Effects of lateral diffusion on the fluorescence anisotropy in hexagonal lipid phases

I. Theory

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ABSTRACT It is shown that fluorescence anisotropy from lipidlike probes in the hexagonal H_{II} phase gives information of (*a*) orientational order parameters, (*b*) the wobbling diffusion constant, and (*c*) the hopping diffusion constant of the probe, D_H, equals D_L/R^2, the lateral diffusion constant over the square of the radius of the hexagonal tubes. Here we consider only lipidlike probes having the absorption transition moment and/or the emission transition moment along the long axis of the molecule. Three models are introduced for analysis of time-resolved data: the "WOBHOP," the "reduced WOBHOP," and the "P2P4HOP" model. The fluorescence anisotropy in response to a very short excitation pulse in each of the three models is a constant plus a number of exponentials. The WOBHOP and reduced WOBHOP models have 3 and 2 exponentials, respectively, and both contain four fitting parameters: r_0 (the fundamental anisotropy), $\langle P_2 \rangle$ (the second rank orientational order parameter), D_w (the wobbling diffusion constant), and D_H (the hopping diffusion constant). The P2P4HOP model has eight exponentials and five fitting parameters: the four parameters listed above and $\langle P_4 \rangle$ (the fourth rank orientational order parameter). Analysis of fluorescence anisotropy data in the hexagonal H_{II} phase using one of these models allows for obtaining the hopping diffusion constant, and, if the lateral diffusion constant is known, the radius of the hexagonal tubes. Substitution of $D_H = 0$ in each of the three models yields an expression for the fluorescence anisotropy that is used in the literature for lamellar (L_α or L_β) phases. The fluorescence anisotropy in coexisting L_α/H_{II} phases is discussed.

INTRODUCTION

In the inverted hexagonal (H_{II}) phase the lipids form cylindrical tubes containing an aqueous phase. The walls of these cylindrical channels are two lipids thick. The polar head groups of the lipids face the inside of the tubes which are parallel to each other and their centers form a two-dimensional hexagonal lattice in a cross-section perpendicular to the cylinders. The H_{II} phase is of interest not only because of its unique structure and symmetry (1), but also because of its relevance for understanding certain membrane functions such as fusion (1, 2, 3, and 4) and protein-mediated ion transport (5, 6). X-Ray diffraction has provided detailed information on the structure of this phase (1, 7, 8). Time-resolved (9, 10) and angle-resolved (11) fluorescence anisotropy yields structural as well as dynamic information on this phase. One of the results of this paper and the accompanying paper (10) is that the combined information from fluorescence anisotropy and lateral diffusion measurements in the H_{II} phase allows one to estimate the radius of the hexagonal tubes.

The change in orientation of a fluorescent lipid hopping around a tube in the hexagonal H_{II} phase during the fluorescence lifetime is $(D_L\tau)^{1/2}/R$ (in radians), if the lipid is constrained to have its long axis perpendicular to the lipid-water interface. Here D_L is the later diffusion constant, τ is the fluorescence lifetime, and R is the radius of curvature of the lipid tubes (see Fig. 1). Using $D_L \approx 0.01$ nm^2/ns (10), $R \approx 1$ nm (8), and $\tau \approx 7$ ns (10) an order of magnitude estimate of this change in orientation yields ~16 degrees corresponding to a depolarization of a factor, $1.5 \cos^2 16° - 0.5 = 0.89$, which is appreciable. This estimate indicates that fluorescence depolarization can be employed to measure D_L/R^2. The present paper is a theoretical evaluation of such effects of lateral diffusion on the fluorescence anisotropy in hexagonal lipid phases. Three models are introduced which we will call the "WOBHOP," the "reduced WOBHOP," and the "P2P4HOP" models. Formulas are derived for analyzing time-resolved data both for experiments in the frequency-domain and for studies using the pulse method. The fluorescence anisotropy in coexisting L_α/H_{II} phases is examined. The implications of the present analysis and the underlying assumptions are considered, and the relevance of the hexagonal phase for membrane research is discussed briefly.

GLOSSARY

FA	Fluorescence Anisotropy
t	time
r_0	fundamental FA = FA in the absence of motion
r	steady-state FA

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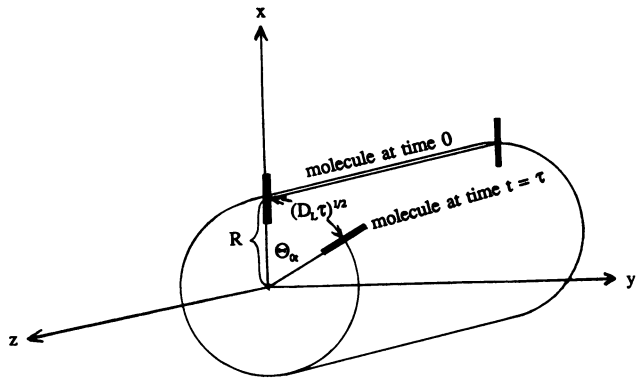

FIGURE 1 A lipid probe, represented as a solid bar, changes its orientation when diffusing around a hexagonal tube in a direction perpendicular to the z-axis, the axis of the tube. When it diffuses parallel to this axis, however, it will not change its orientation. Here R is the radius of curvature of the lipid–water interface, D_L is the lateral diffusion constant, τ is the fluorescence lifetime, and Θ_{0t} is the angle between the molecular axis at time zero, the time of the excitation flash, and that at a later time $t = \tau$. This angle equals $(D_L\tau)^{1/2}/R$ (in radians) for diffusion in a direction perpendicular to the z-axis.

