Rotational Relaxation of the “Microviscosity” Probe Diphenylhexatriene in Paraffin Oil and Egg Lecithin Vesicles*

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The rotational relaxation of the widely used “microviscosity” probe, 1,6-diphenyl-1,3,5-hexatriene, was examined by the technique of nanosecond time-resolved fluorescence depolarization. The decays of the emission anisotropy were determined at five temperatures in the range 3–31° in both a reference paraffin oil and in sonicated egg lecithin vesicles. These decays were complex in both media. Marked qualitative as well as quantitative differences were observed in the rotational behavior of the probe in the complex bilayer medium as opposed to the homogeneous reference solvent. The results are discussed in relation to the structure of the hydrophobic bilayer membrane interior and the concept of its “microviscosity.”

In recent years, the technique of fluorescence depolarization which, following the classic work of Weber (1, 2), had found wide application in determination of the shape and size of labeled biological macromolecules in aqueous solution (3, 4), has been utilized to study the fluidity of a variety of bilayer membranes (5–19). The method has provided information regarding phase transitions and phase separations in liposomes (8–14) and with more complex cell membrane systems (15–19).

The fluidity of the hydrocarbon bilayer interior has been described in terms of an apparent “microviscosity” by comparing the steady state fluorescence depolarization of the probe in a reference oil with that observed in the membrane system. Phase transitions are readily identified from the behavior of the depolarization or of the derived “microviscosity” as a function of temperature. In some cases, it has been possible to define a thermodynamic parameter for the system, the “fusion activation energy,” e.g. Refs. 5, 7, 8, 11, 15, 19.

The extent of depolarization of the emission of a fluorophore reflects the degree to which a population of photoselected excited fluorophores loses its initial selective orientation and becomes randomized. In these applications, a modified form (16) of the Perrin equation (20) has been used to describe the reference oil data and, by implication, also that of the membrane system.

$$\frac{r_n}{\langle r \rangle} = 1 + C(\langle r \rangle) \frac{T}{\gamma}$$

where $\langle r \rangle$ is the observed steady state emission anisotropy (21, 22) defined by:

$$\langle r \rangle = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

in which $I_\parallel$ and $I_\perp$ are the intensities of components of the emission polarized parallel and perpendicular to the electric vector of plane polarized excitation. The zero-point emission anisotropy $r_0$ is the limiting value observed in the absence of rotational motion on the nanosecond time scale, while $\eta$ represents the ”microviscosity” of the system and $\tau$ the excited state lifetime at temperature $T$. $C(\langle r \rangle)$ contains volume and shape factors for the fluorophore plus any associated solvent shell and also, as will be indicated below, any other factor contributing to curvature in the Perrin plot of the reciprocal of the emission anisotropy against the factor $(T\tau/\eta)$ in Equation 1.

When the decays of both the emission anisotropy and the total emission are monoexponential, $C(\langle r \rangle)$ is a constant and the Perrin plot is linear. The former obtains for a spherical rotor or any solid of revolution in which the absorption and/or emission transition dipole is oriented along the symmetry axis. If the latter also holds then, in the first case, the constant is equal to $k$, the Boltzmann constant, divided by the volume of the spherical rotor; in the second it is also a function of the axial ratio (23–25). However, the rotational relaxation is usually more complicated than indicated above and may in general be represented by a multiexponential decay law, e.g. Ref. 26:

$$r(t) = \sum \beta \exp[-t/\phi_i]$$

where $\phi_i$ are the rotational correlation times and the zero point emission anisotropy is determined as the sum of the pre-exponentials $\beta$. In addition, the decay of the emission itself may be complex, again generally representable as a sum of exponentials:

$$I(t) = \sum \alpha_i \exp[-t/\tau_i]$$
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Both these effects lead to a nonlinear Perrin plot. However, under the condition that all emission lifetimes \( \tau_r \) are much smaller than any rotational correlation time \( \phi \), (limit of small rotations (27-29)), Equation 1 is well approximated by:

\[
\frac{\tau_r}{\tau(\phi)} = 1 + \frac{\tau_r}{\tau(\phi)}
\]

where the average lifetime is defined by:

\[
\langle \phi \rangle = \int_0^\infty t \phi(t) dt / \int_0^\infty t dt = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i
\]

and the average rotational correlation time is a weighted harmonic mean:

\[
\phi = \sum \frac{\alpha_i}{\phi_i} \left( \sum \alpha_i / \phi_i \right)^{-1}
\]

Thus, only in the range of \( (T/n) \) for which \( \tau_r \ll \phi \), will the Perrin plot be linear. Outside this range, the nonlinearity will depend not only on the complexity of the rotational process but also on that of the emission kinetics.

