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## Dynamic Structure of Lipid Bilayers Studied by Nanosecond Fluorescence Techniques<sup>†</sup>

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**ABSTRACT:** Molecular motions in liposomes of dipalmitoylphosphatidylcholine (DPPC) were studied by nanosecond fluorescence techniques. As a fluorescent probe for the hydrocarbon region, 1,6-diphenyl-1,3,5-hexatriene (DPH) was used. Time courses of fluorescence intensity  $I_T(t)$  and emission anisotropy  $r(t)$  of DPH embedded in DPPC liposomes were measured at various temperatures. The value of the fluorescence lifetime  $\tau$  obtained from a single exponential decay of  $I_T(t)$  was somewhat higher than that in liquid paraffin below the transition temperature  $T_i$  and decreased above  $T_i$ . Higher values of  $\tau$  below  $T_i$  indicate the almost complete hydrophobic environment. The decay curves of  $r(t)$  were separated into two phases: an initial fast decreasing phase of the order of one nanosecond and a second almost constant phase. This indicates

that the orientational motion of DPH in the hydrocarbon region is described by a wobbling diffusion restricted by a certain anisotropic potential. The results were analyzed on the model that the wobbling diffusion is confined in a cone with a uniform diffusion constant. Though temperature dependence of the cone angle was sigmoidal, that of the wobbling diffusion constant was like the exponential function. The change in the cone angle at  $T_i$  was sharper than that in the wobbling diffusion constant at  $T_i$ . Estimated values of the viscosity in the cone were an order of magnitude smaller than the values of "microviscosity" which were estimated from the steady-state emission anisotropy without considering the restrictions on the rotational motion.

Structure and dynamics of lipids in biological membranes have been recognized as the essential factors in their functions and organization (Inesi et al., 1973; Racker and Hinkle, 1974). Phospholipids in model membranes such as aqueous dispersions or liposomes are known to be in a bilayer structure and the cooperative melting of their hydrocarbon chains, at the crystalline-liquid-crystalline phase transition, takes place at a certain temperature (Träuble and Eibl, 1974; Sackmann et al., 1973; Chapman, 1975; Jacobson and Papahadjopoulos, 1975). Studies of dynamic properties or molecular motion of lipids in model bilayers, however, have not resolved several important questions.

Although the use of spin-label techniques for the dynamic studies of lipid bilayers is widespread, the information regarding molecular motion is restricted in most investigations. In describing the characteristics of molecular motion of

membranes, Hubbell and McConnell (1971) have used the "order parameter", the orientational degree of hydrocarbon chain, which can be obtained from the electron spin resonance spectra assuming rapid anisotropic rotation of the probe. Recently, Israelachvili et al. (1975) proposed a method to analyze the line shapes of ESR<sup>1</sup> spectra assuming both tumbling within the confines of a cone and restricted rotations about the long axis of a probe. Rotational correlation times of tumbling and of restricted rotations of spin-labeled molecules embedded in lecithin bilayers were estimated by the method.

Fluorescence depolarization techniques are also useful to investigate dynamics of lipid bilayers (Badley et al., 1973). From the emission anisotropy measured under constant illumination, Shinitzky et al. (1971) estimated the "microviscosity" of membrane. The treatment to estimate "microviscosity", however, is not appropriate when the rotational diffusion of a fluorescent probe is restricted within a certain region such as a cone.

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<sup>1</sup> Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, DL- $\alpha$ -dipalmitoylphosphatidylcholine; ESR, electron spin resonance.

In this paper we attempt to describe molecular motions in DL- $\alpha$ -dipalmitoylphosphatidylcholine (DPPC) liposomes in detail, using time-resolved nanosecond fluorescence techniques (Tao, 1969). As a fluorescent probe, we used 1,6-diphenyl-1,3,5-hexatriene (DPH), which has been established as a probe for the hydrocarbon regions (Shinitzky and Barenholz, 1974; Lentz et al., 1976a,b; Andrich and Vanderkooi, 1976). DPH has an *all-trans*-polyene structure and has a rod-like shape. The absorption and fluorescence transition moments lie along the major axis of the molecule. Therefore, when DPH is excited by pulsed light at the last absorption band, time dependent emission anisotropy reflects the molecular motion of hydrocarbon chains around DPH.

### Experimental Procedures

**Materials.** DL- $\alpha$ -Dipalmitoylphosphatidylcholine (DPPC, Fluka Puriss grade) and 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich Puriss grade) were used without further purification. Fluorescein and rhodamine B were purchased from Tokyo Kasei.

