Modulation of Neuraminidase Activity by the Physical State of Phospholipid Bilayers Containing Gangliosides Gd_{1a} and Gt_{1b}†

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ABSTRACT: The thermotropic behavior of large unilamellar dipalmitoylphosphatidylcholine vesicles containing the disialoganglioside Gd_{1a} and the trisialoganglioside Gt_{1b} on their outer surface has been studied as a function of the ganglioside molar fraction and Ca^{2+} concentration by using high-sensitivity differential scanning calorimetry and steady-state fluorescence spectroscopy. These studies indicate that both gangliosides have an ordering effect on the hydrocarbon region of the bilayer and that this effect is enhanced by the presence of Ca^{2+} ions. The calorimetric experiments also indicate that ganglioside Gt_{1b} has an intrinsic tendency to phase separate into compositional-rich ganglioside domains even in the absence of Ca^{2+}. Ganglioside Gd_{1a}, on the other hand, only phase separates at Ca^{2+} concentrations equal to or higher than 10 mM. These studies have allowed us to identify and evaluate the factors affecting the rates of hydrolysis of gangliosides by the soluble neuraminidase from *Clostridium perfringens*. The data presented in this paper indicate that the rates of hydrolysis of membrane-bound gangliosides are correlated to the physical state of the membrane and the state of aggregation of the ganglioside molecules within the lipid bilayer. For membrane-bound gangliosides, maximal activation energies were found at temperatures slightly below the lipid phase transition temperature. The rates of hydrolysis of the soluble substrate sialyllactose or that of the micellar ganglioside is independent of Ca^{2+} concentration, whereas the rates of hydrolysis of membrane-bound ganglioside are inhibited by Ca^{2+} especially under conditions in which the clustering effect of Ca^{2+} is maximal. These studies suggest that the soluble neuraminidases from *Clostridum perfringens* prefer ganglioside substrates that are dispersed within the membrane and not forming part of largely aggregated clusters.

Gangliosides are complex sialic acid containing glycosphingolipids located in the outer surface of plasma membranes and in the synaptic membranes of the central nervous system (Ledeen, 1978). They have been implicated in a variety of cell surface related processes such as in the recognition machinery of the cell, as receptors for protein hormones (e.g., thyroid stimulating hormone), bacterial toxins (e.g., cholera-toxin, tetanus toxin), interferon, and fibronectin, and in the mechanism of viral infection (Van Heyningen, 1974; Fishman & Brady, 1976; Lai, 1980; Rodgers & Snyder, 1981; Markwell et al., 1981). It has also been reported that the malignant transformation of cells is sometimes associated with an altered ganglioside composition (Wallach, 1975). Metabolic disorders resulting in the accumulation of certain gangliosides such as Tay-Sachs disease or GM_1 gangliosidosis result in mental retardation (Brady, 1982).

Despite the important role of gangliosides in cell-surface phenomena, very little is known regarding the way they function, the molecular mechanisms of association with ligand molecules, their interactions with other membrane components, and the modulation of these interactions by membrane structural and physical parameters. Since gangliosides interact with a variety of extramembranous substrates and enzymes like sialyltransferases and neuraminidases, it is of primary importance to understand how these interactions are affected by membrane physical parameters such as lipid composition, lipid fluidity, or the formation of ganglioside compositional domains along the membrane surface. In this paper, we present the results of a study directed to elucidate the influence of those membrane parameters on the activity of the enzyme neuraminidase from *Clostridium perfringens*.

Recently (Felgner et al., 1981), we have developed an experimental protocol directed to incorporate ganglioside molecules into the outer surface of preformed single lamellar phospholipid vesicles, thus mimicking the asymmetric distribution of gangliosides found in the plasma membrane. In this paper, we present the results of a systematic characterization of the properties of large unilamellar phosphatidylcholine vesicles containing the disialoganglioside Gd_{1a} and the trisialoganglioside Gt_{1b} in the outer surface. These vesicles have been characterized with regard to their thermotropic behavior as a function of the ganglioside mole fraction and Ca^{2+} concentration by using high sensitivity differential scanning calorimetry and fluorescence spectroscopy. These experiments have allowed us to identify and evaluate the membrane physical parameters involved in the modulation of the activity of the enzyme neuraminidase from *Clostridium perfringens*.

Materials and Methods

Vesicle Preparation. Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Biochemicals (Birmingham, AL) and used without further purification. All the vesicle preparations used for these experiments were fused unilamellar vesicles prepared essentially as described by Schullery et al. (1980). DPPC was first dried from a chloroform solution and lyophilized overnight. The dried lipid was suspended in 50 mM KCl containing 0.02% sodium azide to give a concentration of 50 mg/mL. The lipid suspensions were sonicated by using a bath sonicator (Model G112 SP1G, Laboratory Supplies, Hicksville, NY) and then centrifuged at 15000*g* for 60 min above the lipid phase transition temperature to pellet any residual multilamellar vesicles. The sonicated vesicles were then incubated at 4 °C for 1 week before use. This low-temperature incubation triggers a spontaneous fusion process and produces a homogeneous population of single lamellar vesicles of ~900 Å diameter (Wong et al., 1982). The size and homogeneity of the vesicle preparation were checked by negative-staining electron microscopy. The incorporation of ganglioside Gd_{1a} or Gt_{1b} into the lipid bilayer was achieved by adding the desired amounts of ganglioside, from an aqueous stock containing 20 mg of ganglioside/mL, to diluted aliquots of the vesicle preparations (~3 μmol/mL total lipid) followed by incubation at 45 °C

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for 90 min. This procedure results in the formation of a stable population of lipid vesicles containing ganglioside molecules asymmetrically located in the outer membrane surface (Felgner et al., 1981). Phospholipid concentrations were estimated from phosphate analysis by a modification of the Bartlett procedure as described by Marinetti (1962). Ganglioside concentrations were estimated from sialic acid determination using the method described by Cassidy et al. (1966). When required, Ca^{2+} was added to the vesicle preparations from a $CaCl_2$ stock solution, and the vesicles were incubated for 60 min at 41 °C to allow equilibration of the divalent cation across the membrane.

