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Fluorospectroscopic Studies of Various Ganglioside and Ganglioside–Lecithin Dispersions. Steady-State and Time-Resolved Fluorescence Measurements with 1,6-Diphenyl-1,3,5-hexatriene†

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ABSTRACT: Molecular motions of 1,6-diphenyl-1,3,5-hexatriene (DPH) in gangliosides (GM3, GM2, GM1, GD1a, and GD1b), GA1 glycosphingolipid, and dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC)–ganglioside mixed dispersions were studied by using techniques of steady-state and nanosecond time resolved fluorescence measurements in the temperature range of 20–50 °C. The total fluorescence decay $s(t)$ was approximated to a best-fit curve of double-exponential decays, and two fluorescence lifetimes were obtained. The values of the shorter fluorescence lifetime in dispersions composed of a single glycosphingolipid component approached those of the longer one on addition of DPPC. The molecular arrangement or microheterogeneity of the hydrocarbon region surrounding DPH molecules changed depending on the ratio of DPPC to ganglioside molecules and on the temperature. The steady-state anisotropy r_s in dispersions composed of a single glycosphingolipid component exhibited smooth changes, not abrupt ones, in the temperature range, in contrast to that in DPPC liposomes. In the various glycosphingolipid dispersions studied, the motion of DPH molecules was the most restricted in the GA1 dispersion. Sialic acid linked to the neutral sugar backbone influenced the hydrophobic region and increased the motion of DPH molecules. In the gangliosides tested, the motion of DPH molecules in the hydrophobic region of GM1 ganglioside was found to be the most restricted. These comparative studies indicate that the ultimate and/or penultimate carbohydrate moieties of the neutral sugar backbone of gangliosides and the topographical difference in the locations of the sialic acid linkage influence the integrity of the membranes including the hydrophobic region.

Glycophingolipids are known to be present in especially high concentration in plasma membranes (Dod & Gray, 1968; Klenk & Choppin, 1970; Weinstein et al., 1970; Gahmberg, 1971; Keenan et al., 1972; Yogeeswaran et al., 1972). This characteristic, together with the great structural variety of their hydrophilic carbohydrate portion, makes them ideally suited for participation in diversified cell surface recognition functions, such as hormonal and immunological regulatory mechanisms of cellular activities, cell growth, cell behavior, and cell to cell interactions [Lee et al., 1976, 1977; Mullin et al., 1976; Besancon et al., 1976; Vengris et al., 1976; Holmgren et al., 1973; King & van Heyningen, 1973; Pierce, 1973; Cuatrecases, 1973; van Heyningen & Mellanby, 1959; Helting et al., 1977; Yogeeswaran & Hakomori, 1975; cf. Hakomori (1975); Yamakawa & Nagai, 1978]. In spite of increasing attention to their significance, our knowledge of their physical properties as membrane forming constituents is still poor compared with that of phospholipids. Only a few glycosphingolipids, such as galactocerebroside (Clowes et al., 1971; Abrahamsson et al., 1972; Oldani et al., 1975; Sharom et al., 1976), globosides (Tinker et al., 1976), and ganglioside mixtures (Gammack, 1963; Hill & Lester, 1972; Abramson et al., 1972; Curatolo et al., 1977; Sillerud et al., 1978; Formisano et al., 1979), have been studied. One reason for this is the difficulty in obtaining sufficient amounts of highly purified compounds for studies. However, we recently developed a procedure for isolating and purifying gangliosides in sufficient amounts for such studies (Momoi et al., 1976; Iwamori & Nagai, 1978). This prompted us to investigate their physicochemical properties. Fluorospectroscopy has been useful for investigating the physical properties of the hydrocarbon region of the membranes through the motion of fluorescent probe molecules (Faucon & Lussan, 1973; Cogan et al., 1973; Shinitzky & Inbar, 1974, Shinitzky & Barenholz, 1974; Galla & Sackmann, 1975; Lentz et al., 1976a,b; Kawato et al., 1977; Dale et al., 1977; Martin & Foyt, 1978). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was used as a fluorophore, and the motions of this compound embedded in vesicular lipid membranes consisting of either various ganglioside molecular species or ganglioside–DPPC[1] mixtures were investigated. Steady-state fluorescence measurements give information on the average motion of the fluorophore, while time-resolved fluorescence measurement provides much information on the fluorescence lifetime and the motion of the fluorophore.

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[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DEAE, diethylaminoethyl; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane. The ganglioside designation conforms to the nomenclature of Svennerholm (1964). The structures of glycosphingolipids used in this work are shown in Table I.

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Table I: Structures of Gangliosides and GA1 Glycosphingolipid

GM3	Galβ1→4Glcβ1→1 ceramide 3 ↑α2 NeuNAc
GM2	GalNAcβ1→4Galβ1→4Glcβ1→1 ceramide 3 ↑α2 NeuNAc
GM1	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1 ceramide 3 ↑α2 NeuNAc
GD1a	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1 ceramide 3 3 ↑α2 ↑α2 NeuNAc NeuNAc
GD1b	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1 ceramide 3 ↑α2 NeuNAcα2→8NeuNAc
GA1	Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1 ceramide

Experimental Procedures

Materials. Dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) of more than 99% purity was a gift from Nippon Shoji (Osaka, Japan). Crude asialo-GM1 ganglioside (GA1 glycosphingolipid) was prepared by hydrolysis of bovine gangliosides by the method of Svennerholm et al. (1973). The crude GA1 fraction was freed from unreacted gangliosides by DEAE-Sephadex (A-25, acetate form) column chromatography as described by Ledeen et al. (1973) and then purified by silica column chromatography using Iatrobeads (6RS-8060, Iatron Lab., Tokyo, Japan) (Ando et al., 1976). Molecular species of ganglioside (Table I) were prepared from human brain gangliosides as reported previously (Momoi et al., 1976; Iwamori & Nagai, 1978). All the glycosphingolipids prepared were extensively dialyzed against distilled water to remove low molecular substances and then lyophilized. The purity of glycosphingolipids was checked by thin-layer chromatography using chloroform–methanol–water, 60:40:9 (v/v/v), and chloroform–methanol–3.5 N ammonia, 60:40:9 (v/v/v), as developing solvents, with resorcinol and anthrone reagents and H_2SO_4 charring for detection of spots. Examination of samples of more than 100 μg of glycosphingolipid showed that they were homogeneous. The purity of the glycosphingolipids was also checked by structural analysis with respect to fatty acid, sphingosine, and carbohydrate moieties, using gas chromatography–mass spectrometry as described previously (Iwamori & Nagai, 1978). The fatty acid and sphingosine compositions of the glycosphingolipids are shown in Table II.