r_∞	limiting FA = FA response to a very short pulse, a long time after the pulse
$r(t)$	FA response a time t after a very short pulse
τ	fluorescence lifetime
R	radius of hexagonal tubes = radius of curvature of lipid-water interface
D_W	wobbling diffusion constant of lipidlike probe
D_L	lateral diffusion constant of lipidlike probe
D_H	hopping diffusion constant of lipidlike probe = D_L/R^2
Q_W	depolarization factor due to wobbling
Q_H	depolarization factor due to hopping
$\langle P_2 \rangle$	2nd rank orientational order parameter of lipidlike probe
$\langle P_4 \rangle$	4th rank orientational order parameter of lipidlike probe
τ_W	$(1 - \langle P_2 \rangle^2)/(6D_W)$
τ_H	$1/(4D_H)$
Θ_{0t}	the angle between the long molecular axis at time t and that at time 0
$\langle\langle 3\cos^2\Theta_{0t} - 1)/2\rangle\rangle$	the ensemble average of $(3\cos^2\Theta_{0t} - 1)/2$
$\varnothing_0$	the azimuthal angle of the long molecular axis at time 0
$\varnothing_t$	the azimuthal angle of the long molecular axis at time t
$g(\varnothing_0 \mid \varnothing_t, t)$	the conditional probability of a molecular azimuthal angle being equal to $\varnothing_t$ at time t while having the value $\varnothing_0$ at time 0
N	the number of exponentials in $r(t)$
g_i	pre-exponential coefficients in $r(t) = r_\infty + (r_0 - r_\infty)\,\Sigma\, g_i \exp[-A_i t]$ (i: 1 to N)
A_i	exponential coefficients in $r(t) = r_\infty + (r_0 - r_\infty)\,\Sigma\, g_i \exp[-A_i t]$ (i: 1 to N)
WOBHOP model	model for the FA in the H_{II} phase based on the assumption that hopping and wobbling give rise to independent depolarization factors
$r_{WH}(t)$	expression for $r(t)$ in the WOBHOP model having $N = 3$ and fitting parameters: r_0, $\langle P_2 \rangle$, D_W, and D_H
Reduced WOBHOP	same as the WOBHOP model but with the additional assumption that τ_H is much larger than τ_W
$r_{RW}(t)$	expression for $r(t)$ in the Reduced WOBHOP model having $N = 2$ and fitting parameters: r_0, $\langle P_2 \rangle$, D_W, and D_H
P2P4HOP model	model for the FA in the H_{II} phase based on an extension of the "second approximation" (14) for lamellar phases
$r_{PH}(t)$	expression for $r(t)$ in the P2P4HOP model having $N = 8$ and fitting parameters: r_0, $\langle P_2 \rangle$, $\langle P_4 \rangle$, D_W, and D_H
DC	time-independent contribution to sinusoidally modulated intensity
AC	amplitude of fluctuating contribution to sinusoidally modulated intensity
F	modulation frequency
M_V	modulation factor of the vertically polarized component of the fluorescence
M_H	same for the horizontally polarized component of the fluorescence
$\varnothing_V$	phase angle of the vertically polarized component of the fluorescence
$\varnothing_H$	phase angle of the horizontally polarized component of the fluorescence
r_w	frequency-dependent FA = $[M_V - M_H]/[M_V + 2M_H]$
p	fraction of hexagonal phase lipids in coexisting L_α/H_{II} phases

WOBHOP MODEL

How does hopping, that is, lateral diffusion, affect the depolarization of fluorescence from lipid-like probes? To illustrate the effect of hopping let us consider a macroscopically isotropic sample in the H_{II} phase where the lipids and the probes are exactly perpendicular to the lipid-water interface. Restricting ourselves to probes having the absorption and/or emission dipole along the molecular axis, the fluorescence anisotropy after excitation with a

very narrow pulse is

$$r(t) = r_0 Q_H \quad Q_H = \langle\langle(3\cos^2\Theta_{0t} - 1)/2\rangle\rangle, \tag{1}$$

where $r(t)$ is the fluorescence anisotropy (FA) at time t, r_0 is the fundamental anistropy (r_0 is the FA in the absence of motion) and $\Theta_{0t} = \varnothing_0 - \varnothing_t$ is the angle between the molecular axis at time zero, the time of the flash, and that at a later time t (12, 13). The double brackets indicate an ensemble average. As is indicated in Fig. 1 diffusion in the z-direction will not change the orientation of a molecule. The z-axis has been chosen along the local cylinder axis. Hopping in a direction perpendicular to the z-axis along the surface, however, does change the orientation of the molecule which is constrained to keep its axis perpendicular to the cylindrical surface. In this case of perfect orientational order the correlation function in Eq. 1 reduces to

$$\begin{aligned} Q_H &\equiv \langle\langle 1.5\cos^2\Theta_{0t} - 0.5\rangle\rangle \\ &= \langle\langle 0.25 + 0.75\cos(2\varnothing_0 - 2\varnothing_t)\rangle\rangle \\ &= [1/(2\pi)]\int_0^{2\pi} d\varnothing_0 \int_0^{2\pi} d\varnothing_t g(\varnothing_0|\,\varnothing_t, t) \\ &\quad \cdot \{0.25 + 0.75\cos(2\varnothing_0 - 2\varnothing_t)\}, \end{aligned} \tag{2}$$

where $g(\varnothing_0 \mid \varnothing_t, t)$ is the conditional probability of a molecular azimuthal angle being equal to $\varnothing_t$ at time t while having the value $\varnothing_0$ at time zero. This conditional probability is the solution of the equation for diffusion on a cylindrical surface (part for the azimuthal angle). This solution can be shown to read,

$$\begin{aligned} &g(\varnothing_0|\,\varnothing_t, t) \\ &= (1/\pi)\{0.5 + \sum_{n=1}^{\infty}\cos(n\varnothing_t - n\varnothing_0)\exp(-n^2 D_L t/R^2)\}, \end{aligned} \tag{3}$$

where D_L is the lateral diffusion coefficient for the lipidlike probe and R is the radius of curvature of the cylindrical surface. Evaluating the integrals in Eq. 2 gives

$$Q_H = \{1 + 3\exp(-4D_L t/R^2)\}/4. \tag{4}$$

Substituting Eq. 4 into Eq. 1 yields

$$r(t) = r_0\{1 + 3\exp(-4D_L t/R^2)\}/4. \tag{5}$$

Assuming mono-exponential decay of the fluorescence intensity, the steady-state FA for the H_{II} phase in this case of perfect orientational order, is

$$\begin{aligned} r &= \int_0^{\infty} dt\, r(t)\exp(-t/\tau)/\int_0^{\infty} dt\exp(-t/\tau) \\ &= r_0[1 + D_L\tau/R^2]/[1 + 4D_L\tau/R^2]. \end{aligned} \tag{6}$$

