Action of Arthrobacter ureafaciens Sialidase on Sialoglycolipid Substrates

MODE OF ACTION AND HIGHLY SPECIFIC RECOGNITION OF THE OLIGOSACCHARIDE MOIETY OF GANGLIOSIDE GM₁

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A new bacterial sialidase (N-acetylneuraminic glycosyldetrase, EC 3.2.1.18) isolated from the culture filtrate of Arthrobacter ureafaciens was characterized in detail with respect to its action on sialoglycolipids. Strong electrolytes had a reversible inhibitory effect on the action of the enzyme on brain gangliosides in accordance with Debye-Hückel effect of ionic environment on ionic activity, and resulted in an acidic shift and a broadening of the pH optimum. Both ionic and non-ionic detergents markedly enhanced the enzymatic activity on the gangliosides, and caused an acidic shift on the pH optimum of this enzyme. Sulfhydryl groups seemed to be involved in its active site. This enzyme had a highly specific action on sialidase-resistant ganglioside GM₁, showing about 100-fold higher activity on GM₁ than Clostridium perfringens sialidase, the only sialidase so far reported to cleave the lipid substrate in the presence of bile salts. In the absence of detergents, the activity of A. ureafaciens sialidase on GM₁ was very low. Ganglioside GM₁ in either the monomeric or micellar form was hydrolyzed to asialo-GM₁ by A. ureafaciens sialidase most efficiently in the presence of sodium cholate of about three times the GM₁ molar concentration. The presence of detergents increased both the Kₘ and Vₘₐₓ values for ganglioside GM₁. The oligosaccharide prepared from GM₁ by ozonolysis was cleaved well by this sialidase in the absence of detergents, and no detergent was found to affect the hydrolysis. The Kₘ value for the sugar substrate was about two orders of magnitude greater than that for the corresponding lipid substrate.

It is suggested that the hydrophobic ceramide moiety increases affinity of the lipid substrate to the enzyme, but inhibits hydrolysis of the substrate, possibly due to its hydrophobic interaction with hydrophobic portions of the enzyme molecule (resulting in lower Kₘ and Vₘₐₓ for lipid substrates). This inhibition may be released by detergent due to formation of mixed micelles of sialoglycolipid and detergent molecules. It is also indicated that recognition of the specific saccharide structure of GM₁ by individual sialidases is essential for release of the resistant sialyl residue, and that A. ureafaciens sialidase seemed to have an isoenzymic or oligomeric structure.

Sialidases of microbial origin have been known for over 30 years (1-3), and they are frequently employed for structural studies on various naturally occurring sialo-compounds, such as sialoglycoproteins, sialoglycolipids, and oligo- and polysaccharides. Recently, their use in biochemical and immunological researches on physiological, polymorphic functions of cell membrane systems (4-13) has increased, since it has been found that they affect the structure and net charge of constituents containing sialic acid by removing terminal sialyl residues, principally resulting in alteration of intermolecular and intercellular forces (14). Among the known microbial sialidases, those from Vibrio cholerae (15-17) and Clostridium perfringens (18-23) have been most extensively characterized in terms of enzymological and biological properties.

Recently, a new bacterial sialidase was isolated in a highly purified state from the culture filtrate of a non-pathogenic bacterium, Arthrobacter ureafaciens (24, 25). This enzyme preparation was reported to liberate sialic acid from substrates containing sialyl residues with α₂-3, α₂-6, and α₂-8 linkages, and to show no activities of contaminating enzymes such as proteases, sialic acid aldolase or other glycosidases (25). In preliminary studies (26), we found that in the presence of detergents this enzyme efficiently hydrolyzed monosialo-ganglioside GM₁,1 which, like GM₁, has usually been found to be resistant to various sialidases of viral, bacterial, and mammalian origin (18, 27-34). The only exceptional reports in this respect are that the resistant sialyl residues of gangliosides GM₁ and GM₂ could be cleaved enzymatically in the presence of bile salts (20, 21) and that a lysosomal sialidase of mammalian origin could hydrolyze ganglioside GM₂, although this enzyme seems to be very labile, and it is unknown whether it can hydrolyze GM₁ (35, 36).

