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The release of *N*-acetyl- and *N*-glycolloyl-neuraminic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases

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A series of substrates, sialyl(2→3)lactose, sialyl(2→6)GalNAc and ganglioside G_{M3}, containing either *N*-acetylneuraminic acid (AcNeu) or *N*-glycolloylneuraminic acid (GcNeu), has been prepared. The trisaccharide GcNeu(2→3)lactose was prepared by ozonolysis of GcNeu-G_{M3}, and the disaccharides AcNeu(2→6)GalNAc and GcNeu(2→6)GalNAc were isolated from bovine submandibular-gland mucin by alkali elimination. Sialidases from Newcastle-disease virus, fowl-plague virus, influenza virus A_2, *Clostridium perfringens*, *Vibrio cholerae*, *Arthrobacter ureafaciens* and human liver lysosomes were studied with the above substrates and all showed poorer cleavage of GcNeu-containing substrates when compared with the corresponding AcNeu-containing compounds. This was reflected in the K_m and $V_{max.}$ values of these sialidases. Differences between viral and bacterial sialidases could be detected on the basis of their kinetic constants and time curves of sialic acid release. Preferred release of AcNeu relative to GcNeu was also observed with bovine submandibular gland mucin and a mixture of human and porcine erythrocytes, macromolecular substrates containing both AcNeu and GcNeu. The significance of differential cleavage of AcNeu and GcNeu by sialidases is considered together with examples of the role of GcNeu in physiological systems.

The sialidases are widespread in micro-organisms and animal tissues and catalyse the enzymic release of sialic acid from a wide range of glycoconjugate substrates (Corfield *et al.*, 1981; Drzeniek, 1973). The efficiency of cleavage of sialic acids by sialidases has been shown to depend on the source of the enzyme, the nature of the substrate (e.g. glycoprotein, glycolipid or oligosaccharide), the nature of the glycosidic linkage to the penultimate non-reducing sugar in the oligosaccharide chain and the nature of the sialic acid(s) in the substrate (Drzeniek, 1973; Tuppy, 1974; Corfield *et al.*, 1981). Under this final category the reduction in cleavage rate due to the presence of *O*-acetyl groups at positions 4, 7, 8 or 9 of the sialic acid molecule has been documented (Schauer & Faillard, 1968; Veh *et al.*, 1979).

Less information is available concerning the influence of the type of *N*-substitution occurring in nature, i.e. AcNeu or GcNeu. Previous reports with viral and bacterial sialidases demonstrated a decrease or loss of cleavage with synthetic *N*-substituted sialic acid α-glycosides. Thus a decreased cleavage with *N*-propionyl- and *N*-formyl-sialic acid α-glycosides was found relative to *N*-acetylsialic acid α-glycosides (Meindl & Tuppy, 1966; Brossmer & Nebelin, 1969), whereas *N*-butyryl-, *N*-benzoyl- and *N*-benzyloxycarbonyl-sialic acids were not cleaved at all (Meindl & Tuppy, 1966; Faillard *et al.*, 1969). Preferential cleavage of AcNeu relative to GcNeu was suggested by Jameson & Levine (1965) with viral sialidases. By using purified sialidase preparations from different sources we report kinetic data in this respect for the first time

Abbreviations used: GcNeu, *N*-glycolloylneuraminic acid; Cer, ceramide; AcNeu-G_{M3}, AcNeu(α2→3)Gal(β1→4)Glc(β1→1)Cer; GcNeu-G_{M3}, GcNeu(α2→3)Gal(β1→4)Glc(β1→1)Cer; G_{M1}, Gal(β1→3)GalNAc(β1→4)Gal(3←2αAcNeu)(β1→4)Glc(β1→2)Cer. Abbreviations for gangliosides are those recommended by Svennerholm (1963).

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and confirm here the preferred cleavage of AcNeu relative to GcNeu with highly purified substrates from natural sources. A preliminary report of this work has been published previously (Corfield *et al.*, 1980).

Materials and methods

Substrates

The (2→3)- and (2→6)-isomers of *N*-acetylneuraminyl-lactose were isolated from bovine colostrum by chloroform/methanol extraction by the method of Öhmann & Hygstedt (1968), gel filtration and a new ion-exchange chromatographic method (R. W. Veh, J.-C. Michalski, A. P. Corfield, M. Sander & R. Schauer, unpublished work). The products were homogeneous on silica-gel t.l.c. in solvent 1 and showed unimolar ratios of AcNeu, galactose and glucose on g.l.c. analysis.