**Preparation of Liposomes and Fluorescence Labeling.** A desired amount of DPPC was dissolved in chloroform-methanol (5:1 v/v) solution and was lyophilized under reduced pressure. The product was suspended in phosphate buffer of ionic strength 0.6, pH 7.2, to yield a final concentration of 3 mg/mL.

Fluorescence labeling was achieved by diluting a solution of 1 mM DPH in tetrahydrofuran with 200-fold vigorously stirred aqueous suspension. The amount of dye added was equivalent to approximately 1 molecule per 800 molecules of lipids. Under these conditions, undesirable fluorescence depolarization due to nonradiative or radiative energy transfer between dye molecules was practically eliminated.

The suspension was then subjected to high intensity ultrasonic irradiation under nitrogen gas, for about 30 min in a jacketed vessel maintained above the transition temperature, and the clear suspension was obtained. A 10-kc sonifier (Choonpa Kogyo Model USV-150V) with a tip of 9 mm diameter was used at 50% full power. To prepare liposomes without ultrasonic irradiation (nonsonicated liposome), the labeled suspensions were vigorously stirred for 30 min above the transition temperature.

**Fluorescence Measurements.** Time courses of fluorescence depolarization were measured by nanosecond fluorometer, described in detail elsewhere (Kinosita et al., 1976). Sample solutions were excited by light pulses and time courses of fluorescence decay were measured by the single photon counting technique. The excitation wavelength used for DPH was 360 nm. Time courses of the emission from the solution were analyzed into vertical and horizontal components,  $I_V(t)$  and  $I_H(t)$ , with an analyzer. The wavelength of the fluorescence was selected above 420 nm by cut-off filters.

Steady-state measurements under constant illumination were performed by the same system changing the light source to a 500-W xenon lamp. Steady-state intensities,  $I_V^S$  and  $I_H^S$ , were measured as numbers of detected photons per unit time interval.

Anisotropic sensitivity of the detection system as to the direction of polarized light was corrected using a DPH solution in chloroform. In the nanosecond fluorescence measurements, solutions of high concentration of liposomes (3 mg/mL) were used and the contribution of scattered exciting and emitting lights to the anisotropy could not be neglected. It was corrected according to the following method. The steady-state emission anisotropy  $r^S$  was measured under the same conditions but at

dilute concentration (0.3 mg/mL) where the scattering of exciting and emitting lights could be neglected. Then, the emission anisotropy  $r(t)$  was corrected so that its average value  $\langle r \rangle$  was equal to  $r^S$  where  $\langle r \rangle$  is defined as

$$\langle r \rangle = \frac{\int I_D(t) dt}{\int I_T(t) dt} \quad (1)$$

Fluorescence spectra of dyes were measured with the Hitachi MPF-3 fluorescence spectrophotometer.

**Analysis.** From the observed intensity of  $I_V$  and  $I_H$ , the total fluorescence intensity  $I_T$ , the difference of intensity between vertical and horizontal components  $I_D$ , and the emission anisotropy  $r$  are obtained by

$$I_T = I_V + 2I_H \quad (2)$$

$$I_D = I_V - I_H \quad (3)$$

$$r = I_D/I_T \quad (4)$$

Let us imagine an ideal case where the excitation pulse is infinitely short and designate the corresponding fluorescence intensity and emission anisotropy by  $I_T^\delta(t)$  and  $r^\delta(t)$ . Then the observed intensity  $I_T(t)$  and  $I_D(t)$  are related to  $I_T^\delta(t)$  and  $r^\delta(t)$  by the following convolution products

$$I_T(t) = \int_0^t g(t') I_T^\delta(t - t') dt' \equiv g(t) * I_T^\delta(t) \quad (5)$$

$$I_D(t) = \int_0^t g(t') I_T^\delta(t - t') r^\delta(t - t') dt' \\ \equiv g(t) * [I_T^\delta(t) r^\delta(t)] \quad (6)$$

where  $g(t)$  is the response function of the apparatus.