Most commonly, a lifetime measurement has been made at a single temperature and relative intensities used to estimate the lifetimes at different temperatures from the Perrin relationship (8, 30):

\[
\tau_i / \tau_2 = I_1 / I_2
\]

Equation 8 is strictly valid only if (a) the emission decay is monoexponential and (b) quenching of the emission is dynamic, i.e., the quenching process competes only for the excited state and there is no temperature-dependent formation of nonfluorescent ground-state complexes (static quenching).

The valid interpretation of steady state fluorescence depolarization data obtained with membrane systems in terms of "microviscosities" depends on two main assumptions: (a) the fluorophore rotates in the same way in the specialized bilayer membrane interior as it does in the isotropic reference fluid, and (b) the decay kinetics of the total emission are first order in the reference solvent and remain so in the membrane system, being therefore accurately represented by a single lifetime in Equation 1, except in the small rotation limit when it may be replaced by the average value defined in Equation 6.

Even in the absence of integral protein components and phase separations such as may occur in natural and model mixed lipid membranes, the resistance to rotational relaxation of the embedded probe might be expected (a) to differ considerably as a function of location across the bilayer (microheterogeneity) and (b) to differ in different directions (anisotropic fluid). Experimental evidence for partial orientation of embedded fluorescent probes with respect to the bilayer plane has been obtained in oriented multibilayer (12, 31) and black lipid membrane (32) systems. These observations are interpretable in terms of a model of free rotational motion within the angular confines of a "cage" of hydrocarbon chains (31). They strongly support the assignment of an anisotropic structure to the membrane interior.

Only a few reports of direct measurements of the decay of the emission anisotropy of membrane-embedded probes have appeared in the literature to date. Thus, the addition of tetracaine to Ehrlich ascites tumor cells labeled with perylene resulted in a 25% decrease in the apparent average rotational correlation time determined from the time dependence of the orthogonally polarized components of the emission (33). A direct examination of the emission anisotropy decays of a number of model membrane systems labeled with the fluorescent lipid derivative 12 (9-anthroyl)stearic acid revealed complex emission anisotropy decay kinetics in some cases (9). On the other hand, a much earlier study of the emission anisotropy decay of 1-anilinonaphthalene-8-sulfonate adsorbed to electroplax membranes (34) indicated the presence of some ultra-rapid (subnanosecond) depolarization process bringing the emission anisotropy down from its limiting value of around 0.36 to near 0.16, after which no further depolarization on the nanosecond time scale could be detected \( (\phi > 700 \, \text{ns}) \). The latter effect was attributed to binding of the probe to relatively immobile proteins present in the membrane, but the origin of the ultra-rapid phase of the depolarization was not clear.

One of the more popular fluorescent "microviscosity" probes has been diphenylhexatriene (8, 11-14, 16-19). This is an elongated fluorophore with a relatively long excited state lifetime of about 10 ns in hydrocarbon reference oils as measured both by phase (8) and pulse (35) fluorometry. It has been reported to exhibit an essentially linear Perrin plot in a reference oil with \( C(\tau) = (8.6 \pm 0.4) \times 10^{-9} \, \text{poise} \, \text{deg}^{-1} \, \text{s}^{-1} \) (16). This observation has been supported by direct determination of the emission anisotropy decay in a different reference oil which appeared to be monoeXponential over about 1½ orders of magnitude (95). On the other hand, a result similar to that indicated above for electroplax membranes was obtained for DPH embedded in an electric field-oriented liquid-crystal matrix, indicating diffuse orientation of the probe in this system (35).

DPH has a number of other spectral properties which are highly desirable in a fluorescence probe (8). The molar extinction coefficient is high, approaching \( 10^5 \, \text{cm}^2 \, \text{mmol}^{-1} \) (8) and is only slightly temperature-dependent in the range of normal use (36). The quantum yield approaches unity in reference hydrocarbon solvents and remains quite high in lipid membrane systems (8). Since the absorption and emission bands are very well separated with little spectral overlap (8, 36) there will be essentially no depolarization introduced either by resonance energy transfer (37) or trivial reabsorption (38, 39) for the low concentrations \( (10^{-4} \, \text{m} \, \text{M} - 10^{-3} \, \text{M} \, \text{phospholipid}) \) at which this probe can be effectively used. The ability to utilize widely separated excitation and emission wavelengths also ensures that contamination of the observed signals with scattered excitation (5, 27) is minimal. In addition, its introduction into the membrane from a stable nonfluorescent aqueous dispersion is readily and rapidly accomplished (8). In terms of the practical measurement, its only disadvantage would appear to be a degree of reversible photobleaching observed in lipid systems but not in the reference oil or other hydrocarbon solvent. This can, however, be minimized and extremely reproducible measurements of the emission anisotropy made, provided that short periods of illumination at low intensity are ensured (8).

