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# Hydrolysis of Di- and Trisialo Gangliosides in Micellar and Liposomal Dispersion by Bacterial Neuraminidases†

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ABSTRACT: The hydrolysis of di- and trisialo gangliosides by bacterial neuraminidases was investigated. Slow rates of hydrolysis were obtained with micellar dispersions of the pure gangliosides; the rates increased considerably with mixtures of ganglioside and phospholipids, such as phosphatidylcholine or sphingomyelin. The greatest rates of hydrolysis were obtained with mixtures containing 5–10 mol % ganglioside and 90–95% phospholipid. With the aid of the nonpenetrating reagent trinitrobenzenesulfonic acid, it was ascertained that this mixture consisted of sealed, unilamellar vesicles in which the ganglioside was distributed symmetrically between the two layers of the liposome. When the relative proportion of the ganglioside was increased, the dispersions contained liposomes admixed with micelles of ganglioside and phospholipid. The rates of hydrolysis of the ganglioside could be correlated with the percentage of sealed vesicles in each mixture. Experiments in which another ganglioside (GM1) or cholesterol was incorporated into the mixed dispersions further supported this conclusion. It is suggested that the rate of hydrolysis is affected predominantly by interactions between the carbohydrate chains of ganglioside molecules. The data emphasize that ganglioside metabolism can be best studied when the latter are part of biological or model membranes.

Gangliosides are glycosphingolipids which contain one or more residues of sialic *N*-acetylneuraminic acid. They are minor components of plasma membranes of many vertebrate cells. Their contribution to the physical and biological properties of the membranes is mainly due to the negative charge of the sialic acid and their large carbohydrate head group. Although their precise biological role has not been established, they were shown to be receptors of bacterial toxins, viruses, hormones, and interferon, and their compositional pattern in cell surface changes drastically following transformation [for a review see Yamakawa & Nagai (1978), Klenk (1973), Wallach (1975), Fishman & Brady (1976), Svennerholm & Mandel (1979), Klenk & Huang (1973), Fishman et al. (1973), Hakomori (1973), and Murray et al. (1973)]. It has been proposed that they might act as chelators for calcium and possibly other divalent ions (Harris & Thornton, 1978).

Hydrolysis of sialyl residues by neuramidases is the key step in ganglioside catabolism (Gatt, 1970). Neuraminidase action reduces the number of NANA residues, thereby affecting their physical and biological activities.

Several mammalian neuraminidases have been described in various subcellular localization including those of plasma membrane, lysomal membrane, synaptic membrane, and a soluble enzyme from the cytosol; some enveloped viruses also have neuraminidase in their membrane [for a review see Dawson (1978), Tettamanti et al. (1978), Rosenberg (1978), Veh & Schauer (1978), Sandhoff et al. (1978), and Venerando (1978)].

## Experimental Procedure

*Materials and Enzymes.* Gangliosides were purified by G. Tettamanti using previously described procedures (Tettamanti

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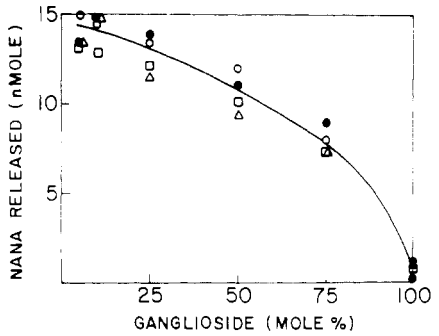


FIGURE 1: Dependence of the initial rates of hydrolysis of gangliosides on their relative content in mixed dispersion with phospholipids. Incubation mixtures, in volumes of 0.25 mL, contained 10 μmol of sodium acetate buffer, pH 5.6, 0.2 munit of *V. cholerae* neuraminidase, 0.125 μmol of $CaCl_2$, 75 nmol of GD1a, and varying concentrations of phospholipid. The tubes were incubated for 10 min at 37 °C, and the released sialic acid was determined as described under Experimental Procedure. (○) GD1a, egg PC; (●) GD1a, sphingomyelin; (□) GD1b, egg PC; (△) GT1, egg PC.

et al., 1973). GD1a,[1] which was used in most experiments, was very pure (and indeed its NANA content was more than 90% of the theoretical value); GD1b and GT1 were highly purified but still contained less than 5% of other gangliosides.

