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Studies on the Interaction of *Clostridium perfringens* Sialidase with Sialic Acid Linked to the Internal Galactose in Monosialogangliotetraosyl Ceramide[1]

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1. Investigation of the action of highly purified *Clostridium perfringens* sialidase on ganglioside II^{3}Neu5Ac-Gg$_4$Cer and its oligosaccharide II^{3}Neu5Ac-Gg$_4$, in the presence and absence of sodium cholate, extend earlier results obtained with impure enzyme fractions.
2. Sialidase labeled with ^{125}I was found to bind to various ganglioside substrate micelles, including II^{3}Neu5Ac-Gg$_4$Cer, and to mixed ganglioside-sodium cholate micelles.
3. No binding occurred between the enzyme and the ganglioside-derived oligosaccharide II^{3}Neu5Ac-Gg$_4$, even when radioactive II^{3}Neu5Ac-Gg$_4$-[^{3}H]ol was used.
4. The binding of sialidase to micellar substrate is a condition for enzymic hydrolysis. Correspondingly, II^{3}Neu5Ac-Gg$_4$Cer and II^{3}Neu5Ac-Gg$_4$Cer-sodium cholate micelles were hydrolyzed by the enzyme but II^{3}Neu5Ac-Gg$_4$ was not.
5. Ganglioside oligosaccharide analogues containing an amino function at the reducing terminus or between two oligosaccharide chains, II^{3}Neu5Ac-Gg$_4$-NH$_2$ and (II^{3}Neu5Ac-Gg$_4$)$_2$NH, were hydrolyzed in the absence of cholate. A synthetic analogue of II^{3}Neu5Ac-Gg$_4$Cer containing only the fatty acid moiety and not the sphingosine residue (I^1-deoxy-I^1-stearamido-II^3-monosialo-gangliotetraitol) behaved as the ganglioside in the presence and absence of sodium cholate.

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Abbreviations: Neu5Ac, *N*-acetylneuraminic acid. Glycolipids and oligosaccharides are abbreviated according to the recommendations of the IUPAC-IUB Lipid Nomenclature document, *Lipids* **12**, 455–468 (1977) (see the table). Sialidase, acylneuraminate hydrolase [EC 3.2.1.18]. CMC, critical micelle concentration.

6. The results are discussed in the light of 500 MHz ^{1}H-NMR data which may suggest less conformational freedom of the internal galactose-linked sialic acid residue in II^{3}Neu5Ac-Gg$_4$ relative to the sialic acid in terminal, linear position of IV^{3}Neu5Ac-, II^{3}Neu5Ac-Gg$_4$. This difference in conformation of the two sialic acid linkages may give an explanation for the resistance of the internal sialic acid towards the action of sialidase.

The sialic acid residues of the following gangliosides almost or completely resist the attack of *Vibrio cholerae* and of most of the other bacterial sialidases tested, *i.e.* II^{3}Neu5Ac-Gg$_3$Cer, II^{3}Neu5Ac-Gg$_4$Cer, IV^{2}Fuc-,II^{3}Neu5Ac-Gg$_4$Cer, IV^{3}Galα-,IV^{2}Fuc-,II^{3}Neu5Ac-Gg$_5$Cer,IV^{3}Neu5Ac-Gg$_5$Cer, and IV^{3}Neu5Ac-,II^{3}Neu5Ac-Gg$_5$Cer (for reviews, see Wiegandt (*1*) and Corfield *et al.* (*2*)). The typically enzyme-resistant sialic acid residues in all these cases are in the branching position at the internal galactose residues neighboring the *N*-acetylgalactosamine residues of the ganglio-oligosaccharide chains (*1–3*). A closer investigation of this phenomenon in the case of *C. perfringens* sialidase revealed that nevertheless a greatly reduced but significant hydrolysis occurred, and that the reaction rate increased below an assumed critical micelle concentration (CMC) of the gangliosides (*4, 5*). It was further found that the addition of bile salts gave improved rates of hydrolysis for the *C. perfringens* sialidase (*4, 6–9*). Sialidases from *Arthrobacter ureafaciens* (*8, 10*) and Sendai virus (*11*) acted on II^{3}Neu5Ac-Gg$_4$Cer even in the absence of bile salt, and in incubations both enzymes were stimulated by the inclusion of sodium cholate (*8, 10, 11*).

In order to explain these data the rate of reaction of *C. perfringens* sialidase was first determined at concentrations where the ganglioside II^{3}Neu5Ac-Gg$_4$Cer substrate was assumed to exist as monomers or micelles, respectively, in the solution (*4, 5, 8, 9*). However, the CMC is now known to be approximately 10^{-8} M and the micellar size is concentration-dependent (Ulrich, B. & Wiegandt, H., unpublished observations). This would lead to variation in enzyme binding to the micelles. Addition of bile salts led to the formation of mixed micelles with a higher CMC and lower molecular weight. Maximum hydrolysis was found to occur at fixed ratios of bile salts to II^{3}Neu5Ac-Gg$_4$Cer (*8, 9*). A dual effect of bile salt was proposed, modifying the dispersion state of the substrate (especially II^{3}Neu5Ac-Gg$_4$Cer) and of the enzyme (*9*).

Earlier work had pointed to a steric hindrance of the branching sialic acid residue in II^{3}Neu5Ac-Gg$_4$Cer due to the neighboring GalNAc residue (*12*). ^{13}C- and ^{1}H-NMR studies of the ganglioside suggested an interaction of the amide-carbonyl group of GalNAc with the glycosidic oxygen and the carboxyl function of the sialic acid linked to galactose, yielding a protected glycosidic bond, which is more or less resistant to sialidase attack (*13, 14*).

In all studies to date on the interaction of *C. perfringens* sialidase with II^{3}Neu5Ac-Gg$_4$Cer, commercial enzyme was used and no details of further purification or contaminants given. In the present study a homogenous sialidase preparation was used (*15*) enabling re-evaluation of the data reported, and allowing a direct analysis of interactions with II^{3}Neu5Ac-Gg$_4$Cer after ^{125}I-labeling of the pure enzyme. The investigation of ganglioside and related oligosaccharide derivatives provides further information on the nature of the hydrolytic reaction.

Information obtained from proton-NMR analysis of lipid-free ganglioside oligosaccharides (Dorland, L., *et al.*, unpublished observations) has given an insight into the conformation of sialic acid residues in these structures and their relation to sialidase action.

Some of the results reported here have been presented in preliminary form (*16, 17*).