In order to determine the ideal response functions,  $I_T^\delta(t)$  and  $r^\delta(t)$ , a curve-fitting procedure was performed, assuming that the ideal response functions were expressed as sums of exponential functions:

$$I_T^\delta(t) = \sum_{i=1}^N I_i \exp(-t/\tau_i) \quad (7)$$

$$r^\delta(t) = \sum_{j=1}^M r_j \exp(-t/\phi_j) \quad (8)$$

First, the values of the parameters  $I_i$  and  $\tau_i$  were so determined as to minimize the function:

$$F_T \equiv \sum_{t_n} \{ [I_T(t_n) - I_T^{\text{calcd}}(t_n)]^2 / I_T(t_n) \} \quad (9)$$

Then, the values of  $r_j$  and  $\phi_j$  were so determined as to minimize the function:

$$F_D \equiv \sum_{t_n} \{ [I_D(t_n) - I_D^{\text{calcd}}(t_n)]^2 / I_D(t_n) \} \quad (10)$$

All calculations were performed on a Hitac 8800/8700 computer, using an iterative least-squares program.

**Determination of the Response Function of the Apparatus.** The response function  $g(t)$  in eq 5 and 6 must be expressed as the following double convolution product:

$$g(t) = P(\lambda_{\text{ex}}, t) * H(\lambda_{\text{em}}, t) * K(t) \quad (11)$$

Where  $P(\lambda_{\text{ex}}, t)$  is the distribution function of the light intensity of excitation flash.  $H(\lambda_{\text{em}}, t)$  and  $K(t)$  are the response functions of the photomultiplier at wavelength  $\lambda_{\text{em}}$  chosen at the emission and of the chain of electronic devices connected to the photomultiplier output. Since the apparatus response function  $g'(\lambda, t)$  obtained from the direct measurements of exciting flash:

TABLE I: Analysis of the Experimental Data Obtained with Glycerin and Liquid Paraffin Labeled with DPH.<sup>a</sup>

Solvent	$T$ (°C)	$\eta$ (P)	$\tau$ (ns)	$r_1$	$\phi_1$ (ns)	$D$ (ns <sup>-1</sup> )	$\langle r \rangle$	$V_e f$ (cm <sup>3</sup> )
Glycerin	-5		5.6	0.395	448.1	$4 \times 10^{-4}$	0.390	
	20	12	3.9	0.392	95.5	$2 \times 10^{-3}$	0.376	$3.2 \times 10^{-23}$
Liquid paraffin	9.2	4	9.8	0.365	18.0	0.009	0.237	$18 \times 10^{-23}$
	19.8	2.0	9.7	0.359	7.5	0.022	0.154	$15 \times 10^{-23}$
	30.0	1.0	9.8	0.346	4.3	0.039	0.106	$18 \times 10^{-23}$
	39.5	0.6	9.9	0.355	2.4	0.069	0.068	$17 \times 10^{-23}$

<sup>a</sup>  $D$  is the rotational diffusion constant in isotropic media,  $D = 1/\phi_1$ .

$$g'(\lambda_{ex}, t) = P(\lambda_{ex}, t) * H(\lambda_{ex}, t) * K(t) \quad (12)$$

is not identical with  $g(t)$  (Wahl et al., 1974), we determined, at least approximately, the response function  $g(t)$  with the following procedure. The quantum yield of fluorescein was known to be constant for a wide range of exciting wavelengths (Weber and Teale, 1957). Moreover its fluorescence spectra measured in NaOH solution were independent of exciting wavelengths between 300 and 530 nm. Therefore, its fluorescence intensity for short exciting pulse  $I_T^\delta(t)$  can be assumed to be independent of exciting wavelength within the above range. The apparent fluorescence intensity  $I_T(t)$  of fluorescein above 520 nm was measured by our present apparatus and the curve  $I_T(t)$  was independent, within the experimental error, of the exciting wavelength,  $\lambda_{ex}$ , between 360 and 470 nm. It can be concluded from these results and eq 5 and 11 that  $P(\lambda_{ex}, t)$  of our apparatus is practically independent of  $\lambda_{ex}$  between 360 and 470 nm. This conclusion was confirmed between 360 and 530 nm by the same method using rhodamine B. Therefore, the apparent dependence of  $g'(\lambda_{ex}, t)$  on  $\lambda_{ex}$  can be attributed to the dependence of  $H(\lambda_{ex}, t)$  on  $\lambda_{ex}$ . So, the true response function  $g(t)$  is reduced to  $g'(\lambda_{em}, t)$  as follows:

$$\begin{aligned} g(t) &\equiv P(\lambda_{ex}, t) * H(\lambda_{em}, t) * K(t) \\ &\simeq P(\lambda_{em}, t) * H(\lambda_{em}, t) * K(t) \equiv g'(\lambda_{em}, t) \end{aligned} \quad (13)$$

## Results

**DPH in Isotropic Media.** Time courses of fluorescence intensity and depolarization of DPH were measured in glycerin and in liquid paraffin at various temperatures. The results were analyzed by the curve-fitting method described in Experimental Procedures. The ideal response functions,  $r^\delta(t)$  and  $I_T^\delta(t)$ , followed a single exponential decay. The parameters of ideal response functions determined by setting  $N = 1$  and  $M = 1$  in eq 7 and 8 are given in Table I. Typical results are shown in a semilogarithmic plot in Figures 1 and 2 where intensity  $I_T(t)$ , anisotropy  $r(t)$ , and apparatus response function  $g'(\lambda_{em}, t)$  are represented by dots, zig-zag curves, and chain lines. Calculated best fit curves corresponding to the parameters given in Table I are shown by thin dashed lines. The fit is almost satisfactory.