Neuraminidase of *Vibrio cholerae* was purchased from Serva and that of *Clostridium perfringens* (type VI) was from Sigma Chemical Co. Pure egg phosphatidylcholine, bovine brain sphingomyelin, and phosphatidylethanolamine were purchased from Makor Chemicals, Jerusalem, Israel. Cholesterol, dicetyl phosphate, and trinitrobenzenesulfonic acid were purchased from Sigma.

*Preparation of Ganglioside Dispersions.* The procedure of Barenholz et al. (1977) was followed by using chloroform–methanol solutions of ganglioside and lipid. The dried lipid mixture was dispersed in the desired buffer and subjected to ultrasonic irradiation as described in this reference. The temperature during sonication was 0–4 °C with PC and 45 °C with SM; these temperatures are at least 5 °C above the phase transition temperatures of the respective lipids. The dispersions were then centrifuged for 1–4 h at 150000*g* at 5 °C (with egg PC) or 25 °C (for SM vesicles). Region III of each tube (Barenholz et al., 1977) was pipetted off carefully and transferred to clean tubes. These were subsequently maintained, at 4 °C, for periods up to about 1 week. When nonsonicated ganglioside–lipid dispersions were desired, buffer was added to the dried mixture, and the tubes were shaken on a cyclomixer and then at 37 °C for 30 min.

*Assay of the Enzymatic Reaction.* The composition of the reaction mixtures is described in the respective legends to the figures. They were incubated at 37 °C and the released NANA was determined according to the procedure of Warren (1959) using only 2 mL of cyclohexanone. One unit of enzyme is defined as that quantity which released 1 nmol of sialic acid from a glycoprotein substrate in 1 min according to the procedure and specification of the respective manufacturer. The specific activity of the enzyme was assumed to be that supplied by the respective manufacturer.

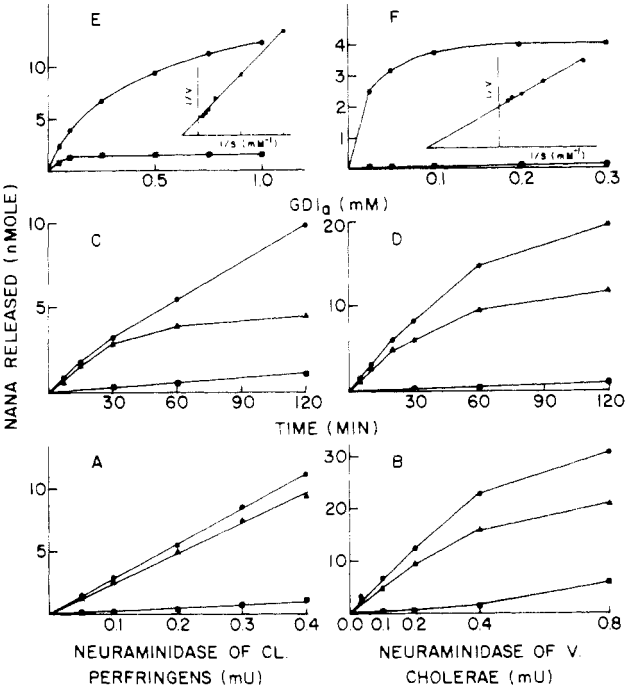


FIGURE 2: Hydrolysis of GD1a dispersions by two bacterial neuraminidases. (A and B) Dependence of the initial rate of hydrolysis on enzyme concentration. (A) Incubation mixtures, in volumes of 0.25 mL, contained 10 μmol of sodium acetate buffer, pH 5.6, 0–0.4 munit of *Cl. perfringens* neuraminidase, and 100 nmol of GD1a. (B) Incubation mixtures, in volumes of 0.25 mL, contained 6.25 μmol of Tris-HCl, pH 6.7, 0.125 μmol of calcium chloride, 0–0.8 munit of *V. cholerae* neuraminidase, and 100 nmol of GD1a. The tubes were incubated at 37 °C for 10 min, and the reaction was terminated as described under Experimental Procedure. (■) Micelles of GD1a; (▲) multilamellar liposomes having 10% GD1a and 90% egg phosphatidylcholine; (●) small unilamellar liposomes having 10% GD1a and 90% egg PC. (C and D) Time course of hydrolysis of GD1a. Conditions were similar to those in (A) and (B), respectively, except that the incubation time was varied and the incubation mixture contained 0.05 munit of each enzyme, 100 nmol of GD1a for the *Cl. perfringens* enzyme, and 75 nmol GD1a for the *V. cholerae* enzyme. (E and F) Effect of substrate concentration. Conditions were similar to those of (A) and (B), respectively, except that 0.2 munit of the neuraminidase of *Cl. perfringens* was used in the experiment of (E) and the incubation time was 30 min. With the *Vibrio* enzyme, 0.05 munit was used and the incubation time was 10 min. The substrate used was 10% GD1a–90% phosphatidylcholine in unilamellar liposomes (●) or micelles of GD1a (■).

