Control of the Action of *Vibrio cholerae* Sialidase on Mammalian Brain Gangliosides by Ionic Strength†

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ABSTRACT: Effect of ionic strength of the medium on activity of *Vibrio cholerae* sialidase with mammalian brain gangliosides was studied. Strong electrolyte reversibly inhibited enzyme activity in accordance with a Debye–Hückel effect of ionic environment on ionic activity. A similar effect was obtained with sodium, potassium, lithium, trishydroxymethylammonium, magnesium, and calcium, as cations, and with chloride and acetate as anions. There was no appreciable shift in pH optimum, near 6.5, in the presence of added strong electrolyte. The latter apparently had little effect upon activity of catalytic groups, since enzymatic hydrolysis of the small molecule, sialyllactose, was not appreciably inhibited by added strong electrolyte. The inhibitory effect with sialolipid operated to change V_0, which is a negative log-square-root function of ionic strength. These results are interpreted to indicate that monomonovalent like-charge ionic interaction on the enzyme may conformationally govern steric availability of the catalytic site.

Detailed explorations of the behavior of lipid-bound glycosides as special substrates for hydrolytic enzymes are rare. In a previous study (Lipovac *et al.*, 1971), we have investigated the effect of concentration-dependent change of physical state of sialyl oligosaccharidyl ceramide substrates (gangliosides) isolated from mammalian brain (Öhman *et al.*, 1970), on the enzymatic action of the sialidase of *Vibrio cholerae*. Sialidase from *V. cholerae* recently has seen wide use in attempts to alter cell surfaces by removal of sialic acid so as to influence cellular immunogenecity (Ray *et al.*, 1971; Currie and Bagshawe, 1967; Schlesinger and Amos, 1971). In the present study, we describe the controlled effect of ionic strength of the reaction medium, in the physiological range, as a potent, hitherto unsuspected, inhibitor of the action of *V. cholerae* sialidase on mammalian brain gangliosides. Such information may eventually be practically applicable to an unraveling of events at the neuronal synapse where gangliosides are concentrated (Schengrund and Rosenberg, 1970), and where ionic environment plays an important role.

Experimental Procedure

Materials

Chromatographically purified beef brain gangliosides and sialidase (*N*-acetylneuraminate glycohydrolase, EC 2.2.1.18) derived from *V. cholerae* were obtained as described in a prior publication (Lipovac *et al.*, 1971). Either purified sialidase from culture filtrates (Rosenberg *et al.*, 1960), "Stage 7," or from Calbiochem, B grade, 500 units/ml, were used. All chemicals were of reagent grade. They were used without further purification.

Analytical Methods

Enzymatically released, free, sialic acid was estimated by the Warren thiobarbituric acid procedure (Warren, 1959), modified by a proportional reduction in reagent volumes. Assay mixtures contained per milliliter the specified quantities of sialidase, usually 1 μg (5 units), and 0.4 mg of gangliosides in 0.01 M Tris-acetate buffer and 0.003 M calcium chloride, at pH 6.8 except for studies on the effect of pH upon activity. For the strong electrolyte studies, the specified quantities of salt were dissolved in the buffer before adding the substrate and enzyme in that order. Assay mixtures were incubated for 35 min at 37° for gangliosides and 10 min for sialyllactose, which was determined experimentally to be within the duration time of the initial rate of reaction for these substrates. The reaction was stopped by carrying out the first step of the Warren procedure (Warren, 1959), *i.e.*, the addition of the acid–periodate solution. All analyses were run in duplicate with corresponding reagent, enzyme, and substrate blanks.