Preparation of Lipid Dispersions and Labeling with DPH. Lipids were dissolved in an appropriate amount of chloroform–methanol, 1:1 (v/v). The solution was evaporated to dryness under a stream of nitrogen, kept in vacuo overnight, and then sonicated to prepare lipid dispersions. All lipid dispersions were prepared at a concentration of 1 mg of lipids in 2.0 mL of 50 mM KCl–10 mM Tris-HCl, pH 7.4 (in glass-distilled, deionized water), by sonication in a bath-type sonicator (Model T-80-80-2-RS, input frequency 80 KHz, Laboratory Supplies Co., Inc., New York). The dispersion of DPPC was sonicated at 50 °C for 10 min, and other dispersions were sonicated at below 40 °C for 5 min. In every case a clear dispersion was obtained. One microliter of 2 mM DPH (Sigma Chemical Co.) in tetrahydrofuran, which had been refluxed over potassium hydroxide and distilled, was added to the lipid dispersions with vigorous stirring (Lenz et al., 1976a). When the mixtures were then incubated at 40 °C for 90 min in the dark, the fluorescence intensity reached a constant level. Under this condition there was little fluorescence depolarization due to resonance energy transfer and reabsorption. (The concentration of DPH was 10^{-6} M in $\sim 10^{-3}$ M lipid.) After measurements, the lipids in the samples used were recovered by extraction with chloroform–methanol (Folch et al., 1957) and examined by thin-layer chromatography to confirm that no degradation had occurred.

Table II: Compositions of Fatty Acids and Sphingosines[a]

		GM3	GM2	GM1	GD1a	GD1b	GA1
fatty acids:	16:0	6.8	3.4	0.5	0.8	2.9	1.3
	18:0	86.3	86.8	89.0	87.8	85.4	95.2
	19:0	0.2	0.3	0.4	0.4	0.4	0.4
	20:0	4.1	8.4	9.1	10.3	10.4	3.0
	22:0	2.6	1.1	1.0	0.7	0.9	nd[b]
sphingosines:	d18:0	3.4	3.5	2.5	1.6	1.8	3.1
	d18:1	82.5	56.5	43.5	45.6	68.4	41.7
	d20:0	trace	2.1	1.6	1.8	1.0	3.2
	d20:1	14.0	37.9	52.4	51.0	28.8	52.0

[a] Data are presented as weight percentages. [b] Not detected.

Fluorescence Measurements. (*A*) *Steady-State Measurement.* Fluorescence anisotropy, r_s, was investigated with a microviscosimeter, Model MW-la (Elscint Ltd., Israel), equipped with a filter for the 365-nm mercury line for excitation and a 400-nm sharp cut-off filter for emission. The fluorescence intensities polarized parallel and perpendicular to the direction of polarization of the exciting light, $I_\parallel$ and $I_\perp$, were measured simultaneously. The steady-state anisotropy, r_s, is defined as

$$r_s = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \tag{1}$$

The temperature was measured by inserting a small thermistor (Shibaura Electronics, and Nihon Kohden, Tokyo, Japan) into the lipid dispersion in a cuvette. The effect of light scattering on the observed r_s values was negligible.

(*B*) *Time-Resolved Measurement.* The time course of change in total fluorescence intensity and anisotropy was studied with an Ortec single-photon counting system. The sample was placed in a quartz cuvette and regulated within 0.1 °C by circulating water. Nanosecond pulsed light was introduced through an interference filter (Nippon Shinku Kogaku, 360 nm), a glass filter, and a polarizer. The emitted light, after passing through the analyzer, was separated from the exciting light with an interference filter (Dell Optics, 430 nm) and glass filters.

The direction of the plane of polarization of the analyzer, either parallel or perpendicular to that of the exciting light, was changed every 16 s and each component [$I_\parallel(t)$ or $I_\perp(t)$] of the emitted light signal was fed into a multichannel analyzer (Canberra 8100). The accumulated data were transferred to a minicomputer (Nova 02/30) for analysis by the following procedures.

The total and difference intensities as functions of time were obtained as

$$s(t) = I_\parallel(t) + 2I_\perp(t)$$
$$d(t) = I_\parallel(t) - I_\perp(t) \tag{2}$$

respectively, and the observed anisotropy $r_{app}(t)$ is given by

$$r_{app}(t) = d(t)/s(t) \tag{3}$$

The real fluorescence intensity decay $S(t)$ is assumed to be

$$S(t) = a \exp(-t/\tau_1) + (1 - a) \exp(-t/\tau_2) \tag{4}$$

where τ_1 and τ_2 are the fluorescence lifetimes and a and $1 - a$ are the contributions of the exponential terms. The real difference decay $D(t)$ can be expressed in terms of $S(t)$ and the real emission anisotropy decay, $r(t)$, as $D(t) = r(t)S(t)$. Following the wobbling in a cone model, DPH molecules in liposomes are assumed to rotate within a limited angle θ_{max} (Kinoshita et al., 1977). Then, $r(t)$ can be written as

$$r(t) = r_0[b \exp(-t/\rho) + (1 - b)] \quad (5)$$

$$r_\infty = \lim_{t\to\infty} r(t) = r_0(1 - b) \quad (6)$$

where r_0 and r_∞ are the anisotropies of $t = 0$ and $t = \infty$, respectively, b is a fraction of the decay component, and ρ is the apparent rotational correlation time (Wahl et al., 1970, 1971).