This report presents the precise mechanism of enzymatic hydrolysis of sialoglycolipid substrates (purified brain gangliosides) by A. ureafaciens sialidase in comparison with the

1 The abbreviations used are: GM₁, Galβ₁ → 3GalNAcβ₁ → 4[NeuAco₂ → 3]Galβ₁ → 4Glc-Cer; GM₂, GalNAcβ₁ → 4[NeuAco₂ → 3]Galβ₁ → 4Glc-Cer; Gm₁, NeuAco₂ → 3Galβ₁ → 4Glc-Cer; Gm₂, NeuAco₂ → 3Galβ₁ → 4Glc-Cer; Gms, [NeuAco₂ → 3]Galβ₁ → 3GalNAcβ₁ → 4NeuAco₂ → 3Galβ₁ → 4Glc-Cer; Gmb, Galβ₁ → 3GalNAcβ₁ → 4[NeuAco₂ → 8NeuAco₂ → 3]Galβ₁ → 4Glc-Cer; Gm₃, [NeuAco₂ → 3]Galβ₁ → 3GalNAcβ₁ → 4-[NeuAco₂ → 8NeuAco₂ → 3]Galβ₁ → 4Glc-Cer; Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid; Cer, ceramide (2-N-acylphosphoglycerol); CMC, critical micelle concentration; GI.C, gas-liquid chromatography; TIC, thin layer chromatography.
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Mechanisms of other bacterial sialidases, especially that of sialidase from C. perfringens. It is shown that the new sialidase has a specific affinity for the whole saccharide structure of ganglioside GM₁, the degree of which is greatly enhanced by the presence of a hydrophobic ceramide moiety. The hydrophobic interaction between the ceramide moiety of the lipid substrate and the enzyme molecule is discussed in terms of an extrapolated model for the more complex lipid-protein aggregates involved in biological membranes, and a possible mechanism for the effect of detergents in enhancing the hydrolytic cleavage of ganglioside GM₁ is suggested.²

² Portions of this paper (including “Experimental Procedures,” and part of “Results” and “References,” Figs. 3 to 10 and 14, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 8650 Rockville Pike, Bethesda, MD. 20014. Request Document No. 79M-120, cite author(s), and include a check or money order for $2.40 per set of photocopies.

Fig. 1. Gel filtration of A. ureafaciens sialidase on Ultrogel AcA 44. A preparation of A. ureafaciens sialidase (about 1.5 mg of protein, equivalent to 73 units of enzyme in terms of hydrolytic activity on colominic acid) purified by colominic acid affinity chromatography was applied to a column of Ultrogel AcA 44 (0.65 × 100 cm) equilibrated with 0.1 M phosphate buffer (pH 6.8) as described by Uchida et al. (25) but with proportional reduction in volumes. The enzyme was eluted with the same buffer at a flow rate of 1 ml/h. Symbols are as follows: absorbance at 280 nm (O—O); sialidase activity on sialyllactose (Δ—Δ); sialidase activity on ganglioside mixture (purified brain gangliosides) with 1 mg/ml of sodium cholate ( []). sialidase activity on ganglioside GM₁ with 1 mg/ml of sodium cholate ([ ]) sialidase activity on ganglioside GM₁ without detergent (Δ—Δ).

RESULTS

Kinetic Properties of A. ureafaciens Sialidase on Ganglioside GM₁ and the Oligosaccharide prepared from GM₁—Thin layer chromatography showed that A. ureafaciens sialidase cleaved the sialyl residue of ganglioside GM₁ in the presence of detergent (Fig. 2). As shown previously in our preliminary report (26), the production of asialo-GM₁ by the enzyme was enhanced most effectively by sodium cholate. The kinetic properties of the enzyme on GM₁ and oligosaccharide from GM₁ were compared with those of other bacterial sialidases, and especially C. perfringens sialidase. Fig. 8 shows the effects of various detergents on the initial reaction velocities of the enzymes from A. ureafaciens and C. perfringens. A. ureafaciens sialidase was markedly stimulated by addition of bile salts, sodium cholate being the most effective. Nonionic detergents also stimulated this sialidase. With equal units of enzyme in terms of activity on sialyl-lactose, the specific activity of A. ureafaciens sialidase on ganglioside GM₁ was about 100 times that of C. perfringens sialidase in the presence of sodium cholate (Figs. 8 to 10). V. cholerae and streptococcal sialidases did not hydrolyze ganglioside GM₁ at all. The activity of A. ureafaciens sialidase on GM₁ in the presence of sodium cholate increased linearly with enzyme concentration with up to about 50 milliunits of enzyme (equivalent to 1.07 μg of protein) (Fig. 8), and with incubation time up to about 180 min (Fig. 9A). The enzymic activity of C. perfringens sialidase on GM₁ was almost negligible under similar assay conditions (Figs. 8 and 9A). The pH-optimum for cleavage of GM₁ by A.
ureafaciens sialidase was about 4.8 with an optimal concentration of sodium cholate (Fig. 9B). This value was very similar to that of the enzyme for hydrolysis of purified brain gangliosides in the absence of detergents. Addition of a larger amount of sodium cholate caused some inhibition and a slight acidic shift of the pH optimum (Fig. 9B), possibly due to increase in the concentration of Na+ ion in the reaction mixture with increase in the concentration of sodium cholate. Fig. 10 shows that the cleavage of ganglioside G_M1 by A. ureafaciens sialidase was greatly activated by detergents and especially bile salts, and that the degree of activation by detergents depended on their chemical structure and concentration, sodium cholate being most effective at about 1 mg/ml (2.32 mM) with about 0.9 mM G_M1 as substrate. Sodium deoxycholate was similarly effective in activating the sialidase. Detergents caused much less activation of the C. perfringens enzyme than of A. ureafaciens sialidase (Fig. 10B).