The integrity of the vesicle preparations after the incorporation of ganglioside was checked by measuring the optical density of the samples at 550 nm. It has been previously reported (Sillerud et al., 1979) that the turbidity of the lipid dispersions abruptly decreases if the phospholipid becomes solubilized by the ganglioside micelles. Under the conditions of our experiments, no detectable change in optical density could be detected by the spectrophotometer.

Ganglioside Purification. Gangliosides were purified by adapting several published procedures (Hakomori & Siddiqui, 1974; Felgner et al., 1981). The upper Folch extract (~8 L) from 3 kg of bovine brain was obtained from Avanti Biochemicals (Birmingham, AL). Four liters of upper Folch was adsorbed to 25 g of DEAE-Sephadex A-25 (acetate form) by stirring at room temperature for 1 h. The DEAE-Sephadex was collected in a Büchner funnel, washed with chloroform–methanol–H_2O (30:60:8) and then with 20 mM ammonium acetate in methanol, packed into a 2.5 × 45 cm column, and washed with additional 20 mM ammonium acetate in methanol. The gangliosides were eluted with 0.2 M ammonium acetate in methanol in 100-mL fractions. Resorcinol reagent was used to identify the ganglioside-containing fractions. The ganglioside fractions were combined and dried in a flash evaporator. The dried material was dissolved in a small amount of water and desalted using Sephadex G-25. The gangliosides were eluted with H_2O and collected in 25-mL fractions. The ganglioside-containing fractions were combined and lyophilized.

Separation of the gangliosides into mono-, di-, tri-, and tetraganglioside fractions was accomplished chromatographically with a 3 × 90 cm column packed with a slurry of Anasil S in chloroform–methanol–water (65:25:4 v/v). The dried gangliosides were dissolved in the same solvent and eluted by using a stepwise gradient of chloroform–methanol–water (65:25:4, 60:30:5, 60:35:8, and 60:40:10 v/v) and finally pure methanol. After elution of the void volume, 20-mL fractions were collected and tested for gangliosides with resorcinol.

Identification of the ganglioside fractions was performed by thin-layer chromatography on silica gel 60 (E. Merck). The plates were developed in 1-propanol–0.2% $CaCl_2$ (8:2 v/v) and sprayed with resorcinol reagent (Kundu, 1981) using authentic ganglioside standards obtained from Supelco (State College, PA) or as a gift from Dr. Thompson's laboratory at the University of Virginia. The ganglioside samples used in the experiments were judged to be better than 99% pure from thin-layer chromatography.

Enzyme Assay. Neuraminidase (type VI) from *Clostridium perfringens* with a specific activity of 6.5 units/mg of protein was obtained from Sigma Laboratories (St. Louis, MO). The enzyme was resuspended in a solution containing 0.01 M phosphate buffer (pH 6), 0.045 M KCl, and 0.3 mg/mL bovine serum albumin (Cassidy et al., 1966). Appropriate dilutions for the assays were made by using the same buffer. *N*-Acetylneuramin lactose (sialyllactose) from bovine colostrum (type I) was purchased from Sigma and used in control experiments. *N*-Acetylneuraminic acid (sialic acid) was also purchased from Sigma and stored at −20 °C at pH 5 in a 0.1 M stock solution.

Neuraminidase activity was measured by monitoring the release of sialic acid as a function of time. The reaction mixture contained 0.15 mL of neuraminidase solution (0.01 unit/mL), 7.5 μL of 1 M sodium acetate buffer (pH 4.5), and 45 μL of 50 mM KCl containing the substrate (typically 0.022 μmol of ganglioside unless otherwise indicated) and was incubated under the desired conditions. Aliquots were taken at 5- and 15-min intervals, and the reaction was stopped by heating at 100 °C for 2 min. Free sialic acid was assayed spectrophotometrically with thiobarbituric acid (TBA) following the procedure of Cassidy et al. (1966) by using 0.025 M periodic acid in 0.125 N H_2SO_4, 2% sodium arsenate in 0.5 N HCl, 0.1 M 2-thiobarbituric acid (pH 9.0), and 1-butanol containing 5% 12 N HCl. All enzymatic measurements were repeated at least twice; in all cases, the resulting rates were reproducible to better than 5%.

High-Sensitivity Scanning Calorimetry. All calorimetric experiments were performed with a Microcal MC1 differential scanning calorimeter. The sensitivity and precision of the basic calorimetric unit have been improved by the use of two separate Keithley amplifiers connected to the heat capacity and temperature outputs of the instrument and interfaced to a TEC86 microcomputer system for automatic data collection and processing. The calorimetric data are digitized by a TM-AD212 A/D converter operating at 40-kHz conversion rate and stored in floppy disks at 0.05 °C intervals for subsequent analysis. In this way, it is possible to perform experiments by using very dilute biological materials. With pure lipid dispersions, concentrations smaller than 0.5 mg/mL can be used with a total sample volume of 0.7 mL. For these studies, the concentration of lipid in the calorimetric cell was ~2 mg/mL. All the calorimetric scans were performed at a scanning rate of 15 °C/h.

