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The fidelity of response by 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene in time-resolved fluorescence anisotropy measurements on lipid vesicles

Effects of unsaturation, headgroup and cholesterol on orientational order and reorientational dynamics

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Abstract. Time-resolved fluorescence anisotropy measurements on 1-[4-(tri-methylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) molecules in lipid vesicles of palmitoyloleoylphosphatidylcholine (POPC), PC extracted from egg yolk (EggPC), dioleoyl-PC (DOPC), dilinoleoyl-PC (DLPC), phosphatidylglycerol extracted from egg yolk (EggPG), dioleoyl-PG (DOPG), sulfoquinovosyldiacylglycerol (SQDG) and digalactosyl-DG (DGDG) with and without cholesterol are presented. The observed intensity decay curves are analyzed simultaneously in terms of the Brownian rotational diffusion model. The analysis thus yields the isotropic fluorescence decay, the initial anisotropy $r(0)$, the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ as well as the diffusion coefficient of the long molecular axis. It is shown that increasing unsaturation in the acyl chains of the PC lipids results in an increase in the rotational diffusion rates of the probes and a decrease in the order parameter $\langle P_2 \rangle$. However, the value of $\langle P_4 \rangle$ remains unchanged. The corresponding orientational distribution function of the probes is bimodal, with fractions lying preferentially parallel and perpendicular to the local vesicle surface. Surprisingly, the fraction of probe molecules lying with their long axes parallel to the bilayer surface increases with increasing unsaturation with a concomitant narrowing in the width of the distribution of the fraction lying perpendicular to it. As expected, cholesterol is found to increase the order parameters in all the systems and to suppress the tendency of the molecules to lie parallel to the bilayer surface. Furthermore, the rotational diffusion coefficients of the probes is found to increase in all the systems except for DLPC. Interestingly, the effects of unsaturation on the reorientational dynamics of TMA-DPH molecules in the vesicle systems are opposite to those found in the corresponding planar multibilayers (Deinum et al. 1988), whereas the same cholesterol effect is observed for the two systems. Nevertheless, the TMA-DPH molecules exhibit higher diffusion coefficients in the vesicle than in the planar multibilayer systems. In addition, a unimodal distribution of the probe molecules is found in the multibilayer systems. The differences between the two systems are ascribed to the differences in the radius of curvature and the hydration of the bilayers. Lastly we rationalize the bimodal distribution of the TMA-DPH molecules in the vesicles in terms of their observed partition between the lipid and aqueous phases.

Key words: Lipid bilayer vesicles, time-resolved anisotropy, TMA-DPH, order parameters, reorientational dynamics

The role played by lipid molecules in the function and structure of biological membranes has been widely investigated by a variety of physical techniques covering a broad range of timescales. It is now accepted that the chemical nature of the lipids is important in determining the dynamic properties of the lipid bilayer, as well as modulating their macroscopic organization (de Kruijff et al. 1985; Killian and de Kruijff 1988). Much of our understanding of lipid bilayers on the molecular scale has been derived from the use of vesicle systems as models for biological membranes.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH 1-[4-(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene; POPC, palmitoyloleoylphosphatidylcholine; EggPC, PC extracted from egg yolk; DOPC, dioleoyl-PC; DLPC, dilinoleoyl-PC; EggPG, phosphatidylglycerol extracted from egg yolk; DOPG, dioleoyl-PG; SQDG, sulfoquinovosyldiacylglycerol; DGDG, di-galactosyl-DG; HPTLC, high performance thin layer chromatography

Fluorescence depolarization techniques are particularly suitable for studies of the molecular orientational order and dynamics of lipid vesicles on the nanosecond timescale. These experiments utilize the time-resolved fluorescence anisotropy elicited from probe molecules embedded in the vesicles. The probe molecules 1,6-diphenyl-1,3,5-hexatriene (DPH) and its cationic analogue TMA-DPH are commonly used because of their favourable photophysical properties. DPH is a sensitive probe for the hydrocarbon region of the bilayer and it has now been established that this probe exhibits a bimodal orientational distribution (van de Ven and Levine 1984; van Ginkel et al. 1986; Pottel et al. 1986; Best et al. 1987). Two populations of the molecules exist, one with their axes oriented preferentially perpendicular to the bilayer surface and the other parallel to it. This latter fraction is probably located in the centre of the bilayer. TMA-DPH, however, is expected to be anchored with its charged group at the lipid-water interface (Prendergast et al. 1981) so that it reflects the properties of the upper region of the bilayer. This probe is also used to monitor exocytosis phenomena in membrane systems (Bronner et al. 1986) and exhibits a partition equilibrium between the aqueous buffer, where it does not fluoresce, and the membranes, where it has a high quantum yield (Donner and Stoltz 1985; Kuhry et al. 1985; Duportail, personal communication).

We have recently shown (van Langen et al. 1987 a) that the model-dependent (global) analysis of time-resolved fluorescence anisotropy from these two probe molecules in lipid vesicles is not straightforward. Two statistically equivalent solutions have been found with essentially the same value of the order parameter $\langle P_2 \rangle$ but with markedly different values of the order parameter $\langle P_4 \rangle$. The order parameters are given by $\langle P_2 \rangle = \langle 3\cos^2\beta - 1 \rangle/2$ and $\langle P_4 \rangle = \langle 35\cos^4\beta - 30\cos^2\beta + 3 \rangle/8$ where β is the angle between the long molecular axis and the normal to the local bilayer surface and $\langle \ldots. \rangle$ denotes an average over all the probe molecules. Each solution thus corresponds to a different orientational distribution of the molecules in the bilayer. It was argued on the basis of the reconstructed orientational distribution function that only the solution with the high value of $\langle P_4 \rangle$ was consistent with X-ray diffraction data.