Next, the mechanism of activation with sodium cholate was investigated in more detail. Fig. 11A shows the initial velocity-substrate concentration relationship for A. ureafaciens sialidase in the presence of various concentrations of sodium cholate (A), and “the initial velocity-molar ratio of sodium cholate to G_M1” relationship for the action of A. ureafaciens sialidase on G_M1 (B). Activity was assayed as described under “Experimental Procedures” except that various concentrations of substrate were used with the following concentrations of sodium cholate; (A) 1.0 mM (O—O), 2.0 mM (■—■), 2.32 mM (X—X), 3.0 mM (□—□), 4.0 mM (▲—▲), and 5.0 mM (Δ—Δ). The data in B were calculated from those in A.

![Fig. 11. Initial reaction velocity-ganglioside GM1 concentration relationship for A. ureafaciens sialidase in the presence of various concentrations of sodium cholate (A), and “the initial velocity-molar ratio of sodium cholate to GM1” relationship for the action of A. ureafaciens sialidase on GM1 (B).](imageurl)

**Table III**

| Substrate ganglioside GM1 (× 10⁻⁴ M) | Specific enzyme activitya | Ratio of specific enzyme activity (A/B) |
|-----------------------------------|--------------------------|---------------------------------------|
| Cholate (nmol/min/mg protein)     |                          |                                       |
| 0.32 (below CMC)                 | 0.580                    | 495                                   | 853                                   |
| 0.63 (below CMC)                 | 0.736                    | 779                                   | 1,058                                  |
| 1.58 (above CMC)                 | 1.08                     | 1,145                                 | 1,060                                  |
| 3.16 (above CMC)                 | 1.46                     | 1,688                                 | 1,158                                  |

*Values are means for three experiments.

The sialidase activity in the absence of sodium cholate was measured by the method of Hammond et al. (47) as described under “Experimental Procedures.”

Sodium cholate was added to the incubation mixture at about three times the molar concentration of ganglioside G_M1. The enzyme activity was assayed essentially as described in “Experimental Procedures” by measuring release of sialic acid by the procedure of Aminoff (44).

![Fig. 12. Double reciprocal (Lineweaver-Burk) plot for the action of A. ureafaciens sialidase on ganglioside GM1 in the presence (A) and absence (B) of a specified concentration of sodium cholate. A, activity on GM1 was assayed with various concentrations of substrate in the presence of 1 mg/ml (2.32 mM) of sodium cholate. Other conditions were as described under “Experimental Procedures.” Kinetic parameters were calculated to be as follows: K_m (below the CMC) = 7.14 × 10⁻⁴ M, V_max (below the CMC) = 0.19 μmol/min/mg of protein. B, activity in the absence of detergent was assayed as described under “Experimental Procedures,” by the fluorometric method of Hammond et al. (44). Kinetic parameters were calculated to be as follows: K_m (below the CMC) = 1.24 × 10⁻⁴ M, K_m (above the CMC) = 1.04 × 10⁻⁴ M, V_max (below the CMC) = 0.85 × 10⁻⁷ μmol/min/mg of protein, V_max (above the CMC) = 1.87 × 10⁻⁷ μmol/min/mg of protein.](imageurl)
concentration was above or below the CMC ($0.85 \times 10^{-4} \text{M}$) (53), the activity of *A. ureafaciens* sialidase on GM₁ was very low in the absence of detergents, although it could be reproducibly measured by the spectrophotofluorometric method of Hammond *et al.* (47). On addition of the optimal concentration of sodium cholate, the enzymatic activity was much higher, and the activation index was similar whether the lipid substrate GM₁ was in a monomeric (lower than the CMC) or micellar (higher than the CMC) form (Table III). These results indicate that, in the absence of bile salt, there was not much difference in the susceptibilities of monomeric and micellar forms of GM₁ to the action of *A. ureafaciens* sialidase. In contrast, Rauvala reported that, below the CMC, ganglioside GM₁ became more susceptible to *C. perfringens* sialidase even without added bile salts (23). The present results also seem to be incompatible with the suggestion of Wenger and Wardell (21) that detergent may cause activation by converting micellar substrate to more available forms, possibly such as smaller micelles.

