Dynamic Structure of Biological Membranes As Probed by 1,6-Diphenyl-1,3,5-hexatriene: A Nanosecond Fluorescence Depolarization Study[†]

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ABSTRACT: A fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene, was incorporated in four different biological membranes, the purple membrane of *Halobacterium halobium*, human erythrocyte membrane, rabbit sarcoplasmic reticulum membrane, and rat liver mitochondrial membrane. Time-resolved fluorescence depolarization of the probe suggested that the rotational Brownian motion of the probe in the membranes was restricted in the angular range. The motion of the rod-shaped, lipophilic probe molecule, expected to reflect closely the motion of neighboring lipid hydrocarbon chains, was analyzed in terms of the wobbling-in-cone model in which the major axis of the probe was assumed to wobble freely in a cone of semiangle θ_c with a wobbling diffusion constant D_w. At 35 °C, D_w in the four membranes, in the above order, ranged between 0.048 and 0.15 ns^{-1} and θ_c between 31 and 53°. From the rotational rate D_w, the viscosity against the wobbling motion was calculated to be 0.9–0.3 P. When the temperature was raised from 10 to 35 °C, D_w in all membranes increased approximately 3-fold, corresponding to activation energies of 7–8 kcal/mol, and θ_c increased by about 10°, except for the purple membrane in which the angular range remained narrow. The same characteristic temperature dependence has been found in many model membrane systems that contain unsaturated lecithins, suggesting an important role of unsaturated phospholipids in the dynamic structure of the lipid hydrocarbon chain region of biological membranes at physiological temperatures. Comparison with model systems suggests that proteins and cholesterol act mainly as barriers that narrow the angular range.

Knowledge of the motional properties of constituent molecules in biological membranes is important for the understanding of the mechanisms of various membrane functions as well as such phenomena as membrane fusion, deformation, etc. Among several physical techniques that detect molecular motions, time-resolved optical anisotropy decay measurements (fluorescence or phosphorescence depolarization, transient absorption dichroism) enable one to follow the process of molecular reorientation in the time domain. Thus, both the rate and range of rotational motion can be readily estimated from a single measurement. The separate determination of the rate and range is particularly important because, in an anisotropic environment such as the membrane, the orientation of a molecule is in general restricted, and the motion tends to be wobbling and/or uniaxial rotation rather than free three-dimensional rotation.

It has been shown, by the nanosecond time-resolved fluorescence depolarization measurements, that the rod-shaped, lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] embedded in synthetic lecithin membranes exhibits rotational Brownian motion of which the angular range is, on the average, restricted (Chen et al., 1977; Kawato et al., 1977; Dale et al., 1977; Lakowicz & Prendergast, 1978). The motion could be approximated by the wobbling-in-cone model in which the major axis of the probe was assumed to wobble freely in a cone of semiangle θ_c around the normal of the bilayer membrane with a wobbling diffusion constant D_w (Kawato et al., 1977; Kinosita et al., 1977). The motion characterized by the two experimentally determined parameters, D_w (rate) and θ_c (range), was expected to reflect closely the motion of neighboring lipid hydrocarbon chains because the size of the rod-shaped DPH approximates that of a lipid hydrocarbon chain. Similar restricted rotation has been implicated for DPH in various biological membranes (Senê et al., 1978; Glatz, 1978; Hildenbrand & Nicolau, 1979; Parola et al., 1979) or in microsomal lipid extracts (Martin & Foyt, 1978).

In order to relate the dynamic structure of the membrane interior, as probed by DPH, with the composition of the membrane, investigations have been made on model systems with simple and defined compositions: saturated lecithin bilayers (Chen et al., 1977; Kawato et al., 1977; Lakowicz et al., 1979) and lecithin–cholesterol systems (Veatch & Stryer, 1977; Kawato et al., 1978; Hildenbrand & Nicolau, 1979; Lakowicz et al., 1979). Recent studies in this laboratory include reconstituted membranes consisting of purified cytochrome *c* oxidase and synthetic lecithin (Kinosita et al., 1981) and lecithin bilayers with various degrees of unsaturation (Stubbs et al., 1981). Here, we report an analysis of DPH wobbling motion in four different biological membranes with known compositions: human erythrocyte membrane, rabbit sarcoplasmic reticulum membrane, rat liver mitochondrial membrane, and the purple membrane of *Halobacterium halobium*. The results are compared with those obtained in the above model system.

Experimental Procedures

Preparation of Membranes. Human erythrocyte membranes were prepared essentially according to Dodge et al.

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[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, dimyristoyl-3-*sn*-phosphatidylcholine; DPPC, dipalmitoyl-3-*sn*-phosphatidylcholine; DOPC, dioleoyl-3-*sn*-phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-3-*sn*-phosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; fwhm, full width at half-maximum.

0006-2960/81/0420-4270$01.25/0

(1963) from freshly drawn heparinized blood. The erythrocytes were washed 3 times with an isotonic saline containing 145 mM NaCl and 10 mM sodium phosphate, pH 7.4, while buffy coats were carefully removed. Hemolysis was achieved by injecting the washed cells into 100 volumes of vigorously stirred 10 mM sodium phosphate buffer, pH 7.4, at 0 °C. The membranes were washed twice with the hypotonic buffer and then twice with the isotonic saline by centrifugation at 12000*g* for 1 h at 4 °C and finally resuspended in the isotonic saline.

Fragmented sarcoplasmic reticulum was prepared from rabbit white skeletal muscles by the method of Ebashi & Lipmann (1962) with some modifications (Ogawa, 1970). The membranes obtained were suspended in a medium containing 100 mM KCl and 20 mM Tris-HCl, pH 7.0, quickly frozen with liquid nitrogen, and stored at -80 °C. Fluorescence measurements were made on thawed samples after dilution with the same medium.