The concentration of phosphorus was determined according to the procedure of Bartlett (1959). The total content of phosphatidylethanolamine and its relative concentration in the outer layer of the liposomal dispersion were determined as described elsewhere (Barenholz et al., 1977).

## Results

Gangliosides (GD1a, GD1b, or GT1) and phospholipids (phosphatidylcholine or sphingomyelin) were mixed, subjected to ultrasonic irradiation, and incubated with neuraminidase of *V. cholerae* (see Experimental Procedure). Figure 1 shows the initial rates of hydrolysis of the *N*-acetylneuraminyl linkages as a function of the mole percent of the gangliosides in the respective mixed dispersions. In each of the four mixtures used, the concentration of the ganglioside was maintained at a fixed value of 0.3 mM, while that of the lipids was varied. It is evident that the rates of hydrolysis of the gangliosides, in the presence of phospholipid, were considerably greater than in their absence. Thus, without phospholipid (i.e., mole percent of ganglioside = 100; see Figure 1) the respective

[1] Abbreviations used: GM1, galactosyl-*N*-acetylgalactosaminyl-galactosyl-(*N*-acetylneuraminyl)glucosylceramide; GD1a, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminylgalactosyl-(*N*-acetylneuraminyl)glucosylceramide; GD1b, galactosyl-*N*-acetylgalactosaminyl-galactosyl-(*N*-acetylneuraminyl-*N*-acetylneuraminyl)glucosylceramide; GT1, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminylgalactosyl-(*N*-acetylneuraminyl-*N*-acetylneuraminyl)glucosylceramide; NANA, *N*-acetylneuraminic acid; PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; SM, sphingomyelin; TNBS, trinitrobenzenesulfonic acid.

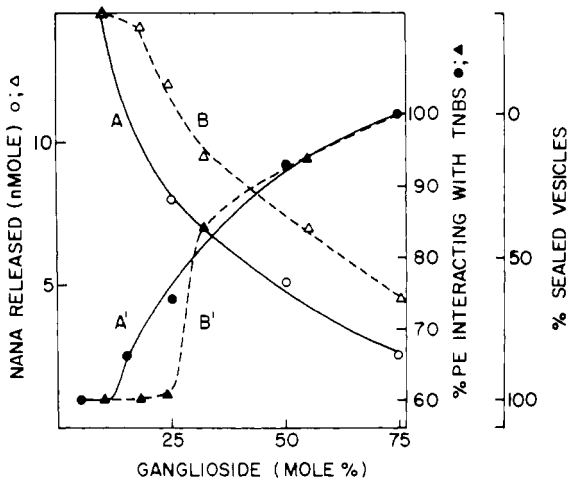


FIGURE 3: Correlation of the relative content of sealed vesicles and the rate of hydrolysis of GD1a in mixed dispersion with phospholipids. (A and A′) Mixtures of GD1a and phospholipid (composed of egg PC and egg PE having a fixed ratio of 8:1 (mol/mol)) were dispersed by ultrasound, in 40 mM acetate, pH 5.6, as described under Experimental Procedure. Aliquots containing 75 nmol of GD1a and phospholipid [whose content can be calculated from the equation: lipid (PC + PE, nmol) = 75 × (mol % GD1a)/(100 − mol % GD1a)] were pipetted into tubes containing 0.2 munit of *V. cholerae* neuraminidase and 0.125 μmol of $CaCl_2$; the volume was 0.25 mL. After 10 min at 37 °C the sialic acid content was determined. Separate aliquots of the GD1a–phospholipid dispersions were interacted with trinitrobenzenesulfonic acid (Barenholz et al., 1977; these data are shown in curve A′). (B and B′) Conditions were similar to those of (A) and (A′) except that GM1 in varying proportions was included in the mixed dispersion containing 0.075 μmol of GD1a, 0.6 μmol of PC, and 0.075 μmol of PE. The GM1 content in nanomoles can be calculated from the equation 750 × (mol % GM1)/(100 − mol % GM1). Curve B describes the rate of hydrolysis and curve B′ describes the percent of sealed vesicles.