For elongated fluorophores

$$\frac{r_\infty}{r_0} = 1 - b = \left[\frac{1}{2}\cos\theta_{max}(1 + \cos\theta_{max})\right]^2 \quad (7)$$

$$D_w = \langle\sigma\rangle/\rho \quad (8)$$

where D_w is the diffusion constant within θ_{max} and $\langle\sigma\rangle$ is a constant which depends on θ_{max}. It should be noted that eq 7 can apply only to rods whose emission moment is parallel to the long axis of the molecule. The DPH molecule has an *all-trans*-polyene structure with absorption and fluorescence transition moments that lie along the major axis of the molecule, and the hydrodynamic shape of DPH can be simulated by a 13-Å rod in harmony with its fluorescence polarization properties (Shinitzky & Barenholz, 1974). The calculated intensity decay $s_c(t)$ and difference decay $d_c(t)$ are respectively given by

$$s_c(t) = \int_0^t E(t')S(t - t')\,dt'$$

$$d_c(t) = \int_0^t E(t')D(t - t')\,dt' \quad (9)$$

where $E(t)$ is the profile of the exciting light. The parameters τ_1, τ_2, a, r_0, ρ, and b can be obtained by using a nonlinear least-squares method (Grinvald & Steinberg, 1974) to fit $s_c(t)$ to $s(t)$ and $d_c(t)$ to $d(t)$.

The normalized residues $Xs(t')$ and $Xd(t')$ were defined as

$$Xs(t') = \frac{s_c(t') - s(t')}{s_c(t')^{1/2}}$$

$$Xd(t') = \frac{3^{1/2}[d(t') - d_c(t')]}{[2s_c(t') + d_c(t')]^{1/2}} \quad (10)$$

The averaged residues σ_s and σ_d were defined as

$$\sigma_s = \frac{1}{n}\sum[Xs(t')]^2$$

$$\sigma_d = \frac{1}{n}\sum[Xd(t')]^2 \quad (11)$$

The simulations were performed to minimize σ_s and σ_d. n is the channel number.

Judgement of the accuracy of the fluorescence lifetimes, the cone angles, and the diffusion constants is very difficult when the fluorescence lifetime is not single. We thought these parameters reasonable when they satisfied the following conditions: (1) no large oscillations in $Xs(t)$ and $Xd(t)$; (2) σ_s and $\sigma_d \lesssim 10$; (3) systematic tendency when the temperature and molecular species were changed. As shown in Figure 1, the simulated curves were greatly improved when two decays in fluorescence intensities were employed. Moreover the three conditions shown above were satisfied by employment of two decays. The situation was also true in simulating $d(t)$. Therefore, we concluded it reasonable to simulate $s(t)$ and $d(t)$ by eq 4 and 5.

Errors in the calculated parameters a, τ_1, τ_2, b, ρ, r_∞, D_w, and θ_{max} are estimated as follows (cf. error estimation in τ_1). First, P was calculated as

$$P = \frac{\prod_{t=0}^{m}\frac{S(t)^{s(t)}}{s(t)!}\exp[-S(t)]}{\prod_{t=0}^{m}\frac{S_{max}(t)^{s(t)}}{s(t)!}\exp[-S_{max}(t)]} \quad (12)$$

where $S_{max}(t)$ is the fluorescence intensity calculated by using the most probable parameter, a_m, τ_{1m}, or τ_{2m}. Then errors in τ_{1m}, $\Delta\tau_{1'm}$, and $\Delta\tau_{1''m}$ were calculated as

$$\frac{\sum_{\Delta\tau_1=\tau_{1m}-\Delta\tau_{1'm}}^{\tau_{1m}+\Delta\tau_{1''m}} P(\tau_1)\Delta\tau_1}{\sum_{\Delta\tau_1=0}^{\infty} P(\tau_1)\Delta\tau_1} = 0.8 \quad (13)$$

Results

Comparison of GA1 Glycosphingolipid and Gangliosides. According to Table III, in gangliosides the contribution of the fluorescence with the shorter lifetime, a, was larger than that of the fluorescence with the longer lifetime, $1 - a$. The lifetimes τ_2 gave similar values to those previously reported in phosphatidylcholine liposomes and biological membranes (Shinitzky & Barenholz, 1974; Lentz et al., 1976a; Dale et al., 1977; Martin & Foyt, 1978; Senê et al., 1978; Hildenbrand & Nicolau, 1979). On the other hand, in the GA1 glycosphingolipid the contribution of the shorter lifetime fluorescence, a, was smaller than that of the longer one, $1 - a$.

The time-resolved anisotropy $r(t)$ was analyzed as described under Experimental Procedures. The anisotropy at a sufficiently long time after excitation, r_∞, did not vanish but remained constant (Figure 1). Therefore, the motion of DPH in the lipid dispersions could be analyzed successfully by using eq 5 and 6. The steady-state anisotropy, r_s, was smaller in ganglioside dispersions than in the GA1 glycosphingolipid dispersion. In accordance with this result, the cone angle, θ_{max}, was larger in ganglioside dispersions than in the GA1 glycosphingolipid dispersion. On the other hand, this correlation was not obtained between r_s and D_w (compare GA1 and GM1 in Figure 2 and Table III). This is consistent with the view that r_s and D_w are dependent on static and dynamic effects, respectively.

Comparison of Molecular Species of Gangliosides. The steady-state anisotropy, r_s, of DPH in dispersions composed of a single component of glycosphingolipids gradually decreased with an increase of temperature, as shown in Figure 2, and no drastic changes were observed in the measured temperature range. In DPPC liposomes, however, the anisotropy changed sharply at the transition temperature, as shown in Figure 3 and reported previously (Lentz et al. 1976a). A tendency for gradual change in the anisotropy was observed in both sialic acid bearing and nonbearing glycosphingolipid dispersions. The steady-state anisotropy in the GA1 glycosphingolipid dispersion was larger than that in ganglioside dispersions.