Rat liver mitochondrial membranes were obtained as "sonic particles" as defined by Pedersen et al. (1978): First, mitochondria were prepared by the "fast, low-yield procedure" of Pedersen et al. (1978) from female Sprague-Dawley rats (4 weeks old) after overnight fasting. The mitochondria were suspended in a medium containing 220 mM D-mannitol, 70 mM sucrose, and 2 mM Hepes, pH 7.4, and sonicated with a Branson Ultrasonics sonicator, Model W185, with a small tip at maximum power for 2 min at 0 °C. The resultant suspension was centrifuged at 100000*g* for 80 min. The pellet was resuspended in the same medium and centrifuged at 3500*g* for 1 min. The supernatant was centrifuged at 100000*g* for 80 min and the final pellet resuspended in the same medium.

Halobacterium halobium strain R_1M_1 was grown, and the purple membranes were isolated and purified according to Oesterhelt & Stoeckenius (1974). Sucrose-gradient centrifugation in the purification procedure gave two closely apposed bands, of which the lower one was used in the present experiments. The membranes, after further centrifugal washings in water, were passed through a membrane filter (Nuclepore) with a pore size of 0.4 μm in order to remove possible aggregates. For fluorescence measurements, the membranes were resuspended in 50 mM sodium phosphate buffer, pH 7.4.

Protein concentrations in the samples were determined either by the method of Lowry et al. (1951) or by the biuret method (Gornall et al., 1949). The concentration of bacteriorhodopsin in the purple membrane suspension was estimated from the molar extinction coefficient at 568 nm of 63 000 for the light-adapted form (Oesterhelt & Hess, 1973).

L-α-Dimyristoyllecithin and L-α-dipalmitoyllecithin were obtained from Sigma. Lipid vesicles were prepared by sonicating a lipid suspension with a tip sonicator (Choonpa Kogyo, Model USV-150V) at 150 W for 30 min at about 40 °C. The clear suspension obtained was then centrifuged at 1000*g* for 10 min in order to remove titanium dust.

DPH (obtained from Aldrich) was incorporated into the membranes by adding a few microliters of a concentrated solution of DPH in tetrahydrofuran to a few milliliters of the membrane suspension so that the molar ratio of lipid to DPH was greater than 500:1 (protein/lipid weight ratios in the biological membranes were assumed to be between 2 and 4). The suspension was then incubated for 1 h at 30 °C (erythrocyte, sarcoplasmic reticulum, and mitochondrial membranes), for 2 h at 50 °C (purple membrane), for 1 h at 35 °C (DMPC), or for 1 h at 50 °C (DPPC), with occasional mixing. The samples were protected from light during the incubation and immediately subjected to fluorescence measurements.

Fluorescence Measurements. The nanosecond time-resolved fluorescence depolarization measurements were made by a computer-aided multipath nanosecond fluorometer to be described elsewhere. The light source was a free-running discharge lamp filled with hydrogen at 11–13 atmospheres. It emitted light pulses of duration less than 0.8 ns (fwhm) at a frequency between 6 and 11 kHz. Light at 360 nm was selected by a grating monochromator and two Hoya U-340 filters in tandem, polarized vertically with a Glan prism, and focused onto the sample in a thermostated cell holder. Fluorescence emitted at 90° to the right and left of the exciting beam was detected by two separate photomultiplier tubes (Hamamatsu TV, R943-02), one observing the vertically polarized component (I_V) and the other the horizontal one (I_H), through a Polaroid HNP'B sheet polarizer and Hoya L-39 and L-42 filters (transmission above 420 nm). The time-dependent fluorescence intensities, $I_V(t)$ and $I_H(t)$, were recorded by the single photon counting technique. An auxiliary circuitry enabled simultaneous measurement of $I_V(t)$ and $I_H(t)$, with the two photomultiplier tubes connected to a single time-to-amplitude converter. The slight difference between the responses of the two tubes was cancelled out by rotating the sheet polarizers and thus alternating the roles of the two photomultiplier tubes for every 5×10^6 excitations. The apparatus response function, $g(t)$, was measured at 455 nm (Kawato et al., 1978) with a dilute Ludox (Du Pont) suspension as light scatterer. Since the mitochondrial and the purple membranes gave weak fluorescence in the absence of DPH, the blank fluorescence was measured under identical conditions and subtracted from the corresponding data. The peak intensity of the blank fluorescence was less than 5% of that of DPH fluorescence in mitochondrial membranes and about 10% of that in purple membranes. (The absolute intensity of the purple membrane fluorescence was only a few times the buffer level and thus not much different from the blank levels in the other two biological membranes. Very weak DPH fluorescence in the purple membrane, however, necessitated the correction.)

Steady-state fluorescence intensities, I_V^s and I_H^s, were measured by the same fluorometer operated in an ordinary photon counting mode. Excitation light from a 150-W xenon lamp was attenuated with Zeiss neutral density filters to achieve the low light level required for the photon counting. In temperature scan experiments, the sample was heated at a rate of 15 °C/h, and the temperature was monitored with a tip thermistor immersed in the sample. All operations of the fluorometer were under the control of a DEC LSI-11/2 microcomputer system.

