a Sep-Pak C_{18} column prewashed in succession with methanol, chloroform, methanol, and water, according to Williams and McCluer (13). After washing the column with 25 ml of water, the gangliosides were eluted with 10 ml of chloroform–methanol 2:1 (v/v). This treatment results in quantitative formation of GM1(H^+) or 4-OAc-Neu5Gc-GM3(H^+) as ascertained by determination by the ratio of Neu5Ac to NH_4^+ [resorcinol (12) and ninhydrin reagents (14), respectively].

GM1(NA), GM1(NA/GalNH_2), lysoGM1(NA) and lysoGM1(NA/GalNH_2) were prepared according to Taketomi and Kawamura (15) and Nores et al. (16). Briefly, 100 mg of GM1(NH_4^+) was hydrolyzed in 0.1 M

Fig. 1. TLC of GM1 and GM1 derivatives. 1, Starting GM1 ganglioside; 2, GM1(NA); 3, GM1(NA/$GalNH_2$); 4, lysoGM1(NA); 5, lysoGM1(NA/$GalNH_2$); 6, products obtained after alkaline hydrolysis of GM1 ganglioside. The chromatogram was developed with chloroform–methanol–0.25% $CaCl_2$ 60:35:8 (v/v/v) and the plate was charred with 20% H_2SO_4.

KOH in n-butanol–water 9:1 (v/v) at 117°C for 2 h. The preparation was neutralized with HCl and evaporated to dryness with N_2. The sample was resuspended in 20 ml of water and applied to a Sep-Pak C_{18} column precoated according to Williams and McCluer (13). After washing with water, the derivatives were eluted with methanol and chloroform–methanol 2:1 (v/v). The eluates were pooled, evaporated under N_2, and purified on Iatrobeads 6RS8060 column previously equilibrated with chloroform–methanol–water 65:20:2 (v/v/v) and eluted with a gradient of chloroform–methanol–water 60:20:2 (v/v/v) to 60:35:8 (v/v/v). Alternatively, GM1(NA) was purified from total bovine brain gangliosides according to Hidari et al. (17). TLC of the GM1 derivatives is shown in **Fig. 1.**

GM1 and GM1 derivatives were converted to the methylesters by the method described previously (18). Briefly, 10 mg of GM1 or GM1 derivatives (NH_4^+ salt) were dissolved in 500 µl of dimethylsulfoxide, 100 µl of methyliodide was added to the solution, and the mixture was left for 15 min at room temperature. The reaction mixture was diluted with 20 ml of water and applied to a Sep-Pak C_{18} column that was prewashed as described by Williams and McCluer (13). After washing the column with 25 ml of water, the esters were eluted with 5 ml of methanol and 25 ml of chloroform–methanol 2:1 (v/v). Eluates were pooled and evaporated.

Spectrometry

^{1}H-NMR spectra (200.13 MHz) of 10^{-2} M solutions of GM1(H^+) or GM1(NH_4^+) in deuterated water (D_2O) were recorded at room temperature on a Bruker AC 200 pulse spectrometer operating in the Fourier transform mode. The pulse width was 6 µsec, the acquisition time was 2.713 sec, and the number of transients was 1000; signals were assigned with reference to the central signal of deuterated water at 4.63 ppm relative to trimethyl silane. IR-transmission spectra were recorded using dry GM1(H^+) and GM1(NH_4^+) as KBr-pellets, about 2 mg of lipid per 100 mg KBr was used. The spectra were recorded at room temperature on a Nicolet 5SXC FTIR.

Counterion exchange with DEAE-Sephadex A-25 and Dowex 50WX8

Solutions (2% w/v) of GM1(H^+) or GM1(NH_4^+) in methanol–water 1:1 (v/v) were adsorbed in batch, at room temperature and under continuous stirring, to DEAE-Sephadex (Cl^- form) at different ratios of Neu5Ac/DEAE-Sephadex equivalents. The samples were centrifuged and aliquots of the supernatant were taken at different times for determination of unbound ganglioside by HCl-resorcinol reagent (12). Solutions (0.1% w/v) of GM1 or GM3 (H^+ or NH_4^+ forms) in distilled water were treated in batch, at room temperature and under stirring, with Dowex 50WX8 (H^+ or NH_4^+) at a ratio of Neu5Ac/Dowex 1/10. After centrifuging, aliquots of the supernatant were taken at different times for ammonium determination with ninhydrin reagent (14).