Fluorescence Experiments. Steady-state fluorescence depolarization experiments were performed by using a Perkin-Elmer LS-5 spectrofluorometer equipped with 3M 105ML glass polarizers in the excitation and emission beams. The temperature of the cuvette was controlled with a Neslab RTE-8 refrigerated bath circulator and the temperature monitored within ±0.1 °C with a Keithley digital thermometer. 1,6-Diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Junction City, OR) was dissolved in acetonitrile and added to the vesicle suspensions at a ratio of 1 probe molecule per 500 phospholipid molecules. All the samples were incubated for 1 h at 45 °C prior to the experiments in order to assure complete equilibration of the fluorescent probe. The total lipid concentration for these experiments was 0.2 mg/mL. The samples were excited at 360 nm, and the emission intensity was measured at 430 nm parallel and perpendicular to the plane of excitation. Anisotropy was calculated as described by Barenholz et al. (1976).

Results

Scanning Calorimetry of DPPC–Gd_{1a} Vesicles. Figure 1 shows a series of calorimetric scans for large unilamellar DPPC vesicles containing increasing mole fractions of ganglioside Gd_{1a} in the outer surface. In the absence of ganglioside, the gel–liquid-crystalline phase transition of these vesicles is characterized by a phase transition temperature (T_m) of 41.3 °C, an enthalpy change (ΔH) of 8.4 kcal/mol of DPPC, a half-height width ($\Delta T_{1/2}$) of 0.3 °C, and a cooperative unit

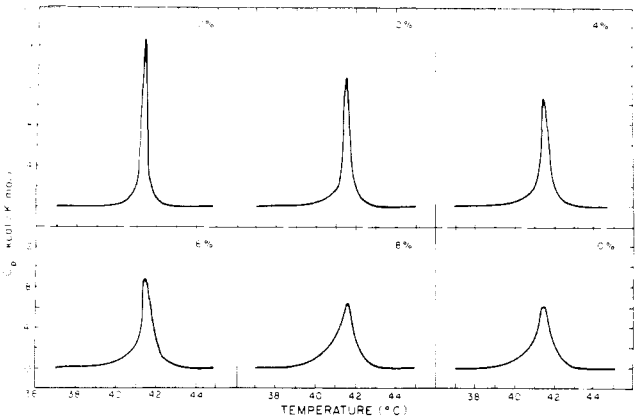

FIGURE 1: Excess heat capacity function vs. temperature for large unilamellar DPPC vesicles containing increasing mole fractions of ganglioside Gd_{1a} in their outer surface.

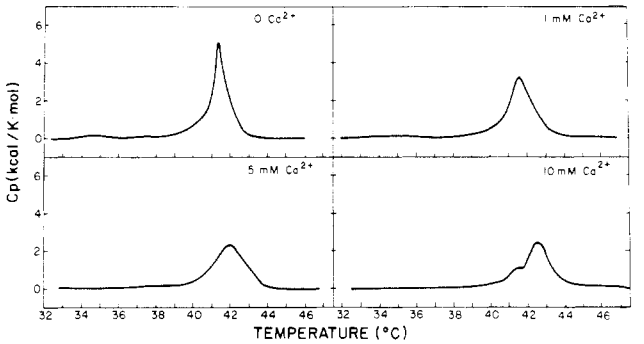

FIGURE 2: Excess heat capacity function vs. temperature for large unilamellar DPPC vesicles containing 10% ganglioside Gd_{1a} and increasing Ca^{2+} concentrations.

size of 190 lipid molecules. The addition of ganglioside Gd_{1a} up to a mole fraction of 0.10 has a relatively small effect on the thermotropic behavior of these vesicles; it broadens the heat capacity function and induces a small shift in T_m to higher temperatures but leaves the transition enthalpy unchanged. At 10 mol % Gd_{1a}, the transition temperature (temperature of the maximum in the heat capacity function) is 41.6 °C, and the cooperative unit size is only 67 lipid molecules. The addition of Ca^{2+} (Figure 2) further shifts the T_m to higher temperatures and at concentrations higher than 10 mM induces some degree of phase separation as judged by the appearance of two distinguishable but strongly overlapped peaks in the heat capacity function. At 10 mM Ca^{2+}, the lower peak is centered at 41.5 °C whereas the higher peak is centered at 42.6 °C. The high-temperature peak appears to contain a large fraction of the total area, suggesting that the phase separation process involves mixed ganglioside–phospholipid domains rather than pure ganglioside domains. For all the Gd_{1a} concentrations studied, the pretransition centered at ~35 °C was present even though at high Ca^{2+} concentrations it became very broad until it could not be detected by the calorimeter at Ca^{2+} concentrations higher than 5 mM.

