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ANGLE RESOLVED FLUORESCENCE DEPOLARIZATION EXPERIMENTS ON ORIENTED LIPID MEMBRANE SYSTEMS

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SUMMARY: It is demonstrated that angle resolved steady state fluorescence depolarization experiments on oriented lipid membrane systems can be an useful alternative to time-resolved fluorescence depolarization experiments on vesicles. It is shown that some basic assumptions underlying time-resolved experiments are not always valid. The usefulness of the measurement of an additional order parameter, $< P_4 >$ is demonstrated.

Fluorescence depolarization (FD) methods are widely used to study the molecular ordering and dynamic behaviour of the lipid bilayers in a biomembrane [1-4]. Here, fluorescent molecules, embedded in the bilayer are used to monitor these properties. In order to interpret FD data in terms of membrane system parameters, a thorough knowledge of the probe properties is required. In particular, the angle between the absorption- and emission moment, which in time-resolved FD experiments is closely related to the fluorescence anisotropy at t = o, r_o, and the assumed reorientational model are important. For one popular probe, DPH, the use of time-resolved FD techniques results in many conflicting reports of the value r_o [1,2,4], whereas the validity of the chosen reorientation model has hardly been discussed. One reason for this might be that in these experiments the problem of obtaining unambiguous curve fits is far from trivial due to the necessity of using deconvolution procedures.
We have recently shown that measurement of the angle resolved steady state fluorescence depolarization (AFD) of DPH in a macroscopically ordered lipid mem-

Abbreviations used: DPH: 1,6 diphenyl -1,3,5 hexatriene; DMPC: dimyristoyl phosphatidylcholine; DPPC: dipalmitoylphosphatidylcholine.

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brane system is a useful alternative to time-resolved FD in vesicle systems: r_o can be straightforwardly determined, and moreover, besides the rotational correlation time τ_o and the second rank order parameter $< P_2 >$ (=S), the fourth rank order parameter $<P_4>$ can be determined. (For a definition of $<P_2>$ and $<P_4>$, see ref. 12). In addition the method provides a validity check on the molecular reorientation model used.

Previously, we have found [5] that AFD experiments are in good agreement with their theoretical description [6]. In this paper we will present some results which can be obtained by AFD. To this end, the ordering and motion of DPH in DMPC and DPPC model membrane systems, with and without cholesterol, was studied at different temperatures. Further, we will demonstrate that measurement of $< P_2 >$ and $< P_4 >$ may yield new information on the molecular ordering which cannot be provided by knowledge of only $< P_2 >$.

MATERIALS AND METHODS

DMPC, DPPC and cholesterol were purchased from Sigma; DPH was obtained from Aldrich. They were used without further purification. The molar lecithine/probe ratio was $\approx$ 250. The molar lecithine/cholesterol ratio in samples containing cholesterol was $\approx$ 5, the water conc. was $\approx$ 20 weight %. Macroscopically ordered multibilayers were prepared as described elsewhere [5].
Angle resolved fluorescence depolarization experiments were carried out on a home built fluorimeter [5]. DPH was excited at λ = (365 ± 8) nm and fluorescence was observed at λ = (432 ± 9) nm. Theoretically [6] and experimentally [5] the method is described in detail elsewhere. In brief, in AFD experiments, the polarization of the fluorescence is measured as a function of two angles which describe, respectively, the direction of the incoming and outgoing beam relative to the membrane symmetry axis. A fit of the experimental AFD profile to the theoretical predictions yields 5 experimental parameters. From these the 3 system parameters $< P_2 >$, $< P_4 >$ and τ_o are calculated under the following assumptions [5]: (a) DPH is an effectively cylindrically symmetric probe; its absorption moment is parallel to its symmetry axis (b) the molecular motion can be described by the strong collision model.
One of the 2 remaining parameters is used to determine the angle α between the absorption and the emission moment of DPH. The other one is used as a consistency check, to test the validity of the strong collision model.