Acid–base titration of ganglioside derivatives

Titration of the different GM1 derivatives was carried out with a Mettler DL21 (Mettler Corp., NJ) autotitrator (1 ml burette) with HCl approx. 0.05 M in bidistilled water or with carbonate-free NaOH approx. 0.05 M. The titrant solutions of HCl or NaOH were standardized, after degassing, on the same day of the experiments with recrystallized sodium carbonate or potassium biphtalate, respectively, under N_2. The titrations (1.5–4 µmol of lipid in bidistilled water with 0.005 M NaCl) were carried out in a 1.5-ml cuvette under continuous stirring at room temperature in a thermostatized cell block (SPR-5 with KPC-5 temperature controller-programmer, Shimadzu Corp. Tokyo, Japan).

Release of sialic acid by sialidase

Sialidase activity was routinely determined in 0.2 ml of either 50 mM sodium acetate buffer, pH 5.3 (*Cl. perfringens* and *A. ureafaciens* sialidases), 50 mM sodium acetate buffer containing 4 mM $CaCl_2$ and BSA 100 µg/ml (*V. cholerae* sialidase), or 10 mM phosphate, pH 7.4 (all sialidases). The assay mixture contained total gangliosides, GM1(NH_4^+), or GM1 derivatives and enzyme, usually 5–10 milliUnits in terms of N-acetyl-neuraminyl-lactose hydrolysis, and were incubated at 37°C for specified periods of time. Glycerol (5–15% v/v) or DMSO (15% v/v) were included in some of the assays

as mentioned in the Results section. After incubation the mixtures were immediately cooled in an ice bath. In mixtures not containing glycerol, the sialic acid released was determined by thiobarbituric acid reagent (19); the mixtures containing glycerol were passed through Sep-Pak C_{18} columns pre-treated according to Williams and McCluer (13) and the free sialic acid eluted was determined by HCl-resorcinol reagent (12).

RESULTS AND DISCUSSION

^{1}H-NMR and IR spectra

Figure 2 shows the IR spectra of GM1. The following absorption bands are observed either in GM1(H^+) or GM1(NH_4^+): amide I band (between 1609 and 1651 cm^{-1}) corresponding to the carbonyl vibration of N-acetyl groups in GalNAc, Neu5Ac, N-acyl group in ceramide, and to ionized carboxyl groups of Neu5Ac, respectively. Amide II band (between 1550 and 1557 cm^{-1}) corresponding to vibrations of –NH and C–N linkages of N-acetyl and N-acyl groups. Only GM1(H^+) (Fig. 1B) shows an absorption band at 1730 cm^{-1} that corresponds to the vibration of the un-ionized carboxyl group of Neu5Ac (20).

Previous studies indicated interactions between the N-acetyl moiety of GalNAc and the carboxylate group of Neu5Ac. These involve hydrogen bonding between the nitrogen –H of the N-acetyl moiety of GalNAc and the carbonyl group of the Neu5Ac carboxylate (5). **Figure 3** shows a low resolution ^{1}H-NMR spectra of GM1. In GM1(H^+) a resonance peak occurs at 14 ppm that is absent in GM1(NH_4^+). The position of this peak corresponds to the proton of the undissociated carboxyl group of Neu5Ac stabilized by hydrogen bonding to the carbonyl group of the N-acetyl moiety of GalNAc (21) (note that the signal of this peak is weak because it represents the resonance of a single proton compared to the rest of the spectral signals resulting from a multitude of hydrogen resonances).

Acid hydrolysis of the Neu5Ac glycosidic bond

Figure 4 shows the release of sialic acid from gangliosides by acid hydrolysis (5.6 mM formic acid, pH 2.8, 80°C). Total gangliosides were hydrolyzed to GM1 (68.63% w/w), GA1 (11.5% w/w), and free Neu5Ac (19.90% w/w). The maximum hydrolysis was reached at 6 h, with no further increase.