As shown in Table III, D_w was larger in the GM3 ganglioside dispersion than in the GM1 ganglioside dispersion, which was consistent with the results shown in Figures 2 and 3 (the r_s value of the GM1 ganglioside dispersion was larger than

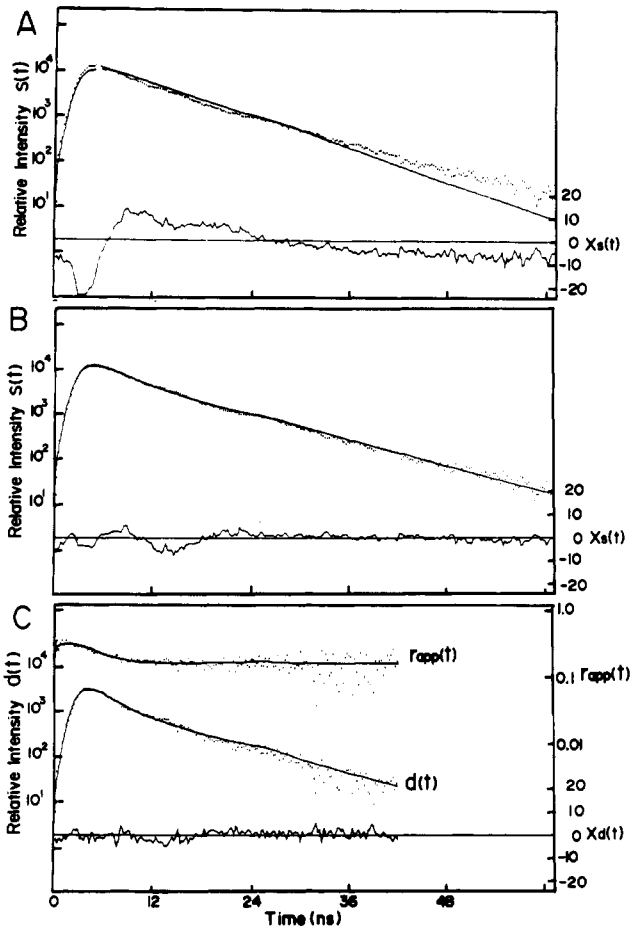

FIGURE 1: Experimental and approximated fluorescence decay curves for DPH in GM2 dispersion at 35 °C. Dots represent measured values and solid lines represent best-fit curves. (A) Single-exponential approximation of total fluorescence intensity $s(t)$: $s_c(t) = \exp(-t/7.0)$; $Xs(t) = [s_c(t) - s(t)]/s_c(t)^{1/2}$; $\sigma_s = 59.7$. (B) Double-exponential approximation of total fluorescence intensity $s(t)$: $s_c(t) = 0.64 \exp(-t/2.9) + 0.36 \exp(-t/9.1)$; $Xs(t) = [s_c(t) - s(t)]/s_c(t)^{1/2}$; $\sigma_s = 5.1$. (C) Approximation of difference fluorescence intensity $d(t)$ and apparent fluorescence anisotropy $r_{app}(t)$: $d_c(t) = [0.64 \exp(-t/2.9) + 0.36 \exp(-t/9.1)][0.35(0.54 \exp(-t/1.4) + 0.46)] = s_c(t)[0.35(0.54 \exp(-t/1.4) + 0.46)]$; $Xd(t) = 3^{1/2}[d(t) - d_c(t)]/[2s_c(t) + d_c(t)]^{1/2}$; $\sigma_d = 1.47$.

that of the GM3 ganglioside dispersion). The observation means that the wobbling motion was more rapid in the GM3 ganglioside dispersion than in the GM1 ganglioside dispersion.

GD1a and GD1b gangliosides, disialogangliosides, contain the same carbohydrate backbone as GM1 ganglioside but differ from each other with regard to the location of one more sialic acid residue. The motion of DPH was less restricted in these two disialoganglioside dispersions than in the GM1 ganglioside dispersion.

Comparison of DPPC–GM1 Ganglioside Mixed Dispersions. The steady-state anisotropy and the results obtained by nanosecond fluorometry on DPH in DPPC–GM1 ganglioside mixed dispersions are presented in Figure 4 and Table III. The changes of steady-state anisotropy in DPPC and GM1 ganglioside dispersions were very different from each other. The anisotropy in DPPC–GM1 ganglioside (80:20 and 50:50 w/w) mixed dispersions became larger with an increase of the GM1 ganglioside content and was larger than that in DPPC liposomes. The apparent transition of the two dispersions was shifted to a higher temperature with an increase in the content of GM1 ganglioside. When the content of GM1 ganglioside increased to 75 wt %, the transition was no longer clear.

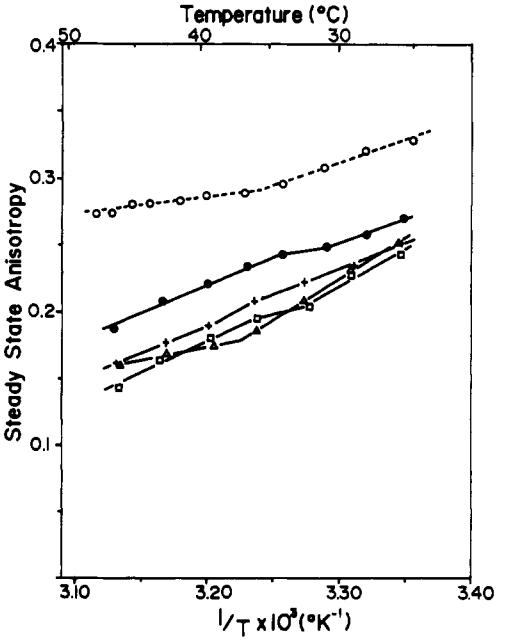

FIGURE 2: Steady-state anisotropy, r_s, of DPH as a function of the reciprocal of the absolute temperature for dispersions composed of a single glycosphingolipid component. GM1 (closed circles); GM3 (open triangles); GD1a (plus signs); GD1b (open squares); GA1 (open circles).

