Kinetics of *Vibrio cholerae* sialidase action on gangliosidic substrates at different supramolecular-organizational levels

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G_{D1a} , G_{D1b} and G_{T1b} gangliosides were dispersed in the following membrane-mimicking systems: (a) homogeneous micelles; (b) mixed micelles with G_{M1} ganglioside (which is resistant to the enzyme action), Triton X-100 or bovine serum albumin; (c) small unilamellar vesicles of egg phosphatidylcholine. The effect of dispersion on sialic acid release by *Vibrio cholerae* sialidase was studied. As reference substrates freely interacting with the enzyme the lipid-free carbohydrates of G_{D1a} and 3'-sialosyl-lactose were employed. The apparent V_{max} of the enzyme was, with all the gangliosides, dependent on the type of ganglioside dispersion. It was lowest for homogeneous micelles and mixed micelles with ganglioside G_{M1} , and increased about 6-fold for ganglioside/bovine serum albumin lipoprotein micelles, 15-fold for mixed-ganglioside/Triton X-100 micelles (optimal molar ratio 1:7.5) and 30-fold for phosphatidylcholine vesicles containing 2.5 mol% ganglioside (this proportion was optimal for enzyme activity on the vesicles). For ganglioside G_{D1a} , the activity on Triton X-100 mixed micelles and on mixed vesicles was even greater (3- and 6-fold respectively) than that displayed on G_{D1a} lipid-free carbohydrate. With each of the used gangliosides the apparent K_m values were very similar values for homogeneous micelles and vesicular dispersions, but showed marked increases for Triton X-100 mixed micelles, approaching the values exhibited by reference oligosaccharides. Triton X-100 micelles and phosphatidylcholine vesicles did not appreciably alter the kinetics of sialidase action on 3'-sialosyl-lactose and on G_{D1a} lipid-free carbohydrate, indicating that the above effects are dependent on the intrinsic characteristics of the membrane-like systems containing gangliosides.

Sialidases are enzymes releasing α -glycosidically linked and terminally positioned sialic acid from a variety of sialosyl-glycoconjugates. These enzymes are widely distributed in Nature and occur, especially in mammalian tissues, in different forms both soluble and membrane-bound (Rosenberg & Schengrund, 1976).

The regulation and substrate specificity of sialidases is a complex matter. Many parameters have been shown to affect the specificity and kinetics of

the enzyme action (see the recent review by Corfield *et al.*, 1981): (a) the linkage anomerity of sialosyl residues (α - or β -); (b) the nature of the sialosyl derivative acting as substrate (oligosaccharide, glycoprotein, glycolipid); (c) the type of linkage between sialic acid and the neighbouring sugar (2 $\rightarrow$ 3, 2 $\rightarrow$ 4, 2 $\rightarrow$ 6, 2 $\rightarrow$ 8); (d) the nature of the neighbouring sugar (D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, sialic acid); (e) the type of sialic acid involved (N-acetyl, N-glycosyl, N-acyl, O-acetyl derivatives); (f) the possible occurrence of interactions between sialic acid and adjacent monosaccharides (especially in branched-chain oligosaccharide units); (g) the availability of the sialic acid carboxy group in the free form; (h) the characteristics of the side chain of sialic acid.

An aspect of sialidase-substrate interaction that deserves special attention, because of its biological implications, is the membrane-bound nature of some

Abbreviations used: BSA, bovine serum albumin; TNBS, 2,4,6-trinitrobenzenesulphonate; G_{M1} , $II^3NeuAc-GgOse_4Cer$; G_{D1a} , $II^3NeuAc-IV^3NeuAc-GgOse_4Cer$; G_{D1b} , $II^3(NeuAc)_2-GgOse_4Cer$; G_{T1b} , $II^3(NeuAc)_2-IV^3NeuAc-GgOse_4Cer$. This paper follows the ganglioside nomenclature of Svennerholm (1964) and the IUPAC-IUB recommendations on Lipid Nomenclature (1977).