GcNeu(2→3)lactose was prepared from horse erythrocyte GcNeu-G_{M3} by the method of Wiegandt & Bücking (1970). The ganglioside (1.25 g, 1 mmol) was dissolved in 200 ml of chloroform/methanol (2:1, v/v) and cooled to −30°C. Ozonolysis was carried out using an ozone generator (model OZ II; Fischer, Bad Godesberg, Germany) with a flow rate of 30 litres of O_2/h corresponding to approx. 1 g of ozone. The sample was then brought slowly to room temperature and excess ozone was removed by bubbling N_2 through the solution for 30 min. The sample was taken down to dryness on a rotary evaporator, dissolved in 200 ml of 0.1 M-Na_2CO_3 and maintained at 45°C for 3 h for the β-elimination of the oligosaccharide chain. The solution was neutralized with 3 ml of acetic acid. Residual lipid micelles were removed by ultrafiltration through an Amicon PM 30 filter and the clear solution containing the oligosaccharide desalted on a Sephadex G-25 column (8 cm × 60 cm) in 2 mM-acetic acid adjusted to pH 5 with pyridine. The product was finally purified by ion-exchange chromatography as above and yielded 250 mg after freeze-drying.

Bovine submandibular-gland major mucin was prepared by the method of Tettamanti & Pigman (1968) and was saponified by treatment with 0.05 M-NaOH at 4°C for 30 min. The solution was neutralized with 2 M-HCl and dialysed for 10 h against 100 vol. of water. The saponification resulted in the loss of 0.18% of the total sialic acid content of the starting material (0.29 mg of sialic acid/mg dry wt.) measured with the Orcinol/Fe^{3+} method. The product was freeze-dried and stored at 4°C.

The disaccharides AcNeu(2→6)GalNAc and GcNeu(2→6)GalNAc were prepared from bovine submandibular-gland mucin purified as far as the ethanol precipitation step by the method of Tettamanti & Pigman (1968). The mucin (50 g) was dissolved in 2 litres of 0.1 M-NaOH and maintained at room temperature for 24 h. During this time 20% of the oligosaccharide chains were β-eliminated. After dialysis against two changes of 10 litres of water each for 24 h, the combined diffusates were applied to a column (10 cm × 25 cm) of Dowex 2 (X8; formate form; 200–400 mesh). After elution with 1 M-formic acid, the sialic acid-positive fractions were pooled and freeze-dried, yielding approx. 3 g of material. Portions (1 g) were dissolved in a minimum amount of n-butanol/n-propanol/water (1:2:1, by vol.) and applied to a cellulose column (Macherey and Nagel; MN 2100 ff; 4 cm × 180 cm). The column was eluted with this solvent. Sialic acid-containing fractions were analysed by t.l.c. on cellulose plates in solvent 2, before and after mild acid hydrolysis (0.1 M-HCl at 80°C for 60 min). Pure fractions were pooled, freeze-dried and the sialic acid and hexosamine contents were analysed.

Ganglioside AcNeu-G_{M3} was prepared from human liver by the method of Seyfried *et al.* (1978) and GcNeu-G_{M3} from horse erythrocytes as described by Hakomori & Saito (1969).

Human erythrocytes from outdated blood-bank stock, and fresh porcine erythrocytes were each washed twice with a buffer containing 4 mM-diethylbarbituric acid, 0.15 M-NaCl, 40 mM-EDTA and 1 g of gelatine/litre, pH 7.2, and twice with isoosmotic NaCl (9 g/litre) solution before use. A mixture of 1.8 ml of packed human erythrocytes and 3.2 ml of packed porcine erythrocytes in 5 ml of a buffer containing 50 mM-sodium acetate, 9 mM-$CaCl_2$ and 0.154 M-NaCl, pH 5.5, was incubated with 75 m-units of *Vibro cholerae* sialidase. This mixture was chosen to contain equivalent amounts of erythrocyte-bound AcNeu (human) and GcNeu (porcine) releasable by *Vibrio cholerae* sialidase after 60 min under the conditions of incubation given above. These releasable sialic acids were determined in separate experiments containing either human or porcine erythrocytes only.