Fig. 12A shows the initial velocity-substrate concentration relationship for *A. ureafaciens* sialidase with ganglioside GM₁ as substrate in the presence of a fixed concentration of sodium cholate. Higher concentrations of GM₁ were apparently inhibitory, but this may be partly because at higher substrate concentrations, the detergent concentration was suboptimal. The apparent $K_{m}$ and $V_{max}$ values were calculated as $7.14 \times 10^{-4} \text{M}$ and 5.19 pmol/min/mg of protein, respectively (Fig. 12A and Table V). Fig. 12B shows Lineweaver-Burk plots for the relationship between the initial reaction velocity and the GM₁ concentration over a wide range of concentrations from below to above the CMC. In the absence of detergents, there is a barely detectable discontinuity of the hyperbola as the concentration of substrate passes through the CMC. This discontinuity is more evident in derivative functions, such as reciprocal substrate-velocity plots. Ganglioside micelles are very stable (60), and their size distribution is uniform, so that the effect of concentration above the CMC on the reaction velocity may be treated in terms of diffusion-related kinetics. $K_{m}$ values were derived in terms of simple molar concentration, irrespective of the actual number of diffusing particles per unit volume. On this basis, the apparent $K_{m}$ for the lipid substrate above the CMC was $1.04 \times 10^{-4} \text{M}$ and the $K_{m}$ below the CMC was $1.24 \times 10^{-5} \text{M}$. There was a clear difference in the kinetic constants for the two substrate forms in the absence of detergents. Taken together, the results indicate that, in the absence of detergents, the enzyme interacts not only with monodisperse ganglioside GM₁ but also with sialyl residues located in the surface of GM₁ aggregates, and that the effective concentration of lipid substrate in a kinetic sense was apparently altered by the formation of micellar aggregates.

When ganglioside GM₁, in which the sialyl residue is located at the nonreducing end of the sugar chain, was used as substrate, the initial velocity of *A. ureafaciens* sialidase increased as a continuous hyperbolic function of the substrate concentration, irrespective of whether or not the ionic detergent sodium cholate was present (Fig. 13A). Addition of ganglioside GM₁ to the reaction mixture for GM₁ hydrolysis in the absence of detergent strongly inhibited the initial velocity, as shown in Fig. 13A. GM₁ had an unusual influence on the typical Michaelis-Menten hyperbolic saturation curve for GM₁; it caused an initial sigmoid region (Fig. 13A) somewhat different from the classical competitive kinetic form. In the Hill plot (Fig. 13B), there are two slopes, i.e. two interaction coefficients of 1.42 and 0.80, with the break between the two at about twice the CMC of gangliosides, suggesting a mixture of positive and negative cooperativity in the interaction of oligomeric enzymes on GM₁ in the presence of GM₁.

For analysis of the interaction between the enzyme molecule and the hydrophilic moiety of ganglioside GM₁, the oligosaccharide was prepared from the sialoglycosphingolipid by ozonolysis (39). As shown in Table IV, the sugar substrate was found to be highly susceptible to *A. ureafaciens* sialidase in the absence of detergent. It was much less susceptible to hydrolysis by *C. perfringens* sialidase. Detergents had no significant effect on the hydrolytic activity of either enzyme on the oligosaccharide, in contrast to their effect in activating (in terms of activity on sialyllactose) in the presence or absence of ganglioside GM₁ (0.1 mg) without any detergents. Data in A are shown as a Hill plot (Hill, A. V. (1910) *J. Physiol. (Lond.)* 40, IV-VIII) in B. The Hill coefficients were calculated to be as follows: 1.0 (without GM₁); 1.42 and 0.80 (with GM₁). The apparent $K_{m}$ and $V_{max}$ values without GM₁ were calculated to be $3.4 \times 10^{-4} \text{M}$ and 23.4 pmol/min/mg of protein, respectively.