Analysis of Fluorescence Data. From the observed intensities I_V and I_H, the total fluorescence intensity I_T, the difference intensity I_D, and the fluorescence anisotropy r were calculated as follows:

$$I_T = I_V + 2SI_H \tag{1}$$

$$I_D = (I_V - SI_H)/D \tag{2}$$

$$r = I_D/I_T \tag{3}$$

where S is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light and was taken as equal to I_V/I_H obtained with horizontally polarized excitation (the alternation of the two photomultiplier tubes was properly taken into account). The demoninator D in eq 2 represents the extent of depolarization due to the scattering of excitation and fluorescence lights.[2] For steady-state data,

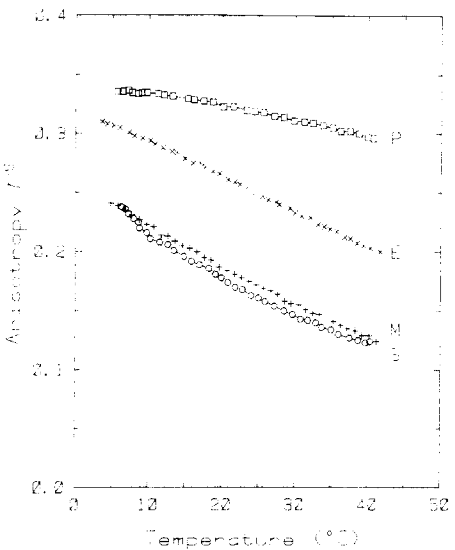

FIGURE 1: Steady-state fluorescence anisotropy of DPH in various biological membranes. P, purple membrane; E, human erythrocyte membrane; M, rat liver mitochondrial membrane; S, rabbit sarcoplasmic reticulum membrane. Concentrations of DPH and protein were 0.02–0.08 μM and 0.03–0.07 mg/mL, respectively. Heating scan at a rate of 15 °C/h.

which were obtained at membrane concentrations on the order of 0.01 mg of protein/mL, D was taken as 1. The nanosecond measurements required an order of magnitude of higher concentrations, resulting in some decrease in r values due to the scattering. D was therefore chosen as

$$D = \left\{\int I_D(t)\,dt \Big/ \left[\int I_T(t)\,dt\right]\right\} \Big/ r^s \tag{4}$$

where r^s is the corresponding steady-state anisotropy.

The nanosecond decay data were analyzed by assuming exponential decays of the following forms:

$$I_T{}^\delta(t) \propto \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \tag{5}$$

$$r^\delta(t) = (r_0 - r_\infty) \exp(-t/\phi) + r_\infty \tag{6}$$

where the superscript δ indicates that these quantities are responses to truely impulsive (δ-function-like) excitation. The parameters α_i, τ_i, r_∞, and ϕ were so determined that the convoluted products, $g(t)*I_T{}^\delta(t)$ and $g(t)*[I_T{}^\delta(t)r^\delta(t)]$, best fitted the observed $I_T(t)$ and $I_D(t)$, respectively (Kinosita et al., 1976; Kawato et al., 1977). The fundamental anisotropy, r_0, of DPH was taken as 0.395 (Kawato et al., 1977). All calculations were done by the LSI-11/2 microcomputer or by a FACOM 230-75 computer.

The cone angle θ_c was calculated from r_∞ by using the equation (Kinosita et al., 1977)

$$r_\infty/r_0 = \cos^2 \theta_c(1 + \cos \theta_c)^2/4 \tag{7}$$

The wobbling diffusion constant D_w was estimated from the following equation, where $x = \cos \theta_c$ (Lipari & Szabo, 1980), which is equivalent to the original numerical result (Kinosita et al., 1977):

$$D_w\phi(r_0 - r_\infty)/r_0 = -x^2(1 + x)^2\{\ln [(1 + x)/2] + (1 - x)/2\}/[2(1 - x)] + (1 - x)(6 + 8x - x^2 - 12x^3 - 7x^4)/24 \tag{8}$$

Results and Discussion

Steady-State Fluorescence Anisotropy. Figure 1 shows the steady-state fluorescence anisotropy, r^s, of DPH in the four biological membranes against temperature. The data for the erythrocyte membrane (E) are in good agreement with the results of Shinitzky & Inbar (1976), and the sarcoplasmic reticulum membrane data (S) agree well with the data of Moore et al. (1978). The anisotropy in the mitochondrial membrane (M) also agrees with that obtained by Shinitzky & Inbar (1976), though our source was rat liver whereas these authors used bovine heart. In the case of the purple membrane (P), our results differ considerably from those of Korenstein et al. (1976). Our r^s is higher and less sensitive to temperature; the break in an Arrhenius plot (log $[(r_0/r^s) - 1]^{-1}$ vs. reciprocal temperature) at 24 °C reported by these authors [also in Sherman & Caplan (1978)] is hardly recognized in Figure 1, though a replot of our data in the same manner gave some indication of a small break at a similar temperature. DPH fluorescence in the purple membrane was much weaker than that in other membranes because of the efficient excitation energy transfer from DPH to the retinal of bacteriorhodopsin (see below). Thus, the discrepancy described above might be ascribed to the presence of the blank fluorescence, which would reduce r^s unless properly corrected for. The origin of the blank fluorescence in our sample was unknown; the excitation spectrum was featureless between 300 and 400 nm.

The steady-state anisotropy depends on both the rate and the range of wobbling motion, as well as on the fluorescence lifetime. Provided the lifetime is constant, higher rate and/or wider range result in a smaller r^s. It thus appears that DPH in the purple membrane, presumably embedded between the crystalline lattice of bacteriorhodopsin (Stoeckenius et al., 1979), is much less mobile, in terms of either the rate or the range, or both, than DPH in sarcoplasmic reticulum or mitochondrial membranes, with the mobility in erythrocyte membrane in between. To draw clearer conclusions, however, one has to resort to time-resolved measurements.