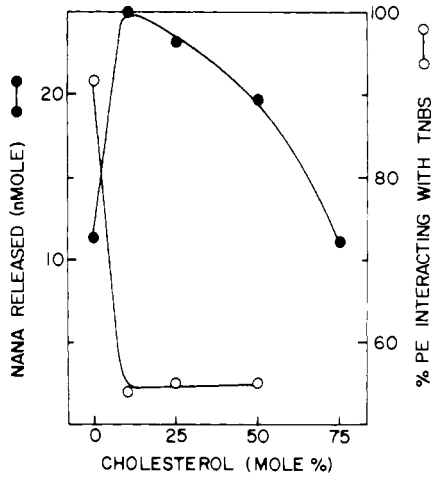


FIGURE 4: Effect of cholesterol on the relative content of sealed vesicles and the rate of hydrolysis of GD1a in mixed dispersion with phospholipids. Conditions were similar to those of Figure 3A,A′ except that cholesterol was also included in the mixed dispersion. For the enzymatic reaction, each tube, in a volume of 0.25 mL, contained 75 nmol of GD1a, 66.7 nmol of PC, and 8.3 nmol of PE, and varying concentrations of cholesterol [whose content (in nanomoles) can be calculated from the equation 150 × (mol % cholesterol)/(100 − mol % cholesterol)]. (●) Sialic acid released; (○) percent of sealed vesicles.

rates of hydrolysis (expressed in nanomoles of NANA released) were as follows: GD1a, 0.9; GD1b, 0.4; GT1, 1.1. Addition of PC or SM increased these to about 15 nmol. It is worth noting that the rates of hydrolysis of the three gangliosides, dispersed with either PC or SM, were practically identical. It was ascertained that in each case the rates of enzymatic hydrolysis were linear with respect to both the concentration of the enzyme and time of incubation and within the range of saturating concentrations of substrate. This is exemplified for mixtures of GD1a and PC in Figure 2B,D,F.

It should be emphasized that the gangliosides and the phospholipids belong to separate lipid classes. Thus, while the gangliosides are "soluble, amphiphilic lipids", which disperse in water as micelles, the phospholipids belong to the class of the "insoluble, swelling amphiphilic lipids", which in aqueous media form bilayered liposomal dispersions (Small, 1968). The considerable effect of varying the relative proportions of ganglioside and phospholipid on the enzymatic hydrolysis (see Figure 1) might therefore be a reflection of the presence of several physical states of lipid dispersions. To test this, we dispersed GD1a with a mixture of egg PC and egg PE. The presence of the PE in the mixed dispersions permitted its interaction with the nonpenetrating reagent trinitrobenzenesulfonic acid (Litman, 1974). The percentage of the total lipid present as sealed, unibilayered vesicles could be calculated from the portion of the total PE that interacted with the above reagent in the absence of detergent (Litman, 1974).

Curves A and A′ of Figure 3 compare the effect of varying the ratio of ganglioside to phospholipid on two parameters: the initial rate of GD1a hydrolysis and the percent PE interacting with TNBS. The latter data are also recalculated and presented as percentage of sealed, unilamellar vesicles present in each mixture. The data of curve A show that the greatest rate was obtained by using dispersions consisting of 5–10 mol % GD1a. When the molar fraction of this substrate increased to 75%, the reaction rates decreased about sixfold. Curve A′ describes the percent PE which interacted with TNBS. When the mole percent of GD1a was 5–10, 60% of the total PE interacted with TNBS, suggesting the sole presence of sealed, nonleaky bilayered vesicles (Litman, 1974). When the mole percent of GD1a was raised, the percentage of sealed vesicles decreased correspondingly. Figure 3, curves A and A′, demonstrate a parallelism and practical equivalence between the percent GD1a degraded in each mixed dispersion and the percentage of sealed vesicles.

The correlation between the integrity of the ganglioside-containing vesicles and the rate of enzymatic hydrolysis was further tested by incorporating two other lipids into the mixed dispersions. These were monosialo ganglioside (GM1) and cholesterol. Because of its similarity to the substrate (GD1a), GM1 (in itself not a substrate for the enzyme) should affect not only the hydrophobic moiety of the bilayer of the liposome but also that portion which interacts with the enzyme, namely, the polar region of GD1a. In contrast, incorporation of cholesterol should affect the hydrophobic part of the bilayered vesicles but should have no direct influence on the polar region (Lee et al., 1974).