This ambiguity in the interpretation arises from the fact that the anisotropy decay function $r(t)$ is a sum of three exponential decay terms, which cannot be separated uniquely into its individual components. A further difficulty in the interpretation is the assignment of the initial value of the anisotropy decay function $r(0)$, which reflects the relative orientation of the absorption and emission transition moments. It is common practice to fix this value in the numerical analysis to values in the range 0.40–0.38, corresponding to an angle between the two moments of between 0° and 10°. However, analyses of the experiments with this angle treated as a free parameter, have yielded angles of up to 20° (van Langen et al. 1987 a; van Langen et al. 1988 a, b). Steady-state experiments on macroscopically ordered multibilayers have also shown the two moments not to be parallel (van Ginkel et al. 1986; van Langen et al. 1987 b; Deinum et al. 1988). The importance of the value of $r(0)$ lies in the fact that it determines to a large extent the initial time derivative of the anisotropy decay which is proportional to the diffusion constant (Zannoni et al. 1983; van der Meer et al. 1984; Szabo 1984). We have indeed demonstrated a significant correlation between the value of $r(0)$ and the model parameters $\langle P_4 \rangle$ and $D_\perp$, the diffusion coefficient of the long molecular axis (van Langen et al. 1988 a, b). This correlation is a consequence of the attempt to extract both the orientation of the emission dipole moment in the molecular frame and the diffusion constants from the anisotropy decay. Nevertheless, it was shown that an objective analysis of the anisotropy decay may be accomplished on treating $r(0)$ as a free parameter even though this increases the total number of adjustable parameters entering the fit procedures.

Here we present a time-resolved fluorescence anisotropy study of the effect of the lipid composition of vesicle systems on the orientational order and dynamics monitored by TMA-DPH molecules. The analysis of the experiments is carried out within the framework of the Brownian diffusion model (Nordio and Segre 1979; Zannoni et al. 1983) with the molecules confined to a generalized angle-dependent potential well. It is generally accepted that unsaturation decreases the molecular orientational order and that this trend is reversed by cholesterol. However, controversy still exists over the influence of these factors on the molecular dynamics.

The effects of unsaturation, temperature and cholesterol on the behaviour of TMA-DPH in vesicle system has recently been studied by Straume and Litman (1987 a, b) using phase and modulation techniques. However, these authors constrained their analysis to the case of parallel transition moments and to a unimodal orientational distribution function with the molecular axes preferentially oriented perpendicular to the bilayer surface, that is parallel to the acyl chains of the lipids.

We have investigated the behaviour of TMA-DPH molecules in small unilamellar vesicles of a number of diacylphosphatidylcholine (PC) derivative with a varying degree of unsaturation: POPC, EggPC, DOPC and DLPC. In addition results obtained from vesicles consisting of the galactolipid DGDG and the polar lipids DOPG, EggPG and SQDG are reported. The effect of cholesterol (25% by weight) on the

behaviour of TMA-DPH in these systems has also been studied. The lipids SQDG and DGDG are found in thylakoid membranes and contain 40% and 80% respectively of 18:3 polyunsaturated acyl chains (Douce and Joyard 1980; Quinn and Williams 1983). The polyunsaturated mono- and digalactosyldiglycerols are involved in the structural and functional organization of thylakoid membranes in higher plants (Sprague 1987).

We have found that increasing unsaturation in the acyl chains of the PC lipids results in an increase in the rotational diffusion rates of the probes and a decrease in the order parameter $\langle P_2 \rangle$. However, the value of $\langle P_4 \rangle$ remains unchanged. The corresponding orientational distribution function of the probes is bimodal, with fractions lying preferentially parallel and perpendicular to the local vesicle surface. Surprisingly, the fraction of probe molecules lying with their long axes parallel to the bilayer surface increases with increasing unsaturation with a concomitant narrowing in the width of the distribution of the fraction lying perpendicular to it. As expected, cholesterol is found to increase the order parameters in all the systems and to suppress the tendency of the molecules to lie parallel to the bilayer surface. Furthermore, the rotational diffusion coefficients of the probes is found to increase in all the systems except for DLPC. Interestingly, the effects of unsaturation on the reorientational dynamics of TMA-DPH molecules in the vesicle systems are opposite to those found in the corresponding planar multibilayers (Deinum et al. 1988), whereas the same cholesterol effect is observed for the two systems. Nevertheless, the TMA-DPH molecules exhibit higher diffusion coefficients in vesicles than in the planar multibilayer systems. In addition, a unimodal distribution of the probe molecules is found in the multibilayer systems. The differences between the two systems are ascribed to the differences in the radius of curvature and the hydration of the bilayers. Lastly we rationalize the orientational behaviour of the TMA-DPH molecules in the vesicles in terms of their observed partition between the lipid and aqueous phases.

Theory

In a time-resolved fluorescence anisotropy experiment the probe molecules are excited with a short pulse of plane polarized light and the decay of the polarized fluorescence emission is monitored. The time dependent intensities of the emitted light polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization direction of the excitation pulse are given by (Gilbert 1983; Zannoni et al. 1983; Ameloot et al. 1984)

$$I_{\parallel}(t) = (1/3)\,F(t)\,\{1 + 2\,r(t)\}$$
$$I_{\perp}(t) = (1/3)\,F(t)\,\{1 - r(t)\} \qquad (1)$$

Here $F(t)$ is the intrinsic fluorescence decay of the probe molecules, while $r(t)$ describes the anisotropy decay function. This function contains the information about the reorientational dynamics of the probe molecules in the vesicle. It is generally accepted that TMA-DPH is effectively cylindrically symmetric with its absorption transition moment direction coinciding with the long molecular axis (van Langen et al. 1987 a; Best et al. 1987). In this case $r(t)$ is given as a sum of three correlation functions $G_0(t)$, $G_1(t)$ and $G_2(t)$ (Zannoni et al 1983; van der Meer et al. 1984; van Langen et al. 1987 a)

$$r(t) = r(0)\,\{G_0(t) + 2\,G_1(t) + 2\,G_2(t)\} \qquad (2)$$

These correlation functions describe the correlation between the orientation of the long molecular axis at the time of absorption and its orientation at the time of emission. They are an average over all the possible orientations of the molecular axes in the vesicle system. The initial anisotropy, $r(0)$, is independent of the dynamic behaviour of the molecule and is related to the relative orientation $\alpha_{\mu\nu}$ of the absorption (μ) and emission (ν) transition moments as (Szabo 1984)

$$r(0) = (2/5)\,P_2(\cos\alpha_{\mu\nu}) \qquad (3)$$

where the second rank Legendre polynominal is defined by $P_2(\cos\alpha_{\mu\nu}) = (3\cos^2\alpha_{\mu\nu} - 1)/2$. The initial and final values of the correlation functions at times $t = 0$ and $t = \infty$ respectively can be expressed in terms of the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ as

$$G_0(0) = 1/5 + 2\langle P_2\rangle/7 + 18\langle P_4\rangle/35$$
$$G_1(0) = 1/5 + \langle P_2\rangle/7 - 12\langle P_4\rangle/35$$
$$G_2(0) = 1/5 - 2\langle P_2\rangle/7 + 3\langle P_4\rangle/35$$
$$G_0(\infty) = \langle P_2\rangle^2$$
$$G_1(\infty) = G_2(\infty) = 0 \qquad (4)$$

The order parameters are defined as $\langle P_2\rangle = \langle 3\cos^2\beta - 1\rangle/2$ and $\langle P_4\rangle = \langle 35\cos^4\beta - 30\cos^2\beta + 3\rangle/8$ where β is the angle between the long molecular axis and the normal to the local bilayer surface and $\langle \ldots \rangle$ denotes an ensemble average over all the probe molecules.