For several cases,  $I_T(t)$  or  $r(t)$  were analyzed by double exponential approximation setting  $N = 2$  or  $M = 2$  in eq 7 and 8. Best fit curves thus obtained are shown by thin solid lines. No significant progress in curve fitting can be observed. As DPH has an elongated ellipsoid body and its absorption and fluorescence transition moments lie along its longest axis, only the rotational motion of the longest axis can be detected by the fluorescence anisotropy. The observed single relaxation time corresponds to the averaged relaxation time of the rotational motions around its shorter axes because the rotational motion

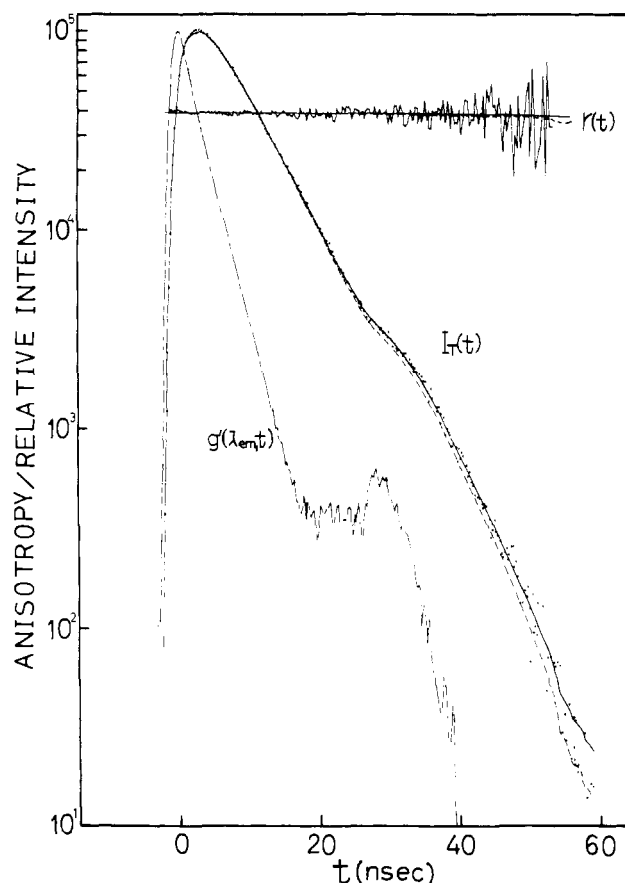


FIGURE 1: Time dependence of the emission anisotropy  $r(t)$  (zig-zag curve) and of the total fluorescence intensity  $I_T(t)$  (dots) of DPH in glycerin at  $-5^\circ\text{C}$ . The apparatus response function  $g'(\lambda_{em}, t)$  was measured at  $\lambda_{em} = 455\text{ nm}$ . The concentration of DPH was  $4 \times 10^{-6}\text{ M}$ . Thin solid line: the best fit curve for double exponential approximation. Thin dashed line: the best fit curve for a single exponential approximation. Thin chain line:  $g'(\lambda_{em}, t)$ .

of DPH around the longest axis should be much faster than other rotational motions.

As shown in Figure 1,  $r(t)$  measured in glycerin at  $-5^\circ\text{C}$  remains practically at a constant value. The corresponding  $\phi_1$  value is almost infinity, indicating that the rotational motion of DPH is almost perfectly inhibited. Then the value of fluorescence anisotropy  $r_0$  of immobilized DPH is expected to be this constant value. In fact in Table I,  $r_1$  and  $\langle r \rangle$  at  $-5^\circ\text{C}$  in glycerin have the same value within the experimental error. All these values together with the value of  $r_1$  at  $20^\circ\text{C}$  in glycerin converge at  $0.395 \pm 0.01$ . The same limiting value is expected from the value of  $r_1$  in liquid paraffin, where the