MATERIALS

Sialidases—*C. perfringens* sialidase was purified to homogeneity as described by Nees *et al.* (*15*). The preparation showed only one enzyme protein band on polyacrylamide gel electrophoresis and the specific activity was 26.8 U/mg protein

with II^{3}Neu5Ac-Lac as substrate. *A. ureafaciens* sialidase was a product of Nakarai Chemicals Ltd., Kyoto, Japan. It contained only enzyme protein and had a specific activity of 40.4 U/mg protein with II^{3}Neu5Ac-Lac as substrate. *V. cholerae* sialidase was a product of Behringwerke, Marburg, FRG, and had a specific activity of 11.0 U/mg protein. Iodination of *C. perfringens* sialidase was by the chloramine T method of Greenwood *et al.* (*18*). Sialidase (134 μg, 3.59 U) was labeled with 1 mCi ^{125}I (Amersham International, Amersham, UK) and the sample applied to a column (30 × 1 cm) of Sephadex G-25 in 0.125 M Tris-HCl buffer, pH 8.1. The column was eluted with the same buffer and aliquots monitored for radioactivity and sialidase activity (with II^{3}Neu5Ac-Lac). The fractions containing enzyme activity were pooled and had a specific radioactivity of 0.76 μCi/μg enzyme protein (28.6 μCi/U). The enzyme was stored at −15°C in the same buffer as above in small (50–100 μl) aliquots. Enzymic activity was monitored at weekly intervals.

Gangliosides and Derivatives—A monosialoganglioside fraction with II^{3}Neu5Ac-Gg$_4$Cer and the disialoganglioside fraction containing IV^{3}Neu5Ac-, II^{3}Neu5Ac-Gg$_4$Cer, and II^3(Neu5Ac)$_2$-Gg$_4$Cer, were isolated from human brain (*19*). The II^{3}Neu5Ac-Gg$_4$Cer preparation was purified by treatment with *V. cholerae* sialidase to remove minor disialoganglioside contaminants, dialysis against distilled water and lyophilization, and by high-pressure liquid chromatography using silica gel H (Merck, Darmstadt) and chloroform, methanol, *n*-hexane, water 60 : 35 : 10 : 4 (v/v) as running solvent. The oligosaccharide II^{3}Neu5Ac-Lac was isolated from bovine colostrum (*20*). II^{3}Neu5Ac-Gg$_4$ (for structure see the table) was prepared from the parent ganglioside and purified by preparative paper electrophoresis (*21*). Reduction of this oligosaccharide to yield II^{3}Neu5Ac-Gg$_4$-[^{3}H]ol was essentially as detailed by Frisch and Neufeld (*22*). The product eluted from Biogel P-2 had a specific radioactivity of 7.5 mCi/mmol and showed only one radioactive component

TABLE I. Structures of monosialoganglioside II^{3}Neu5Ac-Gg$_4$Cer and its derivatives.

Structure	Denomination	Abbreviation
Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc-Cer | α(2-3) Neu5Ac	II^3-monosialo-gangliotetraosyl ceramide	II^{3}Neu5Ac-Gg$_4$Cer
Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc | α(2-3) Neu5Ac	II^3-monosialo-gangliotetraose	II^{3}Neu5Ac-Gg$_4$
Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Sorbitol-NH$_2$ | α(2-3) Neu5Ac	II^3-monosialo-gangliotetraityl-amine	II^{3}Neu5Ac-Gg$_4$-NH$_2$
Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Sorbitol-NH- | α(2-3) Neu5Ac Sorbitol(4-1β)Gal(4-1β)GalNAc(3-1β)Gal | α(2-3) Neu5Ac	bis(II^3-monosialo-ganglio-tetraityl)amine	(II^{3}Neu5Ac-Gg$_4$)$_2$NH
Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Sorbitol-NH-CO- | α(2-3) Neu5Ac (CH$_2$)$_{16}$CH$_3$	I^1-deoxy-I^1-stearamido-II^3-monosialo-gangliotetraitol	—

in two thin-layer chromatographic systems, which corresponded with the orcinol/Fe^{3+} stain (*23*) on the plate. The oligosaccharides Gg_4 and Gg_4-[^{3}H]ol were prepared from II^{3}Neu5Ac-Gg_4 and II^{3}Neu5Ac-Gg_4-[^{3}H]ol, respectively, by incubation in 0.2 M HCl at 90°C for 45 min and passage through columns of Dowex 1×8 (formate, 200–400 mesh). The water wash was pooled and lyophilized and contained the neutral oligosaccharides.

The following ganglioside analogues, the structures and abbreviations of which are shown in the Table I, were prepared as detailed earlier (*24, 25*): I^1-deoxy-I^1-stearamido-II^3-monosialo-gangliotetraitol, II^3-monosialo-gangliotetraityl-amine, and bis(II^3-monosialo-gangliotetraityl)amine.

METHODS

Sialidase Incubations—In all experiments with gangliosides and sodium cholate, chloroform–methanol (2 : 1, v/v) solutions were evaporated to dryness in incubation tubes and 0.05 M sodium acetate of pH 5.1 (incubation buffer) added. Non-ganglioside substrates were added as aqueous solutions. Reaction was initiated by the addition of sialidase and, unless otherwise stated, terminated by mixing with an appropriate volume of periodate reagent for the thiobarbituric acid assay as described (see below and Ref. *26*). All assays were in duplicate.

a) Influence of time, substrates and their concentration: Concentrations of each substrate (II^{3}Neu5Ac-Gg_4Cer or II^{3}Neu5Ac-Gg_4) measured as sialic acid were made up in 700 μl incubation buffer; 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, and 1 mM in the presence and absence of 1.75 mM sodium cholate. Incubation was at 37°C and was initiated by the addition of 8 mU *C. perfringens* sialidase in 20 μl incubation buffer. Aliquots (100 μl) were removed at 30 min, 1 h, 2 h, 5 h, 10 h, and 25 h and mixed with 20 μl periodate reagent. Blanks were incubated under identical conditions but contained no enzyme.

Synthetic analogues of II^{3}Neu5Ac-Gg_4Cer and its oligosaccharide were made up to 650 μl in incubation buffer at sialic acid concentrations of 0.5 mM and 1 mM in the presence and absence of 1.75 mM sodium cholate. Incubations were initiated by the addition of 30 mU of *C. perfringens* sialidase in 25 μl incubation buffer and run at 37°C. Blanks without enzyme were run under the same conditions. Aliquots of 100 μl were withdrawn and assayed for free sialic acid as described above at time intervals up to 8 h.