The decay of the correlation functions $G_k(t)$, $k = 0$, 1, 2, can be calculated from the Brownian rotational diffusion model (Nordio and Segre 1979; Zannoni et al. 1983). In this model the probe molecules are assumed to undergo small step diffusion subject to an angle-dependent ordering potential $U(\beta)$

$$U(\beta) = k\,T\,\{\lambda_2\,P_2(\cos\beta) + \lambda_4\,P_4(\cos\beta)\} \qquad (5)$$

Here $P_L(\cos\beta)$ is the Legendre polynomial of rank L. The rotational diffusion in this potential well is described by the diffusion coefficients $D_{\perp}$ and $D_{\parallel}$ of the long molecular axis and about this axis respectively. As

the absorption transition moment coincides with the long axis only $D_\perp$ can be monitored in our experiments. The decay of the correlation functions is obtained from a numerical solution of the diffusion equation (Nordio and Segre 1979; Zannoni et al. 1983) as an infinite sum of exponential terms:

$$G_k(t) = \sum_m b_{km} \exp(-a_{km} D_\perp t), \quad k = 0, 1, 2 \qquad (6)$$

The decay amplitudes b_{km} and decay coefficients a_{km} are complex functions of the parameters λ_2 and λ_4 which describe the ordering potential. In most practical cases the slowest decay term in (6) dominates the time dependence of each correlation function (Nordio and Segre 1979). Useful approximations for the decay amplitudes and coefficients in terms of $\langle P_2 \rangle$, $\langle P_4 \rangle$ and $D_\perp$ have been given by van der Meer et al. (1984).

The pair of order parameters $\{\langle P_2 \rangle, \langle P_4 \rangle\}$ is uniquely related to the coefficients $\{\lambda_2, \lambda_4\}$ characterising the ordering potential. The orientational distribution function $f(\beta)$, expressing the probability of finding a probe molecule oriented with its long axis at an angle β relative to the normal to the bilayer, takes the form (Nordio and Segre 1979; Kooyman et al. 1983; Mulders et al. 1986)

$$f(\beta) = N \exp\{-U(\beta)/kT\} \qquad (7)$$

where N is a normalization constant. The number density of the molecules oriented at the angle β is now given by $f(\beta) \sin \beta$.

Materials and methods

POPC, DOPC, EggPC, DOPG, EggPG and cholesterol were purchased from Sigma Chemical Company (St. Louis, Mo.). The lipids were used as received, but random purity checks were carried out with HPTLC. DLPC, SQDG and DGDG were obtained from Lipid Products (Surrey, U.K.). The latter two lipid species were extracted from green plants and were tested for purity and oxidation (Koole et al. 1984) before use.

The probe TMA-DPH was purchased from Molecular Probes, Inc. (Junction City, Or.) as a p-toluenesulphonate salt.

Sample preparation

Small unilamellar vesicles were prepared as follows. The lipids, with or without cholesterol, were dissolved in 100% ethanol under a nitrogen atmosphere. TMA-DPH in ethanol from a fresh stock solution was added to the lipids to make a 1:250 mixture on a molecular basis. The solvent was removed by a flow of nitrogen gas and subsequent storage under vacuum for 4–6 h.

The lipid/probe mixture was hydrated by the addition of 6 ml of 20 m*M Tris* buffer, pH 8.0, containing 7.5 μ*M* EDTA. The resulting mixture was homogenized with a vortex mixer followed by sonication in a bath type sonicator for 15–45 min. The clear vesicle suspension was subsequently centrifuged for 1 h at 150,000 *g* in a Beckman L2-65B ultracentifuge. All the preparative steps were carried out as much as possible in the dark under a nitrogen atmosphere. The lipid concentrations in the samples were obtained from phosphorus determinations (Chen et al. 1956) on the phospholipids, galactose determinations on DGDG (Dubois et al. 1956) and sulphate determination on SQDG (Spencer 1960). The phospholipid suspensions of POPC, DOPC, DLPC and DOPG contained 1.1 ± 0.2 mg lipid/ml buffer and 0.75 ± 0.20 mg lipid/ml buffer for EggPC and EggPG. The vesicle suspensions of DGDG and SQDG contained 0.60 ± 0.05 mg lipid/ml buffer.

Time-resolved anisotropy measurements were performed within a week after the preparation of the samples. Blank vesicle suspensions were also prepared for the measurement of elastic scattering.

Time-resolved measurements

The time-resolved anisotropy measurements were carried out with the Synchrotron Radiation Source (SRS) in Daresbury (U.K.). Light from a single bunch of electrons circulating in a storage ring is particularly suitable for experiments with nanosecond time resolution (Munro and Schwentner 1983). The light is highly polarized, has a stable pulse shape independent of wavelength and is tunable over a wide spectral range. The timing characteristics are determined by the 250 ps pulse width and the 3 MHz repetition rate.

The 358 ± 5 nm excitation light was selected by a monochromator (SPEX). The fluorescence wavelength was defined with a low cut-off filter (LF 40, Schott) and a 438 ± 7 nm interference filter (Balzers). A Glann Thompson prism and a Polaroid sheet analyzer were used to define the polarizations of the excitation and fluorescence light respectively.

A standard electronic setup was used in the single photon counting experiments. The time registration utilized a time-to-pulse height converter (TPHC, Ortec 457) whose output was connected to an analogue-to-digital converter and stored in a multichannel analyzer system (INO-TEC 5400); a channel width of 80 ps was used. The start pulse of the TPHC was generated from the first photon detected by the photomultiplier tube (PMT, Philips XP2020Q) Peltier cooled to $-20\,^\circ$C and operating at $-2{,}700$ V. The stop pulse was provided by a ring pulse generated by the circulating electrons. Both electronic signals were

properly defined by constant fraction discriminators (CFD, Ortec 473a and 583) and one of them was delayed with a delay box (EG & G DB 463).