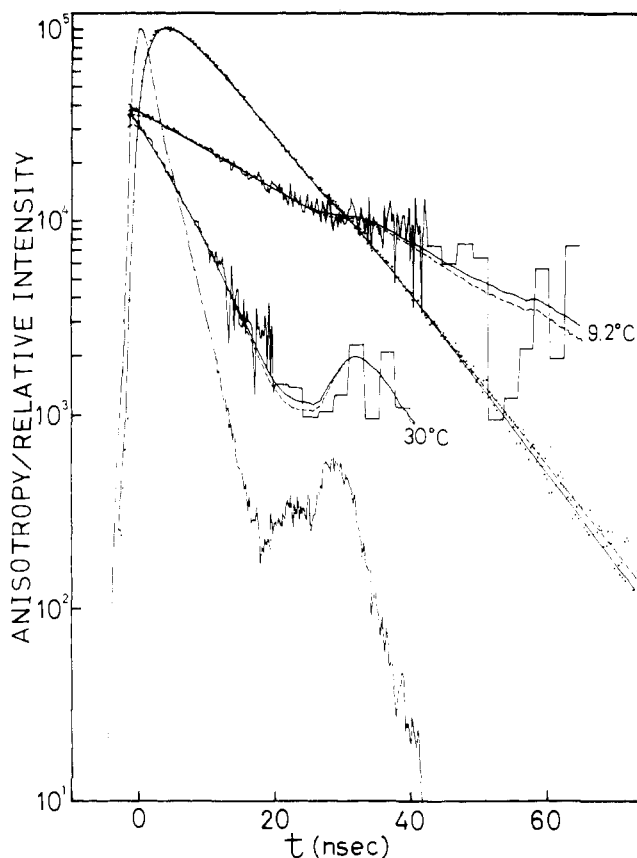


FIGURE 2: Time dependence of the emission anisotropy  $r(t)$  at different temperatures (zig-zag curves) and of the total fluorescence intensity  $I_T(t)$  (dots) at 9.2 °C of DPH in liquid paraffin. The concentration of DPH was  $5 \times 10^{-6}$  M. Thin solid line: the best fit curve for double exponential approximation. Thin dashed line: the best fit curve for a single exponential approximation. Thin chain line:  $g'(\lambda_{em}, t)$ ,  $\lambda_{em} = 455$  nm.

process of extrapolation to time zero induces a wide error. Therefore, the value of  $r_0 = 0.395 \pm 0.01$  was used in the following analysis.

The fluorescence lifetime  $\tau$  of DPH in liquid paraffin, hydrophobic solution, is much larger than that in glycerin, hydrophilic solution. It is interesting that the lifetime  $\tau$  is independent of temperature in completely hydrophobic environment, but it depends on temperature in hydrophilic environment.

**DPH in Liposome.** Time courses of fluorescence intensity and depolarization of DPH embedded in DPPC liposomes were measured at various temperatures. Typical decays of  $I_T(t)$  and  $r(t)$  measured with sonicated liposomes are shown in semi-logarithmic plots in Figure 3. Similar results were obtained with unsonicated liposomes when the effect of scattering was corrected. A significant feature of decay curves is that the curves  $r(t)$  do not follow a single exponential decay but can be separated into two phases: an initial fast decreasing phase and a second almost constant phase. Similar decay curves have been observed in an excitable membrane (Wahl et al., 1971). On the other hand, the curves  $I_T(t)$  follow a single exponential decay as pointed out by Lentz et al. (1976a). The curves  $I_T(t)$  were analyzed by setting  $N = 1$  and 2 in eq 7, and the calculated best fit curves are shown in Figure 3. No significant progress in curve fitting was observed by setting  $N = 2$ .

The curves  $r(t)$  were analyzed by setting

$$r^{\delta}(t) = (r_0 - r_{\infty})e^{-t/\phi_1} + r_{\infty} \quad (14)$$

The best fit curves are shown by thin solid lines in Figure 3.