Colorimetric methods

Sialic acids were determined by a micro-periodic acid/thiobarbituric acid method (Schauer, 1978), making a correction for interference by measuring the chromogen at 549 and 532 nm as described by Warren (1959), or by the orcinol/Fe^{3+} method (Schauer, 1978). Protein was measured by the method of Lowry *et al.* (1951).

Chromatography

T.l.c. was carried out using Merck pre-coated silica-gel 60 plates, developed in ethanol/n-butanol/acetic acid/water/pyridine (100:10:3:30:10, by vol.; solvent 1) for oligosaccharides, or on Merck pre-coated cellulose plates developed in n-butanol/n-propanol/0.1 M-HCl (1:2:1, by vol.; solvent 2)

for sialic acids. Mono- and oligosaccharides were detected by using H_2SO_4/methanol (1:1, v/v) or orcinol/Fe^{3+} reagent sprays (Schauer, 1978).

Monosaccharides were determined by g.l.c. after methanolysis and trimethylsilylation as described by Kamerling *et al.* (1975) with a Packard 428 chromatograph. Sialic acids were identified as their trimethylsilyl-derivatives, as described previously (Schauer, 1978).

Sialidases

Sialidase from *Clostridium perfringens* was purified to homogeneity as described by Nees *et al.* (1975), sialidase from *Vibrio cholerae* was purchased from Behringwerke, Marburg, Germany, and *Arthrobacter ureafaciens* sialidase was a gift from Professor Y. Uchida, Marukiu Shoyo Co., Kyoto, Japan. The activities of these preparations were 26.8 units/mg of protein 11.0 units/mg of protein and 40.4 units/mg of protein respectively, with AcNeu(2→3)lactose as substrate. Each of these enzyme samples contained no glycosidase, phospholipase C, proteinase or acylneuraminate pyruvate lyase activities. The preparations from *Clostridium perfringens* (Nees *et al.*, 1975) and *Arthrobacter ureafaciens* (Uchida *et al.*, 1977) showed only enzyme protein bands on polyacrylamide-gel electrophoretic analysis. Viral suspensions were used as sialidase preparations from Newcastle-disease virus (Italian strain), fowl plague virus (Rostock strain) and influenza A_2 virus and were generously given by Professor Dr. R. Rott, University of Giessen, Giessen, Germany. Sialidase activities were 6.70 units/mg of protein, 1.83 units/mg of protein and 4.7 units/mg of protein respectively, with AcNeu(2→3)lactose as substrate.

Human lysosomal liver sialidase was prepared from autopsy liver, kindly given by Dr. H. Stein, Department of Pathology, University Clinic, Kiel, Germany, as follows. Liver tissue was homogenized in 3 vol. of 0.154 M-KCl with two strokes of a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 1000 *g* and 4°C in a Sorvall SS34 rotor for 15 min. The pellet was resuspended in 1 vol. of 0.154 M-KCl, rehomogenized and centrifuged as above. The supernatants were combined and centrifuged at 65000 *g* for 30 min in an R 30 Spinco rotor at 4°C. The supernatant solution and upper fluffy layer were discarded. The pellet (lysosomal/mitochondrial fraction) was resuspended in 1 vol. of 10 mM-sodium acetate buffer, pH 4.5, with one stroke of a Potter homogenizer and stirred for 30 min at 4°C to achieve lysosomal lysis. The suspension was then centrifuged at 2000 *g* and the milky supernatant was removed. The pellet was resuspended in 1 M-NaCl, stirred for 30 min at 4°C and again centrifuged at 2000 *g*. After resuspension in 0.1 M-sodium acetate buffer, pH 4.5, the suspension was centrifuged at 65000 *g* for 30 min in an R 30 Spinco rotor. The resulting pellet was used as a sialidase preparation. The active preparation showed 4 μ-units/mg of protein with AcNeu(2→3)lactose as substrate.

Sialidase incubations

The incubation buffers for sialidases were as follows: *Clostridium perfringens*, 50 mM-sodium acetate (pH 5.1); *Vibrio cholerae*, 50 mM-sodium acetate/9 mM-$CaCl_2$/0.154 M-NaCl (pH 5.5); *Arthrobacter ureafaciens*, 50 mM-sodium acetate (pH 4.8); Newcastle-disease, fowl-plague and influenza A_2 viruses, 50 mM-sodium acetate (pH 5.5); human liver, 0.1 M-sodium acetate (pH 4.5).