Incubations with II^{3}Neu5Ac-Gg_4-[^{3}H]ol contained 0.1 μCi (13.4 nmol) of the labeled oligosaccharide, 15 mU *C. perfringens* sialidase and incubation buffer in a final volume of 50 μl and were of 24 h duration at 37°C. Similar incubations with 10 mU *A. ureafaciens* sialidase and 0.1 μCi tritium-labeled oligosaccharide were in 0.05 M sodium acetate of pH 4.8 for 5 h at 37°C. Blanks without enzyme were incubated under the same conditions. The reaction was stopped by diluting the incubation with 1 ml of ice cold distilled water and application to a small (0.8×5 cm) column of Dowex 1×8 (formate, 200–400 mesh). The column was washed with five 2 ml aliquots of water and eluted with five 2 ml aliquots of 0.5 M formic acid. Samples were mixed with 15 ml scintillation fluid and radioactivity measured.

The action of sialidases on II^{3}Neu5Ac-Gg_4-[^{3}H]ol was further evaluated on silica gel thin-layer plates (see below) after incubation of 15 mU *C. perfringens* sialidase with 0.5 μCi ^{3}H-labeled oligosaccharide in incubation buffer (100 μl) for 24 h at 37°C. *A. ureafaciens* sialidase (10 mU) was incubated with 0.5 μCi [^{3}H]oligosaccharide in 100 μl 0.05 M sodium acetate buffer, pH 4.8. Blanks without enzyme were also assayed. Reaction was terminated by cooling in an ice bath and immediate application of the sample to the thin-layer plate. Samples were developed in two different solvent systems (see below). After drying the thin-layer plates were scanned for radioactivity and oligosaccharides visualized as described below.

b) Influence of pH: The influence of pH on the action of *C. perfringens* sialidase on II^{3}Neu5Ac-Gg_4Cer and II^{3}Neu5Ac-Gg_4 was studied using 0.05 M sodium acetate buffer with the pH range of 4–5.5 and 0.05 M sodium phosphate buffer in the range of 6–8. Sialidase (8 mU) was incubated with either substrate at 0.5 mM sialic acid for 5 h at 37°C in the presence or absence of 1.75 mM sodium cholate. The total volume of the incubation was 100 μl. Blanks were run without enzyme addition. Reaction was terminated by mixing with 20 μl periodate reagent for the determination of free sialic acid.

c) Influence of sodium cholate: Variation of sodium cholate concentration between 0.47 mM and 5.8 mM with $II^3Neu5Ac\text{-}Gg_4Cer$ (0.5 mM and 1 mM) and $II^3Neu5Ac\text{-}Gg_4$ (0.5 mM and 1 mM) was in 100 μl of incubation buffer. *C. perfringens* sialidase (8 mU) was added to start the reaction for 5 h at 37°C, and termination was by 20 μl of periodate reagent. Blanks were incubated without enzyme.

d) Influence of $II^3Neu5Ac\text{-}Gg_4$ on C. perfringens sialidase action: Five concentrations of $II^3Neu5Ac\text{-}Gg_4$ (0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, and 1 mM) were incubated with $II^3Neu5Ac\text{-}Lac$ (1.5 mM) in 100 μl incubation buffer with or without 1.75 mM sodium cholate. Reaction was initiated by the addition of 3.6 mU sialidase and terminated with 20 μl periodate reagent after 10 min. Similar experiments with 0.5 mM $II^3Neu5Ac\text{-}Gg_4Cer$ and 1.75 mM sodium cholate were for 5.5 h at 37°C. Blanks contained no enzyme.

e) Influence of C. perfringens sialidase concentrations: Sialidase concentrations between 2.5 mU and 80 mU/100 μl were included in 250 μl incubations with 0.5 mM $II^3Neu5Ac\text{-}Gg_4Cer$ containing 1.75 mM sodium cholate. After 5 h at 37°C reaction was stopped by the addition of 50 μl periodate reagent. Incubations with 0.01 mM $II^3Neu5Ac\text{-}Gg_4Cer$ were in 500 μl incubation buffer and terminated with 100 μl of periodate reagent after 1 h at 37°C.

f) Gel filtration of sialidase-ganglioside mixtures: ^{125}I-labeled *C. perfringens* sialidase (100 μl = 0.1 μCi and 3.5 mU) was incubated in 500 μl buffer containing $II^3Neu5Ac\text{-}Gg_4Cer$ or other substrates at 1 mM concentration, with or without 1.75 mM sodium cholate. Incubation was routinely for 20 min at 20°C followed by 10 min at 4°C, but 1 h and 5 h assays at 37°C were also performed. These incubations were chromatographed on Sephadex G-200 superfine (1.25 × 20 cm) in 0.05 M sodium acetate buffer pH 5.1 at 4°C and 0.7 ml/min. The included and excluded volumes were determined before and after each batch of sialidase samples, using blue dextran and sodium azide. In some runs 0.4 M NaCl was included in the eluting buffer and in others the pH of the 0.05 M sodium acetate buffer was 3.5. In each case the column was equilibrated for 16 h in the eluting buffer before use.

Thin-Layer Chromatography—Thin-layer chromatography on precoated silica gel 60 plates (Merck, Darmstadt) was used to separate gangliosides, developing in butan-1-ol : pyridine : water (9 : 6 : 4, v/v) containing 1 mg KCl/ml; solvent 1. Oligosaccharides were separated on silica gel 60 precoated plates in two solvent systems, ethanol : butan-1-ol : pyridine : glacial acetic acid : water (100 : 10 : 10 : 3 : 30, v/v; solvent 2) and butan-1-ol : glacial acetic acid : water (2 : 1 : 1, v/v; solvent 3). Components were visualized by the orcinol/Fe^{3+} spray reagent (*23*) or by spraying with H_2SO_4 : methanol (1 : 1, v/v), with heating at 120°C for 10 min.

Radioactive compounds were identified using a Berthold LB 2733 radio-thin-layer scanner.

Sialic Acid Determination—Free sialic acid was measured using a microadaptation of the periodate/thiobarbituric acid assay and reading the absorbance at two wave-lengths (*23*). Total sialic acid was measured by a microadaptation of the orcinol/Fe^{3+} method (*23*).

RESULTS

Release of N-Acetylneuraminic Acid from Ganglioside $II^3Neu5Ac\text{-}Gg_4Cer$ and Its Derivatives by Clostridium perfringens Sialidase—a) pH Optimum: A well defined pH optimum for *C. perfringens* sialidase action with $II^3Neu5Ac\text{-}Gg_4Cer$ was observed in the region of pH 5 in the presence of 1.75 mM sodium cholate. Without cholate a maximum was reached at pH 5–5.5, which remained constant in the range of pH 6–8 in phosphate buffer.