Wobbling Motion of DPH in Biological Membranes. Since the temperature profile of r^s did not show large irregularities for the four membranes, the nanosecond fluorescence depolarization measurements were made at two temperatures, 10 and 35 °C. As seen in the typical data shown in Figure 2, measured $r(t)$ in all cases was biphasic, consisting of an initial decrease followed by an almost constant phase. Similar results have been reported for $r(t)$ in various biological membranes (Sené et al., 1978; Glatz, 1978; Hildenbrand & Nicolau, 1979; Parola et al., 1979). Since the biphasic decay of DPH anisotropy in model systems has successfully been interpreted in terms of the wobbling-in-cone model (Kawato et al., 1977, 1978; Hildenbrand & Nicolau, 1979), we applied the same analysis to $r(t)$ in the biological membranes. The wobbling diffusion constant D_w and the cone angle θ_c thus obtained are summarized in Table I. In view of the probable heterogeneity in the motional properties of DPH in a biological membrane, these quantities should be considered as (weighted) averages over various wobbling motions in the membrane. Equations 7 and 8 tell us that the nature of the averaging is such that components with extreme θ_c values (≃0° or ≃90°), if present,

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[2] If we put a superscript 0 to quantities that would be obtained in the absence of light scattering, we can show from symmetry considerations that $I_V = (1 - \delta)I_V{}^0 + \delta I_H{}^0$ and $I_H = (1 - \delta')I_H{}^0 + \delta' I_V{}^0$ where δ and δ' are constants that depend only on the extent of scattering. Then, $I_D = I_V - I_H = (1 - \delta - \delta')I_D{}^0$ and $I_T = I_V + 2I_H = I_T{}^0 + (2\delta' - \delta)(I_V{}^0 - I_H{}^0)$. For small δ and δ', i.e., if the scattering is not very severe, we can show that $\delta \simeq 2\delta'$. Thus, light scattering reduces I_D (and therefore r) by a constant factor that is practically independent of the value of r. The factor D in eq 2 in the text corresponds to $1 - \delta - \delta'$. Note that here we are not concerned with the scattered excitation light that reaches the photocathode, which can usually be eliminated by a suitable combination of filters. Values of D in the present study ranged between 1.0 and 1.1.

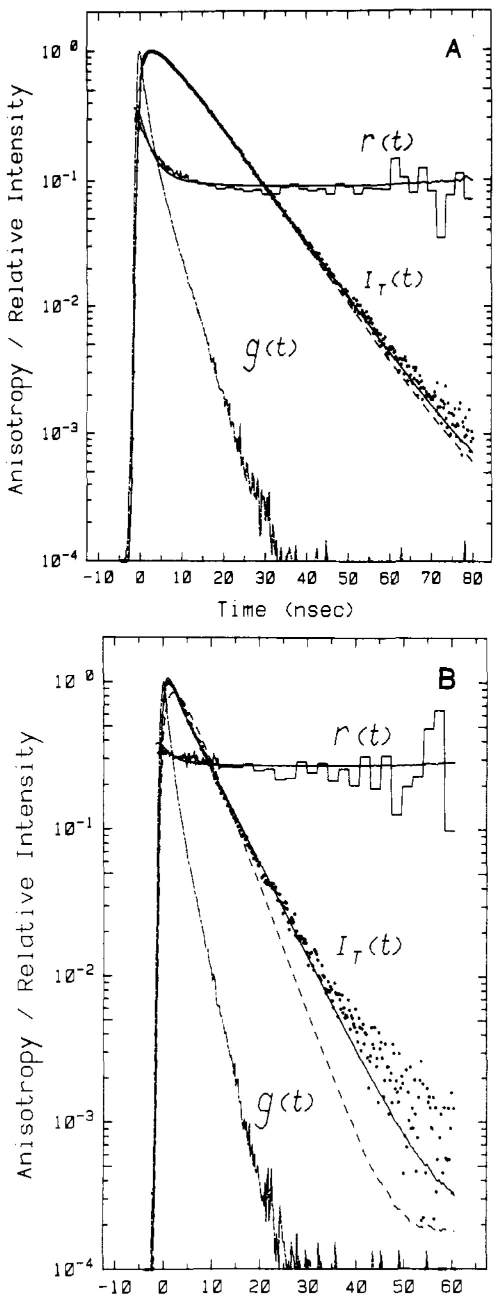

FIGURE 2: Typical fluorescence decays of DPH in biological membranes at 35 °C. (A) In sarcoplasmic reticulum membrane, [DPH] = 0.3 μM, [protein] = 0.2 mg/mL; (B) in purple membrane, [DPH] = 0.25 μM, [bacteriorhodopsin] = 10 μM. $g(t)$, the instrumental response function; $I_T(t)$, the total fluorescence intensity (dots, experimental data; dashed and solid lines, calculated best-fit curves for single- and double-exponential approximations); $r(t)$, fluorescence anisotropy (zigzag lines, experimental; smooth lines, calculated best-fit curves for the exponential-plus-constant approximation).

make somewhat smaller contributions to the average θ_c, and components with larger θ_c values have greater weights in the average D_w. When multiple fluorescence lifetimes exist with short ones preponderant, as in the purple membrane, the interpretation becomes ambiguous as will be discussed below.

The first thing to note in Table I is that, in spite of the large differences in compositions of the four biological membranes as will be discussed below, temperature dependences of D_w and θ_c in the membranes are quite similar. Thus, when the temperature was raised from 10 to 35 °C, the rate of wobbling motion, D_w, in all four membranes increased about 3-fold, corresponding to activation energies of 7–8 kcal/mol. At the same time, the range of motion, θ_c, increased by about 10° in all membranes, except the purple membrane in which θ_c remained small. The similarity of the temperature dependence is more easily seen in Figure 3, where D_w and θ_c at 10 °C (closed symbols) and those at 35 °C (open symbols with letters) are connected with a dotted line.