The time course of sialidase action was followed by using incubations containing approx. 300 μg of substrate sialic acid per ml and sialidase in the above buffers at 37°C. Portions (100 μl) were removed at intervals and mixed with 20 μl of periodate reagent at room temperature for subsequent analysis by the thiobarbituric acid assay. Human liver sialidase was assayed with individual incubations containing 1 mM-substrate and 10 μ-units of enzyme in 0.25 ml for times up to 4 h.

Incubations in the above buffer systems were employed to determine the K_m and $V_{max.}$ values for the respective sialidases and each substrate. Different substrate concentrations and sialidase in a total volume of 100 μl were incubated at 37°C for times between 10 and 60 min giving 5–20% cleavage of the substrate. The incubation was stopped by addition of the periodate reagent for the thiobarbituric acid assay as noted above.

Incubations of influenza A_2 virus and *Vibrio cholerae* sialidases with saponified bovine submandibular gland mucin were under conditions described above, except that the sialic acid content was 1 mg/ml of assay mixture. Incubations were run for times up to 24 h for influenza A_2 virus and for 1 h for *Vibrio cholerae* sialidases. Each incubation was cooled in an ice bath and dialysed for 14 h against 100 ml of water at 4°C. The mucin remaining in the dialysis bag was subjected to mild acid hydrolysis and the sialic acids were separated by dialysis. Both pools of sialic acids were purified by ion-exchange chromatography and analysed by t.l.c. in solvent 2 and by g.l.c.

Erythrocyte mixtures were incubated with 75 m-units of *Vibrio cholerae* sialidase for 15 or 60 min at 37°C with gentle rotation. After incubation the cells were sedimented at 13000 *g* in an Eppendorf centrifuge, the supernatant removed, diluted 150-fold with water and applied to a column (4 cm × 0.8 cm) of Dowex 2 (X8; formate form; 200–400 mesh) and washed successively with 10 ml of water, 8 ml of 0.1 M-formic acid, 2 ml of 0.5 M-formic acid, and the sialic acids were eluted in 12 ml of 0.5 M-formic acid. An erythrocyte mixture was

also hydrolysed with formic acid at pH 2 for 60 min at 70°C by the method of Schauer (1978). After hydrolysis, the mixture was ultrafiltered using an Amicon ultrafiltration cell with a PM 10 membrane at an N_2 pressure of 0.5 MPa, freeze-dried and taken up in 50 ml of water before application to a Dowex 2 (X8) ion-exchange column for purification of the sialic acids as described above.

The resulting sialic acid mixtures were applied to cellulose thin-layer plates and developed with solvent 2. The sialic acids were detected by using the orcinol/Fe^{3+} spray reagent.

Results

Substrate preparation

The GcNeu(2→3)lactose, prepared by ozonolysis, showed equimolar ratios of glucose, galactose and sialic acid on analysis by g.l.c. after methanolysis. The product contained only GcNeu, as analysed by g.l.c. and t.l.c. on cellulose plates in solvent 2 after mild acid hydrolysis. The oligosaccharide ran as a single band on silica-gel t.l.c. plates in solvent 1, showing an $R_{AcNeu(2\rightarrow3)lactose}$ of 0.93, identical with authentic GcNeu(2→3)lactose isolated from bovine colostrum.

The structure of the disaccharides AcNeu(2→6)-GalNAc and GcNeu(2→6)GalNAc were confirmed by 360 MHz n.m.r. spectroscopy by Dr. J. Haverkamp, FOM Institute for Atomic and Molecular Physics, Amsterdam, The Netherlands (van Halbeek *et al.*, 1980).

The gangliosides AcNeu-G_{M3} and GcNeu-G_{M3} each showed a single band on t.l.c. in standard ganglioside solvent systems as described for their preparation. The nature of the sialic acid for each ganglioside was analysed by g.l.c. and t.l.c. on cellulose plates in solvent 2 after mild acid hydrolysis. Each ganglioside yielded only one sialic acid residue. Analysis of the sialic acid components of all substrates by g.l.c. and t.l.c. revealed only AcNeu or GcNeu; no *O*-acetylated sialic acids were detected.