The presence of the acetyl group in GalNAc increases considerably the resistance to acid conditions of the Neu5Ac bound to the internal galactose. GM1(NH_4^+), GM1(H^+), or GM1(NA), in which only the sialic acid is de-acetylated, are not hydrolyzed. By contrast, a quantitative release of sialic acid is observed for

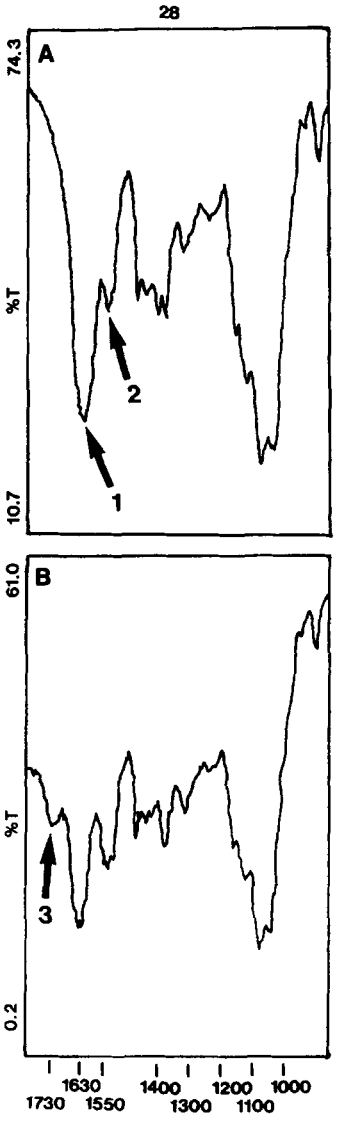

Fig. 2. Infrared spectra of GM1(NH_4^+) (A), and GM1(H^+) (B). Arrows: 1, amide I band; 2, amide II band; and 3, absorption band that corresponds to un-ionized carboxyl group of Neu5Ac.

lysoGM1(NA/$GalNH_2$) and GM1(NA/$GalNH_2$) at 5 h and 6 h of incubation, respectively. As the latter derivatives are de-acetylated in both Neu5Ac and GalNAc, there is no possibility in these compounds for establishing hydrogen bonding interactions between the Neu5Ac carboxylate and the GalNAc carbonyl. The presence of an undissociated or partially ionized carboxyl group of Neu5Ac in GM1, possibly stabilized by hydrogen bonding with the carbonyl of GalNAc, may impair the hydrolysis of the sialic acid. Methyl-esterification of the carboxyl group of Neu5Ac in lysoGM1(NA/$GalNH_2$) or GM1(NA/$GalNH_2$) completely abolishes the hydrolysis of sialic acid (Fig. 4, inset). Chemical modifications such as alkyl-esterification, alkyl-amidation, or reduction of the carboxyl group

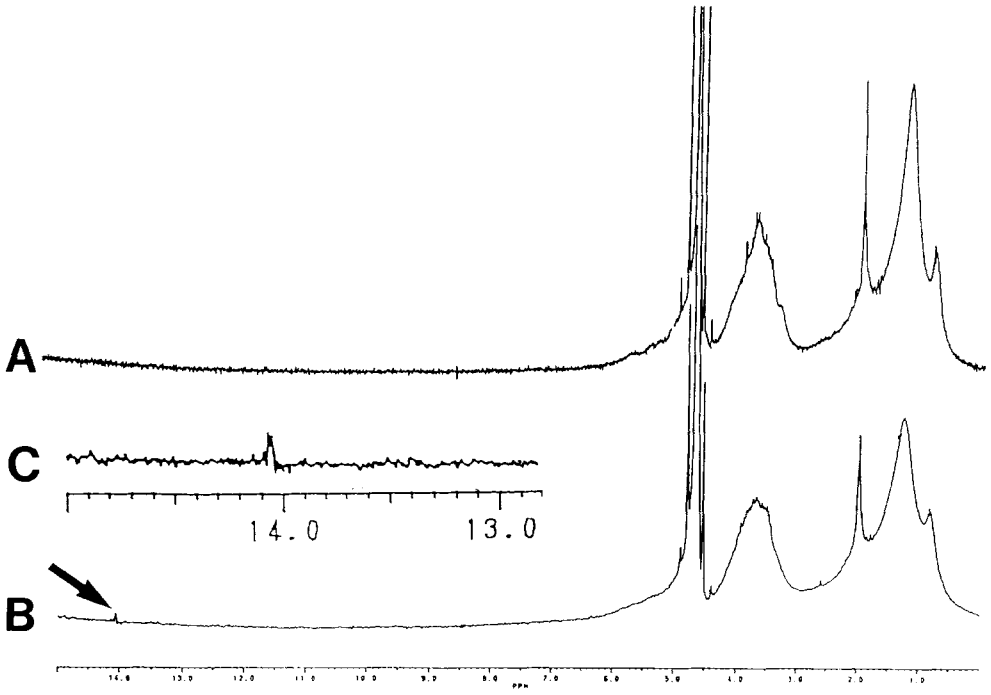

Fig. 3. ^{1}H-NMR spectra of GM1(NH_4^+) (A), and GM1(H^+) (B). (C) represents the enlargement of the peak at 14 ppm present in the GM1(H^+) spectra (see arrow).

of Neu5Ac abolish any possibility for ionization of the carboxyl group of sialic acid and confer resistance of this residue to hydrolysis. The sialic acid of GM1 is completely released under strong acid conditions (1 M formic acid, 80°C, 60 min). Again, methyl-esterification of the carboxyl group of Neu5Ac in GM1 prevents its hydrolysis (Fig. 4, inset).