(Compare this result with the steady-state FA for a spherical micelle of radius R in the case of perfect orientational order, which reads

$$r = r_0/(1 + 6D_L\tau/R^2). \tag{6a}$$

Note that r in Eq. 6a goes to zero whereas r in Eq. 6 goes to 1/4, if D_L goes to infinity.) In deriving Eq. 5 perfect orientational order has been assumed and the FA contains only one depolarization factor namely Q_H, the depolarization factor due to hopping. If the orientational order is not perfect, wobbling causes depolarization as well. A model for the depolarization effect of wobbling in the absence of hopping (14, 15) is

$$r(t) = r_0 Q_W, \tag{7}$$

where Q_W is

$$Q_W = \langle P_2\rangle^2 + (1 - \langle P_2\rangle^2)\exp[-6D_W t/(1 - \langle P_2\rangle^2)]. \tag{8}$$

Here D_W is the wobbling diffusion constant and $\langle P_2\rangle$ is the second rank orientational order parameter (14). In this model it is assumed that the FA can be approximated by one exponential plus a constant. The correlation time $\tau_W = (1 - \langle P_2\rangle^2)/(6D_W)$ has been chosen such that the time derivative of $r(t)$ at $t = 0$ is in agreement with that following from the wobbling diffusion equation ("first approximation" in reference 14). A simple model that takes into account the combined effect of hopping along a cylindrical surface and of wobbling within an ordering potential can be derived by assuming that the hopping and wobbling factors are independent depolarization factors a la Soleillet (16, 17):

$$\begin{aligned} r(t) &= r_{WH}(t) \\ &= r_0 Q_W Q_H = 0.25 r_0\{\langle P_2\rangle^2 + 3\langle P_2\rangle^2\exp[-t/\tau_H] \\ &\quad + (1 - \langle P_2\rangle^2)\exp[-t/\tau_W] \\ &\quad + 3(1 - \langle P_2\rangle^2)\exp[-(\tau_W + \tau_H)t/(\tau_W\tau_H)]\}, \end{aligned} \tag{9}$$

where Q_W is given by Eq. 8 and Q_H by Eq. 4, τ_H is an abbreviation for $0.25/D_H = 0.25R^2/D_L$, and τ_W has been defined above. We will call this model the "WOBHOP" model. It contains the molecular parameters D_W (wobbling diffusion constant), $\langle P_2\rangle$ (the second rank orientation order parameter), and the hopping diffusion constant $D_H = D_L/R^2$. It also contains the fluorescent probe parameter r_0, the fundamental anisotropy.

REDUCED WOBHOP MODEL

Experiments presented in reference 10 suggest that τ_H is of the order of 10 to 20 ns and τ_W is of the order of 1 ns. Consequently for a large range of time values the last

term in Eq. 9 can be approximated as follows,

$$3(1 - \langle P_2\rangle^2) \exp[-(\tau_W + \tau_H)t/(\tau_W\tau_H)] \approx 3(1 - \langle P_2\rangle^2) \exp[-t/\tau_W].$$

In this approximation $r(t)$ becomes:

$$r(t) = r_{RW}(t) = 0.25r_0\{\langle P_2\rangle^2 + 3\langle P_2\rangle^2 \exp[-t/\tau_H] + 4(1 - \langle P_2\rangle^2) \exp[-t/\tau_W]\}. \quad (10)$$

We will call this model for the FA the "Reduced WOBHOP" model. Fig. 2 illustrates that the difference between the WOBHOP and the Reduced WOBHOP model is small if the ratio τ_H/τ_W is large.

P2P4HOP MODEL

If one does not assume that the wobbling and hopping factors are independent, and approximates each correlation function (see Appendix 1) as a constant plus an exponential with a correlation time that follows from the short-time behavior of the correlation function, then one obtains an expression for the FA which we will call the "P2P4HOP" model:

$$\begin{aligned} r(t) &= r_{PH}(t) \\ &= 0.25r_0\{(\langle P_2\rangle^2 + B_0 \exp[-C_0t])(1 + 3 \exp[-t/\tau_H]) \\ &\quad + 4B_1 \exp[+C_1t] (\exp[-0.25t/\tau_H] + \exp[-t/\tau_H]) \\ &\quad + B_2 \exp[-C_2t](3 + 4 \exp[-0.25t/\tau_H] \\ &\quad + \exp[-t/\tau_H])\}, \end{aligned} \quad (12)$$

where B_0, C_0, B_1, C_1, B_2, and C_2 are given in Table 1. The derivation of this model is presented in Appendix 1 (see below). Fig. 3 demonstrates the dependence of $r_{PH}(t)/r_0$ on time and the hopping diffusion constant.

SUMMARY FLUORESCENCE ANISOTROPY MODELS

The three models introduced in this paper, the Reduced WOBHOP, WOBHOP, and P2P4HOP model all contain a constant and N (N = 2, 3, or 8) exponentials having the following form:

$$r(t) = r_\infty + (r_0 - r_\infty) \sum g_i \exp[-A_it], \sum g_i = 1, \quad r_\infty = 0.25r_0\langle P_2\rangle^2. \quad (13)$$

The models are summarized in Table 1.