Results

Inhibitory Effect of Strong Electrolyte. Strong electrolyte inhibited enzymatic action on gangliosides. The inhibition was reversible by removal of the strong electrolyte by dialysis and restoral of the amount of calcium ion (Stone, 1947) required for activity of the enzyme. The inhibitory activity appeared to relate to ionic strength of the medium. It was not markedly specific for any particular ionic species. This was true for calcium ion as well, although a low concentration of this ion is required for the catalytic hydrolysis of sialyl residues by *V. cholerae* sialidase (Rosenberg *et al.*, 1960). The effect of strong electrolyte was in accordance with the predicted influence of ionic atmosphere on ionic activity according to the formulation of Debye and Hückel (1923). The results are shown in Figure 1. There is exponential decrease in enzyme activity towards gangliosides (expressed as a percentage of the highest value for the initial velocity and obtained without addition of salt in the 0.01 M Tris-acetate buffer alone) relative to the square root of the ionic strength provided by increasing concentrations of strong electrolyte in the reaction medium. A similar effect, somewhat more pronounced for divalent cations, was observed whether the cation was trishydroxymethylammonium, sodium, potassium, lithium, magnesium, or calcium, as was also the case for the anions tested: acetate, chloride, and phosphate. This is not to say that an independent effect related to the chemical nature of the cation may not

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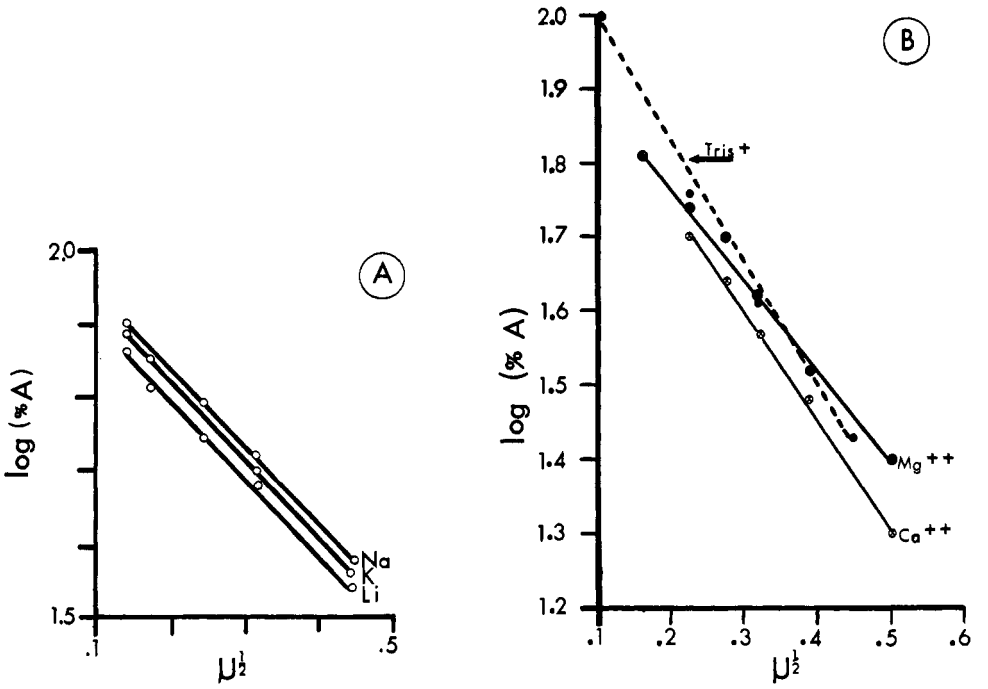

FIGURE 1: The Debye–Hückel (1923) effect of sodium, potassium, lithium, trishydroxymethylammonium, magnesium, and calcium chlorides on the activity of *V. cholerae* sialidase on mammalian brain gangliosides. Activity, *A*, is expressed in terms of the maximum initial velocity. The latter was obtained in 0.01 M, pH 6.8, Tris-acetate buffer without addition of strong electrolyte, and is represented in value as 100%. Assay conditions for enzymatic release of sialic acid are described in the text. Free sialic acid was measured by the Warren (1959) reaction. The substrate, consisting of mono-(nonsusceptible), di-, and trisialoganglioside in the mass ratio: G_{M1}, 14.5; GD_{1A}, 53.7; GD_{1B}, 8.5; G_T, 25.5 (Öhman *et al.*, 1970), was at a concentration of 0.4 mg/ml of assay mixture, which contained 1 μg of enzyme protein (5 units): (A) monovalent cationic electrolyte—sodium, potassium, and lithium chlorides; (B) Tris-chloride and divalent cationic electrolytes—magnesium and calcium chlorides.