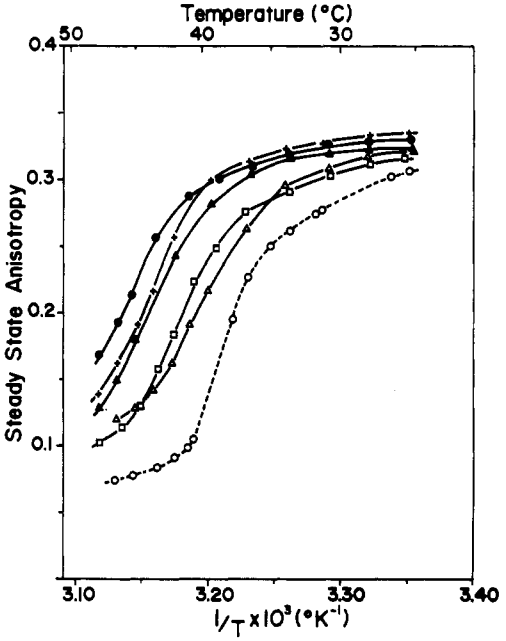

FIGURE 3: Steady-state anisotropy, r_s, of DPH as a function of the reciprocal of the absolute temperature for DPPC–ganglioside (50:50 w/w) mixed dispersions (solid lines) and DPPC liposomes (broken line). GM1 (closed circles); GM2 (closed triangles); GM3 (open triangles); GD1a (plus signs); GD1b (open squares); DPPC (open circles).

At 35 °C, both D_w and θ_{max} in the DPPC–GM1 ganglioside (50:50 w/w) mixed dispersion were smaller than those in the DPPC–GM1 ganglioside (25:75 w/w) mixed dispersion. But conversely at 51 °C, these values in the DPPC–GM1 ganglioside (50:50 w/w) mixed dispersion were larger than those in the DPPC–GM1 ganglioside (25:75 w/w) mixed dispersion. These changes at the lower and higher temperatures were in accordance with the change in the steady-state anisotropy, r_s.

When the steady-state anisotropy was compared at a temperature below 44.5 °C, it increased on addition of GM1 ganglioside to DPPC until the ratio of ganglioside to DPPC