FREQUENCY-DOMAIN EQUATIONS

The information acquired by studying the fluorescence response to short pulses can be obtained by Frequency-Domain Fluorescence Spectroscopy as well (see, for example, reviews by Gratton et al. [18] and Lakowicz [19]). In this technique the exciting beam is intensity-modulated at frequency F:

$$\text{EXCITATION proportional to } DC + AC \sin(2\pi Ft), \quad (14)$$

where DC is the time-independent contribution and AC the amplitude of the fluctuating contribution to the intensity. Consequently, the vertical (or parallel) component, I_V, and the horizontal (or perpendicular) component, I_H, of the fluorescence will also be modulated at the

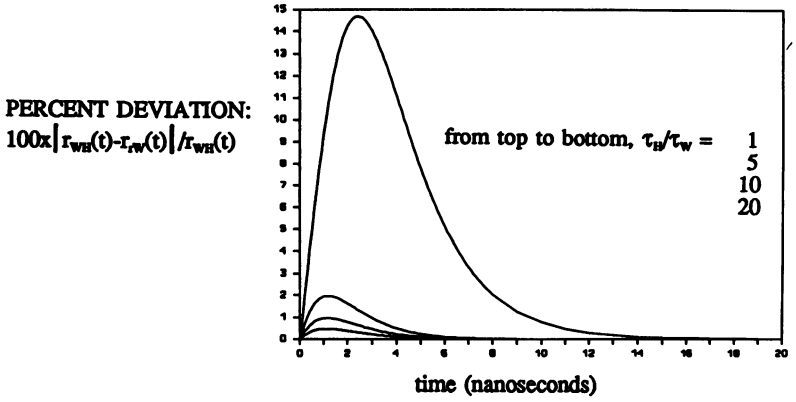

FIGURE 2 The percent deviation, defined as $100|r_{WH}(t) - r_{RW}(t)|/r_{WH}(t)$, vs. the time, t, for $\langle P_2\rangle = 0.85$, $\tau_W = 1$ nanosecond and four different values of the ratio τ_H/τ_W.

TABLE 1 Summary fluorescence anisotropy models

	i	g_i	A_i
Reduced WOBHOP			
$[N = 2, r(t) = r_{RW}(t)]$	1	$3\langle P_2\rangle^2/(4 - \langle P_2\rangle^2)$	$1/\tau_H$
	2	$4(1 - \langle P_2\rangle^2)/(4 - \langle P_2\rangle^2)$	$1/\tau_W$
WOBHOP			
$[N = 3, r(t) = r_{WH}(t)]$	1	$3\langle P_2\rangle^2/(4 - \langle P_2\rangle^2)$	$1/\tau_H$
	2	$(1 - \langle P_2\rangle^2)/(4 - \langle P_2\rangle^2)$	$1/\tau_W$
	3	$3(1 - \langle P_2\rangle^2)/(4 - P_2\rangle^2)$	$(\tau_W + \tau_H)/(\tau_W\tau_H)$
P2P4HOP			
$[N = 8, r(t) = r_{PH}(t)]$	1	$3\langle P_2\rangle^2/(4 - \langle P_2\rangle^2)$	$1/\tau_H$
	2	$B_0/(4 - \langle P_2\rangle^2)$	C_0
	3	$3B_0/(4 - \langle P_2\rangle^2)$	$C_0 + 1/\tau_H$
	4	$4B_1/(4 - \langle P_2\rangle^2)$	$C_1 + 0.25/\tau_H$
	5	$4B_1/(4 - \langle P_2\rangle^2)$	$C_1 + 0.25/\tau_H$
	6	$B_2/(4 - \langle P_2\rangle^2)$	C_2
	7	$4B_2/(4 - \langle P_2\rangle^2)$	$C_2 + 0.25/\tau_H$
	8	$3B_2/(4 - \langle P_2\rangle^2)$	$C_2 + 1/\tau_H$

with:

i	B_i	C_i
0	$0.2 + 2\langle P_2\rangle/7 + 18\langle P_4\rangle/35 - \langle P_2\rangle^2$	$6D_W(0.2 + \langle P_2\rangle/7 - 12\langle P_4\rangle/35)/B_0$
1	$0.2 + \langle P_2\rangle/7 - 12\langle P_4\rangle/35$	$6D_W(0.2 + \langle P_2\rangle/14 - 8\langle P_4\rangle/35)/B_1$
2	$0.2 - 2\langle P_2\rangle/7 + 3\langle P_4\rangle/35$	$6D_W(0.2 - \langle P_2\rangle/7 - 2\langle P_4\rangle/35)/B_2$

$\tau_H = 0.25R^2/D_L$, $\tau_W = (1 - \langle P_2\rangle^2)/(6D_W)$, $\langle P_4\rangle$ = 4th rank orientational order parameter (see Appendix 1).

same frequency but will be shifted in phase,

$$I_V \text{ proportional to } DC + M_V AC \sin(2\pi Ft - \varnothing_V) \quad (15)$$

$$I_H \text{ proportional to } DC + M_H AC \sin(2\pi Ft - \varnothing_H), \quad (16)$$

where M_V and M_H are the demodulation factors of the vertical and horizontal components, respectively, and $\varnothing_V$ and $\varnothing_H$ are the corresponding phase angles. In Appendix 2 it is shown that the differential phase, $\varnothing_H - \varnothing_V$, and the frequency-dependent anisotropy, $r_w = (M_V - M_H)/(M_V + 2M_H)$ (19), are given by