In order to test some of the assumptions inherent in using this probe to determine the "microviscosity" of bilayer lipid membrane systems and to follow its rotational behavior in detail, the nanosecond emission anisotropy decay kinetics of the fluorophore in both a reference oil and single bilayer egg lecithin vesicles (40) were determined as a function of temperature in the range 3-31°C using the single photon counting delayed coincidence technique.

1 The abbreviation used is: DPH, all-trans-1,6-diphenyl-1,3,5-hexatriene.
Experimental Procedures

Materials—L-a-egg lecithin in chloroform solution was purchased from Sigma Chemical Co. and freed of fatty acid impurities by column chromatography on silica gel (SILICAR CC-4) supplied by Mallinckrodt, Inc. A closely homogeneous population of single bilayer vesicles in buffered solution was obtained by sonication and gel filtration following the procedure of Huang (40). DPH was supplied by Sigma Chemical Co., 98.5% Gold Label tetrahydrofuran and 9-cyanoanthracene by Aldrich Chemical Co., all being used without further purification. The vesicles were labeled by stepwise addition from a micropipette of a total of 5 μl of a 1 mM solution of DPH in tetrahydrofuran to 5 ml of a vortexing suspension of the vesicles in 0.01 mM Tris/HCl buffer containing 0.1 mM NaCl, pH 8.5. The lipid concentration, determined by lipophilic lipid phosphate by the method of Bartlett (41) was 10⁻⁵ M, giving a final probe/lipid ratio of 10⁻⁷. The reference solvent, infrared grade heavy paraffin oil (Mallinckrodt, Inc.) was used as supplied (35). Fluorescence measurements were made on a solution of DPH dissolved directly into this solvent at the same concentration as that employed overall in the vesicle suspension, 10⁻⁵ M.

Viscosity Determinations—Bulk viscosities of the reference paraffin oil were determined as a function of temperature with a Rheotest 2 rotary concentric cylinder viscometer calibrated with an aqueous gelatin solution of known viscosity (42).

Steady State Fluorescence Measurements—The measurements were made in essentially the same way as described in an earlier communication (43). Steady state emission anisotropies were measured as a function of temperature in a Perkin-Elmer MFP-4 spectrofluorimeter in which HNHPB Polaroid dichroic film polarizers sandwiched between quartz plates were mounted in the excitation and observation paths between the thermostated cuvette holder and lenses. A 1” quartz wedge quarter-wave scrambler plate (Dell Optics, North Bergen, N. J.) placed in the excitation path at the monochromator exit slit approximately equalized the vertically (V) and horizontally (H) polarized components of the excitation and minimized the G-factor correction (44) in the expression for the steady state emission anisotropy:

\[ r(t) = \frac{I_{VV}(t) - G}{I_{VV}(t) + 2G} \]  

where \( G = \frac{I_{HV}(t)}{I_{HH}(t)} \) and the first and second subscripts refer to excitation and emission components, respectively. Source intensity fluctuations were eliminated by recording in the ratio mode. Band widths of 8 and 12 nm were used at excitation and emission wavelengths of 550 and 430 nm, respectively, in order to simulate the conditions used in the time-resolved studies. Short illumination periods minimized the degree of photobleaching (8) which had a negligible effect on \( r \).

Nanosecond Time-resolved Fluorescence Measurements—The orthogonally polarized decay curves \( I_{V}(t) \) and \( I_{H}(t) \) were collected simultaneously along with the excitation profile in a single photon counting delayed coincidence fluorescence spectrometer (46) as described previously (48). Five-minute alternation periods ensured compensation for shape and timing drifts during the 4- to 6-h collection. Excitation was accomplished with the output of a thyratron-gated air flashlamp and the band at 355 nm selected with a Baird-Atomic interference filter. Randomization of excitation polarizations was again accomplished with a scrambler plate and the appropriate polarizers in both excitation and emission paths selected by double Polacoat UVWWRM dichroic polarizers. Emission and scatter widths of 8 and 12 nm were used at excitation and emission wavelengths (46, 47), as well as fidelity of zero emission anisotropy at all observable times for effectively completely depolarized fluorescence (45), were determined with the single exponential decay standard 9-cyanoanthracene in ethanol using the same excitation and emission conditions.