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sialidases and many of their natural substrates. The crucial point is whether (and, if so, how) physico-chemical features of the membrane structure influence either the activity of membrane-bound sialidase towards exogenously supplied substrates or the availability of membrane-bound sialosyl derivatives to external sialidases. This field has recently begun to be explored. It was shown, for instance (Sandhoff & Pallmann, 1978; Sandhoff *et al.*, 1981), that a membrane-bound sialidase of mammalian brain origin is sensitive to the variations of the physical state of the membrane. It was also established (Cestaro *et al.*, 1980; Gatt *et al.*, 1981) that bacterial sialidases recognize the state of physical dispersion of gangliosides, which are normal components of vertebrate cell plasma membrane.

In the present investigation we examined the kinetics of interaction between sialidase and membrane-linked substrates. To provide information potentially useful to studies with natural membranes, we decided to adopt a simple but appropriate model system consisting of: (a) a soluble sialidase (from *Vibrio cholerae*) of the highest purity available; (b) a substrate that could be inserted into an artificial layer in such a way as to make it available to the enzyme. For this latter purpose we used gangliosides (sialosylglycolipids), which, owing to their amphiphilic properties, can be arranged in two types of supramolecular organization (micelles or vesicles), providing surfaces and mimicking natural membranes.

The experimental approach consisted of recording the changes in sialidase kinetic parameters resulting from variations of the surface features of membrane-like systems containing gangliosides. As reference substrates that did not interact with the 'membrane' surfaces and that therefore freely interacted with the enzyme, 3'-sialosyl-lactose and the lipid-free carbohydrate component of G_{D1a} ganglioside were employed.

Materials and methods

Chemicals and other products

Commercial chemicals were of analytical grade or the highest purity available. Solvents were distilled before use. The water routinely used was freshly distilled on a glass apparatus. Triton X-100 of g.l.c.-purity grade was purchased from Merck (Darmstadt, Germany). Delipized BSA was purchased from Sigma (St. Louis, MO, U.S.A.); its monomeric form was prepared by the method of Tomasi *et al.* (1980). *Vibrio cholerae* sialidase (1 unit/ml; 1 unit is defined as the amount releasing 1 μ mol of *N*-acetylneuraminic acid/min) was from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.). This enzyme preparation, which was highly homogeneous (it showed a single faint contaminating band on polyacrylamide-gel electrophoresis), tended to

decay on storage after opening the vials. Care was taken to use, especially for comparative studies, new or freshly opened vials of the same stock. On average, the sialidase used throughout the experiments had 80% of the specific activity supplied by the manufacturer. TNBS was purchased from BDH (Milan, Italy); *N*-acetylneuraminic acid was from Sigma; Ultrogel Ac 34, which was used for preparing ganglioside-BSA complexes, was from L.K.B. (Bromma, Sweden); Dowex 2 (X8; 200–400 mesh) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. (this resin was prepared in acetate form by the method of Svennerholm, 1957). Egg phosphatidylcholine and egg phosphatidylethanolamine were purchased from BDH and used without further purification, provided that both of them showed a single spot when assayed by t.l.c. on silica-gel thin-layer plates (HPTLC 60; Merck). The solvent system was chloroform/methanol/water (60:35:4, by vol.; 1 h run at 20°C), and spots were revealed by exposure to I_2 .

Gangliosides G_{M1} , G_{D1a} , G_{D1b} and G_{T1b} were extracted and purified from bovine brain by the method of Tettamanti *et al.* (1973). Their identification, structural analysis and purity were assessed as described by Sonnino *et al.* (1978). The final purity was over 99% for all gangliosides.

Monosialyl-lactose [$\alpha(2\rightarrow3)$ isomer] was prepared from cow colostrum by the method of Öhman & Hygstedt (1968). The lipid-free carbohydrate component of gangliosides G_{M1} and G_{D1a} was obtained from the original gangliosides (50 mg each) after ozonolysis and alkaline treatment as described by Wiegandt & Baschang (1965). They were purified from the reaction mixture by chromatography on a Bio-Gel P2 column (2 cm $\times$ 30 cm) previously equilibrated and eluted with water. The elution profile was monitored by t.l.c. on HPTLC-precoated plates with *n*-propanol/water (7:3, v/v) as the developing solvent; the spots were detected by treatment with a *p*-dimethylaminobenzaldehyde spray reagent and heating at 130°C for 10 min. The purity of all oligosaccharides prepared, assessed by the chemical criteria specified by Sonnino *et al.* (1978), was over 95%.