Table III: Experimental Results of Time-Resolved Analysis

	T (°C)	a	τ_1 (ns)	$1-a$	τ_2 (ns)	b	ρ (ns)	θ_{max} (deg)	D_w (μs^{-1})	σ_s	σ_d
DPPC–GM1, 50:50[b]	35	$0.22^{+0.78}_{-0.20}$	9.4 ± 1.2	$0.78^{+0.20}_{-0.78}$	11.6^{+12}_{-2}	0.05 ± 0.01	$12.7^{+700}_{-4.6}$	10.5 ± 1.1	$1.6^{+0.9}_{-1.6}$	4.9	3.7
	51	$0.92^{+0.08}_{-0.92}$	9.0 ± 0.2	$0.08^{+0.92}_{-0.08}$	9.7 ± 0.2	0.54 ± 0.01	1.8 ± 0.2	39.8 ± 0.6	83.3 ± 9.2	5.9	6.8
DPPC–GM1, 25:75[b]	35	0.66 ± 0.28	$9.0^{+1.4}_{-3.7}$	0.34 ± 0.28	$11.3^{+6.5}_{-1.4}$	0.18 ± 0.01	4.2 ± 0.5	20.6 ± 0.6	9.0 ± 1.0	3.5	4.7
	51	$0.80^{+0.20}_{-0.24}$	$8.2^{+1.2}_{-1.6}$	$0.20^{+0.24}_{-0.20}$	$10.6^{+4.9}_{-1.2}$	0.40 ± 0.02	$2.6^{+0.7}_{-0.2}$	32.7 ± 1.1	$62.5^{+5.2}_{-13.2}$	7.6	6.7
GM1[c]	20	0.74 ± 0.03	$5.2^{+0.7}_{-0.5}$	0.26 ± 0.03	9.9 ± 0.9	0.44 ± 0.02	10.1 ± 0.5	34.7 ± 1.1	9.7 ± 0.5	5.5	4.3
	35	$0.56^{+0.03}_{-0.02}$	$4.7^{+0.5}_{-0.2}$	$0.44^{+0.02}_{-0.03}$	$10.1^{+0.5}_{-0.9}$	$0.42^{+0.00}_{-0.03}$	$7.4^{+0.2}_{-0.7}$	$33.7^{+0}_{-1.5}$	$13.2^{+1.3}_{-0.3}$	2.6	4.1
	51	$0.66^{+0.05}_{-0.12}$	$5.8^{+0.2}_{-0.7}$	$0.34^{+0.12}_{-0.05}$	$10.2^{+3.2}_{-0.7}$	0.62 ± 0.01	4.6 ± 0.7	44.1 ± 0.6	$33.9^{+6.0}_{-4.4}$	5.2	6.2
GM2[c]	20	$0.68^{+0.10}_{-0.30}$	3.6 ± 0.2	$0.32^{+0.30}_{-0.10}$	9.6 ± 0.2	0.34 ± 0.02	$6.7^{+1.8}_{-1.4}$	29.6 ± 1.1	11.0 ± 2.9	4.9	1.7
	35	0.64 ± 0.02	2.9 ± 0.5	0.36 ± 0.02	9.1 ± 0.2	0.54 ± 0.01	1.4 ± 0.2	39.8 ± 0.6	$87.8^{+14.6}_{-13.5}$	5.7	1.4
	51	0.64 ± 0.01	2.4 ± 0.2	0.36 ± 0.01	7.6 ± 0.2	0.78 ± 0.01	1.9 ± 0.3	53.8 ± 0.7	$99.4^{+18.6}_{-13.5}$	5.7	1.3
GM3[c]	20	0.76 ± 0.02	4.2 ± 0.5	0.24 ± 0.02	10.9 ± 0.5	0.30 ± 0.01	3.7 ± 0.2	27.5 ± 0.6	$20.2^{+1.1}_{-1.0}$	5.5	4.1
	35	0.76 ± 0.02	4.5 ± 0.5	0.24 ± 0.02	9.9 ± 0.5	0.56 ± 0.01	3.0 ± 0.5	40.9 ± 0.6	$45.6^{+9.1}_{-6.5}$	5.1	5.6
	51	0.64 ± 0.01	2.6 ± 0.2	0.36 ± 0.01	7.1 ± 0.5	0.84 ± 0.02	3.0 ± 0.5	$58.3^{+1.7}_{-1.6}$	$52.3^{+10.4}_{-7.4}$	5.4	13.1
GD1a[c]	20	$0.64^{+0.03}_{-0.07}$	$4.8^{+0.2}_{-0.5}$	$0.36^{+0.07}_{-0.03}$	$9.6^{+0.9}_{-0.5}$	0.42 ± 0.01	$5.0^{+1.4}_{-0.7}$	33.7 ± 0.6	$18.4^{+2.9}_{-4.0}$	4.8	1.9
	35	$0.56^{+0.08}_{-0.02}$	4.0 ± 0.5	$0.44^{+0.02}_{-0.08}$	$9.6^{+0.5}_{-0.7}$	0.50 ± 0.02	3.8 ± 0.5	37.8 ± 1.1	$30.0^{+4.5}_{-3.4}$	6.7	1.8
	51	0.52 ± 0.02	0.5 ± 0.2	0.48 ± 0.02	7.6 ± 0.2	0.64 ± 0.01	3.6 ± 0.2	45.2 ± 0.6	$41.1^{+2.4}_{-2.1}$	9.9	1.1
GD1b[c]	20	0.80 ± 0.02	5.9 ± 0.5	0.20 ± 0.02	11.8 ± 0.5	$0.53^{+0.01}_{-0.02}$	$8.7^{+0.7}_{-0.5}$	$39.3^{+0.6}_{-1.1}$	$11.6^{+0.7}_{-0.8}$	10.8	6.1
	35	0.50 ± 0.02	4.0 ± 0.5	0.50 ± 0.02	9.7 ± 0.5	0.52 ± 0.02	5.6 ± 0.2	38.8 ± 0.6	24.1 ± 0.8	2.9	5.6
	51	0.88 ± 0.02	5.9 ± 0.2	0.12 ± 0.02	12.3 ± 0.5	0.76 ± 0.02	4.0 ± 0.2	$52.4^{+1.4}_{-1.3}$	$52.5^{+2.7}_{-2.5}$	12.9	11.7
GA1[c]	20	0.40 ± 0.04	2.6 ± 0.5	0.60 ± 0.04	8.5 ± 0.5	0.22 ± 0.04	19.9 ± 0.9	$23.0^{+2.3}_{-2.4}$	0.8 ± 0.1	1.3	2.5
	35	$0.20^{+0.10}_{-0.02}$	$1.8^{+0.9}_{-0.5}$	$0.80^{+0.02}_{-0.10}$	$8.2^{+1.4}_{-1.2}$	0.20 ± 0.02	$3.5^{+1.4}_{-1.2}$	21.8 ± 1.2	$13.1^{+6.8}_{-3.7}$	4.7	3.7
	51	0.30 ± 0.04	1.0 ± 0.5	0.70 ± 0.04	8.4 ± 0.5	0.20 ± 0.02	4.6 ± 0.9	21.8 ± 1.2	$10.1^{+2.4}_{-1.6}$	4.5	3.7

[a] Errors indicate 80% probability (see Experimental Procedures). For DPPC multilamellar liposome τ = 10.6 ± 0.5 ns at 20 °C (Lentz et al., 1976). [b] Weight ratio. [c] 100% glycosphingolipid.

approached 50:50 (w/w), while on further addition it decreased (Figure 4B). Above 44.5 °C, the steady-state anisotropy increased until the ratio of GM1 ganglioside to DPPC approached to 75:25 (w/w) and decreased on further addition of ganglioside.

Discussion

The time-resolved fluorescence decay of DPH, $S(t)$, was approximated to the best-fit curve of biexponential decay and therefore was characterized by two different lifetimes. The anisotropy $r(t)$, could not be represented by a single exponential decay, and at a sufficiently long time after excitation it did not vanish but leveled off at a constant value. In earlier studies on the microviscosity of membranes, the fluorescence lifetime of DPH was estimated to be a single value of ~10 ns, which was similar to the longer lifetime, τ_2, measured in our experiments. However, it has recently been found by more precise analyses that DPH molecules in egg lecithin and dimyristoylphosphatidylcholine liposomes exhibit double-exponential decays (Chen et al., 1977; Dale et al., 1977). These characteristics of the fluorescence decay and the anisotropy have been discussed with regard to either microheterogeneity surrounding DPH molecules in hydrocarbon regions, anisotropic rotation of the probe, or excited state cis–trans isomerization (Chen et al., 1977; Dale et al., 1977). This problem, however, still remains to be elucidated, though, in the case of proteins, multiexponential fluorescence decay kinetics have been interpreted in terms of microenvironmental variability in protein structure (Grinvald & Steinberg, 1976).