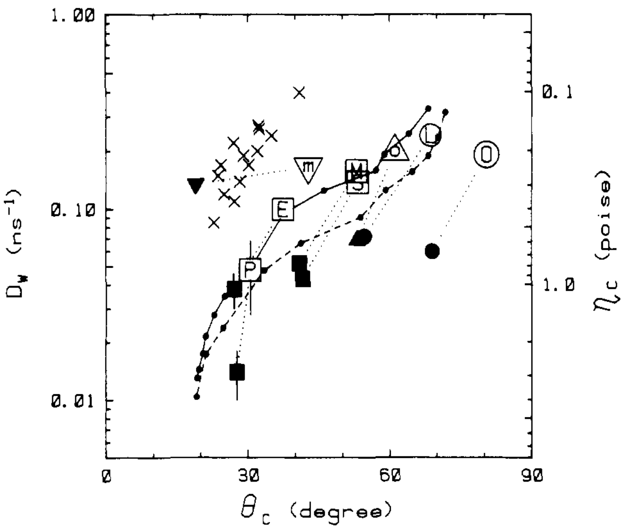

FIGURE 3: Wobbling parameters of DPH in model and biological membranes. Solid curve, sonicated vesicles of DPPC from 5 °C (lower left) to 55 °C (upper right); dashed curve, sonicated vesicles of DMPC from 5 to 50 °C. Data points on the curves (small closed circles) are 5 °C apart, except in the transition region where the intervals are 2–3 °C. Crosses, sonicated DPPC vesicles containing 33 mol % cholesterol between 10 and 60 °C. Where two large symbols are connected with a dotted line, the open symbol with a letter in it denotes D_w and θ_c at 35 or 37 °C, and the closed symbol at 10 °C. P in square, purple membrane; E in square, erythrocyte membrane; M in square, mitochondrial membrane; S in square, sarcoplasmic reticulum membrane; L in circle, multibilayer liposomes of PLPC; O in circle, cholate dialysis vesicles of DOPC, m in inverted triangle, cholate dialysis vesicles of DMPC containing cytochrome oxidase at a heme a/lipid molar ratio of 3/100; o in triangle, cholate dialysis vesicles of DOPC containing cytochrome oxidase at a heme a/lipid molar ratio of 2/100. The right-hand scale for η_c is an approximate one.

The same temperature dependence has been found in many model membrane systems that contain unsaturated lecithins, suggesting an important role of unsaturated phospholipids in the motional properties of the lipid hydrocarbon chain region of biological membranes (see Figure 3 and the next section). Saturated lecithins above the gel to liquid-crystalline phase transition also gave activation energies for D_w of 8–9 kcal/mol. Many naturally occurring saturated phospholipids, however, appear to have a transition above physiological temperatures in their pure state.

The absolute values of D_w and θ_c differed from membrane to membrane. In particular, the results in Table I as well as studies of model systems suggest a strong correlation between θ_c and the composition of the membrane.

Among the four biological membranes, mitochondrial and sarcoplasmic reticulum membranes gave relatively large θ_c values (Table I), the values being intermediate between a θ_c of about 20° obtained in saturated lecithin in the gel phase and about 70° in the liquid-crystalline phase (Kawato et al., 1977). Since cholesterol has been shown to reduce the angular range of wobbling motion dramatically (Veatch & Stryer, 1977; Kawato et al., 1978; Hildenbrand & Nicolau, 1979; Lakowicz et al., 1979), the less restricted wobbling of DPH in the two biological membranes probably relates to the small cholesterol content of these membranes: In the mitochondrial membrane, cholesterol accounts for less than 5% of total lipid (Rouser et al., 1968). The inner mitochondrial membrane

Table I: Fluorescence Parameters of DPH in Various Biological Membranes[a]

	temp (°C)	r^s	ϕ (ns)	r_∞	D_w (ns^{-1})	θ_c (deg)	η_c (P)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	τ_s (ns)
purple membrane	10	0.334	4.9	0.276	0.014	27.6	2.73	0.75	1.2	0.25	7.3	2.6	5.0
			(1.9)	(0.018)	(0.004)	(2.4)		(0.03)	(0.2)	(0.03)	(0.3)	(0.6)	(0.1)
	35	0.302	1.9	0.254	0.048	30.5	0.87	0.78	0.8	0.22	6.8	2.2	4.8
			(1.2)	(0.019)	(0.020)	(2.5)		(0.01)	(0.2)	(0.01)	(0.1)	(0.3)	(0.1)
erythrocyte membrane	10	0.295	1.6	0.280	0.038	27.0	1.01			1.00	11.7	11.7	11.7
			(0.4)	(0.003)	(0.008)	(0.4)					(0.2)	(0.2)	(0.2)
	35	0.219	1.1	0.201	0.100	37.4	0.42	0.08	4.3	0.92	11.8	11.1	11.5
			(0.0)	(0.001)	(0.004)	(0.1)		(0.01)	(0.8)	(0.01)	(0.0)	(0.2)	(0.1)
sarcoplasmic reticulum membrane	10	0.220	2.9	0.169	0.043	41.6	0.89	0.20	2.4	0.80	10.7	9.1	10.3
			(0.1)	(0.001)	(0.001)	(0.2)		(0.01)	(0.3)	(0.01)	(0.0)	(0.1)	(0.1)
	35	0.132	1.3	0.091	0.139	53.2	0.30	0.24	1.5	0.76	9.7	7.7	7.4
			(0.0)	(0.001)	(0.002)	(0.2)		(0.02)	(0.2)	(0.02)	(0.1)	(0.2)	(0.1)
mitochondrial membrane	10	0.223	2.6	0.168	0.051	41.7	0.75	0.32	2.9	0.68	9.7	7.5	8.8
			(0.2)	(0.008)	(0.003)	(1.1)		(0.06)	(0.5)	(0.06)	(0.1)	(0.6)	(0.2)
	35	0.138	1.3	0.091	0.146	53.1	0.29	0.33	2.8	0.67	9.1	7.0	8.2
			(0.1)	(0.001)	(0.014)	(0.2)		(0.05)	(0.3)	(0.05)	(0.1)	(0.4)	(0.2)

[a] r^s, steady-state anisotropy; ϕ and r_∞, apparent relaxation time and residual anisotropy in anisotropy decay; D_w, wobbling diffusion constant; θ_c, cone angle; η_c, viscosity in the cone; α_1, τ_1, α_2, and τ_2, intensity decay parameters for double-exponential approximation (eq 5 in text); $\langle\tau\rangle \equiv \alpha_1\tau_1 + \alpha_2\tau_2$, average lifetime; τ_s, intensity decay constant (lifetime) for single-exponential approximation. Numbers in parentheses indicate the standard deviations for two to four independent determinations.