Scanning Calorimetry of DPPC–Gt_{1b} Vesicles. Figure 3 shows a series of calorimetric scans for large unilamellar DPPC vesicles containing increasing mole fractions of ganglioside Gt_{1b} in the outer surface. As shown in the figure, the effect of the trisialoganglioside on the thermotropic behavior of the vesicles is different than that of the disialoganglioside. Even in the absence of Ca^{2+}, ganglioside Gt_{1b} induces the appearance of a second peak centered at 42.2 °C, suggesting that this trisialoganglioside possesses an intrinsic tendency to phase separate. The addition of Ca^{2+} further shifts the second peak to

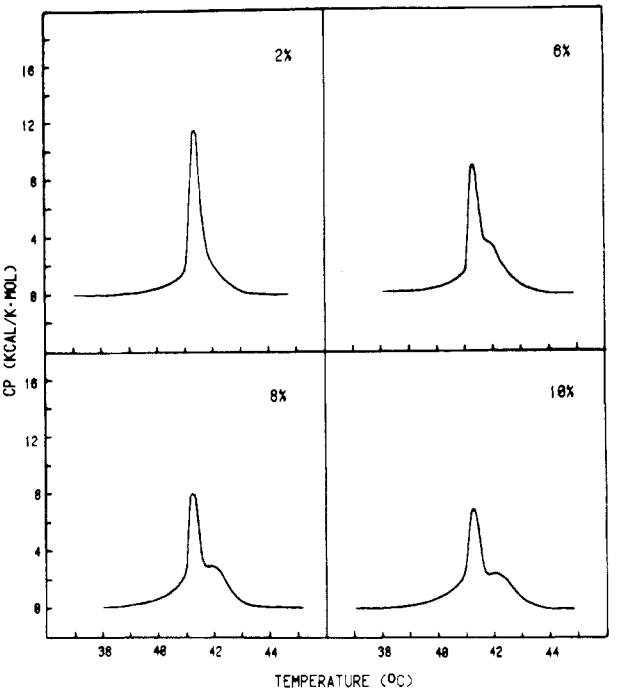

FIGURE 3: Excess heat capacity function vs. temperature for large unilamellar DPPC vesicles containing increasing mole fractions of ganglioside Gt_{1b} in their outer surface.

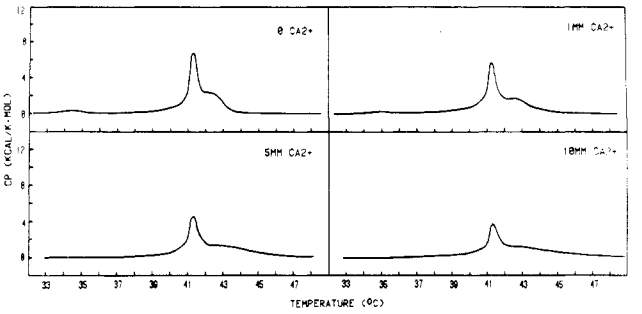

FIGURE 4: Excess heat capacity function vs. temperature for large unilamellar DPPC vesicles containing 10% ganglioside Gt_{1b} and increasing Ca^{2+} concentrations.

higher temperatures and simultaneously broadens the main DPPC peak centered at 41.3 °C, as shown in Figure 4 for vesicles containing 10 mol % ganglioside. Analysis of the heat capacity function in the presence of Ca^{2+} indicates that the area under the high-temperature peak is only ~30% of the total area. If we consider that ganglioside Gt_{1b} is located only on the outer monolayer of the membranes, these results would suggest that the phase-separated domains contain approximately one Gt_{1b} molecule per every two DPPC molecules. Certainly, in the case of Gt_{1b}, the phase-separated domains are more enriched in ganglioside than in the case of Gd_{1a}. As in the case of Gd_{1a}, the pretransition at 35 °C was not abolished by the presence of Gt_{1b} and only disappeared at Ca^{2+} concentrations larger than 1 mM. In all these experiments, the transition enthalpy remained unchanged within experimental error.

Fluorescence Anisotropy. The thermotropic behavior of the DPPC–Gd_{1a} and DPPC–Gt_{1b} vesicles was also examined by measuring the steady-state fluorescence anisotropy of DPH as a function of temperature. Some representative experiments are shown in Figure 5. In agreement with the calorimetric experiments, the fluorescence data also indicate a shift in T_m to higher temperatures when the molar fraction of ganglioside is increased and upon addition of Ca^{2+}. The most distinctive

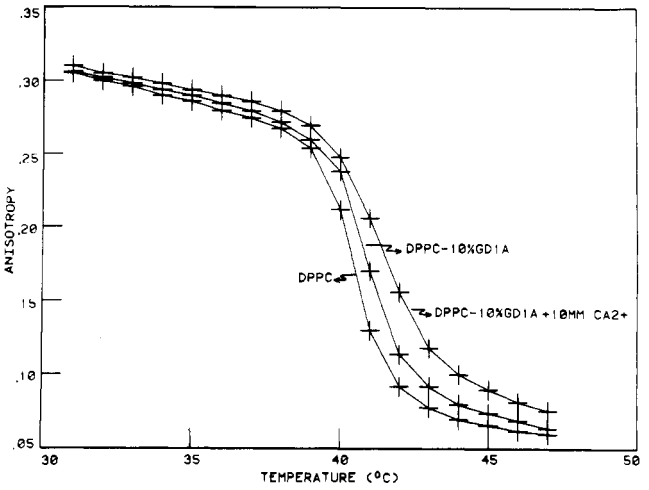

FIGURE 5: Steady-state DPH fluorescence anisotropy of large unilamellar DPPC vesicles, DPPC vesicles containing 10% Gd_{1a}, and DPPC vesicles containing 10% Gd_{1a} and 10 mM Ca^{2+}.