The parallel and perpendicular components of the emitted light were measured in an alternating sequence for 25 s. The number of counts in the maximum of both spectra was always more than 10,000 and a measurement time of less than 20 min allowed sufficient statistics to be collected. The excitation profile was obtained from the elastic scatter of a blank vesicle suspension at the emission wavelength, 438 nm. The instrumental G-factor was determined to be 1.000 ± 0.005.

In all these experiments the count rate was kept at less than 1% of the repetition rate of the excitation pulses to prevent pile-up problems. Furthermore, the intensity in the maximum of scatter from the blank samples was less than 1% of that in the maximum of the fluorescent signal.

The temperature was $20° \pm 2°C$.

Results

Numerical analysis

The experimental decay curves were analyzed using the reiterative non-linear least squares deconvolution technique (Cundall and Dale 1983; Gilbert 1983; Löfroth 1985). In this approach the excitation profile obtained from the elastic scatter at the emission wavelength is considered to be a sum of δ-function excitation pulses each leading to the intensity response functions given in Eq.(1). The observed intensity decays $I_{\perp}(t)$ and $I_{\parallel}(t)$ were fitted simultaneously to the model using the ZXSSQ routine of the IMSL library for the minimal χ^2 search. The intrinsic fluorescence decay was treated as a biexponential function and required four parameters for its description. A further four parameters were necessary to describe the anisotropy decay $r(t)$ as discussed above, Eqs.(2)–(6). The reduced χ^2 minimum was judged by the usual statistical criteria such as visual inspection of the fit to both components, the distribution of the weighted residuals and their autocorrelation function. An example of the simultaneous fit of the observed intensity decays is shown in Fig. 1. We shall only consider the high $\langle P_4 \rangle$ solution obtained from the analysis, in line with our previous findings (van Langen et al. 1987a).

We have, in addition analyzed the experimental data in terms of multiexponential decay components. These fits indicated that the number of significant parameters which can be obtained from this model-independent analysis of the anisotropy decay is limited to six or less. The fit procedure became numerically unstable when more than six parameters entered the description of the time dependence of the anisotropy $r(t)$.

Fluorescence decay

Lifetime measurements on TMA-DPH in a number of vesicle systems indicate that the intrinsic fluorescence function can be described as a biexponential decay (Best et al. 1987; van Langen et al. 1987a; Straume and Litman 1987a, b; Deinum et al. 1988):

$$F(t) = \sum_{i=1}^{2} \alpha_i \exp(-t/\tau_i) \tag{8}$$

The fluorescence decay parameters extracted from our global analysis as well as the minimal reduced χ^2 values are summarized in Table 1. The values reported here are in excellent agreement with those obtained from the analyses of independent determinations of the fluorescence decay under magic-angle conditions (not shown). We have, in fact, found that the parameters describing the time-resolved anisotropy are not affected by the explicit mathematical description of the intrinsic fluorescence decay, as long as it provides a good fit of the lifetime experiment.

Table 1. The reduced χ^2 values and the biexponential fluorescence decay parameters of TMA-DPH in vesicles obtained from simultaneous fit to the parallel and perpendicular observed decay intensities

	χ^2	α_1	τ_1(ns)	α_2	τ_2(ns)	$\langle T \rangle$(ns)[a]
Lipid without cholesterol						
POPC	1.21	0.30	1.20	0.70	4.43	4.09
EggPC	1.21	0.37	1.20	0.63	4.10	3.68
DOPC	1.34	0.45	1.20	0.55	3.79	3.26
DLPC	1.86	0.45	0.92	0.55	3.01	2.59
EggPG	1.18	0.42	1.33	0.58	4.17	3.63
DOPG	1.16	0.38	1.15	0.62	3.77	3.35
SQDG	1.30	0.39	1.13	0.61	3.09	2.72
DGDG	1.65	0.54	1.62	0.46	3.72	3.02
Lipid with cholesterol (25% by weight)						
POPC	1.20	0.34	2.43	0.66	6.66	5.99
EggPC	1.32	0.36	2.67	0.64	6.63	5.90
DOPC	1.54	0.47	2.50	0.53	5.97	5.03
DLPC	1.79	0.54	1.92	0.46	5.07	4.11
EggPG	1.37	0.37	2.26	0.63	6.58	5.84
DOPG	1.32	0.44	2.16	0.56	5.29	4.54
SQDG	1.62	0.46	2.90	0.54	7.04	5.98
DGDG	1.45	0.51	2.35	0.49	5.46	4.49

[a] The average fluorescence lifetime defined in Eq. (9) is calculated.