RESULTS AND DISCUSSION

Molecular properties of DPH. It can be seen from table I that the angle α between the absorption- and emission moments of DPH can be appreciable, and is dependent on both the membrane system studied and the temperature. We can compare these results with values for r_o, the anisotropy at zero time obtained from time resolved FD experiments on DPH doped vesicles. On using [7]

$$r_o = .20(3 \cos^2\alpha - 1) \qquad (1)$$

Table I. Order parameters, rotational correlation times and angles α between absorption and emission moments.

system #	experiments	temp (°C)	α	$< P_2 >$	$< P_4^A >$	$< P_4^B >$	$\omega = \frac{\tau_o}{\tau_o + \tau_f}$	τ_o + (ns)
DMPC	4	9	16	.68 ± .02	.23 ± .08	.24 ± .08	.91 ± 0.02	80
	2	17	0	.78 ± .02	.57 ± .04	.60 ± .04	.65 ± .05	17
	3	23	20	.64 ± .07	.44 ± .05	.63 ± .04	.44 ± .06	7
	2	40	28	.42 ± .02	.25 ± .04	.55 ± .04	.30 ± .06	4
	2	54	34	.40 ± .03	.21 ± .05	.69 ± .07	.28 ± .06	4
DMPC + 20% chol.	3	23	20	.93 ± .03	.84 ± .03	.84 ± .03	.35 ± .06	15
DPPC	2	9	11	.65 ± .02	.16 ± .03	.17 ± .03	>.90	> 80
	3	23	20	.60 ± .03	.12 ± .05	.12 ± .05	>.90	> 80
	4	56	20	.46 ± .13	.24 ± .05	.62 ± .15	.19 ± .06	2
DPPC + 20% chol.	2	23	0	.90 ± .03	.71 ± .04	.75 ± .03	.25 ± .06	3

+ assuming τ_f = 9 ns [17]
T_c (DMPC) = 24 °C
T_c (DPPC) = 42 °C

we find r_o in the range 0.33 - 0.40 for DPPC-systems. This is at variance with experiments on DPPC - liposomes [1], but in general agreement with values found for mixed systems [2]. However, some workers assume standard values r_o = 0.40 [8,9,10] (both moments mutually parallel) or r_o = 0.362 [2,11] which are obtained from experiments on *isotropic, not lipid-like* systems. Contrarily, r_o can be obtained from AFD data in a straightforward manner [5], and our results show that the standard r_o-values cannot be used in anisotropic lipid systems. Rather, r_o-values should be determined for each particular membrane system under study.

Molecular reorientation model. The redundancy of the 5 experimental parameters can be used to calculate $<P_4>$ in two independent ways [5], yielding $<P_4^A>$ and $<P_4^B>$ (see Table I). If $<P_4^A> = <P_4^B>$, the membrane system parameters are

self-consistent and the strong-collision model can be considered to be valid. From Table I a clear picture emerges: membrane systems containing cholesterol behave according to the strong collision model, whereas for the cholesterol lacking systems this only holds for temperatures below the liquid crystalline-gel phase transition temperature T_c. As a consequence, the usefulness of the widely used wobbling-in-cone model [7], which is mathematically equivalent to the strong-collision model, has to be questioned for pure lipid membrane systems above T_c, and one has to use another reorientational model. Additional calculations based upon the somewhat more refined diffusion model [5] showed that $< P_2 >$ and $< P_4^A >$ values are hardly model-dependent. Consequently, the static parameters of the various membrane systems are well reflected by $< P_2 >$ and $< P_4^A >$ given in Table I. Furthermore, values of the corresponding rotational correlation times were found to be in the same order of magnitude as those calculated using the strong collision model.

Molecular ordering. The ordering of lipids in uniaxial membrane systems can be fully described by a so-called angular distribution function $f(\beta)$ in which β is the angle between the membrane symmetry axis and a molecular axis [12]. In its turn $f(\beta)$ can be characterized by an infinite set of so-called order parameters $< P_2(\beta) >$, $< P_4(\beta) >$..... which are the ensemble-averaged Legendre polynomials of order 2,4, ... , respectively [12]. Up to now only $< P_2 >$ has been determined for a number of membrane systems. However, for many cases it can be expected that knowledge of only $< P_2 >$ results in a poor estimate of $f(\beta)$. We have previously argued [5] that an essentially better estimate of $f(\beta)$ can be made if $< P_4 >$ is determined as well and if $f(\beta)$ is reconstructed on the basis of information theory. For the systems studied here this has been done in table II. The important features of $f(\beta)$ are β_{max}, the angle at which $f(\beta)$ attains its maximum and $\beta_{\frac{1}{2}}$, the half-width angle (see figure 1). From table II it can be seen that a molecular tilt ($\beta_{max} \neq 0$) occurs below T_c in cholesterol lacking systems of both DPPC and DMPC. The tilt angle that we find for DPPC is in excellent agreement with results obtained from X-ray diffraction studies [13]. The tilt vanishes if cholesterol is added to the systems, as has been earlier observed [13,14]. The occurence of these

Table II Maximum and half-width of distribution functions.

system	temp (°C)	$\beta_{\frac{1}{2}}$ (°)	β_{max} (°)
DMPC	9	15	20
	17	15	0
	23	15	0
	40	19	0
	54	22	0
DMPC + 20% chol	23	10	0
DPPC	9	8	25
	23	12	25
	56	18	0
DPPC + 20% chol.	23	13	0

tilt angles can only be observed, if $< P_4 >$ is known, which stresses the utility of the AFD-method. $\beta_{\frac{1}{2}}$ can be regarded as a measure of the degree of ordering. The general trend in the systems lacking cholesterol is a decreasing ordering at higher temperatures above T_c, and a more constant behaviour below T_c.