contains almost no cholesterol, and the cholesterol/phospholipid molar ratio in the outer membrane is about 0.12 (Colbeau et al., 1971). The cholesterol/phospholipid molar ratio in sarcoplasmic reticulum membrane has been reported to be below 0.1 from several laboratories (Meissner & Fleischer, 1971; Waku et al., 1971; Sanslone et al., 1971; Madden et al., 1979), though higher values ranging up to 0.2 have also been reported (MacLennan et al., 1971; Drabikowski et al., 1972). On the other hand, erythrocyte membrane is characterized by a high cholesterol content, with a cholesterol/phospholipid ratio of 0.8 to 1 (Van Deenen, 1968; Rouser et al., 1968; Hanahan, 1969). Wobbling of DPH, therefore, is severely restricted (Table I).

The purple membrane contains bacteriorhodopsin as the only protein component, accounting for 75% of the membrane mass. The bacteriorhodopsin molecules form a crystalline lattice in the membrane, with bilayer lipid in between [for a review, see Stoeckenius et al. (1979)]. The wobbling motion of DPH in the purple membrane was notably slow and restricted (Table I). The rigid crystalline lattice apparently hinders and impedes the wobbling motion.

The cone angle θ_c estimated in other biological membranes (Senê et al., 1978; Hildenbrand & Nicolau, 1979) falls within the range of 30–50°. The four biological membranes in the present study thus appear to provide the upper and the lower extremes.

Comparison with Model Systems. Figure 3 summarizes the motional properties of DPH in various model systems analyzed in this laboratory. For comparison, the figure also includes the present results on the biological membranes.

(i) Wobbling motion of DPH in a saturated lecithin, DPPC, has already been reported by Kawato et al. (1977). In the present study, we have reexamined DPPC and DMPC with higher precision. The solid curve from lower left to upper right in Figure 3 shows the temperature-dependent changes in D_w and θ_c in sonicated vesicles of DPPC from 5 to 55 °C, and the dashed curve in sonicated vesicles of DMPC from 5 to 50 °C. The two saturated lecithins are in the gel phase at low temperatures; the vertical portion of the curves at lower left indicate that θ_c remained at about 20° and D_w increased with temperature in the gel phase. Further rise in temperature induced a sharp increase in θ_c, as shown in the relatively horizontal portion of the curves, corresponding to the gel to liquid-crystalline phase transition. The transition occurred at about 38 °C in the DPPC vesicles and at about 22 °C in the DMPC vesicles. Above the transition, change in D_w again dominated over that in θ_c. Note that the whole curves for the two saturated lecithins, differing in the length of the hydrocarbon chains, are almost superimposable with each other, with a slight vertical separation reflecting the difference in the phase transition temperatures. Multibilayer liposomes of DPPC gave basically similar results, except that a sharper transition occurred at 41 °C and that θ_c at all temperatures was smaller than that found in sonicated vesicles (Stubbs et al., 1981). Similar experimental results, though not analyzed in terms of D_w and θ_c, have been reported for DMPC by Chen et al. (1977), and for DMPC, DPPC, and distearoylphosphatidylcholine by Lakowicz et al. (1979).

(ii) Wobbling motions of DPH in bilayers of various unsaturated lecithins were essentially similar with each other (Stubbs et al., 1981). In particular, the activation energies for D_w in all membranes were 7–8 kcal/mol, and the increments of θ_c between 10 and 35 °C were approximately 10°, coinciding with the results on the biological membranes. Figure 3 includes an example, multibilayer liposomes of 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC, two double bonds in position 2 chain). Liposomes of three other unsaturated lecithins with up to four double bonds gave similar D_w–θ_c diagrams in the same region; D_w at 10 °C ranged between 0.06 and 0.1 ns^{-1} and θ_c between 52 and 66°.

(iii) The crosses in the upper left portion of Figure 3 represent D_w and θ_c at temperatures between 10 and 60 °C for DPH in DPPC vesicles containing 33 mol % cholesterol (Kawato et al., 1978). Above the phase transition temperature of DPPC, the addition of cholesterol reduced θ_c whereas D_w was not much affected. Below the phase transition, where pure DPPC was in the gel phase, cholesterol introduced rapid wobbling while θ_c was increased only slightly. We may summarize that the effect of cholesterol is to change θ_c toward that in pure DPPC in the gel phase and D_w toward that in the liquid-crystalline phase, irrespective of temperature. In particular, the main effect on liquid-crystalline lipid is a marked reduction of the angular range of the wobbling motion, as has also been shown by Lakowicz et al. (1979). The effect of cholesterol on bis(dihydrosterculoyl)phosphatidylcholine (Veatch & Stryer, 1977) and on egg yolk phosphatidylcholine and phosphatidylcholine/phosphatidylethanolamine mixtures (Hildenbrand & Nicolau, 1979), all considered to be in the "fluid" state in the absence of cholesterol, was similarly the restriction of DPH wobbling motion without large changes in the rate.

(iv) Cytochrome oxidase, a typical membrane protein, has been incorporated in synthetic lecithin membranes, and the DPH dynamics have been investigated in the reconstituted membranes (Kinosita et al., 1981). Though quenching of DPH fluorescence by heme *a* in the protein hampered precise analysis, the following trend was found at least for those DPH molecules that were not very close (more than or equal to 3.5 nm apart) to heme *a*: When pure lipid (DMPC or DOPC) was in the liquid-crystalline phase, incorporation of the protein reduced the angular range, θ_c, whereas the rate of wobbling, D_w, remained high. Addition of the protein in DMPC in the gel phase did not change θ_c while D_w was apparently increased. Thus, the effect of the protein was similar to that of cholesterol above. Representative results are included in Figure 3; D_w and θ_c for membranes containing cytochrome oxidase are gross indices of the motional properties of "distant" DPH as stated above.