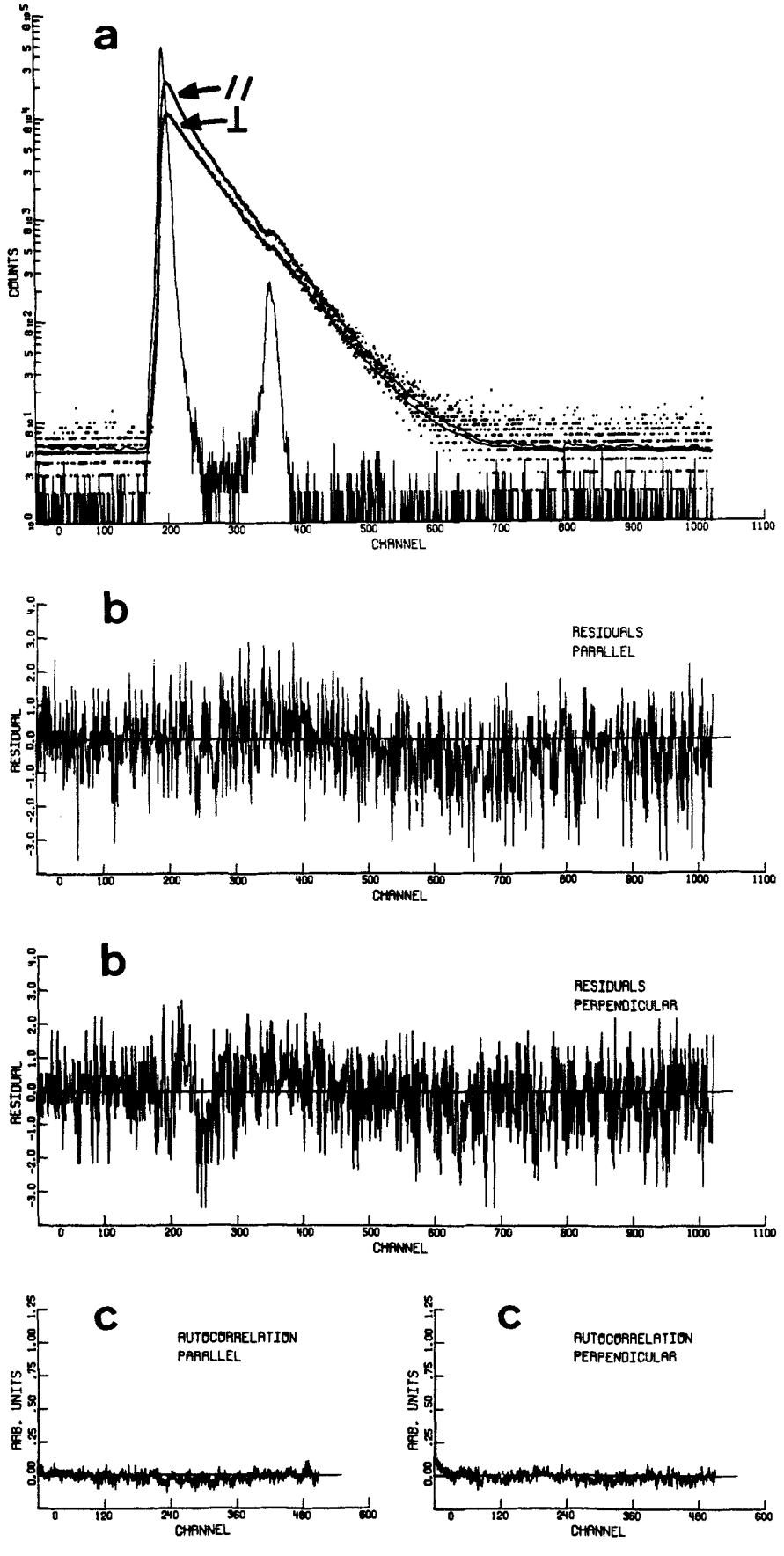

Fig. 1a–c. The simultaneous fit to the observed time dependent intensities $I_{\parallel}(t)$ and $I_{\perp}(t)$ for TMA-DPH in vesicles of EggPC **a** the excitation pulse profile, the experimental data points and the fit; **b** the residuals of the parallel and the perpendicular components and **c** their autocorrelation functions. Time equivalence per channel is 80 ps

Table 2. The parameters describing the anisotropy decay $r(t)$ of TMA-DPH in vesicles

	λ_2	λ_4	$D_\perp(\mathrm{ns}^{-1})$	$\alpha_{\mu v}(^\circ)$	$\langle P_2\rangle$	$\langle P_4\rangle$
Lipid without cholesterol						
POPC	−1.23	−2.26	0.09	13	0.54	0.44
EggPC	−0.94	−2.54	0.12	18	0.49	0.45
DOPC	−0.58	−2.91	0.16	18	0.42	0.45
DLPC	−0.34	−3.32	0.27	18	0.39	0.47
EggPG	−0.63	−3.14	0.13	15	0.47	0.49
DOPG	−0.29	−3.05	0.09	14	0.32	0.43
SQDG	−0.89	−2.77	0.15	14	0.51	0.48
DGDG	−0.31	−3.70	0.17	18	0.43	0.52
Lipid with cholesterol (25% by weight)						
POPC	−1.53	−2.64	0.12	19	0.67	0.54
EggPC	−1.43	−2.72	0.19	18	0.65	0.54
DOPC	−0.99	−3.04	0.29	19	0.58	0.53
DLPC	−1.47	−2.30	0.19	18	0.61	0.48
EggPG	−1.43	−2.72	0.18	18	0.65	0.54
DOPG	−0.99	−2.86	0.10	18	0.55	0.50
SQDG	−2.07	−2.22	0.15	20	0.72	0.54
DGDG	−1.41	−2.76	0.12	21	0.65	0.55

Furthermore, the analysis of the data indicates that the average fluorescence lifetime $\langle T\rangle$ defined as

$$\langle T\rangle = \int_0^\infty t\,F(t)\,\mathrm{d}t \Big/ \int_0^\infty F(t)\,\mathrm{d}t = \sum_{i=1}^{2} \alpha_i \tau_i^2 \Big/ \sum_{i=1}^{2} \alpha_i \tau_i \qquad (9)$$

appears to be independent of the mathematical representation of the fluorescence decay function and may therefore be used for comparison purposes.

Anisotropy decay parameters

The parameters λ_2, λ_4, $D_\perp$ and $\alpha_{\mu v}$, as well as the corresponding order parameters $\langle P_2\rangle$ and $\langle P_4\rangle$, describing the anisotropy decay functions are summarized in Table 2.

Discussion

Fluorescence decay parameters

It is clear from the results shown in Table 1 that the average fluorescence lifetime $\langle T\rangle$ of TMA-DPH molecules in vesicle systems consisting of PC lipid molecules decreases on increasing the degree of unsaturation of the acyl chains from POPC to DLPC. This effect is also evident in the same systems in the presence of cholesterol. We note that the addition of cholesterol results in a significant increase in $\langle T\rangle$. The decay of the probes in EggPC vesicles is similar to that observed in POPC vesicles with or without cholesterol.

A similar behaviour is also found for the vesicles of the PG systems. However, we do not find the expected higher values of $\langle T\rangle$ in vesicles of pure SQDG compared with pure DGDG systems. Nevertheless, the addition of cholesterol to these vesicle systems results in an increase in $\langle T\rangle$ by a factor of two.

The influence of the chemical composition of the lipid system on the average fluorescence lifetime of TMA-DPH molecules found here has also been observed by Straume and Litman (1987b) using phase modulation techniques and by Deinum et al. (1988) for TMA-DPH molecules embedded in macroscopically ordered multibilayer systems.

It is striking, however, that no differences are found in the extracted average lifetimes of TMA-DPH molecules in vesicles of EggPC and EggPG, with or without cholesterol, and for vesicles of pure DOPC and DOPG. It seems that in these systems the influence of the headgroup on the fluorescence decay is negligible. However, the addition of cholesterol to the DOPC and DOPG systems appears to introduce a headgroup effect.