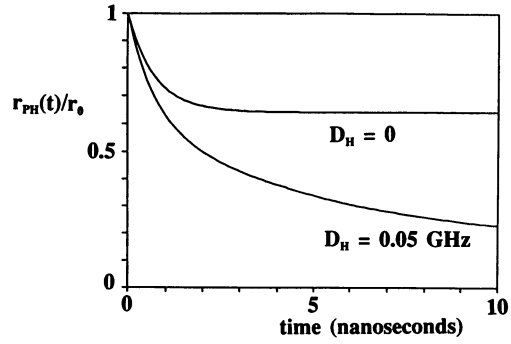

FIGURE 3 The time-dependence of the Fluorescence Anisotropy in the P2P4HOP model for two different values for the hopping diffusion constant. In this simulation of time-resolved data the values for the other parameters were: $\langle P_2\rangle = 0.8$, $\langle P_4\rangle = 0.6$, and $D_W = 0.2$ GHz (GHz = 1/ns).

$$\varnothing_H - \varnothing_V = \tan^{-1}\{3u(r_0 - r_\infty)(G - S)/[(1 - r_\infty)(1 + 2r_\infty)H_1 + (1 - 4r_\infty)H_2 - 2H_3]\} \quad (17a)$$

$$u = 2\pi F\tau \quad (17b)$$

$$H_1 = 1 + u^2 \quad (17c)$$

$$H_2 = (r_0 - r_\infty)(G + u^2S) \quad (17d)$$

$$H_3 = (r_0 - r_\infty)^2(G^2 + u^2S^2) \quad (17e)$$

$$G = \sum_{i=1}^{N} g_i H_1(1 + A_i\tau)/[(1 + A_i\tau)^2 + u^2] \quad (17f)$$

$$S = \sum_{i=1}^{N} g_i H_1/[(1 + A_i\tau)^2 + u^2] \quad (17g)$$

$$r_w = (Y^{1/2} - 1)/(Y^{1/2} + 2) \quad (17h)$$

$$Y = [(1 + 2r_\infty)^2H_1 + 4(1 + 2r_\infty)H_2 + 4H_3]/[(1 - r_\infty)^2H_1 - 2(1 - r_\infty)H_2 + H_3]. \quad (17i)$$

These equations are applicable for all the three models introduced above with the corresponding coefficients g_i and A_i listed in Table 1. Fig. 4 demonstrates the depen-

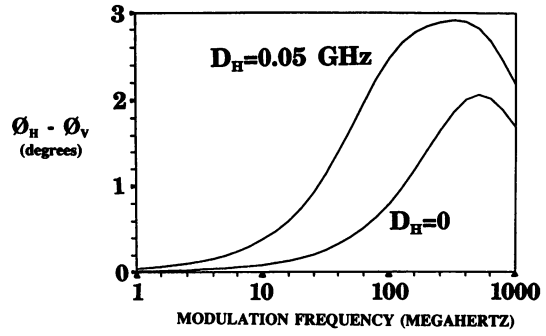

FIGURE 4 Phase angle difference between the horizontal and vertical component versus modulation frequency for the P2P4HOP model with $\langle P_2 \rangle = 0.8$, $\langle P_4 \rangle = 0.6$, and $D_W = 0.2$ GHz.

dence of $\varnothing_H - \varnothing_V$ on modulation frequency and on the hopping diffusion constant for the P2P4HOP model.

FLUORESCENCE ANISOTROPY IN COEXISTING Lα AND H_{II} PHASES

Consider a sample containing lipidlike fluorescent probes incorporated in coexisting lamellar L_α and hexagonal H_{II} phases. According to the Weber-Jablonski addition theorem (20, 21) for anisotropies, the FA of the sample is a linear combination of the FA contribution from the hexagonal phase and that from the lamellar phase. Applying this theorem to the FA response to a short pulse at times much larger than the fluorescence lifetime, we obtain:

$$r_\infty = p_H r_{\infty_H} + p_L r_{\infty_L}, \quad (18)$$

where p_H and p_L are the fractional fluorescence intensities from the hexagonal and lamellar phases, respectively, and $r_{\infty H}$ and $r_{\infty L}$ are the corresponding limiting anisotropies in the two phases. If the extinction coefficients, the lifetimes, the quantum yields, and the solubility of the probe are the same in the two phases, then $p_H = p =$ the fraction of lipids in the hexagonal phase, and $p_L = 1 - p$. If the order parameter $\langle P_2 \rangle$ is also equal in both phases, then $r_{\infty L} = 4r_{\infty H} = r_0 \langle P_2 \rangle^2$, and we obtain:

$$r_\infty = r_0 \langle P_2 \rangle^2 (4 - 3p)/4. \quad (19)$$

This expression differs from the corresponding equation derived by Johanssen and Lindblom (22).

DISCUSSION

The present paper introduces three models for the FA in the hexagonal (H_{II}) phase which take into account the depolarization due to lateral diffusion around the hexagonal tubes. By setting the hopping diffusion constant equal to zero, the models reduce to expressions for the FA in bilayer membranes. Consequently the models can be used for analysis of FA studies of hexagonal-lamellar phase transitions (9, 10).

Equations have been derived for time-resolved fluorescence depolarization studies (both frequency- and time-domain) using probes that have the absorption and/or the emission moment along the long axis of the molecule. However, if the rotational correlation time for rotations about the long molecular axis is much shorter than the correlation time for wobbling, τ_W, and the fluorescence lifetime, then our equations for the fluorescence anisotropy, $r(t)$, would be correct even if both the absorption and emission moment would make an angle with the long molecular axis. In that case r_0 should be replaced by $r_{0\text{-EFFECTIVE}}$, given by

$$r_{0\text{-EFFECTIVE}} = 0.1\,(3\cos^2\Theta_A - 1)(3\cos^2\Theta_E - 1),$$

where Θ_A and Θ_E denote the angles between the absorption moment and the long axis, and between the emission moment and the long axis, respectively. Typically, the rotational correlation time for rotation about the long axis of a lipid is a factor 20–100 shorter than τ_W (25) and the fluorescence lifetime is of the same order of magnitude as τ_W (10). This estimate indicates that the rotation around the molecular axis of a lipid is indeed too fast to observe in a fluorescence depolarization experiment and that our equations could be applicable to the case where Θ_A and Θ_E are both nonzero with the substitution $r_0 = r_{0\text{-EFFECTIVE}}$.