GMI hydrolysis. This oligosaccharide, like GMI, was not hydrolyzed by sialidase from V. cholerae or Streptococcus K 6646 either in the presence or absence of detergents (Fig. 2 and Table IV). These findings indicate that the activities of the two sialidases on GMI reflect their activities on its oligosaccharide moiety. The initial velocity-substrate concentration relationship of A. ureafaciens sialidase on the oligosaccharide from ganglioside GMI was measured (Fig. 14). The apparent $K_m$ and $V_{max}$ values were calculated from Lineweaver-Burk plots to be $2.58 \times 10^{-2}$ M and 2.35 $\mu$mol/min/mg of protein, respectively.

The kinetic parameters of A. ureafaciens sialidase are summarized in Table V. The $K_m$ and $V_{max}$ values varied with the type of substrate in terms of molecular structure and physicochemical state. The apparent $K_m$ and $V_{max}$ values for purified brain gangliosides (ganglioside mixture) were very similar to those for ganglioside GMI. In the absence of detergents, the $V_{max}$ values for ganglioside GMI in either the monomeric or micellar form was very small. The apparent $K_m$ for this sialoglycolipid without detergents was significantly smaller than that with detergents. In general, addition of detergent apparently increased both the $K_m$ and $V_{max}$ values for all of the complex lipid substrates, but had no effect on the values for water-soluble sugar substrates. The $V_{max}$ value for ganglioside GMI was much lower than that for sialyllactose, whereas the $K_m$ values for the two substrates were very similar. Since the sugar moieties of GMI and sialyllactose have the same structure, these results suggest that the hydrophobic ceramide moiety may restrict the cleavage of sialyl residues by some hydrophobic interactions. This restriction may be released by addition of detergents, because the $V_{max}$ value for GMI was greatly increased by sodium cholate. It should be noted that the $K_m$ value for the oligosaccharide from ganglioside GMI was about 2 orders of magnitude greater than that for the corresponding lipid substrate, irrespective of whether the latter was about 2 orders of magnitude greater than that for the corresponding lipid substrate.

**DISCUSSION**

This work was on the enzymatic actions of a new bacterial sialidase on sialoglycolipid substrates, purified from the culture filtrate of a nonpathogenic bacterium, *Arthrobacter ureafaciens* (24, 25). As reported previously (26), the most striking enzymatic property of this bacterial sialidase is its highly specific ability to cleave sialyl residues of ganglioside GMI, which exists in aqueous media as micelles composed of about 225 monomers at above the CMC, and as monomeric, disperse forms below the CMC (53), and which has long been believed to be resistant to sialidases of various origins (18, 27-34). However, as found by TLC (Fig. 2A), this enzyme showed only slight activity on fucosyl-ganglioside GMI, in which the fucosyl residue is linked a-glycosidically to the terminal galactosyl residue of GM1 (51, 52). This finding suggests that *A. ureafaciens* sialidase may recognize the neighboring galactosyl-N-acetylgalactosaminyl residue, resulting in recognition of the whole structure of the saccharide moiety of ganglioside GMI, and that the terminal fucosyl residue of fucosyl-ganglioside GMI may cause steric hindrance of the cleavage of the sialyl residue adjacent to N-acetylgalactosamine located on the galactosyl residue closest to the lipophilic ceramide residue.

The low $pK$ of the carboxyl group of sialic acid, which must have a strong negative charge in the physiological pH range, implies that the sialidase is strongly influenced by the ionic environment, whether the sialoglyco-compounds are watersoluble or whether they form part of superstructural aggregates. The present results indicate that the ionic strength of the medium also affects the action of *A. ureafaciens* sialidase on sialyl lipids (purified brain gangliosides) without markedly affecting the activity of the catalytic center, since the water-soluble sialyl compound sialyl-lactose was readily hydrolyzed under ionic conditions which minimize the availability of the lipid-bound sialyl substrate, as shown previously with the sialidases from *V. cholerae* (17) and *C. perfringens* (22). The effect of the ionic environment on the activity of *A. ureafaciens* sialidase with ganglioside substrates was first detected...
by the appearance of an acidic shift of the pH optimum, and then by a marked decrease of the enzymatic activity over the whole pH range with a broadening of the pH optimum, as observed previously with C. perfringens sialidase (22). The ionic environment had more influence on the new bacterial sialidase than on the C. perfringens enzyme (Fig. 3 and Table I). The size of aggregates of gangliosides changes with increase in concentration of electrolytes and these changes have been measured in the presence of monovalent and divalent cation (60, 61). However, the inhibitory effects of strong electrolytes are probably not chiefly due to salt-induced changes in the degree of aggregation of the ganglioside substrate, because in this work the effect of the ionic environment was studied at substrate concentrations above the CMC to avoid the effect of possible phase transition of the lipid substrate from a monomeric to micellar form. Thus, as suggested by Lipovac et al. (17) for V. cholerae sialidase, strong electrolytes may inhibit the action of A. ureafaciens sialidase on lipid substrates by screening some like-charge interaction on the enzyme that is necessary for maintenance of a conformation of the enzyme in which the catalytic center is available to sialyl residues of bulky lipid molecules and aggregates.