Sialidase action

The time course of sialic acid release from each of the three pairs of substrates by different sialidase preparations is presented in Fig. 1. Similar curves for

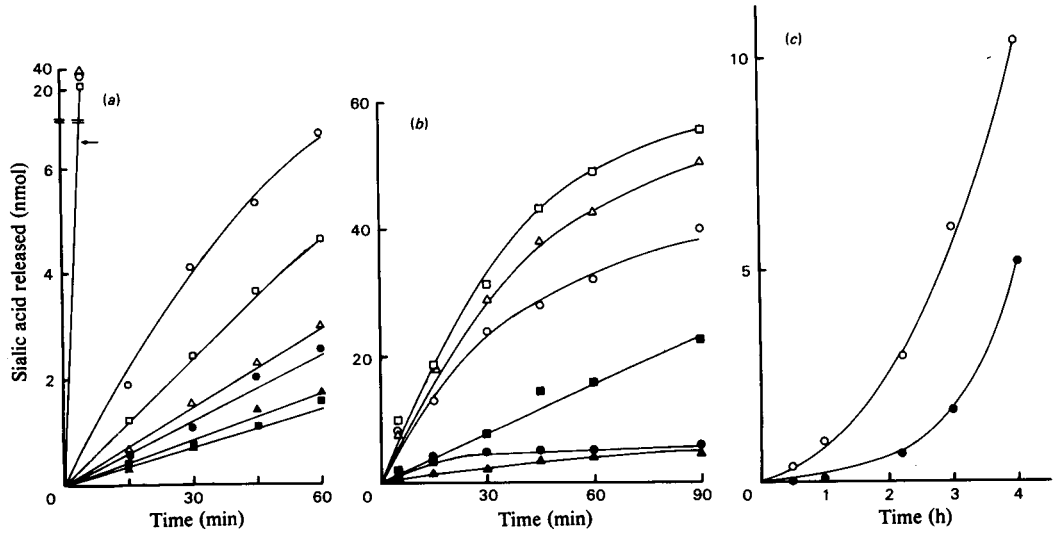

Fig. 1. *Time curve of AcNeu and GcNeu release from substrates by different sialidases*

(*a*) shows the action of viral sialidases on sialyl(2→6)GalNAc (1 mM-sialic acid) in 50 mM-sodium acetate buffer, pH 5.5. Newcastle-disease-virus (□ and ■, 52 m-units/ml), influenza-A_2-virus (△ and ▲, 22 m-units/ml) and fowl-plague-virus (○ and ●, 10 m-units/ml) suspensions were added in the same buffer. The release of AcNeu from AcNeu(2→3)lactose at these enzyme dilutions is also shown for comparison (the line is indicated by the arrow). (*b*) shows the action of bacterial sialidases on sialyl(2→3)lactose (1 mM-sialic acid) in 50 mM-sodium acetate buffers as described in the Materials and methods section. *Vibrio cholerae* (□ and ■, 12 m-units/ml), *Arthrobacter ureafaciens* (△ and ▲, 10 m-units/ml) and *Clostridium perfringens* (○ and ●, 8 m-units/ml) sialidases were added in the respective buffer. (*c*) shows the action of human liver lysosomal sialidase (100 μ-units/ml) on ganglioside G_{M3} (○ and ●) (1 mM-sialic acid) in 100 mM-sodium acetate buffer, pH 4.5. The substrates in each experiment contained either AcNeu (□, △ and ○) or GcNeu (■, ▲ and ●) and the amount of enzyme activity added was measured with AcNeu(2→3)lactose as substrate under standard conditions for each enzyme. The incubation volume was 1 ml in (*a*) and (*b*) and 0.25 ml in (*c*).

the bacterial and viral sialidases were obtained for the substrates not illustrated. These plots indicated a preferred cleavage of AcNeu. The same difference in cleavage rate between AcNeu and GcNeu in sialyl(2→3)lactose was found for all three bacterial sialidases, Newcastle-disease- and influenza A_2 virus sialidases in both 50mM-sodium acetate or 50mM-sodium phosphate buffers, pH 6.0, under otherwise standard conditions. Measurement of the K_m and $V_{max.}$ values for these substrates (Table 1) showed that the K_m values for viral sialidases remained similar for AcNeu- and GcNeu-containing substrates, whereas the $V_{max.}$ was usually higher for the AcNeu derivative. The bacterial sialidases showed the reversed situation, with most K_m values for AcNeu substrates being smaller than for the corresponding GcNeu derivatives. The $V_{max.}$ values for GcNeu substrates were similar or lower than those for the corresponding AcNeu substrate. Exceptions to this general trend were apparent, e.g. Newcastle-disease-virus sialidase showed a lower K_m and higher $V_{max.}$ for AcNeu-G_{M3} relative to GcNeu-G_{M3} and *Clostridium perfringens* enzyme gave a lower K_m for GcNeu-G_{M3}, but a 26-fold lower $V_{max.}$ for this substrate relative to AcNeu-G_{M3} (Table 1).