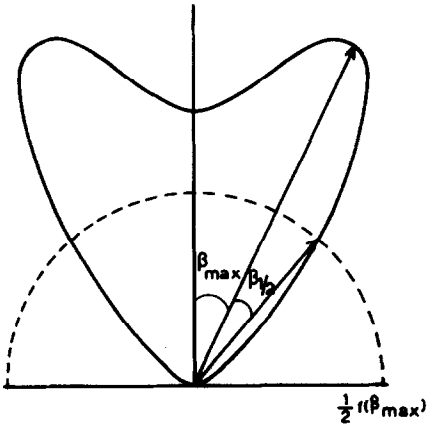
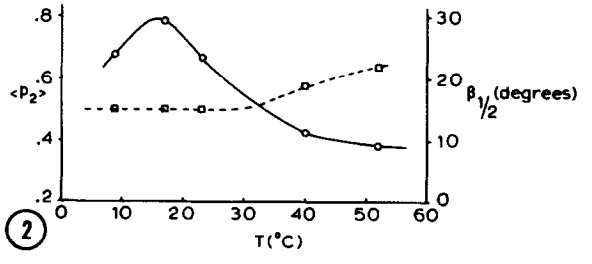


Fig. 1. A typical polar plot of a distribution function defining β_{max} and $\beta_{\frac{1}{2}}$. The vertical axis corresponds to the membrane symmetry axis. The length of a vector making an angle β with the vertical axis is proportional to the fraction of molecules making an angle β with the membrane axis. The dotted semicircle represents the value $\frac{1}{2}f(\beta_{max})$.

This is in agreement with previous FD results [8,9]. The temperature dependence of $\langle P_2 \rangle$ and $\beta_{\frac{1}{2}}$ are shown in figure 2 for DMPC. The different shapes of the curves for $\beta_{\frac{1}{2}}$ and P_2 make it clear that the membrane order is not well reflected in the value $\langle P_2 \rangle$. Additional knowledge of $\langle P_4 \rangle$ separates tilt and ordering effects.

The effect of cholesterol on the membrane order is found to be the inverse of that on the rotational correlation time: cholesterol increases ordering around T_c and has little effect far below it. On the other hand, cholesterol has a profound fluidizing effect, in particular below T_c (cf. DPPC and DMPC at 23°C). Similar results have been reported [8]. Another illustration of the usefulness of measuring $\langle P_2 \rangle$ and $\langle P_4 \rangle$ is provided by inspecting the temperature dependence of $f(90)/f(\beta_{max})$ for DMPC. (see fig. 3). Moreover, this figure demonstrates that on using FD techniques one essentially measures the probe distribution function which does not necessarily correspond to that of the lipid molecules: an increasing fraction of DPH molecules is oriented perpendicular to the lipid chains with increasing temperature. The finding that DPH molecules do not order along the lipid chains has previously been reported in a study on black lipid membranes [15].

Another interesting finding is that the behaviour of DPH in unsaturated lipid membrane systems, such as dioleoyllecithin or galactosylglycerides is completely different from that found for saturated lipids, such as reported here. Details of these findings will be published elsewhere [16].



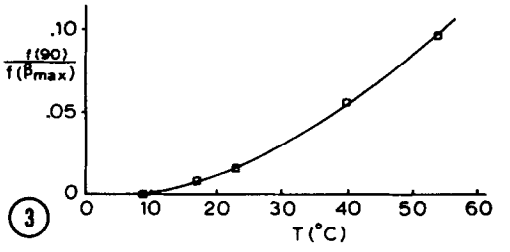


Fig. 2 The order parameter $\langle P_2 \rangle$ (——) and the half-width angle $\beta_{\frac{1}{2}}$ (---) of the distribution function as a function of temperature for DMPC-systems.

Fig. 3 The relative fraction of DPH-molecules lying in the membrane plane $f(90)/f(\beta_{max})$ as a function of temperature for DMPC-systems.

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