The sialidase-catalyzed reactions described here involve a water-soluble enzyme and a lipid-soluble substrate. As expected, various detergents, whether ionic or non-ionic, stimulated the activity of A. ureafaciens sialidase on purified brain gangliosides. Detergents caused less activation of C. perfringens sialidase, in contrast to the report of Wenger and Wardell (21), although their effects were found to be dose-dependent.

More striking than the difference between A. ureafaciens and C. perfringens sialidase in their activations by detergents, was the difference in their initial velocity-pH relationships (Fig. 7): A. ureafaciens sialidase showed an acidic shift of the pH optimum with both ionic and non-ionic detergents, whereas C. perfringens sialidase was activated by non-ionic detergents, but not by ionic ones, only in the acidic pH regions and was slightly inhibited in the neutral pH range, resulting in a marked broadening of the initial velocity-pH curve. The stimulatory effects of detergents on the reaction of A. ureafaciens sialidase with purified brain gangliosides was presumably due to an increase in the solubility of lipid in water or an alteration of the hydrophobic structure of the enzyme, both resulting in a marked increase of the V_max value (Fig. 5). The acidic shift of the pH optimum by detergents was not due simply to the presence of a strong electrolyte, i.e., sodium ion, since it was also observed with non-ionic detergents, such as Triton X-100 and Tween 80. For fuller understanding of the effects of detergents, further studies are required on the hydrophobic interactions of the lipid substrate and the enzyme molecule.

Sulphydryl groups are involved at the active site of A. ureafaciens sialidase, because its activity was inhibited by pCMPS (Table II). It is uncertain why the sialidase was strongly inhibited by pCMPS but not appreciably by other sulphydryl reagents. Similar results have been obtained on nucleoside tetraphosphate hydrolase (62): this enzyme is strongly inhibited by p-chloromercuribenzoate, only moderately by N-ethylmaleimide, and not at all by iodoacetate. The sialidase does not seem to have a serum residue in the active site, because its activity was not inhibited by DFP.

Compounds in which N-acetylgalactosamine is linked glycosidically to position 4 of a galactose residue which bears sialic acid at position 3, such as Gm_3 and Gm_4 gangliosides, are very resistant to sialidases of various origins (18, 27-34). This resistance has been ascribed to steric hindrance of the sialyl group by the substituent on the axial hydroxyl group at position 4 of the galactose residue (63). Lipovac and Rosenberg (64) proposed the existence of competitive inhibition between sialyl and N-acetylgalactosaminyl residues. However, recently, Wenger and Wardell (21) reported that this resistant sialyl residue could be hydrolyzed by C. perfringens sialidase in the presence of bile salt. As we reported previously (26), the new bacterial sialidase from A. ureafaciens hydrolyzes the resistant sialyl residue of ganglioside Gm_1 in the presence of detergents without prior hydrolysis of the terminal galactosyl-N-acetylgalactosaminyl residue. A. ureafaciens sialidase showed much greater activity than the C. perfringens enzyme for hydrolysis of Gm_1 to asialo-Gm_1, in the presence of detergents (Fig. 8). Of the detergents tested, bile salts and especially sodium cholate were effective in increasing the activity of the former enzyme on Gm_1 (Figs. 8 and 10). Since bile salts have a physiological role in lipid absorption from the gut, their physical properties have been extensively studied (65-68). The hydroxyl groups of bile salts are all on one side of the rigid cyclopenteno-phenanthrene ring and the terminalionic group is situated at the end of a short flexible branched aliphatic chain. Thus, they are thought to have a bean-shaped molecule with a polar and an apolar face, and to form small aggregates (from dimers to octamers) in which the molecules lie back to back in water at above a critical concentration (69). At higher counter ion concentrations, larger aggregates may generally form concomitantly with decrease in the CMC, but both the CMC and the aggregation number of trihydroxy bile salts (sodium cholate) have been reported to be resistant to the counter ion concentration (66), having values of 1.3 to 1.5 \times 10^{-2} M, and 2 (dimer) to 4 (tetramer), respectively. Maximal activation by sodium cholate of A. ureafaciens sialidase with ganglioside Gm_1 as the substrate was demonstrated when the molar ratio of the ionic detergent to the lipid substrate was approximately 3 (Fig. 11B). Taken together, these results suggest that single aggregates of sodium cholate (from dimers to tetramers) contain one molecule of ganglioside Gm_1, and that the sialyl residues in these mixed aggregates are the most susceptible to A. ureafaciens sialidase.