No release of sialic acid from the monosialogangliotetraose $II^3Neu5Ac\text{-}Gg_4$ was found under any condition.

b) Time course of reaction: Release of Neu5Ac from ganglioside $II^3Neu5Ac\text{-}Gg_4Cer$ in the absence of sodium cholate reached a maximum between 2 and 5 h of reaction at all concentrations of this substrate (Fig. 1a). After this initial period no further enzyme action occurred up to 25 h. The plateaus observed with increasing substrate concentration showed a decreasing percentual release of total Neu5Ac in the incubation. Maximum release was found at the lowest substrate concentration (0.05 mM) and this was 9% of the total sialic acid (Fig. 1a).

In the presence of 1.75 mM sodium cholate a

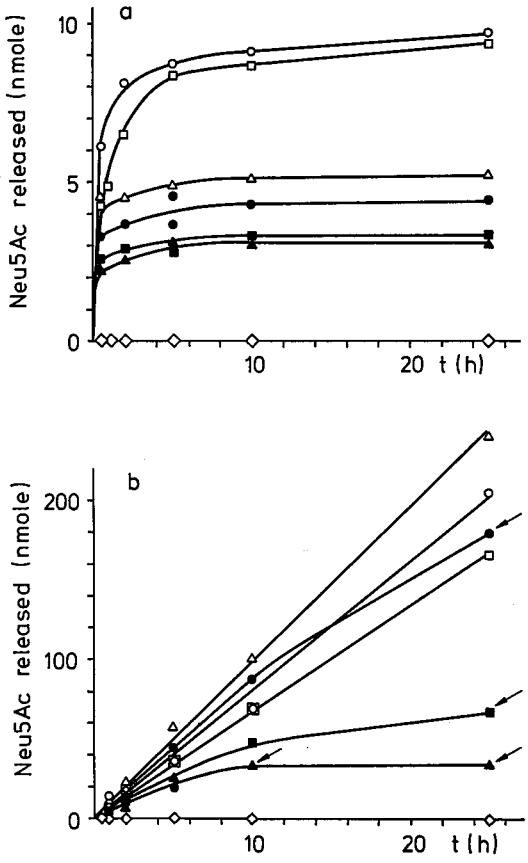


Fig. 1. Influence of time on the release of sialic acid from II³Neu5Ac-Gg₄Cer and II³Neu5Ac-Gg₄ in the absence (a) and presence (b) of 1.75 mM sodium cholate. *C. perfringens* sialidase (8 mU) was added to 700 μl of these substances in 0.05 M sodium acetate buffer of pH 5.1 at the following concentrations: II³Neu5Ac-Gg₄Cer at ○, 1 mM; □, 0.75 mM; △, 0.5 mM; ●, 0.25 mM; ■, 0.1 mM; ▲, 0.05 mM. II³Neu5Ac-Gg₄ at ◇, 1 mM. Aliquots of 100 μl were removed at the times indicated and assayed for free sialic acid as described in "METHODS." In b) the arrows indicate that 100% of available substrate sialic acid has been released.

linear release of Neu5Ac was observed at most ganglioside concentrations (Fig. 1b). A total liberation of sialic acid occurred within 25 h at ganglioside concentrations below 0.5 mM.

No release of Neu5Ac from II³-monosialogangliotetraose was found under any condition.

c) Influence of substrate concentration: In the absence of sodium cholate biphasic curves were obtained with an inflection point at 0.46 mM ganglioside concentration (Fig. 2, a and b). When these data were plotted in a double reciprocal plot the biphasic nature was confirmed and two K_m values were obtained, at 2.5 mM and 0.0345 mM (Fig. 2b).

Inclusion of 1.75 mM sodium cholate modified the mode of action and curves of v against ganglioside concentration were parabolic with a maximum at 0.5 mM. At higher concentrations a decrease in velocity was observed (Fig. 3a). Incubations up to 2 h released a maximum of 30% of total substrate Neu5Ac while those for 5 h and longer reached greater than 70% at lower concentrations. The value of V_{max} did not change in incubations of longer than 1 h in the presence of cholate. The K_m value from double reciprocal plots (Fig. 3b) was 0.45 mM and a V_{max} value of 1.25 nmol/min × μg protein at 1 h and longer was calculated.

No release of sialic acid from II³Neu5Ac-Gg₄ was observed at any concentration.

d) Influence of sodium cholate concentration: Inclusion of sodium cholate in incubations resulted in a significant increase in the liberation of sialic acid, with a maximum at 1.75 mM cholate (not shown).

e) Influence of enzyme concentration: The hydrolysis of substrate was linear with enzyme protein concentration up to 0.4–0.55 μg (10–15 mU) at 0.01 mM and 0.9–1.1 μg (25–30 mU) at 0.5 mM ganglioside, respectively, both in the presence of 1.75 mM sodium cholate (Fig. 4). At higher enzyme concentrations the substrate was hydrolyzed at a much slower rate.

f) Action of sialidases on II³-monosialo-gangliotetraitol: Prolonged incubation of II³Neu5Ac-Gg₄-[³H]ol at relatively high enzyme concentration (15 mU *C. perfringens* sialidase/100 μl) resulted in no hydrolysis of the tritium-labeled sialo-oligosaccharide as judged by TLC. When similar incubation mixtures were applied to Dowex 1 × 8 formate columns, no radioactive, neutral oligosaccharide (gangliotetraitol) could be detected in the water wash. In contrast, when this compound was incubated with *A. ureafaciens* sialidase, hydrolysis of the glycosidic bond of sialic acid occurred. The neutral oligosaccharide observed after incubation with this sialidase migrated on TLC with the same R_f value as authentic Gg₄-[³H]ol in two solvent systems. On silica gel 60 thin-