Preparation of micelle solutions of gangliosides

Each of the gangliosides was dissolved in chloroform/methanol (2:1, v/v). Known portions of the mixture, containing the required amount of ganglioside, were transferred into glass tubes and the solvent was completely removed by a gentle flow of N_2 . The residue, kept under vacuum over P_2O_5 until processed, was dissolved in 0.33 ml of 25 mM-Tris/HCl buffer, pH 6.9 (the buffer used for sialidase assay), briefly vortex-mixed and equilibrated at 37°C for 30 min, before sialidase assay. Mixed micelles of ganglioside G_{D1a} , G_{D1b} or G_{T1b} with either G_{M1} or

Triton X-100 were prepared starting from separate mixtures of each component in chloroform/methanol (2:1, v/v). Known portions of each mixture were mixed in glass tubes and processed as described above. All solutions of micellar, homogeneous or mixed gangliosides were prepared immediately before their use for sialidase assay.

Preparation of ganglioside-BSA complexes (micellar lipoprotein complexes)

Ganglioside-BSA complexes (namely G_{D1a} -BSA, G_{D1b} -BSA and G_{T1a} -BSA) were prepared as described by Tomasi *et al.* (1980) for the G_{M1} -BSA complex. By analogy with this latter complex, two forms of complexes of different molecular weight were obtained for each ganglioside; the low-molecular-weight one (with molecular weight equaling that of one albumin molecule plus one micelle of the individual ganglioside) was employed. The samples, eluted from the Ultrogel Ac 34 column in 50 mM-sodium phosphate buffer, pH 7.5, were dialysed for 1 day at 4°C against 200 vol. of double-distilled water, changed four times, then evaporated *in vacuo* (at 25°C) till almost dry. The residues, dissolved by 25 mM-Tris/acetate buffer, pH 6.9, were equilibrated at 37°C for 30 min before sialidase assay.

Preparation of small unilamellar vesicles containing gangliosides

Small unilamellar vesicles of phosphatidylcholine or phosphatidylcholine-ganglioside were prepared by the procedure of Barenholz *et al.* (1977), which has already been applied to ganglioside-containing systems (Cestaro *et al.*, 1980). The final high-speed supernatant was employed. All specimens were prepared in 0.15 M-NaCl, pH 6.9, at 20–22°C, stored at the same temperature and used within 12 h of preparation. Before sialidase assay the samples were prewarmed at 37°C for 30 min.

Assessment of integrity of the phospholipid-ganglioside vesicles

This was routinely performed by the method of Barenholz *et al.* (1977) by: (a) turbidity measurements; (b) assaying the percentage of amino groups (carried by phosphatidylethanolamine inserted as a marker in the vesicle; about 5 mol%) available to TNBS. The percentage of these amino groups on the outer side of the vesicle was reported to be about 65% for small unilamellar vesicles of phosphatidylcholine (Barenholz *et al.*, 1977). We confirmed this value also in phosphatidylcholine vesicles containing various amounts (up to a given maximum; see the Results section) of ganglioside. All mixtures analysed for this purpose were prepared in 25 mM-sodium phosphate buffer, pH 7.0. The distri-

bution of vesicle-bound ganglioside on the inner and outer sides of the vesicles was determined as follows. A sample of vesicle preparation (carrying about 25 µg of ganglioside G_{D1a} , G_{D1b} or G_{T1b} as bound *N*-acetylneuraminic acid) was incubated under the conditions specified below with an excess of sialidase (15–20 m-units) till no more sialic acid release occurred, and the absorption value was recorded. This corresponded to the number of ganglioside molecules exposed on the outer side of the vesicle and directly available to sialidase. An equal sample, after addition of 0.5% Triton X-100 (final concentration), was vortex-mixed till the mixture became completely clear (a few seconds), indicating complete destruction of the vesicle structure and total exposure of ganglioside (as mixed micelles) to sialidase, and then treated with sialidase as above. The actual degree of ganglioside hydrolysis by sialidase was also controlled by determining formed G_{M1} (which is the end-product of sialidase hydrolysis of the above ganglioside) after extracting gangliosides from the incubation mixture, and separating G_{M1} by t.l.c. (Sonnino *et al.*, 1978).