Figure 5 shows the release of sialic acid of GM1(NH_4^+) or GM1(H^+) under acid conditions in the presence of DMSO. The Neu5Ac residue becomes labile with the increase of concentration of DMSO, a well-known com-

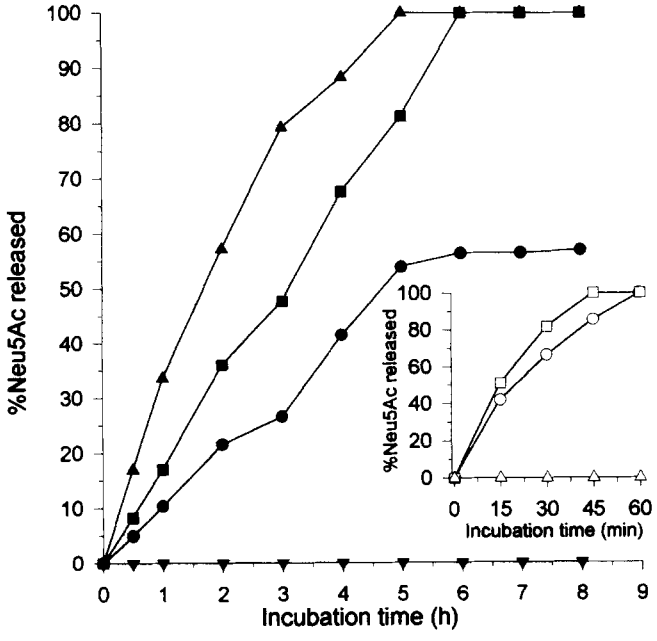

Fig. 4. Release of sialic acid from gangliosides and GM1 derivatives, 5.6 mM formic acid, pH 2.8, 80°C: (●) total gangliosides, (■) GM1(NA/GalNH$_2$), (▲) lysoGM1(NA/GalNH$_2$), (▼) GM1, (▼) GM1-CH$_3$, (▼) GM1(NA), and (▼) lysoGM1(NA/GalNH$_2$)-CH$_3$. Inset: 1 M formic acid, 80°C. (○) GM1, (□) lysoGM1(NA/GalNH$_2$), and (△) lysoGM1(NA/GalNH$_2$)-CH$_3$.

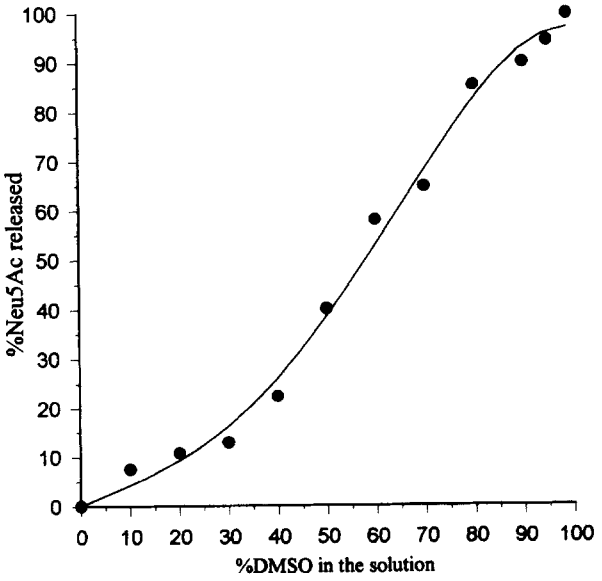

Fig. 5. Release of sialic acid of GM1 under acid condition (5.6 mM formic acid, 80°C for 6 h) in the presence of DMSO.

petitor for hydrogen bonding. In the absence of DMSO, the sialic acid is completely resistant to hydrolysis; at 50% DMSO about 40% of Neu5Ac is released and this reaches 100% in a 99% solution of DMSO. Probably, DMSO competitively disrupts the hydrogen bonding interaction between the sialic acid carboxyl group and the carbonyl group of the N-acetyl residue in GalNAc, thus inducing the full ionization of the carboxyl moiety. This is necessary in order to achieve the intramolecular catalysis required for the hydrolysis of the glycosidic bond of Neu5Ac.