The shorter lifetime, τ_1, in dispersions composed of a single glycosphingolipid component approached τ_2 on addition of DPPC (Table III). The motion of DPH molecules in DPPC–GM1 ganglioside mixed dispersions changed depending on the temperature and the compositional ratio of the two lipids (Table III and Figure 4). These findings may be interpreted as indicating that the molecular arrangement or microheterogeneity of the hydrocarbon region surrounding DPH molecules changes depending on the ratio of DPPC to ganglioside molecules and on the temperature. Similar change was observed on egg lecithin–GD1a ganglioside mixed dispersions by Harris & Thornton (1978) using ^{1}H NMR spectroscopy. This change may be related to the manifestation of a phase difference, as suggested by Tinker et al. (1976) who performed ESR studies on egg lecithin–globoside mixed dispersions.

The cone angle, θ_{max}, of the wobbling motion of DPH molecules in dispersions composed of a single glycosphingolipid

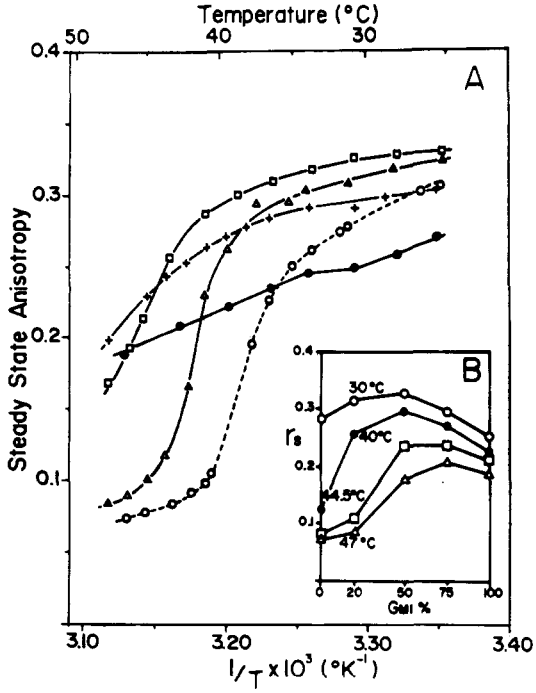

FIGURE 4: (A) Steady-state anisotropy, r_s, of DPH as a function of the reciprocal of the absolute temperature for DPPC-GM1 ganglioside mixed dispersions. DPPC–GM1 ganglioside (w/w): 0:100 (closed circles); 25:75 (plus signs); 50:50 (open squares); 80:20 (open triangles); 100:0 (broken line). (B, inset) Dependency of r_s on compositional changes of GM1 ganglioside in the DPPC–GM1 ganglioside mixed dispersions at 30, 44.5, and 47 °C, respectively. GM1 (%) in abscissa is represented as wt % of GM1 ganglioside in DPPC–GM1 ganglioside mixed dispersions.

component increased with the elevation of temperature, as reported in DPPC liposomes where the phase transition occurred in the measured temperature range (Kawato et al., 1977). The variation in the cone angle in glycosphingolipid dispersions was rather smaller than that in DPPC liposomes, which corresponded to the smaller temperature dependence of the steady-state anisotropy observed in glycosphingolipid dispersions. At 25 °C, the motion of DPH in ganglioside dispersions was less restricted than that in DPPC liposomes, while at 51 °C, the motion of DPH in ganglioside dispersions was more restricted than that in DPPC liposomes. This proposition was also supported by observations on the steady-state anisotropy as well as by time-resolved analysis (see Figure 2 and Table III). The hydrocarbon portion, ceramide, of the glycosphingolipids used consisted of both saturated fatty acyl chains, mainly stearic acid, and d18:1 and d20:1 sphingosines (Table II). Thus the hydrocarbon chains of the glycosphingolipids were longer than those of DPPC, which has a transition temperature near 41 °C (Hinz & Sturtevant, 1972; Shimshick & McConnel, 1973; Lentz et al., 1976a). This may be one reason why gangliosides and GA1 glycosphingolipid show smooth, not abrupt, changes in steady-state anisotropy in the temperature range of 25–50 °C. In the range of 30–40 °C, small, but recognizable, changes of the slope were observed. Two broad transitions of brain ganglioside mixtures with peak maxima at 15 and 39 °C were observed by differential scanning calorimetry (Curatolo et al., 1977). The latter value, 39 °C, was within the temperature range observed in this study. Though more detailed study is necessary, it is suggested that the temperature near 30–40 °C where the slope of the steady-state anisotropy changed might be related to the phase transition. The hydrocarbon region, as demonstrated by the changes in the steady-state anisotropy, may alter at about this temperature from one disordered state

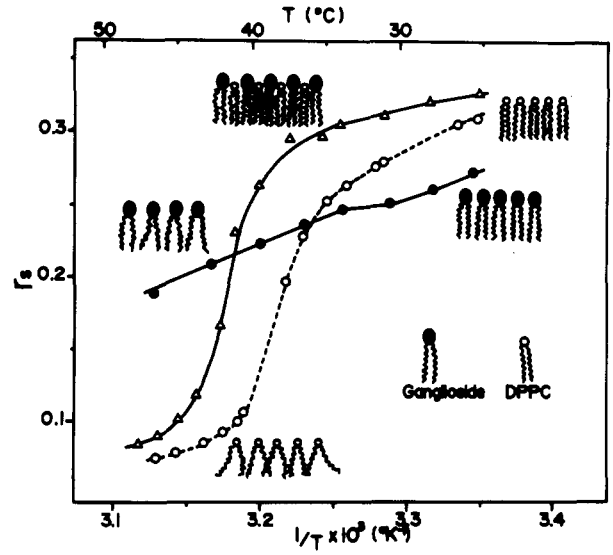

FIGURE 5: Schematic representation of ganglioside–DPPC molecular arrangement in DPPC–ganglioside mixed dispersions. Steady-state anisotropy, r_s, of DPH is represented as a function of the reciprocal of the absolute temperature. DPPC–GM1 ganglioside (w/w): 0:100 (closed circles); 80:20 (open triangles); 100:0 (broken line). The explanation and details are given in the text.

to another, which is presumably more disordered (Tinker et al., 1976; Curatolo et al., 1977).