Sialidase assay

The incubation mixtures contained 0.5–2 µl of sialidase solution, given amounts (see below) of substrate, 25 mM-Tris/HCl buffer, pH 6.9 (which was optimal; see the Results section), in a final volume of 0.35 ml. The incubation mixtures, which were started by addition of the enzyme, were placed in an incubator shaker and incubated at 37°C for 5–10 min. The enzyme reaction was stopped by immersing the tubes in an acetone/solid CO₂ bath. The liberated *N*-acetylneuraminic acid was determined by the chromatographic microprocedure of Caimi *et al.* (1979). The yield by this procedure, checked each time with pure standard *N*-acetylneuraminic acid, was higher than 92% and reproducibility was good ($\pm 2\%$) in the range 3–50 nmol of *N*-acetylneuraminic acid. An absorbance of 0.100 (as corrected absorbance; see Warren, 1963) corresponded to 14–14.5 nmol of free *N*-acetylneuraminic acid. The control incubation mixtures (blanks) were complete incubation mixtures, but lacking the enzyme. K_m and V_{max} values were determined by the method of Lineweaver & Burk (1934).

Other analytical methods

Ganglioside-bound *N*-acetylneuraminic acid was determined by the method of Svennerholm (1957). The protein content of the enzyme preparation was determined by the method of Lowry *et al.* (1951), with BSA as standard.

Results

Of the gangliosides used in the present investigation, G_{D1a} , G_{D1b} and G_{T1b} were affected by

sialidase, whereas G_{M1} , under all conditions, was resistant to enzyme action, in agreement with previous work (Burton, 1963). The entire set of experiments with the different membrane-mimicking systems was carried out with G_{D1a} . Comparative studies were done with G_{D1b} and G_{T1b} under the conditions that were found to be optimal for G_{D1a} . The optimum conditions for sialidase assay on sialyl-lactose and on the lipid-free carbohydrate from G_{D1a} were the same as those reported previously by Drzeniek (1972) and Rosenberg & Schengrund (1976). Preliminary experiments (see also Table 1) established that neither Triton X-100 micelles nor phosphatidylcholine vesicles, within the concentration range used in ganglioside-containing systems, had any influence on the kinetics of sialidase action on the oligosaccharides used in the present study. The lipid-free carbohydrate from G_{M1} was confirmed to be resistant to *Vibrio cholerae* sialidase, as reported by Kuhn & Wiegandt (1963).

Optimal conditions for sialidase action on the different membrane-mimicking systems containing gangliosides

Gangliosidic substrates were employed in two different dispersion systems: micelles and small unilamellar vesicles with phosphatidylcholine (see Fig. 1). Micelles were either homogeneous (that is constituted by a single ganglioside species) or mixed. These latter micelles were made up by one substrate-ganglioside (G_{D1a} , G_{D1b} or G_{T1b}) and either: (a) the non-ionic surfactant Triton X-100 (which is known to give mixed micelles with gangliosides at any molar ratios; Corti *et al.*, 1981), (b) G_{M1} , a 'non-substrate' ganglioside or (c) BSA (lipoprotein micelles).

The optimum pH of sialidase on both micellar and vesicular systems was 6.9. The investigated pH range was 5.0–8.0; lower or higher pH values were not tested, owing to possible perturbation of the physicochemical features of micelles or vesicles. Of a number of different buffer systems (sodium acetate/acetic acid; sodium citrate/citric acid; sodium phosphate; Tris/HCl), Tris/HCl buffer provided the highest enzyme activities. The optimum molarity of this buffer ranged from 10 to 100 mM. In the routine experiments, 25 mM buffer was employed. The rate of enzyme reaction was, with all systems, linear with time up to at least 15 min. The reaction rate was linear with enzyme protein in the range 0.01–0.6 μ g (1 μ l of the commercial enzyme preparation contained 0.04 μ g of protein) with the exception of micelles made up only by gangliosides. In this case the above relationship began being linear from 0.2 to 0.22 μ g of protein. The lack of linearity for lower protein concentrations is probably due to a specific absorption of the enzyme on micelles.