It should be noted that we have considered one wobbling mode only, whereas the combination of internal motion of the fluorophore within the molecule and the wobbling of the molecule as a whole, could give rise to several wobbling modes. At present it does not seem feasible to extract from the data information on various modes of wobbling in addition to parameters on hopping and over-all wobbling.

Two of the three models introduced, the "WOBHOP" and "Reduced WOBHOP" models, are based upon the assumption that the depolarization due to wobbling and that due to hopping (lateral diffusion) are independent depolarization factors. The difference between these two models is that in the Reduced WOBHOP model it is assumed that the rotational correlation time for hopping is much slower than the one for wobbling. When analyzing the FA in detail, however, one finds that the depolarization factors due to hopping and wobbling are only completely independent if the orientational order is complete. The P2P4HOP model is based on such a detailed analysis. It is an extension of the "second approximation" for the FA in lamellar phases introduced in reference 14.

In all three models we have assumed that the deviation from cylindrical symmetry around the normal to the lipid-water interface is small. If the z-axis is chosen along the local hexagonal tube axis, the orientational fluctuations along the x-axis or y-axis must differ from those along the z-axis. We have assumed that such deviations from uniaxial symmetry can be ignored. Van Langen et al. have also made this assumption in their theory of angle-resolved fluorescence depolarization in oriented hexagonal phases (11). Experimental results from Turner and Gruner (23) indicate that deviations from axial symmetry are <10%.

Johansson and Lindblom (22) have analyzed the FA in a system where hexagonal and lamellar phases coexist. They concluded that under certain conditions the limiting FA, r_∞, could abruptly drop to zero in response to relatively small variations in composition or temperature. We believe that this conclusion is not correct as it is based upon the erroneous assumption that the equilibrium orientational distribution function is a linear combination of such functions for the hexagonal and lamellar phases. In our opinion, it is not this distribution function but the FA that is additive as required by the Weber–Jablonski addition theorem (20, 21).

The biological relevance of the onset of nonbilayer phases in membranes has been discussed (see for example, references 1, 2, 4, 5, and 24). To the best of our knowledge the following hypothesis has not yet been proposed: if the composition of biological membrane would be modified, for example, by enriching it with phosphatidylethanolamines, in such a way that certain regions in the membrane would approach a transition to a nonbilayer phase, then the exposition of membrane-bound antigens or receptors in that membrane region could change as a result of an increased curvature of the bilayer in that region. This possible consequence of the onset of the H_{II} phase in membranes could be studied using the fluorescence anistropy methods proposed here and in the accompanying paper (10).

APPENDIX 1

The depolarization factor $\langle\langle 1.5 \cos^2 \Theta_{0t} - 0.5\rangle\rangle$ can be decomposed in correlation functions as follows:

$$\begin{aligned}\langle\langle 1.5 \cos^2 \theta_{0t} - 0.5\rangle\rangle &= \langle\langle P_2(\cos\theta_0) P_2(\cos\theta_t)\rangle\rangle \\ &+ 0.75\,\langle\langle \sin^2\theta_0 \cos 2\varnothing_0 \sin^2\theta_t'' \cos 2\varnothing_t''\rangle\rangle \\ &+ 0.75\,\langle\langle \sin^2\theta_0 \sin 2\varnothing_0 \sin^2\theta_t'' \sin 2\varnothing_t''\rangle\rangle \\ &+ 0.75\,\langle\langle \sin 2\theta_0 \cos\varnothing_0 \sin 2\theta_t'' \cos\varnothing_t''\rangle\rangle \\ &+ 0.75\,\langle\langle \sin 2\theta_0 \sin\varnothing_0 \sin 2\theta_t'' \sin\varnothing_t''\rangle\rangle. \qquad \text{(A1)}\end{aligned}$$

The angles θ and $\varnothing$ denote the polar and azimuthal angle of the axis of a probe molecule, where θ is the angle between a molecular axis and the x-axis, and $\varnothing$ is the angle between the projection of the molecular axis on the y,z-plane and the z-axis. We define a time-dependent coordinate system ($x[t]$, $y[t]$, $z[t]$) having the x-axis along the normal to the interface at the position of the molecule for time t; the z-axis of this coordinate-system is along the local cylinder axis (see Fig. 5). Consequently, the z-axis is constant in time, $z(t) = z(0)$, but the orientation of the x- and y-axes will change with time. The double prime ('') on the angular coordinates for the molecule at time t indicates that these angles are not taken with respect to the coordinate system ($x[t]$, $y[t]$, $z[t]$) but with respect to ($x[0]$, $y[0]$, $z[0]$). The spherical harmonics $P_2(\cos\theta_t'')$, $\sin^2\theta_t'' \cos 2\varnothing_t''$, $\sin^2\theta_t'' \sin 2\varnothing_t''$, $\sin 2\theta_t'' \cos\varnothing_t''$, $\sin 2\theta_t'' \sin\varnothing_t''$ can be expressed in terms of spherical harmonics of θ_t and $\varnothing_t$ as follows:

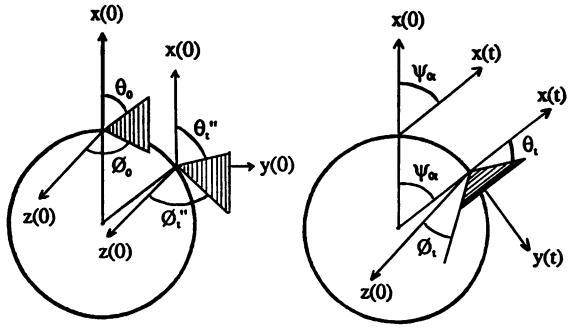

FIGURE 5 The coordinate systems ($x[0]$, $y[0]$, $z[0]$) and ($x[t]$, $y[t]$, $z[t] = z[0]$) used here for evaluating the correlation functions. The z-axis is along the axis of a cylindrical tube. The $x(t)$-axis is perpendicular to the cylinder surface at the position of the molecule at time t.

$$\begin{aligned}P_2(\cos\theta_t'') &= 0.125\,\{[2 + 6\cos 2\psi_{0t}] P_2(\cos\theta_t) \\ &- [3 - 3\cos 2\psi_{0t}] \sin^2\theta_t \cos 2\varnothing_t \\ &+ [6 \sin 2\psi_{0t}] \sin 2\theta_t \sin\varnothing_t\} \qquad \text{(A2a)}\end{aligned}$$

$$\begin{aligned}\sin^2\theta_t'' \cos 2\varnothing_t'' &= 0.25([-2 + 2\cos 2\psi_{0t}] P_2(\cos\theta_t) \\ &+ [3 + \cos 2\psi_{0t}] \sin^2\theta_t \cos 2\varnothing_t \\ &+ [2 \sin 2\psi_{0t}] \sin 2\theta_t \sin\varnothing_t\} \qquad \text{(A2b)}\end{aligned}$$

$$\begin{aligned}\sin^2\theta_t'' \sin 2\varnothing_t'' &= [\cos\psi_{0t}] \sin^2\theta_t \sin 2\varnothing_t \\ &- [\sin\psi_{ot}] \sin 2\theta_t \cos\varnothing_t \qquad \text{(A2c)}\end{aligned}$$

$$\begin{aligned}\sin 2\theta_t'' \cos\varnothing_t'' &= [\cos\psi_{0t}] \sin 2\theta_t \cos\varnothing_t \\ &+ [\sin\psi_{0t}] \sin^2\theta_t \sin 2\varnothing t \qquad \text{(A2d)}\end{aligned}$$

$$\begin{aligned}\sin 2\theta_t'' \sin\varnothing_t'' &= [\cos 2\psi_{0t}] \sin 2\theta_t \sin\varnothing_t \\ &- [\sin 2\psi_{0t}] P_2(\cos\theta_t) \\ &- 0.5[\sin 2\psi_{0t}] \sin^2\theta_t \cos 2\varnothing t. \qquad \text{(A2e)}\end{aligned}$$

The correlation functions to be evaluated have the form: $\langle\langle GH\rangle\rangle$, where G and H are given in Table 2. These correlation functions can be calculated with the "second approximation" using an extension of the method developed in references 13 and 14. The results are:

$$\langle\langle G(\theta_0, \varnothing_0) H(\theta_t, \varnothing_t)\rangle\rangle = B \exp(-At) + C \qquad \text{(A3a)}$$

$$C = \langle G(\theta, \varnothing)\rangle \langle H(\theta, \varnothing)\rangle \qquad \text{(A3b)}$$

TABLE 2 **Factors G and H in the correlation functions $\langle\langle GH\rangle\rangle$**

G	H	G	H	G	H
$P_2(\cos\theta_0)$	$P_2(\cos\theta_t)$	$P_2(\cos\theta_0)$	$\sin^2\theta_t\cos 2\varnothing_t$	$P_2(\cos\theta_0)$	$\sin 2\theta_t\sin\varnothing$
$\sin^2\theta_0\sin 2\varnothing_0$	$P_2(\cos\theta_t)$	$\sin^2\theta_0\sin 2\varnothing_0$	$\sin^2\theta_t\cos 2\varnothing_t$	$\sin^2\theta_0\sin 2\varnothing_0$	$\sin 2\theta_t\sin\varnothing_t$
$\sin 2\theta_0\sin\varnothing_0$	$\sin 2\theta_t\sin\varnothing_t$	$\sin 2\theta_0\sin\varnothing_0$	$P_2(\cos\theta_t)$	$\sin 2\theta_0\sin\varnothing_0$	$\sin^2\theta_t\cos 2\varnothing_t$
$\sin^2\theta_0\cos 2\varnothing_0$	$\sin^2\theta_t\sin 2\varnothing_t$	$\sin^2\theta_0\cos 2\varnothing_0$	$\sin 2\theta_t\cos\varnothing_t$		
$\sin 2\theta_0\cos\varnothing_0$	$\sin 2\theta_t\cos\varnothing_t$	$\sin 2\theta_0\cos\varnothing_0$	$\sin^2\theta_t\sin 2\varnothing_t$		

$$B = \langle G(\theta,\varnothing)H(\theta,\varnothing)\rangle - C \tag{A3c}$$

$$A = -D_w\Big\langle\frac{\partial}{\partial\theta}G(\theta,\varnothing)\,\frac{\partial}{\partial\theta}H(\theta,\varnothing) - \{G(\theta,\varnothing)/\sin^2\theta\}\frac{\partial}{\partial\theta}H(\theta,\varnothing)\Big\rangle\Big/B, \tag{A3d}$$

where the single brackets denote equilibrium averages:

$$\langle F\rangle = \int_0^{2\pi} d\varnothing \int_0^{\pi} d\theta\,\sin\theta f(\theta)F \tag{A4}$$

with $f(\theta)$ denoting the equilibrium orientational distribution function. It follows that all constants B and C vanish except for $G = H$. We find:

$$\langle\langle P_2(\cos\theta_0)P_2(\cos\theta_t)\rangle\rangle = B_0\exp(-C_0t) + \langle P_2\rangle^2 \tag{A5a}$$

$$0.75\,\langle\langle\sin^2\theta_0\cos 2\varnothing_0\sin^2\theta_t\cos 2\varnothing_t\rangle\rangle = 0.75\,\langle\langle\sin^2\theta_0\sin 2\varnothing_0\sin^2\theta_t''\sin 2\varnothing_t''\rangle\rangle = B_2\exp(-C_2t) \tag{A5b}$$

$$0.75\,\langle\langle\sin 2\theta_0\cos\varnothing_0\sin 2\theta_t\cos\varnothing_t\rangle\rangle = 0.75\,\langle\langle\sin 2\theta_0\sin\varnothing_0\sin 2\theta_t''\sin\varnothing_t''\rangle\rangle = B_1\exp(-C_1t). \tag{A5c}$$