Figure 3, curve B, shows that addition of the GM1 decreased the rates of GD1a hydrolysis. Thus, when GM1 was about half that of the entire lipid content, the rate was only 50% of that obtained with the corresponding GD1a-containing dispersion devoid of GM1 (curve A; GD1a = 10 mol %). In the presence of GM1, enzymatic hydrolysis of GD1a again paralleled the degree of exposure of PE to TNBS (i.e., the percentage of sealed vesicles; Figure 3, curves B and B′). The shift of curves B and B′ relative to that of curves A and A′ suggests that GM1 is less effective than GD1a in solubilizing liposomes and converting them to mixed micelles.

Figure 4 shows the effect of increasing the content of cholesterol relative to an equimolar mixture of GD1a and phospholipid. The figure shows that incorporating 10 mol % cholesterol into this mixture, which by itself contains practically no sealed vesicles, transformed the lipid dispersion into

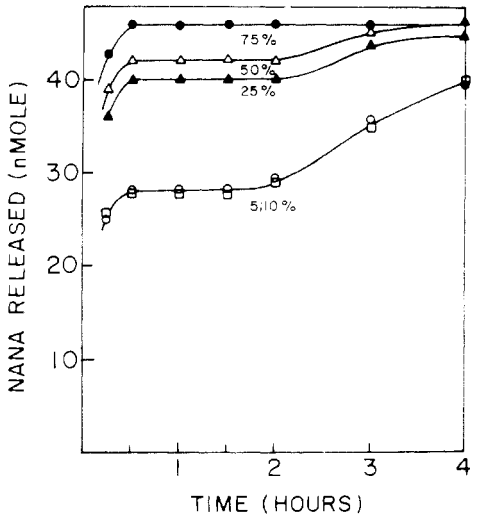


FIGURE 5: Time course of hydrolysis of GD1a in several mixed dispersions with phospholipids. Incubation mixtures, in volumes of 0.25 mL, contained 50 nmol of GD1a, 0.125 μmol of $CaCl_2$, 2 munits of *V. cholerae* neuraminidase, and several concentrations of PC as shown on the respective curves. At the specified times the reaction was terminated and the content of free sialic acid was determined.

unilamellar, bilayered liposomes. Concomitantly, the rates of GD1a hydrolysis increased more than twofold. It is of interest that further enrichment with cholesterol (up to 50%, where GD1a constitutes 37.5% of the total lipid mixture; for calculation, see the legend to Figure 4) resulted in somewhat reduced reaction rates in spite of the presence of fully sealed vesicles.

Figure 5 shows a time curve of hydrolysis of GD1a by a relatively large concentration of neuraminidase of *V. cholerae*, by using mixed dispersions with PC in which the GD1a varied from 5 to 75 mol %. It is evident that with each of the mixed dispersions the reaction rate reached a maximal value after incubating, at 37 °C for 30 min. This value did not change in the following 90 min of incubation. Analysis of the data shows that when the dispersion having 5–10 mol % GD1a was used, the plateau was reached when about 60% of the ganglioside was hydrolyzed, namely, when the dispersion contained mostly sealed, unilamelar vesicles. This suggests that the gangliosides are distributed nearly symmetrically between the outer and inner layers of these vesicles. The respective degrees of hydrolysis using the other dispersion described in Figure 5, which contained mixtures of vesicles and mixed micelles, were as follows: 87, 91, and 100% using 25, 50, and 75 mol % of GD1a, respectively. When the dispersion of sealed vesicles was incubated more than 2 h, the entire GD1a was hydrolyzed by the enzyme. This probably is a consequence of increased penetration of the enzyme into the vesicle, though flipping over of the ganglioside from the inner to the outer monolayer is also possible.

All data of this paper were obtained by using neuraminidase of *V. cholerae*. Figure 2A,C,E shows that replacement of this enzyme with neuraminidase of *Cl. perfringens* resulted in very similar data. Again, ganglioside incorporated into sealed vesicles of phospholipids provided the greatest rates of hydrolysis.

## Discussion

This paper investigated the effect of the dispersion state of gangliosides on their utilization by bacterial neuraminidases. For this purpose di- or trisialo gangliosides were dispersed in an aqueous medium and their rates of hydrolysis were measured by using either the gangliosides themselves or in mixture with other lipids. The data of this paper emphasize the importance of studying the metabolic reactions of gangliosides when these compounds are integral components of a lipid bilayer.