Kinetics of sialidase action on ganglioside micellar systems

The micellar systems constituted by G_{D1a} , G_{D1a}/G_{M1} (in different proportions) or G_{D1a} -BSA complex provided v -[substrate] relationships obeying

Fig. 1. Schematic representation of the different systems used for dispersing gangliosides

Fig. 2. Kinetics of *Vibrio cholerae* sialidase action on different micellar dispersions of G_{D1a} (homogeneous micelles; mixed micelles with non-substrate G_{M1} or BSA). Incubations: 8 min (5 min for the G_{D1a} -BSA complex) at 37°C with 0.24 μ g of enzyme (0.08 μ g for the G_{D1a} -BSA complex). Values are means of four sets of experiments. Units of v are nmol of *N*-acetylneuraminic acid released/min. Symbols: ■, G_{D1a} -BSA complex; ○, G_{D1a} ; ▲, G_{D1a}/G_{M1} (molar ratio 3:1); ●, G_{D1a}/G_{M1} (1:3, molar ratio).

regular hyperbolic kinetics (see Fig. 2). The K_m values were very similar to each other (approx. $40 \mu\text{M}$; see also Table 1). The V_{max} values were lowest with $G_{\text{D1a}}/G_{\text{M1}}$ mixed micelles, highest with G_{D1a} -BSA complex, with differences in the range 5–6-fold (see Table 1). In the presence of Triton X-100 (see Fig. 3), by either varying the concentration of Triton X-100 (at fixed G_{D1a} concentration) or of G_{D1a} (at fixed Triton X-100 concentration) the kinetic profiles were irregular and biphasic. This indicates that the G_{D1a} /Triton X-100 ratio is critical and that, by consequence, fixed G_{D1a} /Triton X-100 ratios have to be used for meaningful kinetic information to be obtained. With fixed G_{D1a} /Triton X-100 proportions (a molar-ratio range from 1:1 to 1:30 was explored), the v -[substrate] curves were always hyperbolic (see Fig. 4). Both K_m and V_{max} values underwent marked variations depending on the G_{D1a} /Triton X-100 molar ratio (see Fig. 5). The apparent highest values of both K_m and V_{max} were observed at a G_{D1a} /Triton X-100 ratio of 1:7.5, at which the K_m and V_{max} values were respectively 23-fold and 15-fold greater than the corresponding ones observed on homogeneous micelles of G_{D1a} (see Table 1).

Table 1. Apparent V_{max} and K_m values of *Vibrio cholerae* sialidase acting on free substances (3'-sialosyl-lactose; G_{D1a} lipid-free carbohydrate) and on different micellar and vesicular dispersions of G_{D1a} ganglioside. Results are means of four sets of experiments. Abbreviations used: PC, phosphatidylcholine; s.u.v., small unilamellar vesicles.

Substrate	K_m (μM)	V_{max} (m-units/mg of protein)
Free substrates		
3'-Sialosyl-lactose	3280	19.7
+ 0.2 mM-Triton X-100	3180	19.9
+ 60 mM-Triton X-100	3320	20.3
+ 0.5 mM-PC, as s.u.v.	3150	19.2
+ 9 mM-PC, as s.u.v.	3210	19.4
G_{D1a} lipid-free carbohydrate		
+ 0.2 mM-Triton X-100	2010	8.3
+ 60 mM-Triton X-100	1930	8.8
+ 0.5 mM-PC, as s.u.v.	1980	8.5
+ 9 mM-PC, as s.u.v.	2050	8.5
	1970	8.2
Micellar dispersions		
Homogeneous G_{D1a}	44	2.0
Mixed $G_{\text{D1a}}/G_{\text{M1}}$ (3:1, molar ratio)	39	1.8
Mixed $G_{\text{D1a}}/G_{\text{M1}}$ (1:3, molar ratio)	38	1.4
G_{D1a} -BSA lipoprotein complex	44	11.0
Mixed G_{D1a} /Triton X-100 (1:7.5, molar ratio)	1020	30.0
Vesicular dispersions (PC s.u.v.)		
G_{D1a} (2.5 mol%)	45	52.0
G_{D1a} (5–15 mol%)	44	38.0

Kinetics of sialidase action on vesicular systems containing gangliosides

The maximum amount of ganglioside in vesicular systems compatible with the maintenance of a sealed