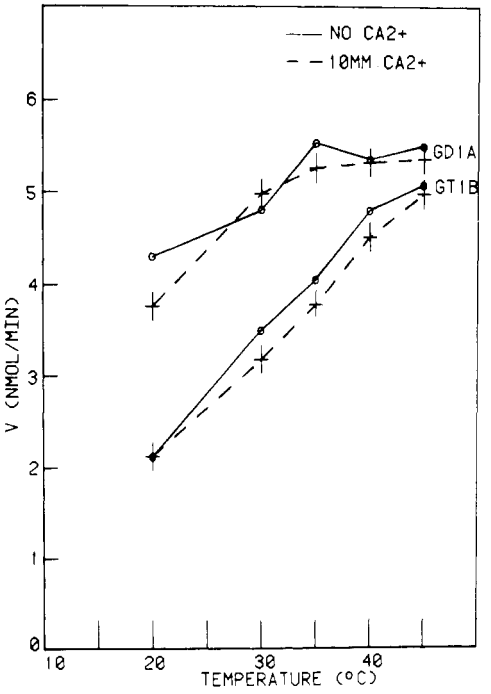

FIGURE 6: Initial rates of hydrolysis vs. temperature of micellar dispersions of gangliosides Gd_{1a} and Gt_{1b} in the absence and in the presence of 10 mM Ca^{2+}, by *Clostridum perfringens* neuraminidase.

feature of these experiments, however, is the increase in the emission anistropy of DPH upon incorporation of ganglioside and again after the addition of Ca^{2+}. These experiments indicate that the presence of disialoganglioside and trisialoganglioside has an ordering effect on the phospholipid bilayer and that the addition of Ca^{2+} further increases the apparent order of the membrane. These experiments are in agreement with the results of Uchida et al. (1981), who also reported an increased anisotropy of DPH for lipid dispersions containing DPPC and gangliosides.

Temperature and Ca^{2+} Dependence of Neuraminidase Activity. Figure 6 shows the initial rates of hydrolysis of micellar dispersions of gangliosides Gd_{1a} and Gt_{1b} as a function of temperature and Ca^{2+} concentration. In all these experiments, the ganglioside concentration was equal to 22 nmol in a total reaction volume of 0.2 mL. Initial velocities were obtained by measuring the sialic acid released after 5 min of incubation under the desired conditions. Several important features are apparent from these experiments. Under all

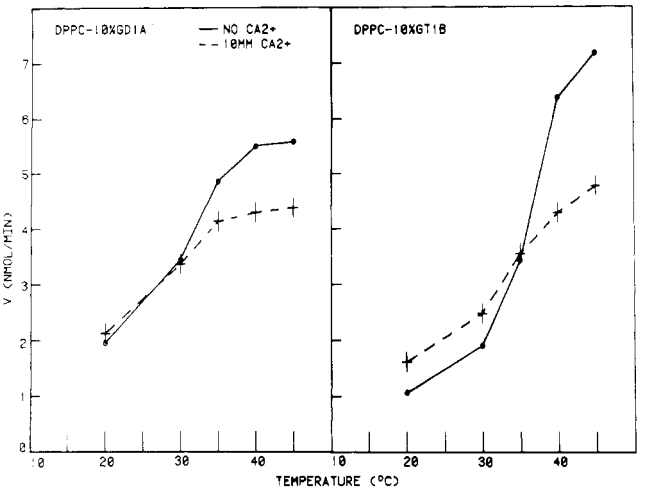

FIGURE 7: Initial rates of hydrolysis vs. temperature of gangliosides Gd_{1a} and Gt_{1b} incorporated into large unilamellar DPPC vesicles at a mole fraction of 0.1.

conditions, the rate of hydrolysis of micellar Gd_{1a} was faster than that of Gt_{1b} even though ganglioside Gt_{1b} is a trisialoganglioside whereas Gd_{1a} is a disialoganglioside. For both gangliosides, the rate of hydrolysis was quite insensitive to the presence of Ca^{2+}; additional experiments (not shown) using sialyllactose as substrate also gave Ca^{2+}-independent results in agreement with the results obtained by Cassidy et al. (1966) for the same neuraminidase. These results indicate that the enzyme per se is Ca^{2+} insensitive and that its activity is also not affected by the binding of Ca^{2+} to the ganglioside molecules. Temperature increases monotonically the rate of hydrolysis as indicated in Figure 6, for both Gd_{1a} and Gt_{1b} micellar dispersions. The effect of temperature, however, is more pronounced for Gt_{1b} than for Gd_{1a}. From Arrhenius plots of the data in the figure, we can estimate activation enthalpies of 6.5 and 2.6 kcal/mol for Gt_{1b} and Gd_{1a}, respectively.

Figure 7 shows the temperature and Ca^{2+} dependence of the rates of hydrolysis of gangliosides Gd_{1a} and Gt_{1b} incorporated into the outer surface of large DPPC unilamellar vesicles at a mole fraction of 0.1. The ganglioside concentration in these experiments was the same as that used Figure 6 for ganglioside micelles. As shown in the figure, the behavior of the enzymatic rates obtained with membrane-bound gangliosides is different from that obtained with micellar ganglioside. Particularly important is the magnitude of the Ca^{2+} effect at different temperatures. At temperatures below 30 °C, there is very little difference in the rates of hydrolysis obtained with or without Ca^{2+}; however, at temperatures higher than 35 °C, the rates of hydrolysis in the absence of Ca^{2+} are much larger than in the presence of Ca^{2+} for both gangliosides. Since the enzyme per se is insensitive to Ca^{2+}, this effect should be mediated by the changes induced by Ca^{2+} on the fluidity and state of aggregation of gangliosides within the membrane surface.

