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# THE EFFECTS OF CHOLESTEROL ON THE TIME-RESOLVED EMISSION ANISOTROPY OF 12-(9-ANTHROYLOXY)STEARIC ACID IN DIPALMITOYLPHOSPHATIDYLCHOLINE BILAYERS

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**The time-resolved fluorescence emission anisotropy of 12-(9-anthroyloxy)stearic acid (12-AS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) have been measured in dipalmitoylphosphatidylcholine liposomes in the presence and absence of 40 mol% cholesterol at temperatures above and below the phase transition temperature (41°C). By using a synchronously-pumped mode-locked frequency-doubled dye laser and single photon counting detection with an excitation response function of 300 picosecond, rotational correlation times down to less than 1 nanosecond could be resolved. Whereas DPH showed only small changes in the limiting anisotropy on the addition of cholesterol, 12-AS showed significant increases in this parameter with the effect being potentiated at higher temperatures. This difference in behaviour has been attributed to a fluorophore-cholesterol interaction that resulted in a change in the fluorophore geometry. Not only do DPH and 12-AS sense different depolarizing rotations due to the different directions of their emission dipoles but also differ in their lipid interactions which alter their limiting anisotropies. The implication is that the comparison of steady-state anisotropy measurements between chemically identical fluorophores in different lipid environments may be complicated by molecular distortions that change the motions to which the steady-state fluorescence parameters will be sensitive.**

## Introduction

The interpretation of the fluorescence emission anisotropy of a fluorescent reporter molecule dissolved in a lipid bilayer must include consideration of both orientational constraint and resistance to the depolarizing rotations of the probe imposed by the surrounding lipid matrix. These two contributions are unresolved in steady-state anisotropy measurements but may be distinguished by time-resolved emission anisotropy measurements as the order parameter and the rotational correlation time, respectively [1–4]. Considerable work on DPH has demonstrated that both these parameters are sensitive to changes in bilayer structure, including alterations in the phase of the lipid [5–8], the degree of lipid unsaturation [5] and the cholesterol content [8,9]. Changes in the steady-state emission anisotropy of DPH can no longer be attributed only to changes in the rate of probe rotation or interpreted simply in terms of a microviscosity parameter as in earlier studies [11,12].

12-(9-Anthroyloxy)stearic acid is one of the set of 9-anthroyloxy-labelled fatty acids which has

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; 12-AS, 12-(9-anthroyloxy)stearic acid; DPPC, dipalmitoylphosphatidylcholine; $S$, order parameter; $\tau$, fluorescence lifetime.

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been used to measure steady-state polarization gradients through lipid bilayers [13,14]. The addition of cholesterol to DPPC bilayers leads to anomalous behaviour of these probes in the gel phase if the steady-state polarization is interpreted directly in terms of lipid fluidity [15,16].

This study uses the time-resolved emission anisotropy methodology to investigate the apparently anomalous behaviour of 12-AS in the presence of cholesterol. Comparison of the effects of cholesterol on the order parameters and rotational correlation times of DPH and 12-AS in DPPC liposomes demonstrates that cholesterol has different effects on the different depolarising rotations of the two probes [15,16].

Part of this study was presented in an abbreviated form at a British Photobiology Association Meeting, June 1980.

## Materials and Methods

L-α-Dipalmitoylphosphatidylcholine was purchased from Koch Light Labs. DPH was obtained from Aldrich Chemical Co. 12-AS was synthesized and purified as previously described [17].

*Preparation and labelling of lipid bilayers*

Multilayers were prepared by vortexing as described elsewhere [17]. Established labelling procedures were used [15], with probe to lipid ratios being 1 : 100 and 1 : 500 for 12-AS and DPH, respectively.

*Time-resolved emission anisotropy*

The theory and method have been described elsewhere [1–5,8]. Briefly the time-resolved emission anisotropy $r(t)$ is defined as:

$$r(t) = \frac{I_V(t) \cdot G - I_H(t)}{I_V(t) \cdot G + 2I_H(t)} \quad (1)$$

where $I_V(t)$ and $I_H(t)$ are the time-resolved components of the fluorescence polarized parallel and perpendicular, respectively, to the plane-polarized incident light and $G$ is a normalization constant to correct for the different sensitivities of detecting $I_v(t)$ and $I_H(t)$ [10]. In our equipment, $G$ was always close to $0.97 \pm 0.01$ S.D. ($n = 10$).