Fig. 3. Effect of Triton X-100 on the hydrolysis of G_{D1a} by *Vibrio cholerae* sialidase

(a) Shows the kinetics at various, fixed, G_{D1a} and varying Triton X-100 concentrations. G_{D1a} concentrations (mM) were: $\bullet$, 0.1; $\circ$, 0.2; $\blacktriangle$, 0.5. (b) Shows kinetics at various, fixed, Triton X-100 and varying G_{D1a} concentrations. Triton X-100 concentrations (mM) were: $\blacksquare$, 0.8; $\blacktriangle$, 16; $\circ$, 4; $\bullet$, 40. Incubations were for 5 min at 37°C with $0.08 \mu\text{g}$ of enzyme. Results are means of four sets of experiments.

Fig. 4. Kinetics of *Vibrio cholerae* sialidase acting on mixed G_{D1a} Triton X-100 micelles at fixed but different molar proportions

Incubations were for 5 min at 37°C with $0.08 \mu\text{g}$ of enzyme. Results are means of four sets of experiments. The G_{D1a} /Triton X-100 molar ratio was: $\square$, 1:1; $\blacksquare$, 1:3; $\blacktriangle$, 1:7.5; $\circ$, 1:15; $\bullet$, 1:30.

Fig. 5. *Vibrio cholerae* sialidase action on mixed G_{D1a} /Triton X-100 micelles at fixed but different molar ratios between constituents

Variations of apparent V_{max} (●) and K_m (○) values with increasing proportions of Triton X-100 in the mixed micelle are shown. Incubations were for 5 min (8 min for the G_{D1a} homogeneous micelles) at 37°C with 0.08 μg of enzyme (0.24 μg for the G_{D1a} homogeneous micelles). Results are means of four sets of experiments.

monolamellar vesicle was 15 mol% for G_{D1a} and 12 mol% for G_{D1b} and G_{T1b} (see also Bertoli *et al.*, 1981). In the G_{D1a} /phosphatidylcholine vesicles, the maximum amount of embedded ganglioside that could be hydrolysed to G_{M1} with excess sialidase was 65–66% of the total, regardless of the proportion of G_{D1a} in the vesicles (from 2.5%, which is the minimum suitable for sialidase studies, to 15%) (see Fig. 6). In addition, the molar proportion between the remaining G_{D1a} and the formed G_{M1} was, after exhaustive incubation with sialidase, 1:2. This means that about two-thirds of G_{D1a} in the vesicle is on the outer side and available to sialidase, the remainder being on the inner side and inaccessible to the enzyme. This value for ganglioside on the outer side is in close agreement with that reported for phosphatidylethanolamine in similar vesicles (Barenholz *et al.*, 1977). Since the incubation with sialidase was carried out for 1 h, the above behaviour also means that no apparent modifications of the vesicle structure take place during at least 1 h of sialidase action.

v -[substrate] relationships with vesicle-bound G_{D1a} obeyed hyperbolic kinetics in the range of G_{D1a} molar proportions examined in the vesicle. The K_m values were unaffected by the proportion of ganglioside in the vesicle and practically overlapped that of homogeneous G_{D1a} micelles (see also Table 1). Instead, the highest values of V_{max} (see Table 1) were attained with 2.5% G_{D1a} in the vesicle. This

Fig. 6. Assessment of 'sidedness' of G_{D1a} in small unilamellar vesicles of phosphatidylcholine containing 2.5 mol% (●) or 15 mol% (○) of G_{D1a}

Small unilamellar vesicles carrying 25 μg of G_{D1a} , as bound sialic acid, were incubated with an excess of *Vibrio cholerae* sialidase (20 m-units) till no more sialic acid release occurred. The amount of G_{D1a} hydrolysed under these conditions (expressed as a percentage of total small-unilamellar-vesicle-bound G_{D1a}) corresponds to the portion of small-unilamellar-vesicle-bound G_{D1a} available to sialidase that is 'outer-sided' (lower curve). The amount of total small-unilamellar-vesicle-bound G_{D1a} (upper curve) was established by exhaustive sialidase hydrolysis after vesicle treatment with 1% (final concentration) Triton X-100 and vortex-mixing. Results are means of four sets of experiments.

value was 26-fold greater than that recorded with homogeneous micelles. In the kinetics studies with vesicular gangliosides, the ganglioside concentration computed was that of the ganglioside available to sialidase (that is the ganglioside on the outer side).