GM1(NH_4^+) or GM1(H^+) incubated in the absence of salt remain stable to acid hydrolysis (5.6 mM formic acid, pH 2.8, 80°C) after 4 h of incubation whereas the presence of high ionic strength (NaCl 1.47 M, equivalent ratio Na^+/Neu5Ac = 228.07/1) leads to about 60% hydrolysis at this time (not shown). This result is in agreement with reports by Li, King, and Li (22) showing that the enzymatic hydrolysis of the sialic acid of GM1 and GM2 by sialidase of *Cl. perfringens* in the presence of bile salts is favored by a high ionic strength in the buffer. Also, in the absence of taurodeoxycholate, it is possible to enzymatically release the sialic acid of GM2 by modification of the ionic strength of the buffer.

Exchange of GM1 counterion

GM1(H^+) is poorly adsorbed to the DEAE-Sephadex A-25 even when the resin is in stoichiometric excess, while GM1(NH_4^+) adsorbs faster and to a greater extent (at comparable times). Similarly, GM1(NH_4^+) stoichiometrically exchanges the NH_4^+ ion for the resin H^+ after 1 h but, even after 2 h, only 12% of GM1(H^+) has exchanged its proton for the NH_4^+ group of the resin. For comparison, GM3(H^+) and GM3(NH_4^+) stoichiometrically exchange their counter-ion with that of the resin. These results (not shown) are in keeping with an impairment of the dissociation of the carboxyl of Neu5Ac in GM1(H^+). Similar to the binding to DEAE-Sephadex, an increase of the amount of resin or of the incubation time leads to an increased exchange to Dowex 50WX8.

Acid–base titration of ganglioside derivatives

Early work on the acid base titration of gangliosides was carried out by Abramsom, Yu, and Zaby (23). The studies suggested that some of the ganglioside carboxyl groups might be unavailable for titration due to undefined hydrogen bonding interaction. In addition, these authors suggested that the presence of mono- and bivalent cations disrupted such interactions and made the carboxyl groups more accessible to protonation. However, at that time these studies were performed with a total native ganglioside mixture and the specific interactions responsible for the changes in the carboxyl group ionization remained undefined.

Table 1 shows the acid titration of the carboxyl group of Neu5Ac or the alkali titration of the available $-NH_2$ groups of the derivatives. Free Neu5Ac can be quantitatively titrated with NaOH. As expected, the titration of GM3, GM1, GD3, and GD1a with NaOH did not reveal any titratable amino groups because all are acetylated. The $-NH_2$ groups of the de-acetylated derivatives GM1(NA) and GM1(NA/$GalNH_2$) were titrated with NaOH with a pK between 8 and 9.5. The titrations carried out with HCl showed that GM3 and GD3 (that do not have the GalNAc moiety) have one and two fully titratable Neu5Ac carboxyl groups per molecule, respectively, as expected. By contrast, in native GM1 and GM1(NA) only a 13% and 9.7%, respectively, of the sialic acid content (resorcinol quantitation) was available for titration. The carboxyl group of Neu5Ac in most of native GM1 appears stabilized by dipole-charge interactions (8, 20) between the acetamide group of GalNAc and the carboxylate moiety of Neu5Ac.

In GD1a that contains two molecules of Neu5Ac per molecule of ganglioside, only one of the sialic acids can be fully titrated while the other is accessible to less than 10%. On the basis of the results obtained with GM1, it is likely that the partially available sialic acid of GD1a is the one linked to the proximal galactose residue in proximity to GalNac, and whose dissociation is impaired as in the monosialoganglioside. In GM1(NA) that is de-acetylated only in the sialic acid, 9.7% of the Neu5Ac is titrated, similar to native GM1. By contrast, in GM1(NA/$GalNH_2$) that is de-acetylated in both the Neu5Ac and GalNAc moieties, 98% of the sialic acid can be titrated with HCl.

