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# REEVALUATION OF THE WOBBLING DYNAMICS OF DIPHENYLHEXATRIENE IN PHOSPHATIDYLCHOLINE AND CHOLESTEROL/PHOSPHATIDYLCHOLINE MEMBRANES

KAZUHIKO KINOSITA, Jr. and AKIRA IKEGAMI

*Institute of Physical and Chemical Research, Hirosawa 2-1, Wako-shi, Saitama 351 (Japan)*

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**The dynamics of lipid hydrocarbon chains in phosphatidylcholine (dimyristoyl- or dipalmitoyl-) and cholesterol/dimyristoylphosphatidylcholine membranes were investigated by nanosecond time-resolved fluorescence depolarization measurements on a lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene embedded in the membranes. In the pure lipid membranes, both the range (amplitude) and the rate of the wobbling motion of the probe increased sigmoidally with temperature reflecting the thermotropic phase transition of the lipid. The rise in the rate slightly preceded the increase in the range, suggesting that the fluctuation of lipid chains is activated to a high level before the ordered array of chains melt into the liquid-crystalline phase. Above the transition temperature, incorporation of cholesterol resulted in a dramatic decrease in the range of wobbling motion while the rate remained high. Below the transition, on the other hand, cholesterol had little effect on the range, whereas the rate was greatly increased. These effects of cholesterol are remarkably similar to the effects of cytochrome oxidase on lipid chain dynamics (Kinosita, K., Jr., Kawato, S., Ikegami, A., Yoshida, S. and Orii, Y. (1981) Biochim. Biophys. Acta 647, 7–17).**

Dynamics of lipid hydrocarbon chains in membranes can be studied by time-resolved fluorescence depolarization measurements on lipophilic fluorescent probe molecules embedded in the membranes [1,2]. A distinct advantage of the time-resolved method, among other physical techniques such as nuclear magnetic or electron spin resonance spectroscopies, is that both the range (amplitude) and rate of reorientational motion can be estimated separately and simultaneously [2,3]. A recent application has been a study of dynamic structure of biological membranes ([4], reviewed in Ref. 5), where roles of various membrane components in the regulation of the rate and range of chain motion have been elucidated using a probe 1,6-diphenyl-1,3,5-hexatriene. Since accurate experimental data on simple, well-defined model membranes are of crucial importance to such studies and also to theoretical studies of membrane dynamics [6,7] or to comparison between different techniques [8,9], we have reexamined our earlier work on the dynamics of diphenylhexatriene in saturated phospholipid membranes [2] and in membranes containing cholesterol [10]. Time-resolved fluorometric investigations on similar systems have also been made by others [1,11–13]. High precision in the present work has allowed elucidation of several interesting features which were not resolved in the previous studies.

Lipid membranes in the present study were in the form of extensively sonicated vesicles. DPPC and DMPC were obtained from Sigma, and cholesterol from Tokyo Kasei. Thin-layer chromatography on silica gel (solvents were chloro-

Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine.

form/methanol/7 M ammonia (230 : 90 : 15, v/v) and hexane/ether/acetic acid (80 : 30 : 1, v/v); iodine stain) did not show impurity spots at a detection limit of less than 1% of the main spot both before and after sonication. The desired amounts of phospholipid and cholesterol were dissolved in chloroform in a test tube and rotary-evaporated to dryness under high vacuum. A solution containing 50 mM sodium phosphate buffer, pH 7.4, was added, and the contents were vortexed at about 50°C. The obtained suspension was sonicated with a tip sonicator (Choonpa-Kogyo Model USV-150V) at 150 W for 30 min under nitrogen stream, and titanium dust was removed by centrifugation. Diphenylhexatriene in tetrahydrofuran was then added at a diphenylhexatriene to phospholipid molar ratio of 1 : 500, and the suspension was incubated for 1 h in the dark at 35°C (DMPC) or at 50°C (DPPC).

Time-resolved and steady-state fluorescence measurements were carried out and the data analyzed as in Ref. 4. Improved instrumentation allowed measurements at concentrations far below those in the previous studies [2,10], eliminating almost completely the depolarizing effect of light scattering. Observed decays of fluorescence anisotropy following pulsed excitation were analyzed in terms of the wobbling-in-cone model [3], in which the major axis of the rod-shaped diphenylhexatriene molecule was assumed to wobble uniformly in a cone of semi-angle $\theta_c$ with a wobbling diffusion constant $D_w$. (Even if the actual orientational distribution of the diphenylhexatriene axis in the membrane is of a Gaussian type, $\theta_c$ properly represents the angular range in which diphenylhexatriene resides for most of the time [14]). The cone angle, $\theta_c$, is related to the 'order parameter' $S$, by $S^2 = \cos^2\theta_c(1 + \cos\theta_c)^2/4$ [3,8]. The rate, $D_w$, may be converted to the 'viscosity in the cone' $\eta_c$, which represents the dynamic friction against the wobbling motion, by assuming Einstein's relation: $D_w = kT/6\eta_c V_e f$, where $k$ is the Boltzmann constant, $T$ the absolute temperature, and $V_e f = 1.7 \cdot 10^{-22}\text{cm}^3$ is the product of the effective volume and shape factor of diphenylhexatriene [2].