Studies carried out on G_{D1b} and G_{T1b} gangliosides (see Table 2) showed that the trend of sialidase action on them paralleled, in the different systems, that displayed on G_{D1a} . With reference to G_{D1a} , the V_{max} values were, in all systems, about 10% greater with G_{T1b} and 60% lower with G_{D1b} ; the K_m values for G_{T1b} were very close to those for G_{D1a} , whereas the values for G_{D1b} were four to five times greater.

Comparative evaluation of the V_{max} and K_m changes with the different membrane-mimicking systems

V_{max} and K_m values obtained with the different systems are reported in Table 1. K_m values for G_{D1a} were lowest, and in close agreement (about 40 μM) in all the systems used, except of G_{D1a} /Triton X-100 mixed micelles, which gave much higher K_m values (up to 1020 μM). This value is of the same order of magnitude as that shown by G_{D1a} lipid-free carbohydrate (2010 μM) and sialyl-lactose (3280 μM). V_{max} values were lowest with micelles containing only gangliosides (1.4–2 m-units), and increased 5–6-fold with G_{D1a} -BSA complex (10 m-units),

Table 2. Apparent V_{max} and K_m values of *Vibrio cholerae* sialidase acting on different micellar and vesicular dispersions of G_{D1b} and G_{T1b} gangliosides

The experimental conditions described for G_{D1a} were employed. For G_{D1b} , a 5-fold greater enzyme content was used. Results are means of three sets of experiments.

Dispersing system	G_{D1b}		G_{T1b}	
	V_{max} (m-units/ mg of protein)	K_m (μM)	V_{max} (m-units/ mg of protein)	K_m (μM)
Homogeneous micelle	0.82	212	2.2	42
Mixed micelle with G_{M1} (1:3, molar ratio)	0.61	188	1.6	39
Mixed micelle with BSA	4.6	205	12.3	41
Mixed micelle with Triton X-100 (1:7.5, molar ratio)	12.5	4865	34.2	953
Phosphatidylcholine small unilamellar vesicles containing 2.5 mol% ganglioside	21.2	209	56.6	42

15-fold with G_{D1a} -Triton X-100 (molar ratio 1:7.5) (30 m-units) and 26-fold with 2.5 mol% vesicular G_{D1a} (52 m-units). The V_{max} value recorded in this latter system was much higher than that displayed with G_{D1a} lipid-free carbohydrate (8.3 m-units) and sialyl-lactose (about 20 m-units).

Discussion

Gangliosides, in water solution, spontaneously form micelles carrying the oligosaccharide chains on the surface; when mixed with phospholipids (e.g. phosphatidylcholine) they give rise to aggregates, the type of which depends on the ratio between the two components. At ganglioside/phosphatidylcholine ratios lower than a certain value (15 mol% for G_{D1a} , 12 mol% for G_{D1b} and G_{T1b} ; Bertoli *et al.*, 1981) the dispersions, obtained by sonication, consist of unilamellar bilayered vesicles (Cestaro *et al.*, 1980), which carry about two-thirds of the embedded ganglioside on the vesicle outer side. Therefore both micellar and vesicular dispersions of gangliosides provide simple models suitable for studying the interactions between sialosyl derivatives exposed on the surface and small, water-soluble, proteins, like *Vibrio cholerae* sialidase. To acknowledge the importance of the surface effect in sialidase-ganglioside interactions, it was of interest to know how the enzyme worked on free ganglioside molecules, that is on monomeric gangliosides. However, this could not be directly explored. In fact no measurable enzyme activity was recorded for ganglioside concentrations lower than 10 μM . On the other hand, gangliosides are known to micellize at about 1 μM (Gatt *et al.*, 1981) or lower (Formisano *et al.*, 1979; Mraz *et al.*, 1980; Corti *et al.*, 1980). Therefore, monomeric gangliosides at any of the concentrations used in the present study were neither present alone nor in large amounts. Owing to the lack of monomeric gangliosides, the lipid-free carbohydrate of G_{D1a} served as an analogous substance freely interacting with the enzyme and directly comparable with micellar and vesicular G_{D1a} .