TABLE 1. Acid–base titration of Neu5Ac, gangliosides, and GM1 derivatives

Specie	Titration	% Titrated	pK
Neu5Ac	OH^-	96	3.94
GM3(Na^+)	H^+	95	3.77
GM1(Na^+)	H^+	13	4.73
GM1(Na^+)	OH^-	–	–
GM1(H^+)	H^+	–	–
GM1(H^+)	OH^-	46.4	4.23
		53.6	5.81
GM1(NA)	H^+	9.7	4.02
GM1(NA)	OH^-	96	8.52
GM1(NA/$GalNH_2$)	H^+	98.4	3.40
GM1(NA/$GalNH_2$)	OH^-	80	9.47
GD3	H^+	97	4.37
GD1a(Na^+)	H^+	46.4	4.53
GD1a(Na^+)	OH^-	–	–

The titration of GM1(H^+) with NaOH shows that 46.4% of the Neu5Ac is titrated with a pK of 4.23, similar to the other derivatives, but 53.6% of Neu5Ac exhibits a pK of 5.81. This increase of pK is in keeping with the stabilization of at least half of Neu5Ac by hydrogen bonding between the carboxyl proton and the carbonyl group of GalNAc which favors the equilibrium toward the un-ionized form of the carboxyl group.

Enzymatic release of sialic acid from ganglioside derivatives

Although in GM1(NH_4^+) and GM1(Na^+) the carboxyl group of sialic acid is in the ionized state, this is not readily hydrolyzed by *Cl. perfringens* or *V. cholerae* sialidases in neutral buffer. It is possible that the structural rigidity conferred to the Neu5Ac residue by the hydrogen bonding between the GalNAc amide proton and the carboxyl moiety of the ionized carboxyl group of Neu5Ac impairs the accessibility of the Neu5Ac α-ketosidic linkage to the enzyme active site. O-acetylation of the Neu5Ac or Neu5Gc markedly interfered with the sialic acid enzymatic hydrolysis; this influence is enhanced when the acetyl group is located in O_4 or O_7 of the sialic acid moiety and causes complete resistance to hydrolysis by sialidases (24). It has been suggested that the electrostatic and steric effects derived from O-acetylation decrease the rate of protonation of the glycosidic oxygen or the stability of the carbonium-oxonium ion intermediate. The steric effect of the O-acetyl group in C_4 or C_7 interferes with the hydrolysis by impairing the chair–half chair change of conformation of the sialic acid which requires rotation along the C_3–C_4 and C_5–C_6 bonds (1). As an acetyl group causes a greater inductive effect than a hydroxyl group (1), the decreased rate of hydrolysis was explained by a reduced stability of the carbonium-oxonium ion intermediate due to the enhanced inductive effect of O-acetylation in C_4 or C_7. Acetoxy substituents in C_8 or C_9 of sialic acid are too far away and unimportant for these effects.

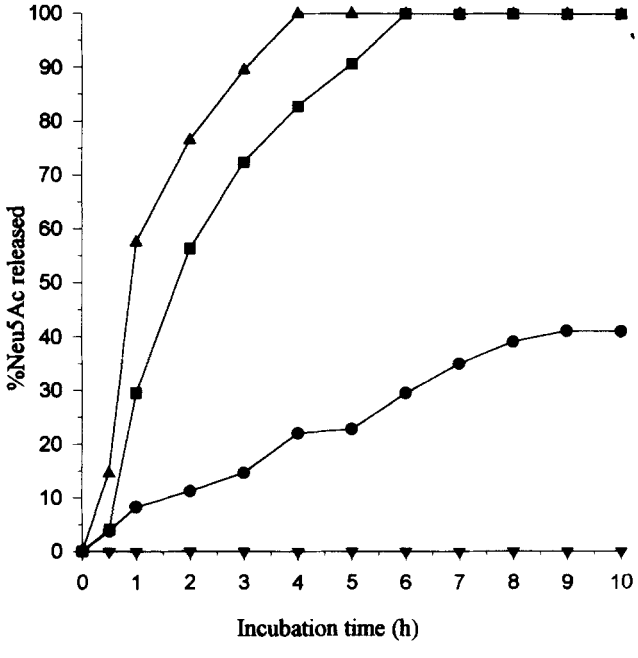

Fig. 6. Enzymatic release of sialic acid from gangliosides and GM1 derivatives with *Cl. perfringens* sialidase (for conditions see Materials and Methods). (●) Total gangliosides, (■) GM1(NA/$GalNH_2$), (▲) lysoGM1(NA/$GalNH_2$), (▼) GM1, (▼) GM1-CH_3, (▼) GM1(NA), (▼)GM1(NA)-CH_3, (▼) GM1(NA/$GalNH_2$)-CH_3, and (▼) lysoGM1(NA/$GalNH_2$)-CH_3.