Although $r(t)$ is distorted from the true emission anisotropy decay due to the excitation response function, deconvolution is unnecessary in our apparatus until very short times because of the short excitation response function (300 ps). This limit is due to the photomultiplier transit time, not the laser pulse duration (5 ps). We found that, for the signal/noise ratio available in our data, deconvolution was not warranted for times greater than 0.5 ns; $r(t)$ has been fitted using [10]:

$$r(t) = r_\infty + (r_0 - r_\infty) e^{-t/\langle\phi\rangle} \quad (2)$$

where $\langle\phi\rangle$ is the mean rotational correlation time of all the depolarizing rotations of the probe and $r_0$ and $r_\infty$ are the limiting emission anisotropies at zero time and times long compared to the fluorescence lifetime, respectively.

The order parameter, $S$, is related to $r_0$ and $r_\infty$ and to the half cone angle $\theta_{max}$ of the 'wobbling-in-a-cone' model [1–3] by:

$$S^2 = \frac{r_\infty}{r_0} = \left[\frac{1}{2}\cos\theta_{max}(1+\cos\theta_{max})\right]^2 \quad (3)$$

This serves as the basis for the interpretation of data from both DPH and 12-AS. The 'spinning-in-equatorial-band' model for 12-AS [1] was not used as it requires that $r_0 \simeq 0.4$ which clearly is not the case in the bilayer system that we used ($r_0 \simeq 0.1$).

*Instrumentation*

Excitation light was produced at 340 nm by frequency doubling using a lithium iodate crystal, of light from a jet dye laser (Rhodamine 101, 5 ps pulses) synchronously pumped using a mode-locked Argon ion laser (514.5 nm line, 80 ps pulses). An electro optic modulator was used to reduce the pulse repetition rate to 90 kHz. Single photon counting detection was used with rotation of the emission polaroid between the two components, $I_V(t)$ and $I_H(t)$, during collection to correct for long term drift in the laser power (8 rotations for each 2 h accumulation) [10]. Scattered light was eliminated using 400 nm cutoff filters on the emission side. Scattering of emitted fluorescence light did not contribute to the emission anisotropy as shown by using water soluble Rhodamine G in a lipid sample. The temperature of the samples

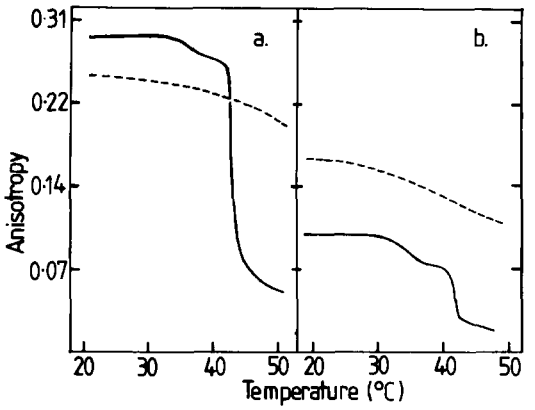


Fig. 1. The steady-state fluorescence anisotropy as a function of temperature for DPH (a) and 12-AS (b) bound to DPPC liposomes in the presence of 0 mol% (———) and 40 mol% (------) cholesterol. The anisotropy axis is non-linear and has been converted from polarization which was measured continuously using a polarization instrument (see Methods).

was thermostatically controlled within a range of ±0.5°C.

Steady-state anisotropy-temperature profiles were recorded as previously described [17].

## Results

Fig. 1 shows the steady-state emission anisotropy-temperature profiles for DPH and 12-AS bound to DPPC multilayers in the presence and absence of cholesterol. Abrupt changes in the emission anisotropy of both probes monitor the main gel-liquid crystalline phase transition at 41.6°C and the broader pretransition at 34–35°C. In the presence of cholesterol, no well-defined transition is detected by either probe, consistent with results from other techniques [19,20]. However, the essential difference between the two probes is that 12-AS shows an increased emission anisotropy at all temperatures whereas that of DPH is decreased below but increased above the main transition on addition of cholesterol. Such results have been reported previously [16] and have been observed in lipid vesicles [21].

Fig. 2 presents the typical time-resolved emission anisotropy data for DPH (Fig. 2 a, b) and 12-AS (Fig. 2 c, d) bound to DPPC liposomes at 20°C and 47°C. The fitted lines are calculated from Eqn. 2 without deconvolution. Tables I and II contain the parameters obtained from the data analyses of the time-resolved emission anisotropy curves presented in Fig. 2 and for similar curves in which 40 mol% cholesterol had been incorporated into the lipid. The $r_0$ values obtained by the curve fitting procedure were always lower than those obtained form the extrapolation of Perrin plots for the probes dissolved in high viscosity solvents [17,22,23]. This is discussed below.