The initial anisotropy $r(0)$

In all systems studied the angle between the transition moments of TMA-DPH molecules lies in the range 13°–21°, corresponding to $r(0)$ values of 0.37–0.32. We have previously shown (van Langen et al. 1988b) that an analysis using a model independent multiexponential representation of the anisotropy decay $r(t)$ yields the same values of $r(0)$ as those extracted from our approach. Thus, $r(0)$ can be treated as a free model parameter despite its correlation with $D_\perp$.

The deviation of $r(0)$ from the value of 0.4 expected for the case of parallel emission and absorption transition moments is probably due to the complex photophysics of diphenylpolyene molecules. It was recently shown (Itoh and Kohler 1987) that these molecules exhibit dual fluorescence as the result of the interaction between the dipole-forbidden 2^1A_g(S1) excited state with the strongly dipole-allowed 1^1B_u(S2) excited state. These levels are separated only by several hundred cm^{-1} and this energy gap is sensitive to the environment of the molecule (Itoh and Kohler 1987). We expect that this interaction will also affect the direction of the emission transition moment in the molecular frame, so that its orientation relative to the absorption moment will be dependent on the environment of the molecule. This expectation is borne out for DPH molecules in planar multibilayers of EggPC, where the relative angle is found to increase

with increasing hydration of the lipid headgroups (van Langen et al. 1987b).

The orientational order

The results presented in Table 2 show that the order parameter $\langle P_2 \rangle$ decreases markedly on increasing the degree of unsaturation in the acyl chains of all the lipid systems studied here. The same trend is also found in the presence of cholesterol, even though higher values of $\langle P_2 \rangle$ are found than in the pure lipid vesicles. The only exception to this appears to be the DLPC vesicles. These findings agree with previous studies using a variety of physical techniques (Lee 1975). Surprisingly, the order parameter $\langle P_4 \rangle$ is found to be insensitive to the degree of unsaturation of the acyl chains in vesicle systems either with or without cholesterol. Nevertheless we note that the addition of cholesterol increases the value of $\langle P_4 \rangle$, an observation consistent with the picture of increasing molecular orientational order. Again an exception is found in the case of DLPC vesicles.

It is interesting to note that similar order parameters are observed for the EggPC and EggPG vesicles, whereas higher values are found for DOPC than DOPG vesicles with or without cholesterol.

The effects of the chemical composition of the lipids on the orientational order of the TMA-DPH molecules in the vesicles is best illustrated by the reconstructed orientational distribution function $f(\beta)$, Eq. (7) or the number density $f(\beta)\sin(\beta)$. The orientational distributions of the probes in vesicles of POPC, EggPC, DOPC and DLPC are shown in Fig. 2a and the corresponding number densities are shown in Fig. 2b. The figures clearly show that the probe molecules possess a bimodal orientational distribution in the vesicle systems, one population lying parallel to the bilayer surface (around 90°) and the other with its axes preferentially along the normal to the surface at 0°. It can be seen that the number of probe molecules lying with their long axes around 90° increases with increasing unsaturation. Surprisingly, however, at the same time the distribution of molecules lying along the normal to the bilayer (i.e. around 0°) becomes narrower. This behaviour was not observed by Straume and Litman (1987a) as they constrained all the probe molecules to be distributed around 0°. Similarly this type of distribution is a priori excluded by the "wobble-in-cone" model (Kinosita et al. 1984).

This behaviour is the consequence of our observation that the order parameter $\langle P_4 \rangle$ is virtually independent of the number of double bonds in the acyl chains. We have thus demonstrated the importance of the knowledge of this order parameter in characterizing the orientational order. We have here shown that the

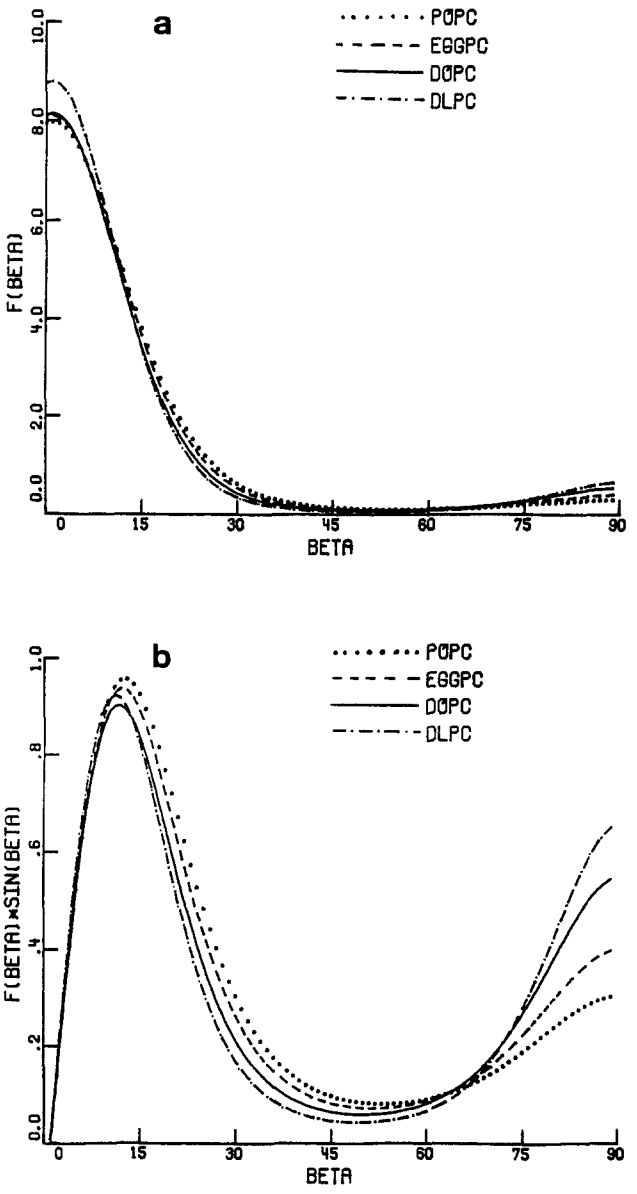

Fig. 2. a Orientational distribution function ($f(\beta)$) and **b** the corresponding number densities ($f(\beta)\sin(\beta)$) for TMA-DPH in vesicles of POPC, EggPC, DOPC and DLPC

observation of a higher value of $\langle P_2 \rangle$ does not of itself indicate that more molecules are oriented along the normal to the bilayer surface at 0°.