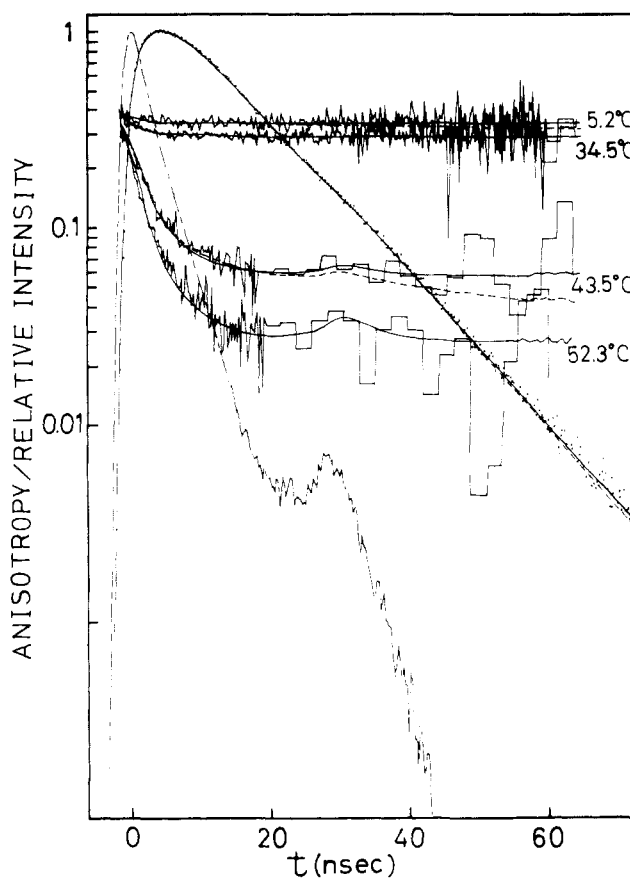


FIGURE 3: Time dependence of the emission anisotropy  $r(t)$  at different temperatures (zig-zag curves) and the total fluorescence intensity  $I_T(t)$  (dots) at 5.2 °C of DPH in DPPC liposomes. The concentrations of DPPC and DPH were 3 mg/mL and  $5 \times 10^{-6}$  M. Thin solid line: the best fit curve of  $I_T(t)$  for double exponential approximation and that of  $r(t)$  according to eq 14. Thin dashed line: the best fit curve of  $I_T(t)$  for a single exponential approximation and that of  $r(t)$  for double exponential approximation. Thin chain line:  $g'(\lambda_{em}, t)$ ,  $\lambda_{em} = 455$  nm.

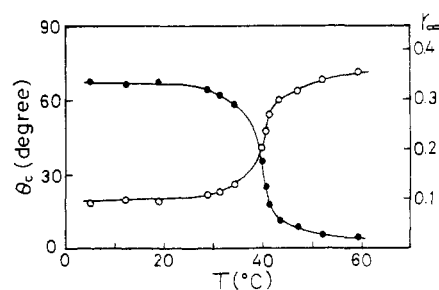


FIGURE 4: Temperature dependence of  $r_{\infty}$  (●) and of the cone angle  $\phi_1$  (○) for DPH in DPPC liposomes.

For several cases,  $r(t)$  was deconvoluted by setting  $M = 2$  in eq 8, that is, two exponential approximation. The best fit curves for  $r(t)$  are shown by thin dashed lines in Figure 3. In all cases examined, values of  $\phi_2$  obtained by deconvolution were very large. Thus,  $r^{\delta}(t)$  can be expressed well by eq 14.

The obvious interpretation of the results is that the orientational distribution of DPH embedded in the hydrocarbon region is anisotropic even at equilibrium state;  $r_{\infty}$  represents the degree of anisotropy in equilibrium distribution of DPH and  $\phi_1$  represents the relaxation time to approach the anisotropic equilibrium distribution of excited DPH. The "order parameter" commonly used in spin-label studies should correspond to the equilibrium anisotropy ratio  $r_{\infty}/r_0$ . As shown

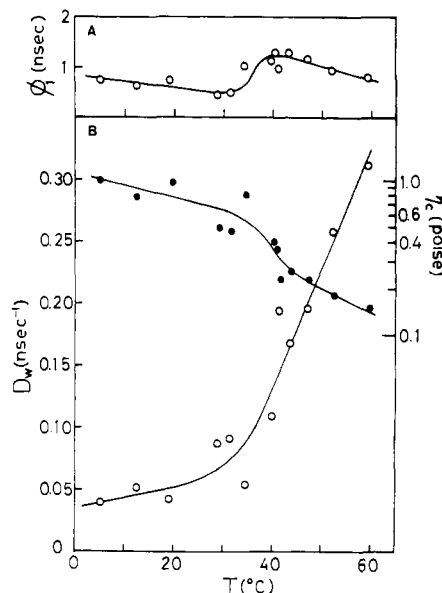


FIGURE 5: (A) Temperature dependence of the relaxation time  $\phi_1$  of DPH in DPPC liposomes. (B) Temperature dependence of the wobbling diffusion constant  $D_w$  (○) and of the viscosity in the cone  $\eta_c$  (●) for DPH in DPPC liposomes.

in Figure 4,  $r_\infty$  decreases sharply near the transition temperature 40 °C. Highly anisotropic distribution of DPH in liposomes below the transition temperature  $T_i$  becomes almost isotropic by the transition. On the other hand, the apparent relaxation time  $\phi_1$  is very short, ranging from 0.4 to 1.3 ns, and markedly "increases" near  $T_i$  as shown in Figure 5.