When the gangliosides, which are "soluble" lipids (Small, 1968), are dispersed in water, they form micellar aggregates whose molecular weights are between 200 000 and 300 000 (Yohe et al., 1976). In comparison, phosphatidylcholine and sphingomyelin belong to the class of the insoluble, swelling lipids (Small, 1968). The type of aggregates obtained by mixing a ganglioside and a phospholipid (e.g., egg PC) would depend on the ratio between the two (Hill & Lester, 1972). This paper shows that at low ganglioside to PC ratio the sonically irradiated dispersions consist of unilamellar, bilayered liposomes; as the ganglioside to phospholipid ratio increases, mixed micelles form. Winsor (1968) and Israelachvili et al. (1976, 1977) presented a theoretical treatment of the type and structure of dispersions obtained by mixing lipids of various classes. We have analyzed several mixed aggregates of ganglioside and lipid and found them to be larger than that of the respective micelle of the ganglioside itself (Barenholz, Cestaro, and Gatt, unpublished experiments). Thus, the molecular weight determined as described by Yedgar et al. (1974) of a mixed vesicle consisting of 10 mol % GD1a and 90% PC was $(2.3 \pm 0.3) \times 10^6$. When the GD1a was 25% of the lipid mixture, the molecular weight was $(1.0 \pm 0.2) \times 10^6$, and, by use of 100% GD1a, the molecular weight was $0.3 \times 10^6$ [the latter value is of magnitude similar to that reported by Yohe et al. (1976)]. It is a common observation, supported by theoretical considerations, that smaller aggregates are more open and hydrated than larger structures (Yedgar et al., 1974; Israelachvili et al., 1976, 1977). Bacterial neuraminidases which are relatively small, water soluble protein molecules interact with the sialyl residue which is located on the furthermost, hydrophilic portion of the ganglioside molecule. It might, therefore, have been expected that the micelles of the pure gangliosides, which, in addition to being open and hydrated, also have the greatest surface concentration of the substrate, would be a better substrate for these enzymes than the mixed dispersions of gangliosides and lipid. The experimental data of this paper did not confirm this. Micelles of pure GD1a, GD1b, or GT1 were rather poor substrates and addition of phospholipids increased the rates of hydrolysis of these compounds. Two separate factors may be responsible for this: the high surface density of the gangliosides in the micelles and, furthermore, the high density of negative charges of the neuraminyl residues may interfere with the interaction with the enzyme.

The most interesting finding of this paper is the excellent correlation between the enzymatic rates of hydrolysis and the relative content of sealed, unilamellar vesicles. The enzymatic activity was maximal when the mixed dispersions consisted of 5–10 mol % ganglioside and 90–95% phospholipids. These mixtures contained only sealed, unilamellar bilayered vesicles in which the ganglioside is distributed symmetrically. The data of Figures 3 and 4 show that compounds affecting the integrity of the vesicles also affected the enzymatic reaction rates. Thus, GM1 (Figure 3B,B′) which decreased the content of such vesicles decreases the reaction rates, while cholesterol, which assisted in formation of sealed vesicles (Figure 4), increased the rates of hydrolysis of GD1a.

In the sealed vesicles, because of charge repulsion and their low content (only 5–10 mol %), the ganglioside molecules are most probably separated from each other. In these structures, interaction between ganglioside molecules is therefore probably minimal. When the percentage of the ganglioside increases,

contact between ganglioside molecules increases, becoming maximal in the micelles consisting of pure ganglioside. Such contact probably induces interaction between the carbohydrate residues of adjacent ganglioside molecules. Using spin-labeled gangliosides, Sharom & Grant (1978) concluded that such interactions indeed occurred. Our own studies using proton magnetic resonance spectrometry of GM1 dispersions (Lichtenberg, Barenholz, and Gatt, unpublished experiments) also support this notion. In comparison, the contribution of the hydrophobic moiety of the bilayer to interaction between ganglioside molecules is probably small. This conclusion is supported by the findings of Figure 1 which show practically no difference in the rates of hydrolysis of GD1a in mixed dispersion with phosphatidylcholine or sphingomyelin, which have very dissimilar dynamic properties (Hertz & Barenholz, 1977; Shinitzky & Barenholz, 1974). Such interactions probably are disadvantageous to hydrolysis of the sialyl residues of the ganglioside molecules by the bacterial neuraminidases. Experiments are planned to test the conclusions of this study using mammalian neuraminidases and gangliosides which are a part of the bilayer of liposomes or of biological membrane.

Acknowledgments

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