Analysis of Polarized Decay Data—Impulse response functions for total emission \( s(t) \) and emission anisotropy \( r(t) \) were recovered from experimental sum and difference decays \( S(t) \) and \( D(t) \), respectively, which were constructed from the data as:

\[ S(t) = I_{V}(t)G + 2I_{H}(t) \]  
\[ D(t) = I_{V}(t)G - I_{H}(t) \]  

An experimental emission anisotropy decay curve may be also constructed from the data according to Equations 11 and 12 together with the identity:

\[ R(t) = D(t)/S(t) \]  

In each case, one of the exponential terms describing \( r(t) \) or \( r(t) \) could be replaced by a constant (equivalent to a decay term with an infinite correlation time). Modifications of the nonlinear least squares search in which the sum parameters were held constant and only the parameters of the emission anisotropy decay adjusted (49) were used to accomplish these analyses. As with the sum analysis, the impulse response functions were convolved onto the emission profile and compared with the data for both these models. The correct statistical (photon-counting) weights in the least squares analysis of the composite sum and difference decay curves are not linear functions of the counts collected in the two polarized decays \( I_{V}(t) \) and \( I_{H}(t) \). However, using synthetic test data to which photon-counting noise was added, the test parameters were recovered with good accuracy for the sum curve using the normal weighting factors, i.e., the reciprocal of the apparent number of counts in \( S(t) \). For the difference curves, however, it was found that application of equal weighting to all points led to good recovery of the test emission anisotropy parameters from \( D(t) \), while the normal weighting procedure consistently returned incorrect parameters and poor fits as judged by the residuals and their autocorrelation functions (45, 48).

Results

Steady state emission anisotropies \( r(t) \) were measured at five temperatures in the range 3–31° both for DPH in the reference paraffin oil and in the vesicle suspension. A background fluorescence of the oil contributing less than 1.5% to any of the polarized components of emission was subtracted. The error that would have been introduced by neglecting this correction was smaller than the standard error of the measurements. The background due to vesicles was always less than 0.2% and could safely be entirely neglected. These results are given in Fig. 1. The error bars on the vesicle data are the standard errors for 6 to 10 determinations on the same vesicle suspension used in the time-resolved studies.

The nanosecond time dependence of the emission anisotropy \( r(t) \) was determined at the same five temperatures for both the reference oil and vesicle suspension. Subtractions of the appropriate small blanks for each of the orthogonally polarized decay curves in the case of the reference oil did not have any significant effect on the final decay parameters obtained.
The decay of the total emission of DPH in paraffin oil did not vary significantly over the temperature range investigated. The impulse response function \(\alpha(t)\) was close to, but not quite, monoexponential as judged by fitting criteria and comparison with the monoexponentially decaying standard, 9-cyanoanthracene in ethanol, measured under the same conditions. The major decay component, which comprised \((98.5 \pm 0.5)\%\) of the emission, had a lifetime of \(9.66 \pm 0.02\) ns, the minor one \(3.3 \pm 0.7\) ns. The average lifetime, defined in Equation 6 was \(9.55 \pm 0.03\) ns compared with \(9.54 \pm 0.03\) ns given by the best single exponential fits to the data. A comparison of the fits for single and double exponential decay laws convolved with the excitation profile \(E(t)\) is presented in Fig. 2 for one of the 16° data sets along with the fractional residuals and autocorrelation function of the weighted residuals (45). These, together with the reduced \(\chi^2\) values for the fits and fidelity of the monoexponential hypothesis for decay of the standard, suggest that a single exponential decay model is not adequate for DPH in the reference oil. It seems possible that the apparent biexponentiality of the decay process may be connected with a reversible excited state reaction e.g. formation of a nonfluorescent isomer of this spectroscopically unusual fluorophore (36, 50).

The decay course of the total emission in the vesicle system was far from consistent with first order kinetics at any of the temperatures examined. The decays were decidedly biexponential at all temperatures. Both lifetimes decreased somewhat with increasing temperature as seen in Table I, in qualitative agreement with relative yield measurements in the literature (8). The ratio of the pre-exponential factors remained remarkably constant, and \((23.4 \pm 0.6)\%\) of that part of the excitation giving rise to fluorescence was associated with the shorter decay time. Comparative fits for the single and double exponential decay model to one of the 16° total emission decay curves are displayed in Fig. 3. Whether the biexponentiality of these decay kinetics arises from two excited state populations differing in their quenching interactions (microheterogeneity), from reversible formation of a dark, i.e. nonfluorescent, photoproduct in the excited state or has a more complex origin is an open question.