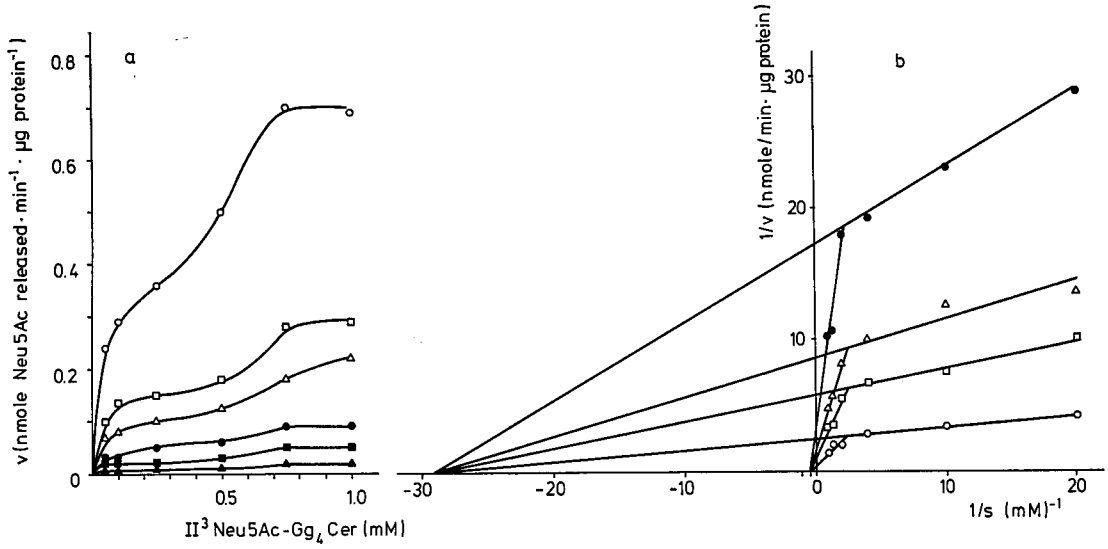
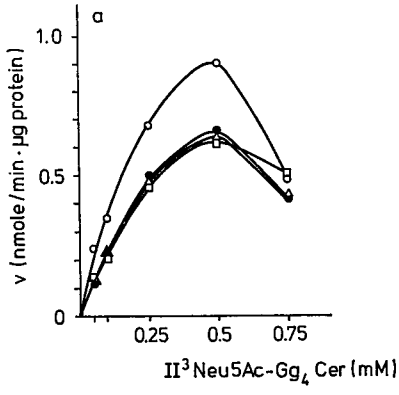


Fig. 2. The data obtained from Fig. 1a are replotted to show the effect of substrate concentration. a) Velocity against the concentration of substrate incubated for different times. b) Double reciprocal plot of the data from a). The incubations were for ○, 30 min; □, 1 h; △, 2 h; ●, 5 h. Other conditions are given as in Fig. 1.



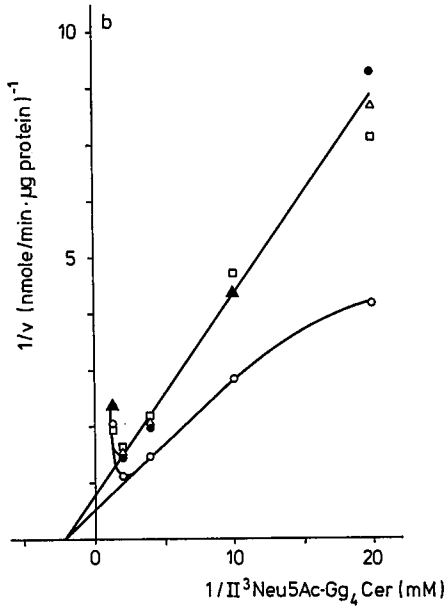


Fig. 3. The data obtained from Fig. 1b are replotted to show the effect of substrate concentration in the presence of 1.75 mM sodium cholate. a) Velocity against substrate concentration in dependence on time. b) Double reciprocal plot of the data in a). The incubations were for ○, 30 min; □, 1 h; △, 2 h; ●, 5 h. Other conditions are given as in Fig. 1.

layer chromatograms in solvent 2 II³Neu5Ac-Gg₄-[³H]ol and Gg₄-[³H]ol migrated with R_f values of 0.63 and 0.60, respectively, while in solvent 3 the R_f values were 0.12 (II³Neu5Ac-Gg₄-[³H]ol) and 0.2 (Gg₄-[³H]ol).

g) C. perfringens sialidase action in the presence of monosialogangliotetraose: The sialidase activity with II³Neu5Ac-Lac and II³Neu5Ac-Gg₄Cer as substrates was not inhibited by the presence of II³Neu5Ac-Gg₄ between 0.01 mM and

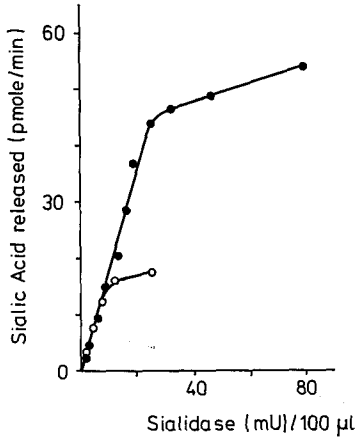


Fig. 4. The influence of sialidase concentration on the value of sialic acid release from II³Neu5Ac-Gg₄Cer in the presence of 1.75 mM sodium cholate. ●, 0.5 mM substrate and sialidase in a total volume of 250 µl, incubation for 5 h. The maximum release of sialic acid represents 13.2% of the total sialic acid in the incubation. ○, 0.01 mM substrate and sialidase in a total volume of 500 µl, incubation for 1 h. The maximum represents 21% of total substrate sialic acid. All assays were in 0.05 M sodium acetate of pH 5.1 at 37°C.

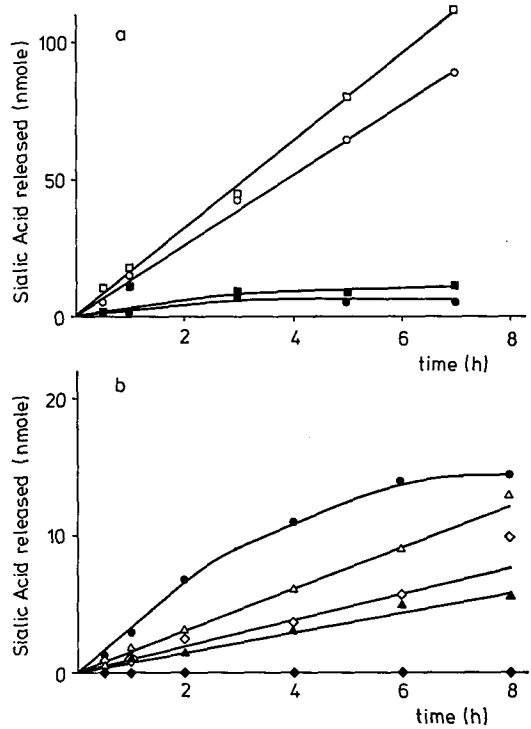


Fig. 5. Action of *C. perfringens* sialidase on II³Neu5Ac-Gg₄Cer and structurally related compounds in 0.05 M sodium acetate buffer pH 5.1 at 37°C. Substances were incubated for different times at the concentrations indicated with 10 mU sialidase in the presence (+) or absence (−) of 1.75 mM sodium cholate and in a total volume of 650 µl. a) ○, II³Neu5Ac-Gg₄Cer (0.5 mM, +); ●, II³Neu5Ac-Gg₄Cer (0.5 mM, −); □, I¹-deoxy-I¹-stearamido-II³-monosialo-gangliotetraitol (0.5 mM, +); ■, I¹-deoxy-I¹-stearamido-II³-monosialo-gangliotetraitol (0.5 mM, −). b) ●, II³Neu5Ac-Gg₄Cer (1 mM, −); △, bis(II³-monosialo-gangliotetraityl)amine (1 mM, + and −); ▲, bis(II³-monosialo-gangliotetraityl)amine (0.5 mM, −); ◇, II³-monosialo-gangliotetraityl-amine (1 mM, −); ◆, II³Neu5Ac-Gg₄ (1 mM, + and −).