The new bacterial sialidase was active on ganglioside Gm_1 even in the absence of detergent (Table III and Fig. 12B). Very recently, Rauvala (23) reported that Gm_1 below the CMC became susceptible to C. perfringens sialidase even without addition of bile salt. However, in our experiment, there was not much difference in the susceptibilities of monomeric and micellar forms of Gm_1 to the action of A. ureafaciens sialidase in the absence of bile salt, and the detergent increased the activities on both forms to almost equal degrees (Table III). These results indicate that detergents may not be essential for release of the sialyl residue from ganglioside Gm_1, but may play an auxiliary role in enhancing the enzymatic activity. In contrast, C. perfringens sialidase is reported to show an absolute requirement for bile salts in cleavage of ganglioside Gm_1 (21).

Our results also indicate that the cleavages of the resistant sialyl residue from the oligosaccharide prepared from Gm_1 by the bacterial sialidases are well correlated with their activities on Gm_1, and that detergents have no effect on hydrolysis of the oligosaccharide (Table IV). These findings suggest that the recognitions of the specific saccharide structure of ganglioside Gm_1 by the respective enzymes are of primary importance for hydrolysis of the sialyl residue of Gm_1. The hydrophobic ceramide moiety of Gm_1 seems to restrict the enzymatic hydrolysis (the V_max value for Gm_1 is very low in the absence of detergent, while the V_max value for the oligosaccharide, des-Gm_1, is high). Detergents may release this restriction possibly by modification of the interaction between the hydrophobic portion of Gm_1 and the enzyme molecule (the V_max value for Gm_1 is high in the presence of detergents) (Table V).

Ganglioside Gm_1 had a marked effect on the enzymatic
cleavage of ganglioside GM$_3$ in the absence of detergents (Fig. 13A). With *A. ureafaciens* sialidase, ganglioside GM$_3$ gave a typical hyperbolic Michaelis-Menten curve, but it gave an unusual curve in the presence of ganglioside GM$_1$. The initial sigmoid region in the presence of GM$_1$ shown in Fig. 13A indicates some degree of deviation from the classical competitive kinetic form. The inhibition of the action of *A. ureafaciens* sialidase on ganglioside GM$_3$ by ganglioside GM$_1$ may involve some type of subunit interaction. The Hill plot (Fig. 16) had two slopes, with a break between them at about twice the CMC of gangliosides (53), again suggesting that some oligomeric enzyme structures exhibit a mixture of negative and positive cooperativity. This phenomenon seems to be compatible with the finding by gel filtration and polyacrylamide gel electrophoresis that the highly purified preparation of *A. ureafaciens* sialidase shows molecular heterogeneity, the different enzyme fractions exhibiting different substrate specificities (Fig. 1).

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Additional Refs. 37 to 69 are found on p. 7853.
Action of *A. ureafaciens* Sialidase on Sialoglycolipids

**Experimental Procedures**

**Materials**: Galactosylceramide was obtained from New Brunswick Biochemicals Co., Inc., New Brunswick, N.J. 08901. Lipid extracts of *A. ureafaciens* (American Type Culture Collection, Rockville, Md.) and *S. sanguis* (American Type Culture Collection, Rockville, Md.) were obtained from Sigma Chemical Co., St. Louis, Mo.

**Methods**: The activity of the sialidase was measured by the increase in viscosity of the reaction mixture by following the release of sialic acid as a function of time. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.4) and 100 μg of enzyme solution. The reaction was initiated by the addition of 100 μg of galactosylceramide at 37°C. The reaction was stopped by adding 10 μl of 0.5 M HCl. The reaction mixture was then heated at 100°C for 10 min. The product was then removed from the reaction mixture by 100 μg of Sephadex G-25. The reaction product was then analyzed by thin-layer chromatography.

**Results**: The sialidase from *A. ureafaciens* was found to be active on galactosylceramide and galactosylganglioside. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.4) and 100 μg of enzyme solution. The reaction was initiated by the addition of 100 μg of galactosylceramide at 37°C. The reaction was stopped by adding 10 μl of 0.5 M HCl. The reaction mixture was then heated at 100°C for 10 min. The product was then removed from the reaction mixture by 100 μg of Sephadex G-25. The reaction product was then analyzed by thin-layer chromatography.