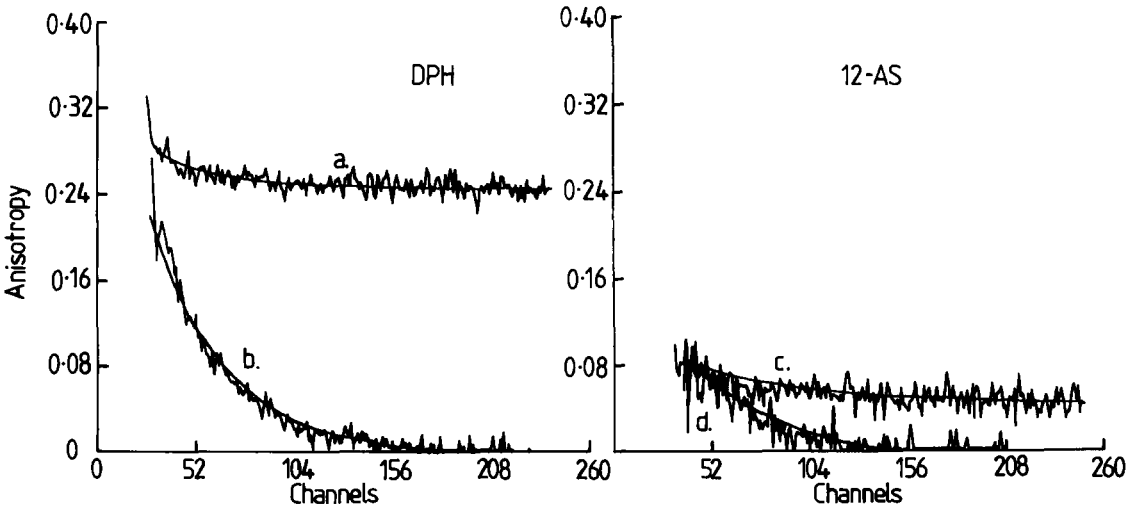


Fig. 2. Time-resolved emission anisotropy curves for DPH and 12-AS bound to DPPC liposomes at above and below the phase transition temperature. (a) DPH, 20°C; (b) DPH, 47°C; (c) 12-AS, 20°C and (d) 12-AS, 47°C. The time calibration was 34.24 channels/ns. Curves are computer fitted as described in Methods.

TABLE I

TIME-RESOLVED EMISSION ANISOTROPY DATA FOR DPH BOUND TO DPPC LIPOSOMES

The data analysis is described under Methods. The fluorescence lifetimes, $\tau$, were fitted to a single exponential function without deconvolution. The range of errors are given in parentheses for duplicate measurements.

| Cholesterol (mol%) | Temperature (°C) | $\tau$ (ns) | $r_0$ | $r_\infty$ | $S$ | Rotational correlation time (ns) $\langle\phi\rangle$ |
|---|---|---|---|---|---|---|
| 0 | 20 | 10.2 (0.1) | 0.22 | 0.21 | 0.95 (0.02) | 6.8 (0.5) |
| 40 | 20 | 10.1 (0.2) | 0.26 | 0.24 | 0.96 (0.02) | 6.5 (0.5) |
| 0 | 47 | 7.8 (0.1) | 0.20 | 0.0 | 0.00 (0.02) | 1.1 (0.2) |
| 40 | 47 | 8.9 (0.1) | 0.23 | 0.11 | 0.70 (0.02) | 1.0 (0.2) |

TABLE II

TIME-RESOLVED EMISSION ANISOTROPY DATA FOR 12-AS BOUND TO DPPC LIPOSOMES

The analyses is described under Methods. The fluorescence lifetimes, $\tau$, were fitted to a double exponential function without deconvolution with the shorter component having about a 30% weighting. The range of errors in triplicate measurements are given in parentheses.