1 mM. In the presence of 1.75 mM sodium cholate the activity of *C. perfringens* sialidase with II³Neu5Ac-Lac was depressed by approximately 50%, but there was no inhibition due to the presence of the monosialogangliotetraose.

h) C. perfringens sialidase action on ganglioside and oligosaccharide analogues: The synthetic sialoglycolipid I¹-deoxy-I¹-stearamido-II³-monosialo-gangliotetraitol was hydrolyzed at a similar rate as II³Neu5Ac-Gg₄Cer, and cholate stimulated the hydrolysis to the same degree as the parent ganglioside (Fig. 5a). Oligosaccharide analogues containing a free amino group (II³Neu5Ac-Gg₄-NH₂), or two identical oligosaccharides linked *via* an amino group ((II³Neu5Ac-Gg₄)₂NH) were hydrolyzed by sialidase, but at rates slower than the ganglioside II³Neu5Ac-Gg₄Cer. This reaction occurred in the absence of cholate, and the bile salt did not influence the hydrolysis (Fig. 5b). In contrast to these compounds, no hydrolysis was observed with the monosialogangliotetraose II³Neu5Ac-Gg₄.

¹²⁵I-Sialidase Interaction with II³Neu5Ac-Gg₄Cer and Other Gangliosides—The enzyme was successfully iodinated, with loss of 20% of the original hydrolytic activity against II³Neu5Ac-Lac. The ¹²⁵I-sialidase preparations lost activity on storage at −20°C; after 40 days 75% of the original activity remained. Two batches of enzyme were prepared and each used for experiments within 20 days of preparation.

The behavior of ¹²⁵I-sialidase on Sephadex G-200 gel filtration gave information on the interaction of the enzyme with substrates and cholate. Ganglioside II³Neu5Ac-Gg₄Cer at 1 mM migrated

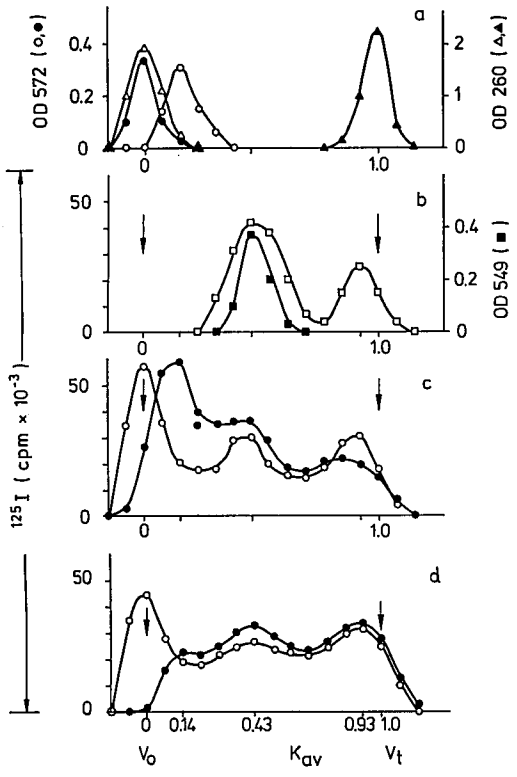


Fig. 6. Gel filtration of ^{125}I-labeled *C. perfringens* sialidase on Sephadex G-200 (1.25×20 cm) in 0.05 M sodium acetate pH 5.1. Incubations were at 4°C for 10 min and contained substrates, bile salt and sialidase (3.5 mU; 0.1 μCi) as indicated, in a total volume of 600 μl. The column was eluted with acetate buffer at 0.7 ml/min and fractions of 1 ml were collected. Incubations contained: a) △, blue dextran; ▲, sodium azide; ●, II^{3}Neu5Ac-Gg$_4$Cer, 1 mM; ○, II^{3}Neu5Ac-Gg$_4$Cer, 1 mM and sodium cholate, 1.75 mM. The ganglioside was detected using the orcinol/Fe^{3+} colorimetric method (*23*). b) □, ^{125}I-sialidase and ^{125}I-iodide; ■, sialidase (8 mU) assayed with II^{3}Neu5Ac-Lac as described in "METHODS." c) ○, ^{125}I-sialidase and II^{3}Neu5Ac-Gg$_4$Cer, 1 mM; ●, ^{125}I-sialidase, II^{3}Neu5Ac-Gg$_4$Cer, 1 mM and sodium cholate, 1.75 mM. d) ○, ^{125}I-sialidase and a mixture of IV^{3}Neu5Ac-, II^{3}Neu5Ac-Gg$_4$Cer, II^3(Neu5Ac)$_2$-Gg$_4$Cer, 1 mM with respect to Neu5Ac; ●, ^{125}I-sialidase, disialylganglioside mixture 1 mM and sodium cholate 1.75 mM. The values of v_0 and v_t as determined using blue dextran and sodium azide, and shown in a) are indicated by arrows in b) to d). The elution position of other components was calculated as K_{av} $(=v_e-v_0/v_t-v_0)$, where v_e is the volume of the peak maximum for each component, $v_0=0$ and $v_t=1.0$.

as micelles at the excluded volume ($v_0=6$ ml), but in the presence of 1.75 mM sodium cholate it was displaced to a mixed micellar complex of lower molecular weight ($K_{av}=0.14$) (Fig 6a). The ^{125}I-sialidase (molecular weight 60,000 (*15*)) eluted at $K_{av}=0.43$ and radioactive iodide at $K_{av}=0.93$ (Fig. 6b). ^{125}I-sialidase and cholate alone gave peaks of K_{av} 0.14 and 0.43, similar to the profile observed in Fig. 6c for enzyme, ganglioside and cholate together.