Temperature dependence of the fluorescence lifetime  $\tau$  is shown in Figure 6. Below  $T_i$ , the value of  $\tau$  is the same or somewhat higher than that in liquid paraffin, indicating almost complete hydrophobic environment. At the transition region,  $\tau$  decreases rather sharply and decreases farther above  $T_i$ .

## Discussion

**Anisotropic Distribution and Wobbling Diffusion.** Results of the emission anisotropy  $r(t)$  summarized by eq. 14 indicate that the orientational distribution of DPH in liposomes in equilibrium is anisotropic. The emission anisotropy at sufficiently long time after the excitation,  $r_\infty$ , does not vanish but remains constant even above the transition temperature. Then, the orientational motion of DPH in liposomes must be described by the wobbling or tumbling restricted by a certain anisotropic potential.

The exact treatment of the wobbling diffusion should be very complicated, for the molecular motion is affected by the shape of potential and local diffusion constants. Here we assume a simple square well potential and a uniform diffusion constant. The wobbling diffusion of the excited DPH is confined within a cone around the normal of the membrane with the cone angle  $\theta_c$  ( $0^\circ \leq \theta_c \leq 90^\circ$ ). The "wobbling diffusion constant"  $D_w$  is constant throughout the cone and the orientational distribution of DPH in the cone is uniform at equilibrium. Even in this simple model, the wobbling diffusion cannot be expressed by a single relaxation time but by many relaxation times (Kinosita et al., 1977). The resultant expression of  $r(t)$  is of the form

$$\frac{r(t)}{r_0} = \sum_{i=1}^{\infty} A_i \exp(-D_w t / \sigma_i) \quad (15)$$

where  $A_i$  and  $\sigma_i$  are constants which depend only on  $\theta_c$ . The

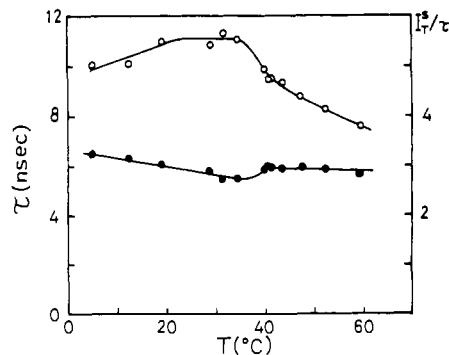


FIGURE 6: Temperature dependence of the fluorescence lifetime  $\tau$  (○) and of the ratio of the steady-state fluorescence intensity to the fluorescence lifetime  $I_T^S \tau$  (●) for DPH in DPPC liposomes.

cone angle  $\theta_c$  is related to the equilibrium anisotropy ratio as

$$A_\infty = r_\infty / r_0 = \left[ \frac{1}{2} \cos \theta_c (1 + \cos \theta_c) \right]^2 \quad (16)$$

Then, the single relaxation time  $\phi_1$  obtained experimentally can be expressed approximately by the average of relaxation times as

$$\phi_1 \cong \langle \phi \rangle \equiv \frac{1}{D_w} \frac{\sum_i A_i \sigma_i}{\sum_i A_i} \quad (17)$$

The average relaxation time  $\langle \phi \rangle$  increases with increase of the cone angle  $\theta_c$  because the time needed for the equilibrium in a wider angle is longer. That is, the apparent relaxation time for the restoration of equilibrium in the cone does not express the "diffusion rate".

**Wobbling Diffusion Constant and Cone Angle.** The "wobbling diffusion constant"  $D_w$  calculated by eq 17 and the cone angle  $\theta_c$  by eq 16 are plotted against temperature in Figures 4 and 5. The change in  $\theta_c$  at  $T_i$  is sharper than that in  $D_w$  at  $T_i$ , and, though the temperature dependence of  $\theta_c$  is sigmoidal, that of  $D_w$  is like the exponential function. Thus, the apparent "increase" in  $\phi_1$  due to the transition in Figure 5 is attributed to the effect of drastic increase in  $\theta_c$  which overcomes the effect of increase in  $D_w$  near  $T_i$ .