operate to change the degree of substrate aggregation and effect concentration. The parallel lines for monovalent cations in Figure 1 do not extrapolate back to log 100, whether for lithium, sodium, of potassium ion. Divalent cations, and Tris, have similar effects on the enzymatic activity.

pH Optimum in the Presence of Salt. The lower activity values obtained in salt are not because of a shift in pH optimum. Figure 2 shows a plot of per cent optimum initial velocity against pH in the presence and in the absence of added sodium chloride. Decrease in activity is greatest around the pH optimum. However, there is no marked shift in optimum pH.

Nature of the Inhibitory Effect of Strong Electrolyte. The major effect of salt may be attributed directly to an effect on the enzyme, rather than to a screening effect on the interaction of substrate and the enzyme, even though the substrate is anionic at all pH's tested. K_M values are relatively unchanged by the addition of strong electrolyte as shown by the inverse substrate–velocity plots in Figure 3. However, V_0 clearly is depressed. A plot of log V_0 against the square root of ionic strength shows a linear relationship, as depicted in Figure 1.

Effect of Salt on the Catalytic Center of the Enzyme. Depression of activity of the *V. cholerae* sialidase toward gangliosides by strong electrolyte is not essentially due to interference with the activity of the catalytic center. Strong electrolyte has a relatively minor effect on the enzymatic hydrolysis of the model trisaccharide substrate, sialyllactose, as shown in Table I. All of the studies on the lipid–sialyl substrate were performed above the critical micelle concentration for the latter, about 0.016%, w/v (Yohe and Rosenberg, 1973). For this reason, the inhibitory effect cannot be ascribed to a radical shift in physical state of the substrate from its monomeric to its aggregate form, and the regularity of the effect produced by mono- and by divalent cationic electrolyte suggests that this effect probably cannot be ascribed to any change in aggregate size for the substrate in salt, with an attendant change in effective substrate concentration, since the considerable binding properties of the calcium ion and, to some extent, magnesium ions with respect to the carboxylate function of the terminal sialyl residues (Behr and Lehn, 1972) in the surface of the micellar aggregates of gangliosides should, in this event, have an obvious influence. The effect of salt over the range of concentrations studied on the aggregate size of gangliosides is minor (Gammack, 1963).

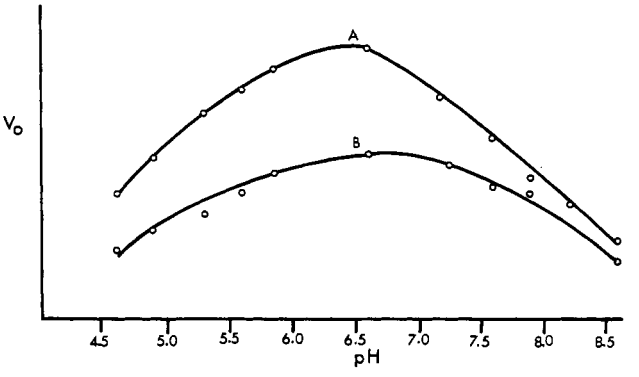

FIGURE 2: Effect of pH and added sodium chloride on the activity of *V. cholerae* sialidase on brain gangliosides. Assays for enzymatically liberated sialic acid were performed as described in the text. Assay mixtures contained 0.4 mg of ganglioside in 0.01 M Tris-acetate buffer and 1 μg (5 units) of enzyme: (A) no added electrolyte; (B) 0.05 mmol of NaCl added.