Comparison of the present results on biological membranes with the above results on model systems suggests that, as long as the motional properties in the lipid hydrocarbon chain region are concerned, a biological membrane may be represented, to a first-order approximation, by the following simple formula: biological membrane $\simeq$ unsaturated phospholipid + protein + cholesterol. Compositions within each term in the right-hand side are insignificant; the important factor is the relative amounts of the three components.

Analyses of fatty acid composition in sarcoplasmic reticulum (Nakamura et al., 1976; Moore et al., 1978), mitochondrial (Colbeau et al., 1971), and erythrocyte [a review by Rouser et al. (1968)] membranes have shown that approximately 35–45% of phospholipid acyl chains in these membranes are saturated. Van Deenen (1968) has shown, however, that in many mammalian membranes saturated fatty acids occur predominantly in position 1, and mostly combined with an unsaturated chain at position 2. In human erythrocyte membrane, for example, only 13% of lecithins are disaturated whereas 1-saturated, 2-unsaturated species account for more than 62%. The percentage of unsaturated phospholipid molecules in biological membranes thus appears to be quite high.

Cholesterol levels in sarcoplasmic reticulum and mitochondrial membranes are low or negligible as already mentioned. Then, these membranes may be considered as unsaturated phospholipid plus protein. Figure 3, in fact, shows that D_w and θ_c in these membranes are close to those in DOPC (diunsaturated) plus cytochrome oxidase vesicles, though a better approximation would be PLPC (1-saturated, 2-unsaturated) plus oxidase. The protein/lipid weight ratio in the model membrane was about 1.2, somewhat lower than the ratios in these biological membranes of about 1.5–2 (Meissner & Fleischer, 1971; MacLennan et al., 1971; Colbeau et al., 1971). Exact comparison, however, is difficult because we would have to discount those portions of proteins which are in the aqueous phase. Erythrocyte membrane contains, in addition to predominantly unsaturated phospholipid and protein [protein/lipid ratio is about 1 according to Dodge et al. (1963)], a large amount of cholesterol. Thus, the D_w–θ_c diagram is displaced further toward the left as compared to that of the low-cholesterol membranes.

The small θ_c in the purple membrane could be attributed to the high protein/lipid ratio of about 3. Lipids in the membranes of the halophilic bacterium, however, are unique in that they contain exclusively dihydrophytyl chains without unsaturation (Kates et al., 1965). Thus, the purple membrane is an exception to the above formula, though the methyl groups along the chain may well act as substitutes for the cis double bonds. The D_w–θ_c diagram for this membrane, though not very reliable as discussed below, is rather close to that for saturated lecithins in the gel phase. The probable tight confinement imposed by the protein lattice may force the lipids into an apparent gel phase. The steady-state anisotropy of DPH suggested that this confinement persisted up to 70 °C (data not shown), above which a restructuring of the protein lattice occurs (Jackson & Sturtevant, 1978).

The three-component description above is only the first-order approximation. Neglection of saturated phospholipids, for example, will not be justified when dealing with details. Moreover, the description with a single pair of D_w and θ_c values may be considered too simplistic if heterogeneity of DPH dynamics in a membrane, the magnitude of which is difficult to estimate from the present experiments, is very large. However, we feel that Figure 3 points to the importance of unsaturated lipids in biological membranes at physiological temperatures. Note that the D_w–θ_c diagrams of all systems containing unsaturated lipids are obtained by simple translation of the diagram of pure unsaturated lecithins (PLPC or DOPC).

Wobbling-in-Cone Analysis. The present analysis assumes that each of the rod-shaped DPH molecules in the biological membranes wobbles around a particular direction, i.e., in a more or less conical region, and does not perform a surface-of-cone type nor an outside-of-cone type rotation. This is an unproved assumption. Moreover, the original theory (Kinosita et al., 1977) postulated a simple square-well potential that delimited the orientations of the DPH axis; the validity of such an approximation may be questioned. However, our recent calculation (K. Kinosita, Jr., and A. Ikegami, unpublished results) has shown that the present wobbling-in-cone analysis yields reasonable information even if the actual orientational distribution is of a Gaussian type, which, according to a theory by Jähnig (1979b), may be more appropriate to describe the distribution in a lipid bilayer. Thus, θ_c approximates to the effective width of the Gaussian distribution θ_e, defined as the angle beyond which the fractional population is $1/e^2 = 0.135$ of the total. D_w obtained by applying the present analysis to the Gaussian distribution agrees with the actual rotational rate when the distribution is narrow and becomes an underestimate with a maximal deviation of 35% for wider distributions.

An alternative way of interpretation is to express the results in terms of the "order parameter", which is the square root of r_∞/r_0 (Heyn, 1979; Jähnig, 1979a; Lipari & Szabo, 1980). For example, the DPH order parameter in sarcoplasmic reticulum and mitochondrial membranes is 0.65 at 10 °C and 0.48 at 35 °C. Less assumptions are required in deriving the order parameter. Comparison between the order parameters of different systems, however, is not very meaningful unless the motional types are similar with each other.

It should be noted that the cone angle or the order parameter (including the one obtained by conventional electron spin resonance spectroscopy) refers to the angular range of the wobbling motion of *individual* molecules in the nanosecond or faster time range. They are not necessarily related to the degree of parallelism among molecular orientations, because the axis of the fast wobbling motion (the cone axis) may differ, depending on the position of the molecule; e.g., the cone axis is likely to coincide with the membrane normal in the lipid bilayer region whereas it may well be inclined along the protein surface. If slow reorientations of the cone axis occur in the micro- or millisecond time range, due to the probable translational motion of the molecule, the overall (long-time) angular range will be much wider than the cone angle estimated from fluorescence. The overall angular range can be estimated, for example, by deuterium magnetic resonance spectroscopy. In a heterogeneous system as biological membranes, therefore, the order parameter obtained by deuterium magnetic resonance spectroscopy will in general be smaller than that estimated from fluorescence or electron spin resonance spectroscopy. Deuterium studies (Seelig & Seelig, 1978; Kang et al., 1979) have shown that, above the lipid phase transition, incorporation of cytochrome oxidase reduces the order parameter of deuterated lipid, whereas the wobbling range of DPH became narrow under similar conditions (Figure 3). This apparent discrepancy may also be explained by the above difference: The nanosecond wobbling range at or near the protein surface is narrow, but the axis of the wobbling motion takes variable directions along the irregular surface of the protein. Then, a slow translational motion can account for the low deuterium order parameter.