**Viscosity in the Cone.** From the wobbling diffusion constant, the "viscosity in the cone" can be estimated. The rotational diffusion constant  $D$  of an ellipsoid is generally expressed by

$$D = \frac{kT}{6\eta V_e f} \quad (18)$$

where  $\eta$  denotes the viscosity of the solvent,  $V_e$  and  $f$  denote the effective volume and shape factor of the probe. The same relation can be applied between the wobbling diffusion constant and the "viscosity in the cone".

Here we assumed that the value of  $V_e$  in hydrocarbon chains of lipids is the same as that in liquid paraffin because of the similar molecular nature of both solvents. Then the "viscosity in the cone",  $\eta_c$ , was estimated using the average value of  $V_e f$  estimated in liquid paraffin (see Table I). The results (see Figure 5) are an order of magnitude smaller than the values of "microviscosity" estimated from the steady-state fluorescence anisotropy of perylene and DPH in DPPC vesicles (Cogan et al., 1973; Lentz et al., 1976a).

The main source of the large difference between  $\eta_c$  and

"microviscosity" should be in the difference between the two analyses. In the present analysis, contributions of the anisotropic equilibrium on  $r(t)$  are considered separately; that is,  $\eta_c$  is the viscosity only in the cone where probes can diffuse practically. The "microviscosity" was estimated from the steady-state anisotropy  $r^S$  without regarding the restrictions on the rotational motion. Rather higher values of the "microviscosity" reported for DPPC suggest restrictions in the rotational motion of perylene in the bilayer. In fact, preliminary results show that  $r(t)$  of perylene in DPPC vesicles follows eq 14 indicating restrictions.

**Fluorescence Lifetime and Steady-State Intensity.** The fluorescence lifetime and the quantum yield  $Q$  are directly related to the rates of emission  $k_e$  and internal quenching  $k_i$  by the relation:

$$Q = \frac{\tau}{\tau_0} = \frac{k_e}{(k_e + k_i)} \quad (19)$$

Therefore the sharp decrease of  $\tau$  at  $T_i$  may be induced by the increase in thermal motion and in polarity around DPH due to the transition, because  $k_i$  is generally affected by these factors.

As the natural lifetime  $\tau_0 \equiv 1/k_e$  is supposed to be constant in these experimental conditions, the quantum yield  $Q$  is proportional to  $\tau$  and the ratio  $I_T^S/\tau$  gives the relative number of DPH embedded in vesicles. The relative number of DPH in the bilayer increases about 10% at  $T_i$  as shown in Figure 6.

**Dynamic Aspects of Lipid Bilayers.** As DPH has a rod-like shape, very similar to a hydrocarbon chain of lipid, DPH in the lipid bilayer should occupy just the same positions of chains and their molecular motion should reflect directly the motion of hydrocarbon chains.

The estimated values of  $\theta_c$  shown in Figure 4 are about 20° even at the crystalline state, sufficiently below  $T_i$ , and about 70° above  $T_i$ . These values indicate that the fairly large space must be attributed to the cone used to describe the wobbling diffusion. It is very unnatural to consider that such a large space leaves a vacuum around each DPH. A major part of the space should be occupied by hydrocarbon chains to reduce void volume. DPH is permanently in collision with these chains and wobbles around according to the thermal motion of hydrocarbon chains, and vice versa. The cone-like potential for wobbling diffusion is a conceptual one which indicates the time-averaged arrangement of chains around DPH. Rather large values of  $\theta_c$  at temperatures above  $T_i$  indicate a highly disordered state of hydrocarbon chains.

The wobbling diffusion constant is determined by the frequency of collisions between DPH and hydrocarbon chains and by the mean free rotational angle between successive collisions. As the density of hydrophobic region is not so much changed by the transition, the temperature dependence of  $D_w$  shown in Figure 5 mainly indicates the change in the frequency of collisions, that is, the rate of chain motion. It should be noted that no drastic change in the rate of chain motion was apparent at  $T_i$  and its temperature dependence was like the exponential function. However, the orientational freedom of chains, or  $\theta_c$ , changed like a sigmoidal transition.

Contrary to this wobbling diffusion constant, a sigmoidal transition in the lateral diffusion constant of lipids in DPPC vesicles at  $T_i$  was reported recently (Naqvi et al., 1974). The rate of lateral diffusion of lipids may be determined mainly by the number of structural defects in the two-dimensional arrangements of lipids. Such defects should increase drastically at  $T_i$  corresponding to the increase of  $\theta_c$ .

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