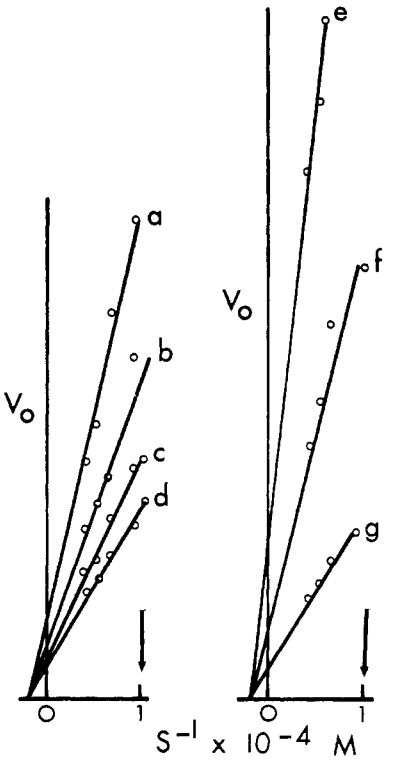

FIGURE 3: Inverse substrate concentration–velocity plots for the action of *V. cholerae* sialidase on brain gangliosides. The legend for Figure 1 gives the substrate composition. Assay conditions are described in the text. Enzymatically released sialic acid was determined by the Warren (1959) reaction. All assays were run in 0.01 M Tris-acetate (pH 6.8). Added strong electrolyte: (a) 0.10 M NaCl, (b) 0.03 M NaCl, (c) 0.01 M NaCl, (e) 0.10 M $CaCl_2$, (f) 0.03 M $CaCl_2$, and (d and g), no added strong electrolyte, *i.e.*, buffer alone. The arrows approximately show the location of the critical micelle concentration for the mixed ganglioside substrate.

Effect of Exposure to Medium of Increased Dielectric Nature. Incubation for 35 min at 37° in 10%, v/v, methanol–water, 10%, v/v, ethanol–water, or pure acetone, followed by recovery of the enzyme by centrifugation, gave values of 1.00, 1.00, and 0.95, respectively, for activity relative to that before treatment, under the conditions of the standard assay. Activity of the ganglioside substrate, however, was affected by such treatment, presumably because of the formation of inverse micellar aggregates with sialyl residues buried rather than at the surface. With treatment, the substrate gave relative activity values of 0.89, 0.74, and 0.63, respectively.

TABLE 1: Effect of Ionic Strength on Activity of *V. cholerae* Sialidase with Sialyllactose Substrate.

Added Electrolyte	$\mu^{1/2}$ [a]	Log (% A_{opt}) [b]
None	*a*	2.00
NaCl	0.141	2.00
NaCl	0.173	2.00
NaCl	0.245	2.00
NaCl	0.316	2.00
NaCl	0.447	2.04
$CaCl_2$	0.223	1.96
$CaCl_2$	0.387	1.95
$CaCl_2$	0.500	1.98
$CaCl_2$	0.707	1.95

[a] $\mu^{1/2}$ = square root of ionic strength of added strong electrolyte. The ionic strength of the pH 6.8, 0.01 M, Tris-acetate buffer was not included in the values listed. [b] A_{opt} = optimum activity, obtained in buffer alone without additional strong electrolyte.