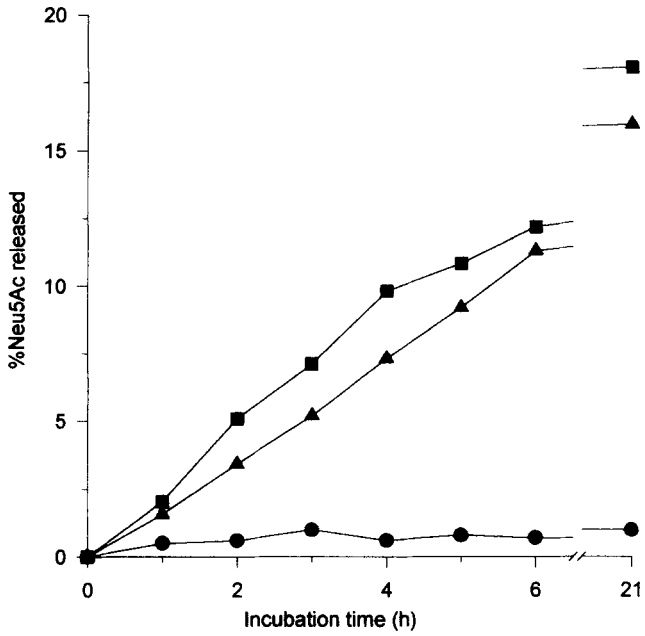

Fig. 7. Release of sialic acid of GM1 by *Cl. perfringens* sialidase in 50 mM sodium acetate buffer in the absence (●), or presence of DMSO (■) or glycerol (▲). Similar results are obtained using buffer phosphate, and with sialidases of *V. cholerae* and *A. ureafaciens* (not shown).

We found that in phosphate buffer at pH 7.4, the carboxyl group of sialic acid in 4-OAc-Neu5Gc-GM3(NH_4^+) is ionized and the enzymatic hydrolysis is complete, while the 4-OAc-Neu5Gc-GM_3(H^+) is resistant (not shown). This is in keeping with a stabilization of the un-ionized form of the sialic acid residue by hydrogen bonding between the carbonyl moiety of the O-acetyl substituent and the proton of the carboxyl group of Neu5Gc. By contrast, in sodium acetate buffer at pH 5.3, either the NH_4^+ or the H^+ form of 4-OAc-Neu5Gc-GM3 are resistant to hydrolysis. This is because the NH_4^+ ion in the 4-OAc-Neu5Gc-GM3 is exchanged by the protons of the medium to form 4-OAc-Neu5Gc-GM3(H^+) (as ascertained by Sep-Pak C_{18} column chromatography after conversion of the NH_4^+ to the acid form in the acetate buffer, see Materials and Methods). Once the sialic acid is protonated, it is stabilized by the above-mentioned hydrogen bonding and becomes resistant to hydrolysis.

The elimination of the N-acetyl moiety of Neu5Ac and of GalNAc in GM1(NA/$GalNH_2$) and lysoGM1(NA/$GalNH_2$) induces complete hydrolysis of sialic acid by *Cl. perfringens* sialidase (**Fig. 6**). Methyl-esterification of the carboxyl group in GM1(NA/$GalNH_2$) and lysoGM1(NA/$GalNH_2$) abolishes the susceptibility of sialic acid to enzymatic hydrolysis. In addition, the pentasaccharide II^3Neu5Ac-GgOSe_4 prepared from GM1 is completely resistant to the action of *Cl. perfringens* sialidase, even in the presence of bile salts (25).

In the presence of DMSO or glycerol (**Fig. 7**) the sialic acid of GM1 can be released with *Cl. perfringens* sialidase, even in the absence of bile salts. The hydrolysis by *V. cholerae* and *A. ureafaciens* sialidase is also increased in the presence of DMSO and glycerol (data not shown). This is consistent with DMSO and glycerol interfering or competing with intramolecular hydrogen bonding between the Neu5Ac carboxyl group and the GalNAc carbonyl moiety. The enzymatic hydrolysis by all of the sialidases requires the integrity of the ionized carboxyl group of Neu5Ac: alkyl-esterification, alkyl-amidation, or carboxylate reduction in GD1a causes that even the Neu5Ac bound to the distal galactose residue becomes completely resistant to hydrolysis (26).

GM1 can be hydrolyzed by sialidase from *A. ureafaciens* with high affinity and low V_{max} compared to the GM1 oligosaccharide portion lacking the ceramide moiety. It has been suggested that this may be the consequence of the ceramide moiety on the micelle-forming capacity of the ganglioside. The lypophilic portion may enhance the substrate affinity for the enzyme while restricting the structural mobility required for catalysis (27).