The results on pure phospholipid vesicles are summarized in Fig. 1, where the wobbling diffusion constant, $D_w$, and cone angle, $\theta_c$, for diphenylhexatriene in DMPC (△) and DPPC (○) vesicles

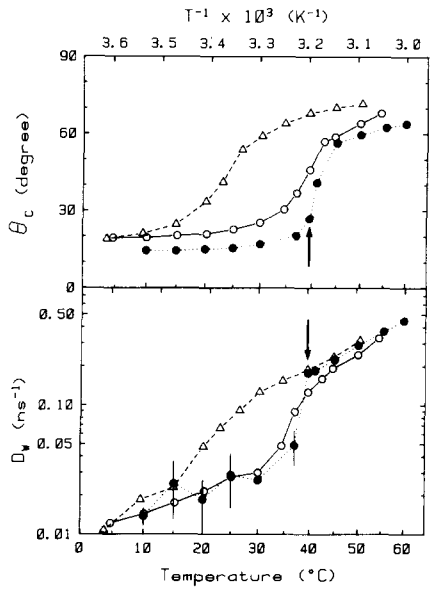


Fig. 1. Temperature dependence of the wobbling diffusion constant, $D_w$, and the cone angle, $\theta_c$, for diphenylhexatriene in DMPC (△) or DPPC (○) vesicles. Concentrations were diphenylhexatriene 200 nM and phospholipid 100 μM. Reproducibility was better than twice the size of the symbols. Data for multilamellar liposomes of DPPC (●) are taken from Ref. 15.

are plotted against temperature. As a reference, data for multilamellar liposomes of DPPC [15] are also included (●). In all samples, $D_w$ and $\theta_c$ increased sigmoidally with temperature, reflecting the gel to liquid-crystalline phase transition: lateral expansion of bilayer at the transition [16] is accompanied by an extensive amplification of both the rate and angular range of wobbling motion. Careful examination of Fig. 1, however, reveals that the rise in $D_w$ preceded that in $\theta_c$, as most easily seen at the points indicated by the arrows. Fluctuations in the ordered array of lipid chains in the gel phase appear to be activated to a high level before the array melts into the liquid-crystalline phase.

The transition in sonicated DPPC occurred at a lower temperature and over a broader range than in multilamellar DPPC. In addition, $\theta_c$ in the small DPPC vesicles was larger than that in the multilamellar liposomes at all temperatures. High curvature in the vesicles apparently introduced extra space for wobbling without much effect on the rate $D_w$. Basic features of the temperature

profile, however, were common to the two forms of membranes.

The $\theta_c$-temperature curves for sonicated DPPC and DMPC in Fig. 1 are superimposable with each other by a horizontal shift. Thus, the cone angle appears to be a function of reduced temperature (deviation from the transition temperature) as has been shown for deuterium order parameter [17]. For $D_w$, however, temperature itself rather than the deviation from the transition temperature appears to be the primary factor. Studies of unsaturated phospholipids [15] have also suggested a similar trend.

At temperatures above the transition, $D_w$ increased with an apparent activation energy of 8–9 kcal/mol, which agrees with the value of 7–8 kcal/mol found in unsaturated phospholipids in the liquid-crystalline phase [15]. A similar value may apply to $D_w$ below the transition, although limited precision precludes definite conclusion. Biological membranes appear to share the same value [4]. The viscosity in the cone, $\eta_c$, was 2.7 poise in DPPC and 2.4 poise in DMPC at 10°C (approx. 200-times the viscosity of water at the same temperature), 0.27 poise in DMPC at 35°C ($\approx 30 \times \eta_{H_2O}$), and 0.18 poise in sonicated DPPC and 0.15 poise in multilamellar DPPC at 50°C ($\approx 30 \times \eta_{H_2O}$).

The effect of cholesterol was studied in sonicated DMPC vesicles. Fig. 2 shows temperature profiles of the steady-state fluorescence anisotropy, $r^s$, at various cholesterol/DMPC ratios. At temperatures where pure DMPC was in the liquid-crystalline phase, $r^s$ increased with cholesterol content indicating slower and/or more restricted wobbling of diphenylhexatriene in the presence of cholesterol. Below the transition temperature, $r^s$ remained high in apparent contradiction to the proposed 'fluidizing' effect of cholesterol in the gel phase [18]. Note that the measurements were made at very dilute concentrations to eliminate depolarization due to scattering. This was important since samples containing cholesterol were slightly turbid at high concentrations.