| Cholesterol (mol%) | Temperature (°C) | $\tau$ (ns) | $r_0$ | $r_\infty$ | $S$ | rotational correlation time (ns) $\langle\phi\rangle$ |
|---|---|---|---|---|---|---|
| 0 | 20 | 2.8, 11.5 (0.1) | 0.08 | 0.05 | 0.79 (0.03) | 1.5 (0.2) |
| 40 | 20 | 2.8, 11.5 (0.1) | 0.13 | 0.06 | 0.68 (0.02) | 0.7 (0.4) |
| 0 | 47 | 1.2, 9.3 (0.1) | 0.09 | 0.00 | 0.00 | 1.5 (0.2) |
| 40 | 47 | 1.2, 9.3 (0.1) | 0.21 | 0.05 | 0.49 (0.03) | 1.1 (0.1) |

## Discussion

It has been well established using a variety of techniques that cholesterol alters the structure of the lipid bilayer by 'fluidizing' the gel phase by disrupting the rigid close packing of the acyl chains while 'condensing' the liquid-crystalline phase by restricting the *trans-gauche* isomerizations of the acyl chains [19,24–27]. The behavior of DPH shown in Fig. 1a is consistent with these effects if a decrease in steady-state emission anisotropy is interpreted as an increase in the rotational freedom of the probe. The trends observed for 12-AS (Fig. 1b) are not readily interpretable due to the increased anisotropy in the presence of cholesterol at all temperatures. Such apparently anomalous behaviour has been previously reported for 12-AS and for other probes and has been attributed to the anisotropic lipid environment [12,15].

We have measured the time-resolved emission anisotropy to allow direct determination of the orientational constraint imposed on the probes by the bilayer. The decay curves for the emission anisotropy (Fig. 2) indicate that $r_\infty$ only falls to zero in the fluid state and only in the absence of cholesterol. The non-zero $r_\infty$ values under other conditions (Tables I and II) imply that the probes have hindered rotational freedom.

At zero time, the emission anisotropy, $r_0$, is significantly lower than the $r_0$ values obtained from high viscosity solvents. Such an observation has been reported for DPH elsewhere [9,18]. No satisfactory explanation for this difference has been given. We have eliminated the possibility of partial

randomization of the planes of polarization of excitation and emission due to scatter and briefringence in the lipid suspension by demonstrating that a freely rotating water soluble dye (Rhodamine G) shows no anisotropy on addition of the vesicle suspension. The possibility remains that there are as yet unresolved very rapid depolarizing motions, although these would be less than 100 ps for our experiments not to have detected them. The lower $r_0$ values may also be due, in part, to the use of slightly different excitation wavelengths by different groups [8,9,18].

An alternative explanation is that the highly rigid bilayer environment not only becomes perturbed by the presence of the probe molecule but the probe geometry also becomes distorted resulting in a realignment of the absorption and emission transition dipoles. DPH has been reported to undergo torsional motions [28]. Such an effect is less speculative for 12-AS for which there is evidence for a conformational change occurring in the fluorophore on excitation which can be restricted in high viscosity media [29,30]. Changes in the vibronic structure of the emission spectrum of 12-AS in bilayers containing cholesterol have been interpreted in terms of such conformational changes [15]. Thus the variation in $r_0$ with changes in environment would not be unreasonable. This explanation, if true, would require the measurement of $r_0$ in each experiment as is done using the time-resolved emission anisotropy technique rather than assuming $r_0$ to be a constant as in steady-state measurements.

The trends in the order parameter of DPH (Table I) with increasing cholesterol content and on changing the temperature are the same irrespective of whether the $r_0$ value was found from the time-resolved emission anisotropy or from the extrapolated Perrin plots. However, as the time-resolved value was always smaller, the order parameters in the gel phase so obtained were always larger in magnitude. We note also that $r_0$ increases slightly in the presence of cholesterol at both 20°C and 47°C. In the gel phase (20°C), the $S$ value of DPH is not affected by the presence of cholesterol, implying that the difference in the steady-state measurements (Fig. 1a) below the phase transition must be due to a decreased rotational correlation time in the presence of cholesterol. The fluorescence lifetimes are constant and the decay times for single exponential decay are given in Table I. These decays were slightly non exponential, the two lifetimes of a double exponential fit being close to that of the single exponential values given. Accurate measurement of the correlation times was not possible for values much less than 1 ns or for large order parameters due to the signal/noise ratio available in our data resulting in the preexponential factor ($r_0 - r_\infty$ in Eqn. 2) being very small. Through the phase transition temperature the change in steady-state anisotropy (Fig. 1a) can be attributed to changes in $S$, the orientational constraint, as well as rotational rates (Table I), a result reported by other workers [5–7]. On addition of 40 mol% cholesterol to the fluid phase (47°C), the orientational constraint was increased with little change in the correlation time. The increase in the steady-state emission anisotropy (Fig. 1a) is thus not a reflection of altered rates of motion but rather increased constraint ($S$) on the depolarizing rotations, an effect also previously reported [9]. Our results for DPH are in agreement with the published literature and give a clear interpretation of the effects predominating in the steady-state fluorescence experiments.