Table 2 shows the kinetic parameters for the enzymatic hydrolysis of GM1, GM1(NA), and GM1(NA/$GalNH_2$) with sialidase from *A. ureafaciens*, and of lysoGM1(NA/$GalNH_2$) and GM1(NA/$GalNH_2$) with *Cl. perfringens* sialidase. The K_m values are practically unaltered compared to those for GM1(NH_4^+) while the V_{max} of GM1(NA) is reduced. This indicates that the recognition of the sialic acid moiety by the enzymes is little affected by de-acetylation of Neu5Ac but the rate of hydrolysis is reduced. De-acetylation of the GalNAc residue restores or increases the susceptibility to enzymatic degradation of Neu5Ac, depending on the derivative.

TABLE 2. Kinetic data for sialidase activity on GM1 and GM1 derivatives as substrates

Sialidase	Substrate	K_m	V_{max}
		mM	*nmol/min*
Arthrobacter ureafaciens	GM1	0.161	1.35
	GM1(NA)	0.163	0.69
	GM1(NA/$GalNH_2$)	0.163	1.44
Clostridium perfringens	GM1(NA/$GalNH_2$)	0.162	1.74
	LysoGM1(NA/$GalNH_2$)	0.163	2.77

TABLE 3. Behavior of GM1 and representative GM1 derivatives treated with *A. ureafaciens*, *Cl. perfringens*, and *V. cholerae* sialidases (37°C for 5 h)

Substrate	Sialidase		
	A. ureafaciens	Cl. perfringens	V. cholerae
	% Neu5Ac released		
GM1	50	0	0
GM1(NA)	25	0	0
GM1(NA/$GalNH_2$)	50	100	30

Table 3 summarizes the behavior of representative derivatives treated with *A. ureafaciens*, *Cl. perfringens*, and *V. cholerae* sialidase. GM1(NA/$GalNH_2$) is the only compound degraded by the *Cl. perfringens* enzyme and, to a much lesser extent, by the *V. cholerae* enzyme. The sialidase from *A. ureafaciens* can hydrolyze the sialic acid of GM1(NH_4^+) (see also Table 2) but, when the Neu5Ac is de-acetylated [i.e., in GM1(NA)], there is a reduction of the enzyme hydrolytic capacity by about 50%. This enzyme probably requires the presence of the N-acetyl moiety in the position C_5 of the sialyl residue as the deaminated sialic acid KDN (2-keto-3-deoxy-D-glycero-D-galacto nonoic acid) present in glycoproteins or some gangliosides is not a suitable substrate for the enzyme (28). When both the Neu5Ac and GalNAc are de-acetylated [i.e., in GM1(NA/$GalNH_2$)] the activity of *A. ureafaciens* sialidase increases to the level observed for GM1(NH_4^+).

CONCLUSIONS

The cumulative results described with the different assays consistently point to an impairment of the full dissociation of the carboxyl group of Neu5Ac in ganglioside GM1, in slightly acid conditions. This is correlated to its capacity to undergo acid and enzymatic hydrolysis. In order to achieve resistance of the Nau5Ac, it is also required that an intact GalNAc residue be present in its adjacent proximity on the oligosaccharide chain. As a result of a poor carboxyl group dissociation, the susceptibility of Neu5Ac to hydrolysis is greatly reduced. The elimination of the acetyl moiety in GalNAc allows the complete acid and enzymatic hydrolysis of the Neu5Ac of GM1. On the other hand, the sole elimination of the acetyl moiety of Neu5Ac does not have such influence. All of our results indicated that the major reason for the insensitivity of the Neu5Ac to hydrolysis in gangliosides having the core oligosacharide GalNAc(β,1 → 4)[Neu5Ac(α,2 → 3)]Gal(β,1 → 4)Glc(β,1 →

1′) is due to the reduced dissociation of the Neu5Ac carboxyl group. This appears to be the result of stabilization by a hydrogen bonding interaction between the partially undissociated carboxyl group in the sialic acid and the carbonyl group of the N-acetyl moiety in the neighboring GalNAc residue. In addition, our results suggest that the enzymatic hydrolysis of the ionized form of sialic acid in GM1(Na^+) and GM1(NH_4^+) is impaired by a second hydrogen bonding interaction between the proton of the acetamide group in GalNAc and the carbonyl moiety of the carboxyl group of the Neu5Ac.

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