Similar bimodal distributions are also found for the other lipid system studied. The orientational distribution and number density functions for DOPG are shown in Fig. 3a and b, together with those of DOPC. It can be seen that a larger fraction of molecules at 90° is found for the former system, presumably reflecting the influence of the lipid headgroups. On the other hand, no significant differences were found between EggPC and EggPG.

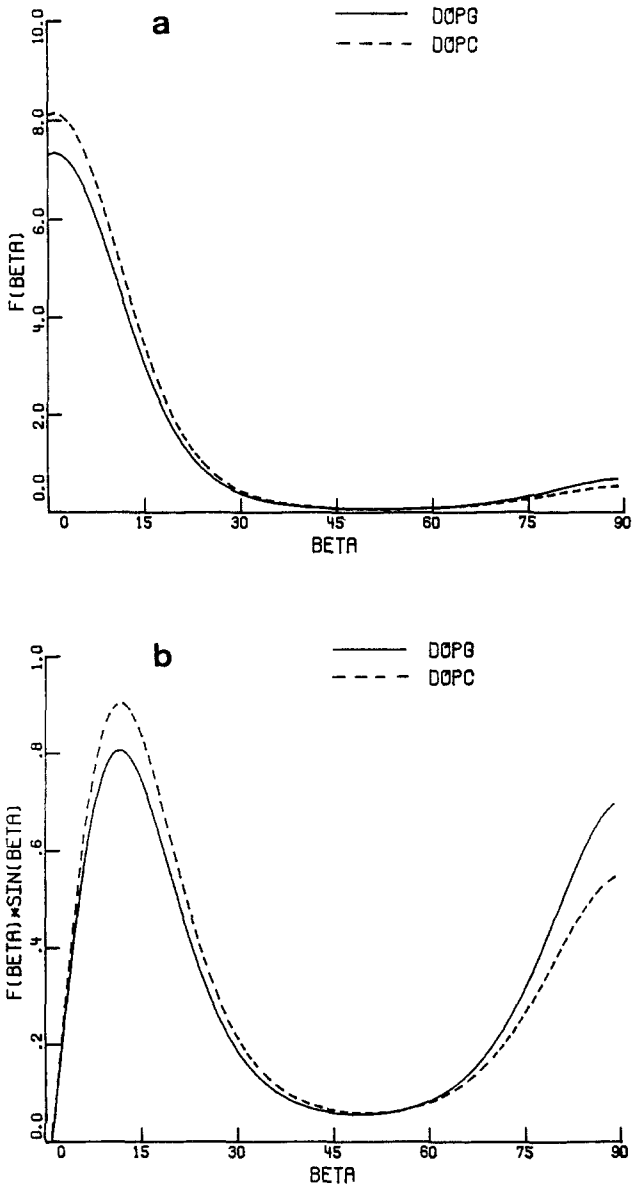

Fig. 3a. Orientational distribution function ($f(\beta)$) and **b** the corresponding number densities ($f(\beta)\sin(\beta)$) for TMA-DPH in vesicles of DOPG and DOPC

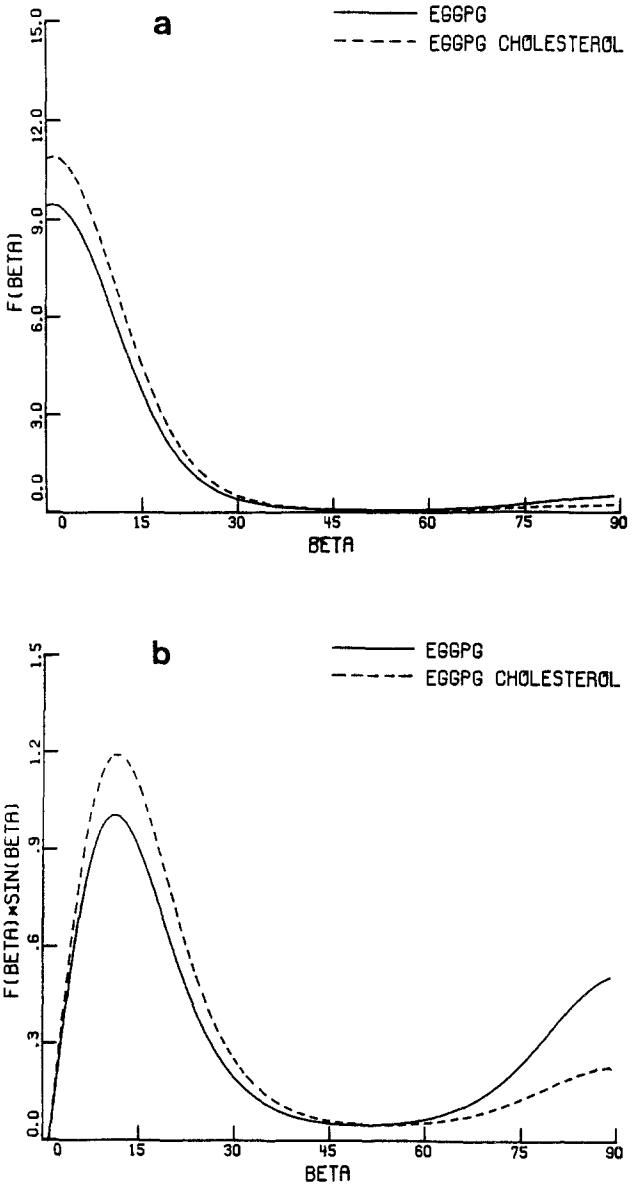

Fig. 4a. Orientational distribution function ($f(\beta)$) and **b** the corresponding number densities ($f(\beta)\sin(\beta)$) for TMA-DPH in vesicles of EggPG with and without cholesterol

Cholesterol was found to suppress the population of TMA-DPH molecules lying around 90° in every system studied here and to enhance the population of molecules aligned along the lipid chains around 0°. This is illustrated by the orientational distribution function Fig. 4a and the number density function Fig. 4b for TMA-DPH in vesicles of EggPG with and without cholesterol.