Discussion

It is not possible to fully understand the modes of enzymatic action on biological membrane components such as the gangliosides without a thorough knowledge of environmental effects which often are predominantly ionic in physiological systems. The current study deals with a well-known and highly purified (Rosenberg *et al.*, 1960) end-group hydrolase which can remove glycosidically bound sialic acid from a number of physiologically important substrates (Wiegandt, 1967) ranging from the simple milk trisaccharide, sialyllactose, through the sialoglycoproteins, which are major blood plasma, intercellular, and cell surface components, to the sialoglycolipids, which appear to a degree to be common to the plasma membranes of all mammalian cells (Schengrund *et al.*, 1971). Although sialic acid is linked α ketosidically in these compounds (Ledeen, 1966), the low p*K* of its carboxyl function, which must be highly ionized in the physiological range of pH, predicates contiguity of an ionic environment where sialyl residues occur in biological compounds, whether the compounds are soluble, or whether they form part of superstructural aggregates. *V. cholerae* sialidase is secreted in substantial quantity into the culture medium (Rosenberg *et al.*, 1960). This enzyme has its counterpart in the sialidases of other anaerobes (Rafelson *et al.*, 1966), the membrane coat of myxoviruses (Rafelson *et al.*, 1966), and the membranes of mammalian cells (Schengrund *et al.*, 1971). The results of this study, which has been circumscribed so as to investigate the effect of ionic atmosphere on the action of *Vibrio* sialidase on the brain gangliosides for the most part, indicate that ionic strength of the medium exerts a powerful control on the action of the enzyme on sialyl lipids without markedly affecting the activity of the catalytic center, since the small water-soluble sialyl compound, sialyllactose, is readily attacked under ionic conditions which minimize the availability of lipid-bound sialyl substrate. *V. cholerae* sialidase splits off all but the sialyl residue adjacent to *N*-acetylgalactosamine located on the galactosyl residue closest to the lipophilic, ceramide, residue of the brain gangliosides. Thus, the end product is monosialoganglioside, G_{M1} (Svennerholm, 1963). The ionic effect upon activity of the enzyme with the lipid substrate cannot be ascribed basically to salt-induced changes in the degree of aggregation of the ganglioside substrate. The work described here was performed at concentrations above the critical micelle concentration so as to obviate the effect of possible phase transition of the substrate from the monomeric form to the micellar aggregate form. The rate of decrease of activity, in accordance with the Debye–Hückel effect, is similar for monomonovalent and for dimonovalent electrolyte. Differences in aggregate size for gangliosides with increasing concentration of electrolyte and differences in the presence of mono- or divalent cation have been measured (Gammack, 1963; Rosenberg and Chargaff, 1959). Yet, the effect of the chemical nature of the strong electrolyte on the inhibition of sialidase activity on gangliosides does not appear to be great. In confirmation, K_M for ganglioside substrate above the critical micelle concentration does not change with electrolyte

concentration. Nor is the inhibitory effect apparently due to a decrease in the activity of the catalytic center, since sialyllactose hydrolysis is not markedly affected by salt. Taken together, these observations indicate that strong electrolyte may screen a like-charge interaction on the enzyme, which may be a necessary factor in the maintenance of a conformation for the enzyme in which the catalytic center is available to sialyl residues when they are attached to bulky lipid molecules and aggregates. A likely possibility, in view of the similar K_M values for sialyllactose and for gangliosides however, is that the release of the products of hydrolysis, *e.g.*, free sialic acid and G_{M1} ganglioside, are hindered for the high-salt conformation of the enzyme, so that reversal of the hydrolytic reaction and net decrease in V_0 may be the result. Lipid aggregates of this sort may be reasonable models for extrapolation to the more complex lipid–protein aggregates which form biological membrane structures. The results of this study may help provide some insight in this regard. Position in the oligosaccharide structure of substrate molecules is known to affect the susceptibility of sialyl residues to enzymatic attack (Wiegandt, 1967). The present work demonstrates the operation of an effect which is due to ionic environment. The principles which govern this effect may have applicability to other enzymatic systems. A calculation of the value of the slope for the negative log V_0–square root of ionic strength function, taking into account the approximate temperature coefficients for the Debye–Hückel equation (Datta and Gryzbowski, 1961), gives an average value of close to 0.5 for the slope of the linear function. This value corresponds to a value for the product of the formal charges on interacting ionic groups of unity. This, plus the negative value of the slope, would suggest that a monomonovalent like-charge interaction controls the availability of ganglioside substrate to the enzyme.

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