In marked contrast to DPH, 12-AS showed significant changes in $r_0$ on incorporation of cholesterol into the liposomes (Table II). This can be interpreted as a distortion in the geometry of the fluorophore. The important implication of this finding is that comparison of steady-state anisotropy measurements such as in Fig. 1b is not valid as the new geometry may sense different depolarizing rotations due to changes in orientation of the emission dipole. Such geometrical distortions imposed by the lipid environment even in the absence of cholesterol may explain, at least in part, the observed steady-state anisotropy gradient through bilayers observed by the 9-anthroyloxy-labelled fatty acids [13,14,16,17]. Certainly the increase in steady-state anisotropy at 20°C for 12-As (Fig. 1b) is not consistent with the decreased orientational constraint and rotational correlation time seen in the presence of cholesterol (Table II). The interpretation of the 12-AS data according to the 'wobbling in a cone' or 'equatorial' model [6,7] must both be treated with caution. A complete description of fluorescence depolarization due to

reorientational motion has recently been published [4] and shows that in general the fluorescence anisotropy could contain very many exponential terms composed of combinations of the molecules diffusion coefficients. Due to experimental signal to noise limitations, however, more than two exponential terms in the fluorescence anisotropy are rarely, if ever, observed. Simplification of the model occurs only if the absorption and emission dipoles are parallel to the rotation symmetry axis. This is not the situation in 12-AS; even if the 12-AS dipoles were perpendicular to the symmetry axis, except in the case of a very prolate molecule, the anisotropy contains more terms than can be determined experimentally. Thus in 12-AS and related compounds, we cannot identify the origin of the rotational correlation time measured, ie. whether it refers to motion about one axis only or is a mean value of several similar motions.

Other features of interest in this data are the non-exponential nature of the fluorescence decay and the increase in $r_0$ on adding cholesterol which is particularly noticeable above the phase transition, Fig. 3. From our fluorescence decay time data we cannot distinguish between a multiplicity of sites for the 12-AS each having its own particular static interaction with the chromophore and hence giving rise to the observed fluorescence decay or a time dependent effect. This latter may be a solvent relaxation and could affect the polarisable carboxyl moiety on the 12-AS and which would generate a time dependent interaction but not one necessarily related to the rotational motion of the chromophore. Time resolved fluorescence spectra could be used to determine the time course for the interaction since for multiple sites with static interactions the fluorescence spectrum will not change with time.

How cholesterol changes the $r_0$ value is unknown, but a change is observed both in steady-state and time-resolved measurements. As the fluorescence decays are largely unaffected by adding cholesterol the interactions changing $r_0$ would appear to be different and additional to those which affect the fluorescence. The present study does not allow us to differentiate between a close 12-AS-cholesterol association due to packing restrictions of the lipid matrix and a lipid-transmitted effect of cholesterol on 12-AS. However, the need for care in the interpretation of steady-state fluorescence measurements of different probes is clearly demonstrated even in simple lipid systems.

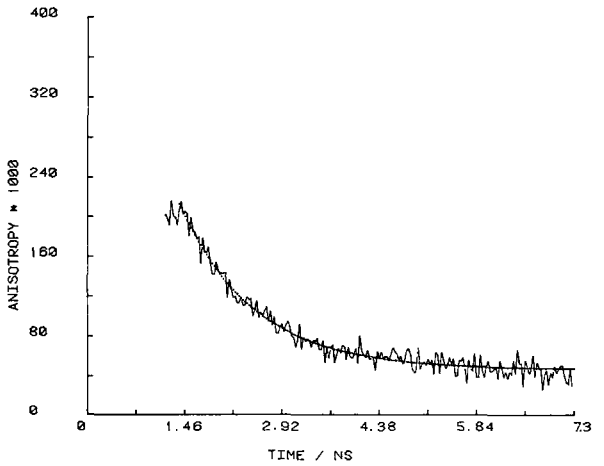


Fig. 3. Time-resolved emission anisotropy curves for 12-AS bound to DPPC liposomes at 47°C and in the presence of 40% cholesterol. Time calibration was 34.24 channels/ns.

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