By using saponified bovine submandibular-gland mucin as substrate g.l.c. analysis of the sialic acids released within short incubation times yielded AcNeu/GcNeu ratios of 12:1 (2h) for influenza A_2 virus and 8:1 (15min) for *Vibrio cholerae* sialidases. With prolonged incubation the ratio of AcNeu to GcNeu changed from these early values to 6:1 (24h) and 3:1 (1h) for influenza-A_2-virus and *Vibrio cholerae* enzymes respectively. After release of the remaining sialic acids by mild hydrolysis the total sialic acids from sialidase and acid hydrolysis gave an AcNeu/GcNeu ratio of 3:1. This ratio was also found for mild-acid-hydrolysed mucin. In addition to the difference in AcNeu/GcNeu release these values also reflect the poor cleavage of (2→6)-linkages prevailing in bovine submandibular-gland mucin by the viral, relative to the bacterial, sialidase.

The human liver sialidase preparation was assayed with AcNeu/GcNeu(2→3)lactose and AcNeu/GcNeu-G_{M3} substrates. No difference in the rate of enzymic cleavage was observed for AcNeu(2→3)lactose and GcNeu(2→3)lactose under the conditions employed, but a marked difference was observed for the ganglioside substrates (Fig. 1*c*), with AcNeu being released more rapidly.

Incubation of a mixture of human and porcine erythrocytes with *Vibrio cholerae* sialidase showed a preferred release of AcNeu from the human erythrocytes in the initial phase of the reaction. After incubation for 15min t.l.c. analysis showed a strong AcNeu spot and only a very faint GcNeu band. After 60min the presence of both sialic acids was clearly visible in a ratio similar to that observed after mild acid hydrolysis of the erythrocyte mixture. This result is similar to that observed for bovine submandibular mucin.

Discussion

The preparation of well defined oligosaccharides or gangliosides as natural substrates for sialidases, containing either AcNeu or GcNeu, has allowed an assessment of the influence of this substitution on sialidase activity. The occurrence of the three substrate pairs used in the present study has been described; in bovine colostrum (Kuhn & Gauhe, 1965) for AcNeu- and GcNeu-(2→3)lactose, in bovine and ovine submandibular-gland mucins (Tettamanti & Pigman, 1968) for AcNeu- and GcNeu-(2→6)GalNAc in glycosidic linkage to the protein backbone and in human liver (Seyfried *et al.*, 1978) and equine erythrocytes (Hakomori & Saito, 1969) for AcNeu- and GcNeu-G_{M3} respectively. The substrates were isolated from these sources except for GcNeu(2→3)lactose, which was prepared by ozonolysis of GcNeu-G_{M3}, and were of high purity as judged by t.l.c., g.l.c. and n.m.r. analysis.

Table 1. *K_m and $V_{max.}$ values for sialidases with AcNeu- and GcNeu-containing substrates*
The K_m values are given as mM-sialic acid and $V_{max.}$ as μmol of sialic acid released/min per mg of protein.

Substrate ...	Sialyl(2→3)lactose				Sialyl(2→6)GalNAc				G_{M3}			
	AcNeu		GcNeu		AcNeu		GcNeu		AcNeu		GcNeu	
Sialidase	K_m	$V_{max.}$	K_m	$V_{max.}$	K_m	$V_{max.}$	K_m	$V_{max.}$	K_m	$V_{max.}$	K_m	$V_{max.}$
Fowl-plague virus	1.52	1.83	1.92	0.67	4.17	2.67	3.33	0.21	5.56	3.20	4.40	1.60
Newcastle-disease virus	1.06	6.70	1.85	0.74	3.33	1.20	2.00	0.92	1.72	7.40	5.60	0.92
Influenza-A_2 virus	1.25	4.70	2.10	0.71	4.54	2.40	5.50	0.21	1.85	4.20	2.10	4.20
Clostridium perfringens	0.40	26.8	6.60	32.4	0.50	11.50	2.60	1.69	1.40	82.3	0.05	3.10
Vibrio cholerae	0.72	11.0	1.46	6.80	1.48	3.20	4.00	4.00	0.22	7.40	0.60	2.55
Arthrobacter ureafaciens	0.19	40.4	3.12	20.8	0.40	64.1	2.85	79.2	0.08	11.7	0.21	1.40