In the absence of Ca^{2+}, the rates of hydrolysis of gangliosides Gd_{1a} and Gt_{1b} are greatly enhanced in the temperature region between 30 and 40 °C. In the presence of 10 mM Ca^{2+}, however, only a small enhancement is observed in this temperature region. This effect is better illustrated in Figure 8 where the derivatives (dV/dT) obtained from a least-squares analysis of the data in Figure 7 have been plotted as a function of temperature. This figure clearly shows that in the absence of Ca^{2+} the temperature activation of the enzymatic reaction is maximal at ~35 °C for both gangliosides. At the temperature of the peak maximum, the activation enthalpies ob-

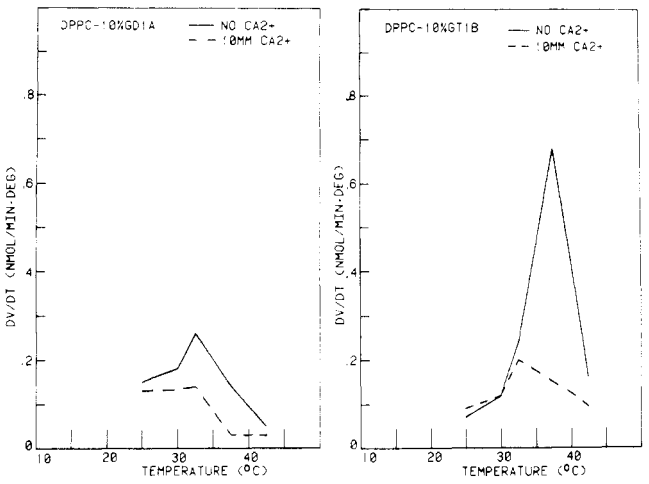

FIGURE 8: Plot of the temperature derivative (dV/dT) of the initial rates of hydrolysis vs. temperature for large unilamellar DPPC vesicles containing 10 mol % Gd_{1a} and Gt_{1b} in the absence and in the presence of 10 mM Ca^{2+}.

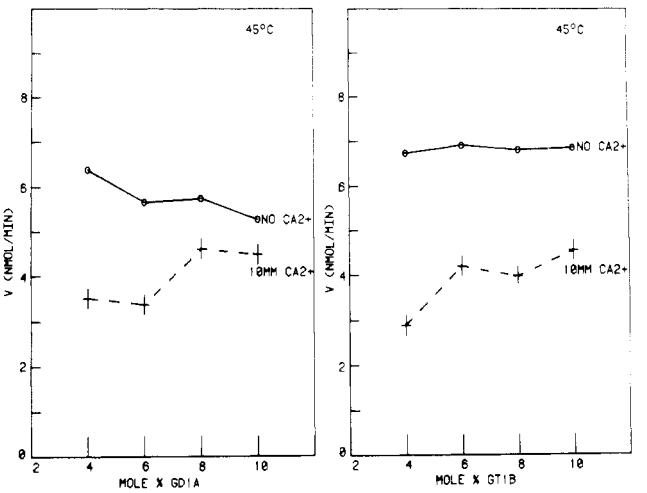

FIGURE 9: Initial rates of hydrolysis at 45 °C vs. ganglioside Gd_{1a} and Gt_{1b} mole percents in the absence and in the presence of 10 mM Ca^{2+}.

tained from this data amount to 29 kcal/mol for Gt_{1b} and 12 kcal/mol for Gd_{1a}. For both gangliosides, these activation enthalpies are almost 5 times larger than those obtained for their micellar forms. In the presence of 10 mM Ca^{2+}, the maximum still appears to be present even though greatly reduced in magnitude. This behavior of membrane-bound ganglioside should be contrasted with the one observed for micellar ganglioside in which the rate of hydrolysis increases monotonically with temperature and is unaffected by the presence of Ca^{2+}.

Dependence of Neuraminidase Activity on Ganglioside Surface Density. In order to examine the dependence of neuraminidase activity on the concentration of ganglioside at the membrane surface, we measured the rates of hydrolysis of gangliosides Gd_{1a} and Gt_{1b} as a function of their molar fraction in DPPC vesicles. In these experiments, the total concentration of ganglioside was kept constant (22 nmol in a total reaction volume of 0.2 mL) and the amount of DPPC varied in order to achieve the desired membrane composition. Figure 9 shows the initial rates of hydrolysis obtained at 45 °C, i.e., at a temperature in which the differences between Ca^{2+}-containing and Ca^{2+}-free samples are maximal. In the absence of Ca^{2+}, the effect of increasing the mole fraction of Gt_{1b} up to 10 mol % was negligible whereas in the case of Gd_{1a} a decrease of ~15% was observed. As indicated in previous paragraphs, the presence of Ca^{2+} decreases the initial rates of hydrolysis at temperatures above 35 °C. As shown in Figure 9, the magnitude of this decrease is inversely proportional to the molar fraction of ganglioside within the membrane surface. At 4 mol % ganglioside, the initial rates of hydrolysis of Gd_{1a} and Gt_{1b} are reduced 55% in the presence of 10 mM Ca^{2+}, whereas at 10 mol % ganglioside, the decrease is only 30% for Gt_{1b} and 14% for Gd_{1a}. At 100 mol % ganglioside (i.e., micellar ganglioside), Ca^{2+} has no effect on the rate of hydrolysis as shown in Figure 6. These results indicate that the activity of neuraminidase from *Clostridium perfringens* is strongly influenced by the state of aggregation of ganglioside molecules and can be modulated by changes in the concentration of Ca^{2+} ions.