The constants C_i, and B_i ($i = 0, 1, 2$) have been given in Table 1 above in terms of D_W, the wobbling diffusion constant, $\langle P_2\rangle$ and $\langle P_4\rangle$, the second and fourth rank orientational order parameters, respectively. These order parameters can be calculated as follows:

$$\langle P_2\rangle = \int_0^{\pi} d\theta\,\sin\theta P_2(\cos\theta)f(\theta) \tag{A6a}$$

$$\langle P_4\rangle = \int_0^{\pi} d\theta\,\sin\theta P_4(\cos\theta)f(\theta), \tag{A6b}$$

where $f(\theta)$ is the equilibrium orientational distribution function, i.e., the probability density of finding a molecule with polar angle θ with respect to the normal to the lipid-water interface. This distribution is normalized:

$$\int_0^{\pi} d\theta f(\theta)\sin\theta = 1. \tag{A7}$$

P_2 and P_4 are the 2nd and 4th rank Legendre polynomials, respectively:

$$P_2(z) = (3z^2 - 1)/2 \tag{A8a}$$

$$P_4(z) = (35z^4 - 30z^2 + 3)/8. \tag{A8b}$$

APPENDIX 2

If the total intensity can be described by a single exponential decay with lifetime τ, then I_V, the fluorescence response to a vertically polarized pulse short compared to τ, and I_H, the fluorescence response to a horizontally polarized pulse short compared with τ, can be written as

$$I_V = I_1\{1 + 2r(t)\}\exp[-t/\tau] \tag{B1a}$$

$$I_H = I_1\{1 - r(t)\}\exp[-t/\tau], \tag{B1b}$$

where I_1 is a constant. Taking for $r(t)$ either $r_{RW}(t)$, $r_{WH}(t)$ or $r_{PH}(t)$ from Table 1, the sine- and cosine-transforms of I_V and I_H can be calculated, yielding:

$$N_V = \int_0^{\infty} I_V\sin(2\pi Ft)\,dt\Big/\int_0^{\infty} I_V\,dt = (u/H_1)\Big\{1 + 2r_\infty + 2(r_0 - r_\infty)\sum_{i=1}^{N}\cdot g_iH_1/[(1 + A_i\tau)^2 + u^2]\Big\}\Big/\Big\{1 + 2r_\infty + 2(r_0 - r_\infty)\sum_{i=1}^{N} g_i/(1 + A_i\tau)\Big\} \tag{B2a}$$

$$N_H = \int_0^{\infty} I_H\sin(2\pi Ft)\,dt\Big/\int_0^{\infty} I_H\,dt = (u/H_1)\Big\{1 - r_\infty - (r_0 - r_\infty)\sum_{i=1}^{N}\cdot g_iH_1/[(1 + A_i\tau)^2 + u^2]\Big\}\Big/\Big\{1 - r_\infty - (r_0 - r_\infty)\sum_{i=1}^{N} g_i/(1 + A_i\tau)\Big\} \tag{B2b}$$

$$E_V = \int_0^{\infty} I_V\cos(2\pi Ft)\,dt\Big/\int_0^{\infty} I_V\,dt = (1/H_1)\Big\{1 + 2r_\infty + 2(r_0 - r_\infty)\sum_{i=1}^{N}\cdot g_iH_1(1 + A_i\tau)/[(1 + A_i\tau)^2 + u^2]\Big\}\Big/\Big\{1 + 2r_\infty + 2(r_0 - r_\infty)\sum_{i=1}^{N} g_i/(1 + A_i\tau)\Big\} \tag{B2c}$$

$$E_H = \int_0^\infty I_H \cos(2\pi Ft)\,dt \Big/ \int_0^\infty I_H\,dt$$

$$= (1/H_1)\left\{1 - r_\infty - (r_0 - r_\infty)\sum_{i=1}^{N} \cdot g_i H_1(1 + A_i\tau)/[(1 - A_i\tau)^2 + u^2]\right\}\Big/$$

$$\left[1 - r_\infty - (r_0 - r_\infty)\sum_{i=1}^{N} g_i/(1 + A_i\tau)\right], \quad \text{(B2d)}$$

where the following abbreviations have been used:

$$u = 2\pi F\tau \quad \text{(B3a)}$$

$$H_1 = 1 + u^2. \quad \text{(B3b)}$$

The observables, $\varnothing_H - \varnothing_V$ (the differential phase) and M_V/M_H (the modulation ratio), or r_w (the frequency-dependent anisotropy) can now be calculated from:

$$\varnothing_H - \varnothing_V = \tan^{-1}[(E_V N_H - E_H N_V)/(N_V N_H + E_V E_H)] \quad \text{(B4a)}$$

$$M_V/M_H = Y^{1/2} \quad \text{(B4b)}$$

$$r_w = [Y^{1/2} - 1]/[Y^{1/2} + 2] \quad \text{(B4c)}$$

$$Y = [N_V^2 + E_V^2]/[N_H^2 + E_H^2] \quad \text{(B4d)}$$

The results are given in Eq. 17.

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