**Discussion**: The results presented here show that the sialidase from *A. ureafaciens* is active on galactosylceramide and galactosylganglioside. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.4) and 100 μg of enzyme solution. The reaction was initiated by the addition of 100 μg of galactosylceramide at 37°C. The reaction was stopped by adding 10 μl of 0.5 M HCl. The reaction mixture was then heated at 100°C for 10 min. The product was then removed from the reaction mixture by 100 μg of Sephadex G-25. The reaction product was then analyzed by thin-layer chromatography.

**Conclusion**: The results presented here show that the sialidase from *A. ureafaciens* is active on galactosylceramide and galactosylganglioside. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.4) and 100 μg of enzyme solution. The reaction was initiated by the addition of 100 μg of galactosylceramide at 37°C. The reaction was stopped by adding 10 μl of 0.5 M HCl. The reaction mixture was then heated at 100°C for 10 min. The product was then removed from the reaction mixture by 100 μg of Sephadex G-25. The reaction product was then analyzed by thin-layer chromatography.
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Fig. 9. Action of A. ureafaciens sialidase on sialoglycolipids from guinea pig brain. The action of the sialidase was measured by the release of sialic acid from sialoglycolipids. The reaction was carried out in the presence of 0.1 M Tris-HCl buffer, pH 7.0. The sialidase was added to the reaction mixture at a concentration of 100 units/ml. The reaction was terminated by the addition of 0.5 M HCl. The liberation of sialic acid was measured by the loss of absorbance at 520 nm.

Fig. 10. Effect of various detergents on the activity of A. ureafaciens sialidase. The activity of the enzyme was measured in the presence of various detergents at a concentration of 1 mg/ml. The activity was expressed as the percentage of the control activity in the absence of detergent. The enzymes were incubated for 30 min at 37°C before the addition of substrate.

Fig. 11. Effect of various pH values on the activity of A. ureafaciens sialidase. The activity of the enzyme was measured at different pH values ranging from 4.0 to 10.0. The enzyme was incubated for 30 min at each pH value before the addition of substrate.

Fig. 12. Effect of various concentrations of sialidase on the activity of A. ureafaciens sialidase. The activity of the enzyme was measured at different concentrations of sialidase ranging from 0.1 to 10 units/ml. The enzyme was incubated for 30 min at each concentration before the addition of substrate.

Fig. 13. Effect of various concentrations of sialidase on the activity of A. ureafaciens sialidase. The activity of the enzyme was measured at different concentrations of sialidase ranging from 0.1 to 10 units/ml. The enzyme was incubated for 30 min at each concentration before the addition of substrate.

Fig. 14. Double reciprocal (Lineweaver-Burk) plot for the action of A. ureafaciens sialidase on sialic acid. The activity was calculated by measuring the rate of substrate hydrolysis. The double reciprocal plot was used to determine the Michaelis-Menten constants for the sialidase.

Fig. 15. Action of A. ureafaciens sialidase on sialoglycolipids from guinea pig brain. The action of the sialidase was measured by the release of sialic acid from sialoglycolipids. The reaction was carried out in the presence of 0.1 M Tris-HCl buffer, pH 7.0. The sialidase was added to the reaction mixture at a concentration of 100 units/ml. The reaction was terminated by the addition of 0.5 M HCl. The liberation of sialic acid was measured by the loss of absorbance at 520 nm.

Fig. 16. Effect of various pH values on the activity of A. ureafaciens sialidase. The activity of the enzyme was measured at different pH values ranging from 4.0 to 10.0. The enzyme was incubated for 30 min at each pH value before the addition of substrate.

Fig. 17. Effect of various concentrations of sialidase on the activity of A. ureafaciens sialidase. The activity of the enzyme was measured at different concentrations of sialidase ranging from 0.1 to 10 units/ml. The enzyme was incubated for 30 min at each concentration before the addition of substrate.

Fig. 18. Effect of various concentrations of sialidase on the activity of A. ureafaciens sialidase. The activity of the enzyme was measured at different concentrations of sialidase ranging from 0.1 to 10 units/ml. The enzyme was incubated for 30 min at each concentration before the addition of substrate.

Fig. 19. Double reciprocal (Lineweaver-Burk) plot for the action of A. ureafaciens sialidase on sialic acid. The activity was calculated by measuring the rate of substrate hydrolysis. The double reciprocal plot was used to determine the Michaelis-Menten constants for the sialidase.
Action of Arthrobacter ureafaciens sialidase on sialoglycolipid substrates. Mode of action and highly specific recognition of the oligosaccharide moiety of ganglioside GM1.

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