Solutions of ^{125}I-sialidase with 1 mM II^{3}Neu5Ac-Gg$_4$Cer with or without sodium cholate gave qualitatively similar results whether incubated for 20 min at 20°C, followed by 10 min at 4°C, or 1 h or 5 h at 37°C. The radioactivity migrated with the ganglioside micelles as observed for the substrate alone, and the displacement due to cholate was also observed with the enzyme present (Fig. 6c). In each case a peak of non-incorporated sialidase eluted at $K_{av}=0.43$ and some iodide was observed near the void volume (Fig. 6c). Identical elution profiles were obtained for the sialidase with ganglioside II^{3}Neu5Ac-Gg$_4$Cer with and without sodium cholate when 0.4 M NaCl was included in the buffer or when the pH of the buffer was adjusted to 3.5. No change in the elution profile occurred when 1 mM of the oligosaccharide II^{3}Neu5Ac-Gg$_4$ was added to incubations of ^{125}I-sialidase with the ganglioside II^{3}Neu5Ac-Gg$_4$Cer. The oligosaccharide substrate II^{3}Neu5Ac-Lac, too, had no influence on the elution of ^{125}I-sialidase, the only radioactive peak having a K_{av} of 0.43. A mixture of the gangliosides IV^{3}Neu5Ac-, II^{3}Neu5Ac-Gg$_4$Cer and II^3(Neu5Ac)$_2$-Gg$_4$Cer behaved similarly to the monosialoganglioside II^{3}Neu5Ac-Gg$_4$Cer with and without cholate (Fig. 6d).

DISCUSSION

Many sialidases of bacterial (*1–3*) and viral (*2, 3, 27*) origin are unable to hydrolyze the sialic acid residue in II^{3}Neu5Ac-Gg$_4$Cer and its oligosaccharide. However, the release of sialic acid from the gangliosides II^{3}Neu5Ac-Gg$_3$Cer and II^{3}Neu5Ac-Gg$_4$Cer by *C. perfringens* sialidase has been described (*4–9*). These data have indicated very low or no release of *N*-acetylneuraminic acid from the internal galactose residue of gangliosides by the enzyme in the absence of bile salts, and signifi-

cantly improved rates of hydrolysis in their presence. These increased rates are still well below values observed for the hydrolysis of sialic acid residues linked to the non-reducing terminus of the gangliotriaose, gangliotetraose, or gangliopentaose units of other ganglioside substrates. In all studies reported to date, commercial *C. perfringens* sialidase preparations were used (*4–9*), and the purity of these preparations was not detailed. The *C. perfringens* sialidase preparation used in the present studies has been purified to homogeneity (*15*), and is thus suitable for iodination experiments with ^{125}I, which would not be meaningful with the commercial enzyme.

The biphasic response of *C. perfringens* sialidase action with the concentration of II^{3}Neu5Ac-Gg$_4$Cer in the absence of bile salt results in a break in the double reciprocal plot at 0.46 mM (Fig. 2b), similar to *A. ureafaciens*, where the break was at 0.085 mM (*8*), and in experiments with *C. perfringens* sialidase, where an increase in hydrolysis occurred below 0.1 mM II^{3}Neu5Ac-Gg$_4$Cer (*4*). The break observed in the curves has been interpreted as being due to a phase transition from the monomer to the micellar state, *i.e.* at the critical micelle concentration (*4*, *8*). More recent reports of the determination of the critical monomer to micellar transition concentration of gangliosides using various independent methods, such as gel permeation chromatography (*28*), sedimentation in the ultracentrifuge (*29*), or small angle light scattering measurement (*30*), however, all arrived at values lower than 1 μM for ganglioside II^{3}Neu5Ac-Gg$_4$Cer, probably in the range of 10 nM. Therefore, the concentration values, at which the rate change of hydrolysis by sialidases was observed, 1.5–2.5 μM (*9*), 25–28 μM (*5*), 85 μM (*8*), and 0.46 mM (present communication), may well correspond to some other as yet unknown transition in the state of aggregation of gangliosides in the aqueous solution.

The biphasic dependence on substrate concentration was found for all assays from 30 min to 25 h (Fig. 2, a and b). Although two unchanging K_m values result from the data, there is a decrease in V_{max} as the transition occurs. Less than 10% of the substrate is hydrolyzed during reaction and the inhibition is not explained by a change in substrate binding to the active site as the K_m values do not change. Measurement of activity at times shorter than 30 min may clarify this situation. The data reported by Rauvala (*5*) were obtained using levels of enzyme about thirty-fold greater than those found here. Approximate calculation of K_m and V_{max} values from Fig. 8 of Rauvala (*5*) indicates that the K_m value is similar to that found here (35 μM) for ganglioside II^{3}Neu5Ac-Gg$_4$Cer, but that V_{max} shows a very large difference, ~0.02 nmol/min × mg as compared with 200 or 1,000 nmol/min × mg (Fig. 2b) at 1 h of incubation, and suggests some difference in the nature of the two enzyme preparations. The measurement of two K_m values with II^{3}Neu5Ac-Gg$_4$Cer has also been found for *A. ureafaciens* sialidase and with values in the same range as those measured here for the *C. perfringens* enzyme (*8*).

In the presence of sodium cholate an increase in sialidase hydrolysis of II^{3}Neu5Ac-Gg$_4$Cer has been documented (*4*, *6–9*, *17*). The presence of bile salt simplifies the velocity-time plot (Fig. 1b) and the dependence on substrate concentration (Fig. 3, a and b), in agreement with the earlier reports. The curve for incubations of 30 min duration in Fig. 3b indicate the possibility of a second type of enzyme reaction in the presence of sodium cholate, and shorter incubation times may give additional information to support this. Optimal concentrations of bile salts have been reported for *C. perfringens* sialidase (*6*, *7*). A closer investigation showed the existence of preferred molar ratios of bile salt to II^{3}Neu5Ac-Gg$_4$Cer (*9*). Similar results have also been described for *A. ureafaciens* sialidase (*8*). The present work confirmed these data, whereby a concentration was found of 1.75 mM sodium cholate at 0.5 mM II^{3}Neu5Ac-Gg$_4$Cer, in the same range as the values reported by Wenger and Wardell (*7*) and Rauvala (*4*).

A sharp pH optimum at pH 5.0 exists in the presence of sodium cholate, whereas a plateau of activity from pH 5–8 is seen without the bile salt (*17*). In general, broad pH optima have been reported for II^{3}Neu5Ac-Gg$_4$Cer hydrolysis within the range of pH 4.6–5.6 (*4–6*, *9*) and incubations have usually been carried out in this pH interval. The work of Saito *et al.* (*8*) with *C. perfringens* sialidase indicates routine analysis of the reaction at pH 7.2, but this pH optimum has not been confirmed by other workers (*4–7*, *9*).