Viscosity in the Cone. Table I also includes the "viscosity in the cone", η_c, calculated from

$$D_w = \frac{kT}{6\eta_c V_e f} \tag{9}$$

where V_e and f denote the effective volume and the shape factor of the probe, respectively, k is the Boltzmann constant, and T is the absolute temperature. $V_e f$ of DPH was taken as 1.7×10^{-22} cm^3, a value estimated in liquid paraffin (Kawato et al., 1977). This viscosity represents the dynamic friction against the wobbling motion and should be distinguished from the "microviscosity" (Shinitzky & Barenholz, 1978), which depends on both the rate and range of the wobbling motion. As has been shown in several studies (Hildenbrand & Nicolau, 1979; Heyn, 1979; Jähnig, 1979a), the steady-state anisotropy r^s, from which the microviscosity is calculated, can be expressed as the sum of a kinetic term corresponding to the time-dependent part of $r^\delta(t)$ in eq 6 and a static term, r_∞. Table I shows that, in the four biological membranes investigated, more than half of r^s is contributed from r_∞, though r_∞/r^s varies considerably among the four membranes. Since r_∞ depends exclusively on θ_c (eq 7), the microviscosity in biological membranes reflects largely the range of motion rather than the rate. It should be noted, however, that the temperature dependences of r^s shown in Figure 1 resulted from the changes in both D_w and θ_c.

D_w and thus η_c values in mitochondrial, sarcoplasmic, and erythrocyte membranes were close to each other. The microviscosity values for these membranes, by contrast, showed a considerable variation, and the values were an order of magnitude larger than η_c (Shinitzky & Inbar, 1976; Moore et al., 1978). The difference between the two viscosities is due to the contribution of r_∞ to the microviscosity as discussed above. The activation energies for microviscosity in various biological membranes have been reported to fall within 6.5–8.5 kcal/mol (Shinitzky & Inbar, 1976), in fortuitous agreement with the activation energies for D_w of 7–8 kcal/mol.

Fluorescence Lifetime. The decay of the total fluorescence intensity, $I_T(t)$, in colorless membranes, erythrocyte and sarcoplasmic reticulum, was practically single exponential, but $I_T(t)$ in the other two membranes was not. We therefore analyzed $I_T(t)$, assuming two fluorescence lifetimes, τ_1 and τ_2 (eq 5), though a perfect fit for $I_T(t)$ in the purple membrane would have required more than two lifetimes. Similar nonexponential decay of DPH fluorescence in the purple membrane has been reported by Sherman & Caplan (1978). The results of the lifetime analysis are included in Table I.

The average lifetime, $\langle\tau\rangle$, differed significantly among the four membranes. The markedly short $\langle\tau\rangle$ in the purple membrane is due primarily to the excitation energy transfer from DPH to bacteriorhodopsin, as has been suggested by Sherman & Caplan (1978), because the fluorescence spectrum of DPH overlaps with the strong absorption bands of the retinal chromophore. In mitochondrial membrane, hemes and other chromophores give a weak brownish color, which also overlaps with the DPH fluorescence. Here again, the energy transfer is at least partially responsible for the relatively short $\langle\tau\rangle$. The other two membranes, erythrocyte and sarcoplasmic reticulum, were practically colorless. The difference in $\langle\tau\rangle$ between the two colorless membranes may indicate a more polar environment for DPH in the sarcoplasmic reticulum membrane than in the erythrocyte membrane. Alternatively, the more rigid environment in the erythrocyte membrane (see above) may provide better protection against thermal quenching. It has been shown that the fluorescence lifetime of DPH in saturated lecithin bilayers decreased upon the gel to liquid-crystalline phase transiton (Kawato et al., 1977); lifetimes in liposomes of unsaturated lecithins were consistently shorter than those in saturated lecithins (Stubbs et al., 1981). The fluorescence lifetime of DPH thus appears to depend, to some extent, on the motional state of the probe.

The nonexponential character of $I_T(t)$ in the colored membranes is ascribed primarily to excitation energy transfer, because its rate depends critically on the distance between DPH and the acceptor chromophore (Förster, 1965). The problem of fluorescence quenching due to energy transfer to acceptors randomly distributed in a plane has been treated in recent studies (Fung & Stryer, 1978; Wolber & Hudson, 1979). The quenching results in a continuous distribution of fluorescence lifetimes. The double-exponential analysis of $I_T(t)$ in the present study was an approximation, adopted because of technical simplicity.

The distribution of fluorescence lifetimes, with shorter ones preponderant, in the purple membrane introduces some ambiguities in the interpretation of D_w and θ_c, because the contribution to $r(t)$ from short-lived DPH decreases rapidly with time. A possibility thus remains that the motion of short-lived DPH may be more restricted than suggested by θ_c in Table I. Moreover, D_w obtained under the approximation of eq 6 may not necessarily be a proper index of the rate of rotation, because the time dependence of $r(t)$ could result from the decreasing contribution from short-lived components if there is a strong correlation between the fluorescence lifetime and the motional properties.

Added in Proof

Heyn et al. (1981) have also reported high steady-state anisotropy values for DPH embedded in purple membrane, ranging from 0.35 to 6 °C to 0.32 at 66 °C.

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