In order to obtain more specific information, time-resolved measurements were made at two temperatures. The effects of cholesterol on the

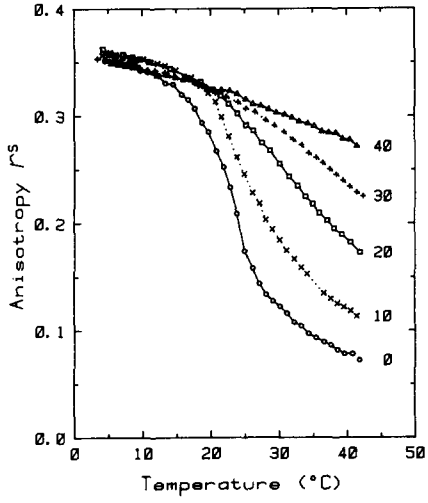


Fig. 2. Temperature dependence of the steady-state fluorescence anisotropy, $r^s$, for diphenylhexatriene in cholesterol/DMPC vesicles. Fluorescence was excited at 360 nm and emission above 420 nm was collected. Concentrations were diphenylhexatriene 40 nM and DMPC 20 $\mu$M. The numbers at the end of the curves denote molar percentage of cholesterol in the membranes. Heating scan at a rate of 30 deg. C/h. Reproducibility of anisotropy values was within $\pm$0.005.

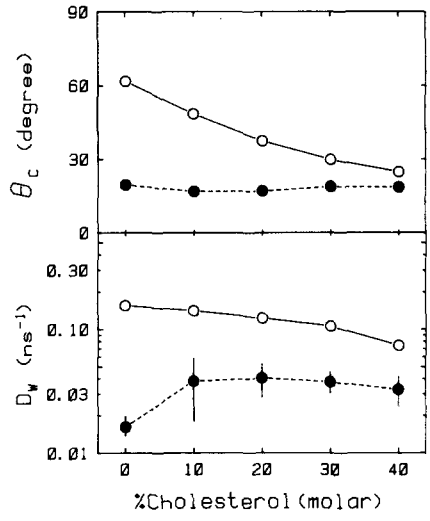


Fig. 3. The wobbling diffusion constant, $D_w$, and the cone angle, $\theta_c$, for the wobbling motion of diphenylhexatriene in sonicated vesicles of DMPC containing cholesterol. ○, at 35°C; ●, at 10°C. Each point is the average of two to four independent measurements, the vertical bar being the standard deviation (where not indicated, the deviation was less than the size of the symbol).

wobbling parameters, $D_w$ and $\theta_c$, are shown in Fig. 3. At 35°C, above the transition temperature of DMPC, addition of cholesterol greatly reduced the range, $\theta_c$, toward the value in pure DMPC in the gel phase. Cholesterol also decreased the rate of motion, $D_w$, although the change was not large in that $D_w$ remained at a high level except at the highest cholesterol content examined. The viscosity in the cone, $\eta_c$, was 0.39 poise at the cholesterol content of 30%. Thus the 'rigidifying' effect of cholesterol in the liquid-crystalline phase [18] manifests itself mainly in the form of reduction in the angular range of wobbling motion. At 10°C in the gel phase, on the other hand, cholesterol had little effect on $\theta_c$. The slight, but significant, decrease in $\theta_c$ at the cholesterol content of 10 and 20% (and the corresponding small increase in $r^s$ in Fig. 2) may be due to the high curvature of the sonicated vesicles; the small increase in $\theta_c$ upon sonication, as seen for DPPC in Fig. 1, may be reversed by the insertion of cholesterol. The major effect of cholesterol in the gel phase was an increase in the rate of motion, $D_w$, which would account for the 'fluidizing' effect. At 10°C, $\eta_c$ of 2.4 poise in pure DMPC decreased to about 1.0 poise in the presence of cholesterol (10–40%). The higher wobbling rate, however, was not reflected in $r^s$, since $r^s$ depends primarily on $\theta_c$ when $\theta_c$ is small.

The effect of cholesterol above is remarkably similar to the effect of cytochrome oxidase on the dynamics of diphenylhexatriene in lipid membranes [19]. In liquid-crystalline phospholipid, incorporation of the membrane protein resulted in a decrease in $\theta_c$ while $D_w$ was unaffected; in the gel phase, the protein increased $D_w$ without changing $\theta_c$. Introduction of basically rigid molecules, such as cholesterol or proteins, in between otherwise vigorously wobbling lipid chains in the liquid-crystalline phase will naturally reduce the angular