The action of sialidases from bacterial, viral and mammalian sources on these substrates shows a general trend with poor cleavage of GcNeu- relative to AcNeu-containing substrates. This is in agreement with previous studies with synthetic sialic acid glycosides (Meindl & Tuppy, 1966; Brossmer & Nebelin, 1969; Faillard *et al.*, 1969). These differences in cleavage are reflected in the K_m and $V_{max.}$ values of each sialidase with the various substrates (Table 1). In general the viral sialidases show similar K_m but lower $V_{max.}$ values for GcNeu- relative to AcNeu-containing substrates, whereas the bacterial enzymes show higher K_m and similar or lower $V_{max.}$ values for the GcNeu derivatives. Exceptions to this generalization occur and demonstrate that more data on the substrate specificity of sialidases are required to evaluate the possibility of a general mechanism of action for viral and bacterial sialidases. This has been reviewed by Corfield *et al.* (1981).

A further factor that may influence kinetic parameters is the nature of the enzyme preparations. The viral sialidases are localized in the membrane of the virion (Drzeniek, 1973) and a viral suspension is used as an enzyme preparation. Thus the possibility of interactions between the substrates, especially the gangliosides and virion membranes, which may influence enzyme action, cannot be excluded. In contrast, the bacterial sialidases used here are all soluble, and occur as extracellular enzymes (Drzeniek, 1973). The bacterial enzymes used in these studies are of high purity, a factor that may have influenced previous work, where commercially available bacterial sialidases still contained significant activities of unwanted enzymes such as proteinases, glycosidases and phospholipase C (Den *et al.*, 1975).

By using substrates containing both *N*-acetyl and *N*-glycolloyl derivatives of sialic acid the preferred release of AcNeu could be demonstrated. This was clearly the case with bovine submandibular-gland mucin, and the results with mixed erythrocytes suggest a similar behaviour, although differences in membrane structure may also play a role.

The differing release of AcNeu and GcNeu imposes a further level of complexity on the specificity of sialidase cleavage in addition to the already documented variations between enzymes with respect to (*a*) the glycosidic linkage, (*b*) the presence of *O*-acetyl groups in the sialic acid moiety, (*c*) the side-chain sialic acids, e.g. in the ganglioside G_{M1}, (*d*) the length of the oligosaccharide chain and (*e*) the composition of the complex sialocarbohydrate. Thus the demonstration of a substrate preference for AcNeu over GcNeu points to a physiologically important recognition site in the sialidase reaction mechanism.

It is noteworthy that the human liver lysosomal sialidase preparation also shows a substrate preference for AcNeu with ganglioside substrates, as human tissues are thought to contain only AcNeu (Ng & Dain, 1976). GcNeu has further been identified as the sialic acid present in gangliosides exhibiting 'serum sickness' antigenicity (Higashi & Naiki, 1977). Further observations relevant to GcNeu function are the absence of virus haemagglutination inhibition by porcine submandibular-gland mucin containing predominantly GcNeu (Gottschalk *et al.*, 1972); a tissue-specific distribution of GcNeu-G_{M3} in rabbit tissues (Iwamori & Nagai, 1978) reflecting a general species- and tissue-specific distribution of GcNeu in nature; the occurrence of GcNeu in canine erythrocyte glycolipids showing a genetic distribution between species that may be related to a canine blood group (Yasue *et al.*, 1978); and finally the correlation of the proportion of cell-surface glycoprotein GcNeu with the proportion of *N*-glycosyl-linked carbohydrate chains in the glycoprotein in an ascites cell TA3 tumour system (Codington *et al.*, 1979). The investigation of substrates, including cell surfaces containing both of these forms of sialic acid, in experiments where desialylation or especially partial desialylation employing sialidase is used, need careful consideration. The possibility of a 'GcNeu concentration effect' due to such treatment may lead to unexpected results, which may be related to biological phenomena similar to those cited above. This deserves particular attention in cell-culture experiments with animal cells possessing both AcNeu and GcNeu in their cellular membranes.

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