The question now arises as to whether the bimodal orientational distribution found in this study is an artefact of the model used in the analysis. In particular, one may question the choice of the ordering potential, Eq.(5) which is symmetric about $\beta = 90°$. This choice was discussed by us previously in some detail (van Langen et al. 1988a, b). It was shown that the simplest ordering potential not possessing this symmetry, $U(\beta) = kT\{\lambda_1 P_1(\cos\beta) + \lambda_2 P_2(\cos\beta)\}$, could not account for the orientational order of TMA-DPH molecules in POPC vesicles with and without cholesterol. Although a more extended potential, incorporating higher order Legendre polynomials could be used, it introduces more parameters into the fitting procedure. We have, however, found that the deconvolution procedures become numerically unstable when more than six model parameters describe the anisotropy decay.

In order to check whether the bimodal distribution found in our analysis arises from uncertainties in the values of the extracted model parameters, we have

fitted the experimental data using the unimodal ordering potential $U(\beta) = kT\lambda_2 P_2(\cos\beta)$ peaked at $\beta = 0°$, but obtained reduced χ^2 values significantly higher (25%) than the ones reported in Table 1, with visibly unacceptable weighted residuals and autocorrelation functions. Interestingly, the distribution functions found again indicated a significant population of TMA-DPH molecules at $\beta = 90°$. It is important to realize, that the application of a Gaussian orientational distribution (Straume and Litman 1987a) or the "wobble-in-cone" model (Kinosita et al. 1984) not only presupposes a unimodal distribution of the probe molecules, but also rules out a population of molecules lying with their axes parallel to the bilayer surface. Consequently, one can only demonstrate a mathematical fit to the data and cannot validate the underlying physical model.

We have, furthermore, attempted an analysis of the data in which the short fluorescence lifetime component was associated with the population of molecules lying with their long axes parallel to the bilayer surface. The TMA-DPH molecules aligned along the lipid chains were correlated with the long lifetime component. Each population was described by its own diffusion constant and the molecules assigned to two distinct Gaussian distributions peaked either at $\beta = 0°$ or $\beta = 90°$. The value of $r(0)$ was taken to be the same for both distributions. In this way only five model parameters were needed to describe the anisotropy decay. The relative weights of the two populations were obtained from the biexponential analysis of the fluorescence decay. The best fits obtained in this way were characterized by reduced χ^2 values up to 10 times higher than those obtained from the analysis above, with correspondingly worse residuals and autocorrelation functions. It is interesting to note in this connection that the addition of cholesterol has a marked effect on the decay times of both components, see Table 1, but has a negligible effect on ratios of their amplitudes. For these reasons we do not believe that this approach provides a physical description of the behaviour of the TMA-DPH molecules.

The fluorescence anisotropy experiments do not identify the location of the probe molecules in the bilayer structure. In view of the finding that TMA-DPH molecules can partition between the membrane and aqueous phases (Kuhry et al. 1985; Bronner et al. 1986), we believe that a fraction of the molecules lie at the water/lipid interface of the vesicle, parallel to the bilayer surface. We note here that only a unimodal distribution of TMA-DPH molecules has been found in multibilayer systems of the same lipids studied here (Deinum et al. 1988). These latter systems have little bulk water between the stacked bilayers, in contrast to the vesicle systems. Consequently we expect the TMA-DPH molecules to be located almost exclusively in the bilayer phase, in agreement with our observations. Interestingly, higher values of order parameters and sharper orientational distributions are found in the planar multibilayer systems than in the vesicles.

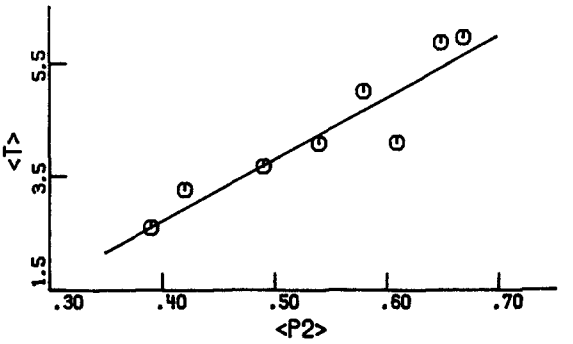

Fig. 5. The linear correlation between the order parameter $\langle P_2 \rangle$ and the average fluorescence lifetime $\langle T \rangle$ for TMA-DPH in PC vesicles

Lastly we note that our measurements reveal a linear correlation between the average fluorescence lifetime $\langle T \rangle$ and the order parameter $\langle P_2 \rangle$ for TMA-DPH molecules in PC vesicles, Fig. 5, with a slope of 11.0 ± 1.7 (ns).

Reorientational dynamics

The influence of the lipid matrix on the reorientational dynamics of TMA-DPH molecules is expressed in terms of the diffusion coefficient $D_\perp$; the larger the coefficient, the faster the motion. The diffusion coefficient increases significantly in the PC and DG vesicles on increasing the unsaturation of the lipid chains. On the other hand, the opposite effect of decreasing $D_\perp$ is found for the PG vesicles. Interestingly, the motion of the TMA-DPH molecules is considerably faster in DOPC than in DOPG vesicles with or without cholesterol, whereas essentially the same motional rates are found for the EggPC and EggPG systems.

The addition of cholesterol to the PC and PG vesicles results in a marked increase in the diffusion coefficient with the exception of DLPC vesicles. In contrast cholesterol appears to reduce the motional rates in DGDG vesicles, but has no effect on the motion of TMA-DPH molecules in vesicles of SQDG.

The diffusion coefficients reported here are significantly lower than those obtained by Straume and Litman (1987a, b). However, this discrepancy arises solely from the different ways the parameter $r(0)$ is treated in the analysis. We have here taken $r(0)$ to be an adjustable model parameter, whereas Straume and Litman (1987a, b) have fixed it at 0.4. We have recently shown (van Langen et al. 1988a, b) that an analysis with the fixed $r(0) = 0.4$ yields a higher diffusion coeffi-

cient and worse fits than those obtained with our approach.

The effect of unsaturation on the reorientational dynamics of TMA-DPH molecules found here is the opposite to that reported by us (Deinum et al. 1988) for planar multibilayer systems of the same lipids. Nevertheless, cholesterol is found to increase the diffusion coefficients in both the vesicle and multibilayer systems. Interestingly, the reorientational motions are considerably faster in the vesicle than in the planar multibilayer systems.

We believe that the explanation for this behaviour must be sought in the effects of high curvature and hydration on the properties of bilayer systems.

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