Discussion

The calorimetric experiments presented in this paper indicate that ganglioside Gd_{1a} and ganglioside Gt_{1b} perturb the thermotropic behavior of DPPC large unilamellar vesicles in a different fashion. In the absence of Ca^{2+}, we could not find evidence for the formation of Gd_{1a} phase-separated domains in the concentration range studied, which is the concentration range in which gangliosides are found in plasma membranes (Ladeen, 1978). On the contrary, ganglioside Gt_{1b} even in the absence of Ca^{2+} had a tendency to induce the formation of phase-separated domains as judged by the appearance of a second peak in the heat capacity function. In the concentration range studied, gangliosides Gd_{1a} and Gt_{1b} did not have a measurable effect on the enthalpy change associated with the lipid phase transition. These results are in agreement with those obtained by Sillerud et al. 1979 with ganglioside GM_1 in the same concentration range. Both gangliosides, however, increased the steady-state anisotropy of the fluorescence probe DPH, in agreement with previous observations (Barenholz et al., (1980); Uchida et al., (1981). These results indicate that gangliosides have an ordering effect in the hydrocarbon region of the lipid bilayer.

The addition of Ca^{2+} in concentrations up to 10 mM affects differently the termotropic behavior of DPPC–Gd_{1a} and DPPC–Gt_{1b} vesicles. In the case of Gd_{1a}, Ca^{2+} induces a broadening of the heat capacity function, and only at concentrations as high as 10 mM, the calorimetric profile appears to be composed of two superimposed peaks. However, these peaks overlap strongly, making it very difficult to perform a quantitative analysis of their relative contributions to the total area. In any case, the area under any of these peaks appears to be too large to contain contributions from ganglioside molecules only, thus implying that if phase separation does actually occur the phase-separated domains would most likely consist of mixed domains containing relatively large amounts of DPPC. The situation is different with ganglioside Gt_{1b}. In this case, the addition of Ca^{2+} shifts the phase-separated peak to higher temperatures. This peak is present even in the absence of Ca^{2+} and contains approximately 30% of the total area, suggesting that the phase-separated domains contain ganglioside Gt_{1b} and DPPC in a 1:2 molar ratio. Thus, even in this case, the phase-separated domains are not pure ganglioside domains but mixed domains containing ganglioside and phosphatidylcholine molecules. The exact composition of these domains appears to be related to the nature of the ganglioside head group as indicated by the different behavior of Gd_{1a} and Gt_{1b}. Another important and still unanswered question is related to the molecular packing of these ganglioside-rich domains and specially to whether or not these domains also involve phospholipid from the inner monolayer of the membrane. Additional calorimetric experiments with ganglioside

GM_1 (B. Goins and E. Freire, unpublished results) indicate that the monosialoganglioside and the trisialoganglioside produce qualitatively, even though not quantitatively, similar heat capacity profiles in the presence of Ca^{2+}. The similarity of the effect of Ca^{2+} on Gt_{1b} and GM_1 as opposed to Gd_{1a} could be due to a "cross-bridging effect" when the ganglioside possesses an odd number of negative charges. In this respect, the disialoganglioside could be electrostatically neutralized by Ca^{2+} without inducing significant amounts of phase separation whereas the mono- and trisialogangliosides would have to be cross-bridged in order to achieve charge neutralization. This or a similar type of effect could help explain the degree of ganglioside phase separation induced by Ca^{2+}. It has also been reported that the carbohydrate residues in the head group of gangliosides are able to form intermolecular hydrogen bonds (Sharom & Grant, 1978). Thus, gangliosides apparently possess a unique combination of an attractive potential provided by their hydrogen-bonding capability and a repulsive electrostatic potential susceptible to being modulated by Ca^{2+} binding.

The results presented in this paper indicate that the rates of hydrolysis of gangliosides by the soluble neuraminidase from *Clostridium perfringens* are affected by the physical state of the membrane as well as the state of aggregation of gangliosides. In agreement with previous results in the literature (Cassidy et al., 1966), we found that this enzyme is not affected by Ca^{2+} and that the rates of hydrolysis of micellar gangliosides are also independent of Ca^{2+} concentration within experimental error. The hydrolysis of micellar Gd_{1a} proceeds faster than the hydrolysis of ganglioside Gt_{1b} in the temperature range studied; however, the activation enthalpy for Gt_{1b} is almost 3 times larger than that of Gd_{1a}. The origin of this effect is not yet clear and could be due to the larger size and polarity of Gt_{1b} or to enhanced intermolecular interactions between the head groups of this ganglioside. The absence of a Ca^{2+} effect is consistent with laser light-scattering experiments (Corti et al., 1980) indicating that Ca^{2+} has no effect on the size and molecular packing of the ganglioside Gd_{1a} micelles.

When the gangliosides are incorporated into the lipid bilayer, the rates of hydrolysis are affected by the physical state and molecular organization of the membrane. Contrary to the case of micellar gangliosides, the initial rates of hydrolysis of membrane-bound gangliosides show characteristic temperature activation curves with a maximum at ~35 °C, as shown in Figure 8. It is interesting to note that the temperature of this maximum is lower than the temperature of the maximum in the heat capacity function. Similar effects have been observed with other membrane enzymes (Thilo et al., 1977), with some permeability rates (Papahadjopoulos et al., 1973), and with the kinetics of incorporation of human high-density lipoprotein A-1 to phospholipid vesicles (Swaney, 1980), in which the maximal rates occur below the transition midpoints. Nagle & Scott (1978) have interpreted this type of phenomenon as arising from enhanced density fluctuations near the phospholipid bilayer phase transition. Another intriguing possibility is that, in the case of gangliosides, the maximum activation is not related to the phospholipid main transition but to the pretransition centered at 35 °C. This possibility is substantiated by the observation that the amplitude of the maximum activation becomes negligible at Ca^{2+} concentrations in which the pretransition disappears. Tillack et al. (1982) have presented evidence obtained by freeze–etch electron microscopy using ferritin–*Ricinus communis* agglutinin labeling that asialo-GM_1 has a tendency to organize in linear arrays at temperatures slightly below the main transition and that this ordered structure disappears above the transition temperature. The formation of these linear arrays has been linked to the so-called rippled phase existing at temperatures between the pretransition and main transition.