Investigation of increased enzyme concentra-

tion on the reaction rate at substrate concentrations above and below 0.1 mM in the presence of sodium cholate revealed a pronounced decrease in hydrolysis rate at both substrate concentrations (Fig. 4). Conditions were chosen to keep substrate hydrolysis within 15–20% of the total to evaluate only the effect of extra enzyme protein. Hence a limit is observed in the amount of enzyme able to interact with the substrate and sodium cholate in mixed micelles. A similar change in reaction rate has been described for *C. perfringens* sialidase at concentrations of II^3Neu5Ac-Gg_4Cer below 0.1 mM and in the absence of bile salt (*5*). In this case the decrease in hydrolysis rate was attained at a ten-fold greater concentration of sialidase.

The organization of the glycolipid substrate in solution resulting from its amphiphilic nature, obviously plays an important role in the level of sialidase activity observed. Indeed the importance of the lipid moiety in the action of *C. perfringens* sialidase on the ganglioside II^3Neu5Ac-Gg_4Cer is demonstrated by the complete absence of hydrolysis of II^3Neu5Ac-Gg_4 or II^3Neu5Ac-Gg_4-[^{3}H]ol under any condition including 24 h incubation, presence of bile salt and high enzyme or substrate concentration. Both radioactive and non-labeled oligosaccharides, however, were hydrolyzed by *A. ureafaciens* sialidase, in agreement with the findings of Saito *et al.* (*8*). Further evidence supporting a complete lack of affinity of this oligosaccharide for the *C. perfringens* sialidase is the absence of inhibition of II^3Neu5Ac-Gg_4Cer or II^3Neu5Ac-Lac hydrolysis by the enzyme in the presence of 1 mM II^3Neu5Ac-Gg_4 and the lack of influence of the oligosaccharide on the elution profile of ^{125}I-sialidase with II^3Neu5Ac-Gg_4Cer.

Interestingly, after introduction of an amine function, the monomeric and dimeric products of reductamination of the II^3-monosialo-gangliotetraose, can once again be cleaved by the *C. perfringens* enzyme, however, without a hydrolysis-enhancing effect of cholate. Following the addition of an aliphatic 18-C hydrocarbon chain by substitution of the II^3-monosialo-gangliotetraitylamine with stearic acid, the ensuing ganglioside analogue can be cleaved not only with *C. perfringens* sialidase, but again shows the cholate effect observed with the parent ganglioside. At present it is not known, whether the presence of a lipid moiety in the sialidase substrate enhances the binding to the enzyme or else facilitates the "unlocking" of an interaction between the sialic acid carboxyl and the *N*-acetylamido function of the neighboring hexosamine due to competition within the tightly packed carbohydrate of the ganglioside micelles. This "unlocking" effect is perhaps further promoted by the cholate carboxyl that comes into close proximity to exert its effect after hydrophobic binding of the steroid. An "unlocking" of the sialic acid carboxyl might also explain why introduction of an amino group has a promoting effect for the hydrolysis of the sialo-oligosaccharide substrates.

The "locking" of the branching sialic acid has been documented in ^{13}C-NMR studies of the ganglioside II^3Neu5Ac-Gg_4Cer (*13, 14*). In 500 MHz ^{1}H-NMR spectroscopy studies of the oligosaccharides including the disialo-gangliotetraose IV^3Neu5Ac-, II^3Neu5Ac-Gg_4 (Dorland *et al.*, unpublished observations) a difference in line width of 40% was observed between the H-3ax signals of the internal II^3Neu5Ac residue (δ1.913) and of the IV^3Neu5Ac residue in terminal, linear position (δ1.798). The linebroadening of the H-3ax signal of the internal II^3Neu5Ac residue is probably due to steric hindrance of its rotational freedom. This may explain the difference in susceptibility to sialidase action.

Similar "locked" and sialidase-resistant sialic acids have been found in human erythrocyte Cad and Sd^a antigen oligosaccharides (*31*) and in trout egg glycoprotein oligosaccharides (*32*), bound in α(2-3) linkage to an internal galactose with a neighboring, non-reducing *N*-acetylgalactosamine (*31*), or linked α(2-3) to an internal *N*-acetylgalactosamine (*32*).

The experiments with ^{125}I-labeled enzyme show that it becomes bound to the substrate micelles or substrate and cholate micelles (Fig. 6). Although the nature of this interaction is unknown and hydrophobic forces may be assumed, non-specific ionic interactions may be ruled out, since similar results on gel filtration were obtained at higher concentrations of NaCl and at a lower pH. The presence of "excess" sialidase, migrating as the native enzyme (K_{av}=0.43) in all cases, may be explained by saturation or exchange phenomena between the micelle and the aqueous medium during incubation. The inclusion of 1 mM II^3Neu5Ac-

Gg_4 in the reaction mixture did not change the results and further illustrates the lack of affinity of this ganglioside-derived oligosaccharide for the enzyme. The substrate II^3Neu5Ac-Lac, readily hydrolyzed by *C. perfringens* sialidase, also had no influence on the elution profile of the sialidase. A disialoganglioside mixture of IV^3Neu5Ac-, II^3Neu5Ac-Gg_4Cer, and $II^3(Neu5Ac)_2$-Gg_4Cer shows that incorporation of the ^{125}I-sialidase into micelles in the absence and presence of sodium cholate occurs. These gangliosides contain sialic acid residues readily susceptible to sialidase action, leading to the formation of II^3Neu5Ac-Gg_4Cer.

The action of microbial sialidases on ganglioside II^3Neu5Ac-Gg_4Cer in the absence of bile salt is limited to *A. ureafaciens* (*8*, *10*) and Sendai virus (*11*) sialidases. Examples of mammalian sialidases acting on II^3Neu5Ac-Gg_3Cer or II^3Neu5Ac-Gg_4Cer are documented, including rat intestine (*33*), rat heart (*34*), human brain and heart (*35*), and human liver (*36*). The rates of hydrolysis of these substrates were in all cases lower than for other ganglioside or glycoprotein substrates with these sialidases (*3*). Since the mammalian enzymes used were crude or partially purified preparations, degradation of the substrates by other glycosidases prior to the action of sialidases cannot be excluded in all cases. Elimination of substrate degradation by other glycosidases prior to sialidase action was ruled out in only one case (*33*) by identification of the reaction products of II^3Neu5Ac-Gg_3Cer as Neu5Ac and Gg_3Cer.

The complete or partial resistance of branch sialic acids in gangliosides towards the action of most sialidases from viral, bacterial, and mammalian origin is believed to be of metabolic, *e.g.* the possibility of resynthesis of a variety of oligosialogangliosides from II^3Neu5Ac-Gg_4Cer, and biological importance, *e.g.* the preservation of II^3Neu5Ac-Gg_4Cer which is known to be involved in manifold cell receptor functions (*1*).

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