From these results the relationship between gangliosides and DPPC are represented schematically as shown in Figure 5. At lower temperature, it is suggested that both DPPC and ganglioside molecules are comixed and not segregated from each other, since the steady-state anisotropy of the DPPC–ganglioside mixed dispersions could be expected to be intermediate between the anisotropy of DPPC liposomes and that of the dispersions composed of a single ganglioside component, if DPPC and ganglioside molecules were segregated. The steady-state anisotropy of the DPPC–ganglioside mixed dispersions, however, was larger than the anisotropy of DPPC liposomes and also that of the dispersions composed of a single ganglioside component. Thus, at lower temperature, the hydrocarbon chains of gangliosides are more loosely arranged, due to different physicochemical properties of their head group, than those of DPPC, and the hydrocarbon region of ganglioside–DPPC (20:80 and 50:50 w/w) mixed dispersions is more solid than those of ganglioside dispersions and DPPC liposomes themselves, because of the close and evenly mixed arrangement of the hydrocarbon of the two molecules. The hydrocarbon regions of gangliosides, DPPC, and ganglioside–DPPC mixed dispersions become more disordered with elevation of the temperature. Among the hydrocarbon chains of these three, above the phase transition temperature of DPPC, those of DPPC are the most disordered and those of gangliosides are the least disordered, which may be ascribed to a difference of the chain length of the head group and/or of the structure of the hydrophobic moiety, ceramide and glyceride. In this connection it is of particular interest to note that sphingomyelin–sphingomyelin interactions are stronger than phosphatidylcholine–phosphatidylcholine interactions (Schmidt et al., 1977; Barenholz et al., 1976). The order of the hydrocarbon region of ganglioside–DPPC (20:80 and 50:50 w/w) mixed dispersions is intermediate, presumably due to the close molecular packing of the two different types of molecule.

In the various glycosphingolipid dispersions studied, the motion of DPH molecules was the most restricted in the GA1 glycosphingolipid dispersion. Probably these differences are due not to compositional differences in the hydrocarbon moiety,

but to the presence or absence of a sialic acid residue in the carbohydrate portion, since the compositions of the hydrocarbons of GA1 glycosphingolipid and gangliosides are very similar (Table II). In the gangliosides studied, the motion of DPH molecules was the most restricted in the GM1 ganglioside dispersion. In GD1a and GD1b ganglioside, disialoganglioside, dispersions, the motion of DPH molecules was less restricted than in the GM1 ganglioside dispersions, which should be ascribed to the effect of the larger number of sialic acid residues in these gangliosides. The hydrocarbon region of GD1b ganglioside is looser than that of GD1a ganglioside probably owing to the fine structural difference in the locations of the sialic acid linkage. Comparison of GM1, GM2, and GM3 gangliosides, which have the same number and location of sialic acid residues, shows that the motion of DPH became less restricted with shortening of the chain of the neutral sugar backbone, suggesting that formation of hydrogen bonds between carbohydrate moieties at the ultimate and/or penultimate positions exerts a significant influence on the hydrocarbon arrangement.

Some of the changes in parameters measured with pure ganglioside solutions may be interpreted as related to phase changes and not to molecular packing, etc., changes within one phase. We performed a negative-staining electron microscopic study on a diluted solution (10^{-5} M) of total gangliosides, GM1 and GD1b, and found that gangliosides most probably exist in a micellar form with an average diameter of 120–180 Å (data not shown). This suggests that different ganglioside molecular species may disperse in a similar micellar state. In the present experiments, however, more concentrated solutions (10^{-3} M, i.e., 100 times as much as the aforementioned one) were used. In such concentrated solution, it is highly possible that gangliosides behave differently compared with those in the diluted solution. Election microscopic analysis seems to be difficult to perform on such highly concentrated solutions. Corti et al. (1980), using a laser light scattering investigation, have recently reported that GM1 and GD1a gangliosides at a concentration below 1×10^{-6} M gave the micellar molecular weight $M_r = 532\,000 \pm 50\,000$ and the hydrodynamic radius RH = 63.9 ± 2 Å for GM1 and $M_r = 417\,000 \pm 40\,000$ and RH = 59.5 ± 2 Å for GD1a and that some change in scattered intensity was observed at a concentration between 10^{-4} and 10^{-6} M.

The results thus can be interpreted in terms of at least two significant parameters, the sialic acid and the hydrogen bonding. The carbohydrate-linked sialic acid influences the hydrocarbon region and facilitates the motion of DPH molecules. The more sialic acid residues that are linked, the larger is the mobility of the probe molecules. Shinitzky & Barenholz (1974) showed that the ζ potential generated by insertion of dicetylphosphate into egg lecithin and sphingomyelin liposomes does not affect the dynamic properties of the hydrocarbon region. Eibl & Blume (1979) reported that phosphatidic acid in the region between the first and the second pK is characterized by only small variations in the transition temperature in spite of the large changes occurring in the surface charge of the membrane. Thus, the influence of sialic acid extending to the hydrocarbon region is unique in contrast with those of acidic phospholipids, dicetylphosphate, and phosphatidic acid and may be caused principally by either its steric bulkiness or its electrostatic repulsion, or by both. From the results, it is suggested that increase of the number of sialic acid residues may allow ganglioside molecules to occupy a greater surface area and thereby make the hydrocarbon region loosely arranged. The significance of hydrogen bonding between carbohydrate moieties in lipid membranes has also been suggested by Sharom & Grant (1978) from studies on spin-labeled gangliosides and by Abrahamsson et al. (1972, 1977). The present findings that the physicochemical behaviors of gangliosides in lipid bilayer membranes are different not only from those of phosphatidylcholines but also from those of nonsialic acid bearing glycosphingolipids should be useful in studies on membrane-related activities of gangliosides in cells.

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