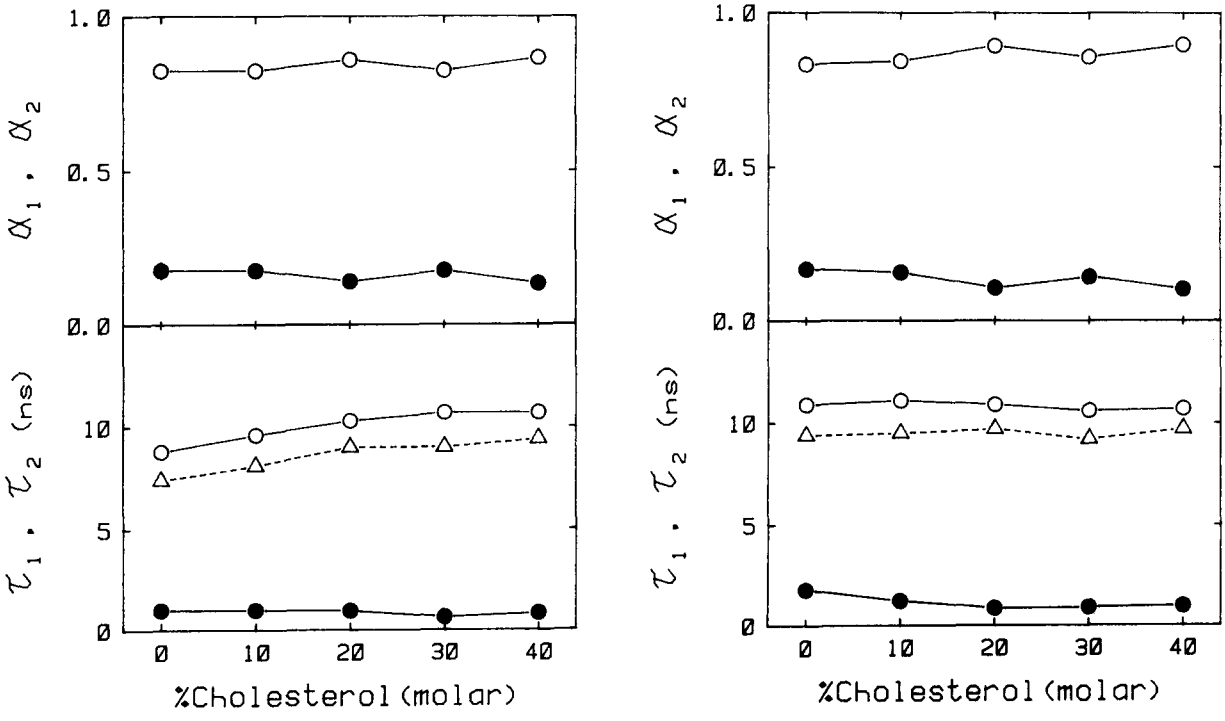


Fig. 4. Fluorescence intensity decay parameters for diphenylhexatriene in cholesterol/DMPC vesicles at 35°C (left) and 10°C (right). The decay of the total fluorescence intensity, $I_T^s(t)$, following a truely impulsive excitation was assumed to consist of two exponential functions: $I_T^s(t) \propto \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$, $\alpha_1 + \alpha_2 = 1$. ●, short-lived component; ○, long-lived component; △, average lifetime, $\langle \tau \rangle \equiv \alpha_1\tau_1 + \alpha_2\tau_2$. Each point is the average of two to four measurements; the maximal standard deviation was less than twice the size of the symbol.

range of the wobbling motion; the wobbling diffusion constant would not change appreciably as long as the major effect remains as a 'rigid wall' effect. In the gel phase, on the other hand, the foreign molecules may disrupt the cooperativity of lipid chain motion, introducing rapid, small-amplitude wobbling motion at the interface. The effect would depend on the closeness of the fit between the foreign molecule and the neighboring lipid chain. In the case of cholesterol, the slightly narrower and flexible tail portion of the molecule may be responsible for the rapid wobbling. Macroscopically, incorporation of cholesterol into lipid membrane in the gel phase reduces the bulk modulus and density of the membrane [20].

Observed decays of the total fluorescence intensity, following the pulsed excitation, of diphenylhexatriene in the membranes were fitted with single- or double-exponential decay functions as previously described [4]. Results of double-exponential analyses for cholesterol/DMPC membranes are plotted in Fig. 4. The component with a longer lifetime (○) accounted for more than 97% of the emission in all samples ($\alpha_2\tau_2/(\alpha_1\tau_1 + \alpha_2\tau_2) > 0.97$): the decays were basically single exponential. Thus, at least the intensity decay did not show any evidence of heterogeneity in the membranes containing cholesterol. At 35°C, inclusion of cholesterol slightly increased the average lifetime of diphenylhexatriene, whereas the lifetime was almost unchanged at 10°C. Comparison with Fig. 3 suggests that small cone angles ensure a long lifetime for diphenylhexatriene fluorescence. Among several biological membranes, erythrocyte membrane with a high cholesterol content and a small $\theta_c$ gave the longest lifetime [4].

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