The addition of Ca^{2+} above the bilayer phase transition temperature decreases the rates of hydrolysis in a manner dependent on the mole fraction of ganglioside within the bilayer. At very low ganglioside mole fractions, the inhibitory effect of Ca^{2+} is maximal; however, as the ganglioside mole fraction increases, the inhibitory effect of Ca^{2+} diminishes. At 100 mol % ganglioside (i.e., micellar ganglioside), Ca^{2+} does not have any significant effects on the rates of hydrolysis. These observations are consistent with the calorimetric experiments indicating that Ca^{2+} induces the formation of ganglioside-rich domains within the bilayer. At low mole fractions, the ganglioside molecules are most likely dispersed within the bilayer, and therefore, the clustering effect of Ca^{2+} has a more dramatic effect than at relatively high mole fractions, in which the ganglioside molecules are already near each other. These experiments also suggest that the soluble neuraminidase from *Clostridium perfringens* prefers ganglioside substrates that are randomly dispersed within the bilayer and not phase-separated ganglioside domains. Previously, Cestaro et al. (1980) have shown that the rates of hydrolysis of gangliosides by *Vibrio cholera* neuraminidase are also larger for membrane-bound than for micellar gangliosides.

In the cell surface, gangliosides are substrates for both neuraminidases and sialyltransferases. Tettamanti et al. (1980) have shown that these enzymes possess different optimal pHs. Our experiments indicate that the rates of ganglioside hydrolysis are also susceptible of being modulated by changes in the physical state of the membrane, as well as changes in the state of aggregation of gangliosides within the membrane surface. Currently, we are investigating the magnitude of these effects in synaptosomal membranes in which Ca^{2+} is known to play an important role. The results of these studies will be presented in a future paper.

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Registry No. DPPC, 2644-64-6; Ca, 7440-70-2; ganglioside Gd_{1a}, 12707-58-3; ganglioside Gt_{1b}, 59247-13-1; neuraminidase, 9001-67-6.

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Phosphoprotein Particles: Calcium and Inorganic Phosphate Binding Structures†

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ABSTRACT: Phosphoprotein particles were isolated in their native state from the physiological fluid of the estuarine clam *Rangia cuneata*, and the characteristics of the mineral ion–protein complex which constitutes the native particle were investigated by using mineral ion binding and mineral ion exchange techniques. The particles are aspartic acid rich, highly phosphorylated proteins containing calcium, magnesium, and inorganic phosphate ions and covalently cross-linked via histidinoalanine residues. Twenty-nine percent of the amino acid residues are phosphorylated. In their native state, the particles contain a protected pool of calcium and inorganic phosphate ions and an exchangeable pool of calcium and magnesium ions. The Ca/PO_4 ratio in the protected pool is about 2.5. The number of binding sites for the protected mineral is unknown, but on the average, the native particles contain about 0.2 inorganic phosphate ion per organic phosphate residue. There is 1.0 exchangeable metal ion binding site per organic phosphate residue, and there is probably a phosphoserine residue at each site. These sites bind calcium with an apparent binding constant (K_{Ca}) of 4×10^3 M^{-1} at 50% saturation under physiological conditions, and K_{Ca}/K_{Mg} is about 1.6. In vivo, about 85% of the exchangeable sites are occupied. The total number of calcium ion binding sites (N) in the phosphoprotein particles is related to the number of organic phosphate residues (P_o) and the number of bound inorganic phosphate ions (P_i) by the equation $N = P_o + 2.5P_i$. The phosphoprotein particles probably serve as both the transporter and reserve source of calcium ions for shell development.

Aspartic acid rich, highly phosphorylated proteins are associated with two very different mineralized tissue structures. Phosphophoryn is a component of vertebrate tooth dentin (Veis & Schlueter, 1964; Dimuzio & Veis, 1978a) where it is concentrated at the mineralization front (Weinstock & Leblond, 1973). Phosphoprotein particles are components of the extrapallial fluid and innermost shell lamella of some species of bivalve mollusks, i.e., clams (Marsh & Sass, 1983). Both phosphophoryn and phosphoprotein particles are postulated intermediates in the mineralization process (Veis, 1978; Marsh & Sass, 1983), largely on the basis of their localization at mineralizing fronts and their ability to sequester large quantities of calcium ions (Lee et al., 1977; Zanetti et al., 1981; Marsh & Sass, 1983).

The invertebrate shell and vertebrate dentin are mineralogically very different; the former is a calcium carbonate (aragonite or calcite) tissue, and the latter is a calcium phosphate (hydroxyapatite) tissue. The associated phosphoproteins have some interesting similarities and differences. Both phosphoprotein particles and phosphophoryn are rich in aspartic acid and phosphoserine residues (Lee et al., 1977; Lyaruu et al., 1982; Termine et al., 1980; Marsh & Sass, 1983). On the other hand, histidine accounts for about 34% of the particle residues but for only about 1% of the phosphophoryn residues. The molecular weight of rat phosphophoryn is about 38 000 (Jontell, 1982); in this study, the molecular weight of the phosphoprotein particles is estimated to be about 54 million. The particles are composed of phosphoprotein monomers of an undetermined size covalently

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