The kinetic studies reported here clearly show that, for G_{D1a} , the K_m and V_{max} values depended on: (a) whether the substrate was free or surface-linked; (b) the kind of physical dispersion (micelle or vesicles) of the ganglioside; (c) the molar ratio between the components, in the case of Triton X-100/ganglioside mixed micelles and of phosphatidylcholine/ganglioside small unilamellar vesicles. In particular, the V_{max} moved from a minimum value with pure ganglioside micelles, through intermediate values with the lipid-free carbohydrate (4–5-fold greater) and with the Triton X-100 mixed micelles (up to about 15-fold greater) to the highest value with phosphatidylcholine/ganglioside vesicles (up to about 30-fold greater); the K_m , lowest with pure ganglioside micelles and all vesicular dispersions, increased by about 23-fold with Triton X-100 mixed micelles and by 45-fold with the lipid-free carbohydrate. Qualitatively parallel results were obtained with G_{D1b} and G_{T1b} gangliosides. This means that both the formation of the enzyme-ganglioside complex and the ganglioside turnover rate are affected by the surface characteristics of the ganglioside-carrying particles. It is noteworthy that micelles of pure Triton X-100 and vesicles of pure phosphatidylcholine do not appreciably modify the kinetics of sialidase action on sialosyl oligosaccharide, excluding any direct influence on the enzyme itself.

The micelle and vesicle features that are responsible for the variations of *Vibrio cholerae* sialidase action on bound gangliosides are not easily characterized. In mixed micelles of ganglioside and detergent (Triton X-100, bile salts), the presence of ganglioside probably affects the size (Gatt *et al.*, 1981; Corti *et al.*, 1981) of the particle and the critical point of consolution ('cloud point') of the system (Corti *et al.*, 1981). These changes, together possibly with concurrent modifications in the curvature and shape of the particle, have previously been demonstrated to be recognized by enzymes (Gatt *et al.*, 1981; Corti *et al.*, 1981). In artificial lipid bilayers, gangliosides induce a decrease in mem-

brane fluidity (Maggio *et al.*, 1980; Bertoli *et al.*, 1981). The strong side-side head-group interactions on the bilayer surface (between ganglioside oligosaccharide chains and between ganglioside and phospholipid polar head groups) lead the lipid chains to assemble in a more rigid fashion. These events are expected to affect some of the basic properties of the bilayer, such as transition temperature of the components, surface pressure, surface charge density, which, interestingly, are known to greatly influence the action of phospholipases on vesicle- or membrane-bound phospholipids (Op Den Kamp *et al.*, 1974; Demel *et al.*, 1975). Particular attention should also be paid to the oligosaccharide portions of gangliosides, which, in the micelle, are forced to pack together by the strong hydrophobic interactions among the lipophilic moieties and by hydrogen bonds involving the hydroxy groups of long-chain bases and the carbonyl oxygen atoms of adjacent fatty acids. This enables the formation of a network of side-side hydrogen bonds and dipole-dipole interactions within the oligosaccharide units and produces an intense surface charge density, owing to the vicinity of sialic acid carboxy groups. The overall packing of ganglioside molecules is not suitable for interaction with enzymes, as previously suggested by Gatt *et al.* (1981). In mixed Triton X-100 micelles and in phosphatidylcholine vesicles, the detergent or phospholipid acts as a 'spacer', separating the ganglioside molecules and decreasing interaction between them, thereby facilitating sialidase binding and action. On the other hand, when G_{M1} , a non-substrate ganglioside, was used as the spacer, since the intercarbohydrate attraction was maintained, the enzyme action remained at the low level expressed with homogeneous G_{D1a} micelles.

In conclusion, the interaction of *Vibrio cholerae* sialidase with a gangliosidic substrate appears to depend on the intrinsic characteristics of the membrane-like system in which the ganglioside is embedded. It would be interesting to know whether the same phenomenon occurs for other enzymes that are physiologically involved in ganglioside metabolism, for the various ligands known to specifically interact with gangliosides and for gangliosides bound to natural membranes.

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