Taking the viscosity of a refractometrically determined \((90.5 \pm 0.5)\%\) glycerol/water (w/w) solution (42) as 170 cp at 25° with an estimated uncertainty of \(\pm 10\) cp (42) for calibration, the viscosity of the reference oil was found to be \(145 \pm 10\) cp at 25° with a fusion activation energy of \(12.3 \pm 0.2\) kcal/mol determined from the linear Arrhenius plot obtained over the range 1.5 to 42.5°. These values are very similar to those in the literature for American White Oil U.S.P. 35, i.e. 185 cp and 12.7 kcal/mol, respectively (27). From the steady state emission anisotropies for DPH in egg lecithin vesicles as well as the above determined viscosity/temperature profile, the value of \(C(\tau)\) was calculated from Equation 1 at the five temperatures examined between 3 and 31°, setting the zero point emission anisotropy \(r_0\) at 0.362 (see below) and using the average emission decay time of 9.55 ns. As seen in the Perrin plot of Fig. 4, \(C(\tau)\) did not approximate a constant in this temperature range. Its value varied from \((14.6 \pm 1) \times 10^5\) poise degree\(^{-1}\) s\(^{-1}\) at 3° to \((10.4 \pm 1) \times 10^5\) poise degree\(^{-1}\) s\(^{-1}\) at 31°. These results appear to be at variance with a previous report that the Perrin plot for DPH in purified American White Oil U.S.P. 35 was essentially linear with a value for \(C(\tau)\) of \((8.6 \pm 0.4) \times 10^5\) poise degree\(^{-1}\) s\(^{-1}\) (16). Using the values of \(C(\tau)\) obtained here along with the steady state emission anisotropies for DPH in egg lecithin vesicles displayed in Fig. 1 and the average emission decay times given

![Fig. 1. Steady state emission anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene in paraffin oil and sonicated egg lecithin vesicles as a function of temperature. Both solutions were 10\(^{-3}\) M in fluorescent hexatriene in paraffin oil and sonicated egg lecithin vesicles as a reference oil. The major decay component, which comprised (98.5 \pm 0.5)\% of the emission, had a lifetime of 9.66 \pm 0.02 ns, the minor one 3.3 \pm 0.7 ns. The average lifetime, defined in Equation 6 was 9.55 \pm 0.03 ns compared with 9.54 \pm 0.03 ns given by the best single exponential fits to the data. A comparison of the fits for single and double exponential decay laws convolved with the excitation profile E(t) is presented in Fig. 2 for one of the 16° data sets along with the fractional residuals and autocorrelation function of the weighted residuals (45). These, together with the reduced \(\chi^2\) values for the fits and fidelity of the monoexponential hypothesis for decay of the standard, suggest that a single exponential decay model is not adequate for DPH in the reference oil. It seems possible that the apparent biexponentiality of the decay process may be connected with a reversible excited state reaction e.g. formation of a nonfluorescent isomer of this spectroscopically unusual fluorophore (36, 50).

![Fig. 2. Experimental and convolved fluorescence decay curves for 10\(^{-3}\) M DPH in paraffin oil at 16°. The excitation wavelength was 340 nm, observation wavelength 430 nm, and the time resolution 0.204 ns/channel. (a), total emission \(S(t) = \int E(t)G \ dt\), compared with the best single exponential fit defined by \(\chi^2 = 3.82\) ns, \(\tau_1 = 3.42\) ns, \(\tau_2 = 9.55\) ns convolved onto the excitation pulse profile \(E(t)\). (b), as (a) but compared with the optimal double exponential fit defined by \(\chi^2 = 2.57\) ns, \(\tau_1 = 3.42\) ns, \(\tau_2 = 9.55\) ns, \(\tau_3 = 9.88\) ns. The fractional residuals are displayed along with the decay curves in each case and the autocorrelation functions of the residuals (45) are pictured in the insets.](https://example.com/fig2.png)
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In Table I, the Arrhenius plot for derived "microviscosities" was linear in the range investigated with a fusion activation energy of 9.35 ± 0.4 kcal/mol in good agreement with previous data (8). On the other hand, the absolute "microviscosity" at 25° of 97 ± 13 cp obtained here is almost twice the value reported previously for this system (8), although it does agree closely with values given for perylene in egg lecithin dispersions (5, 8).

None of the emission anisotropy decays observed in this study were satisfactorily described by first order kinetics. However, on analysis according to Equation 15, a double exponential decay law was entirely adequate to describe the reference oil data at any given temperature studied. The goodness of fit for one of the 16° data sets is displayed in Fig. 5a with respect to the difference curve D(t) and in Fig. 5b with respect to the emission anisotropy decay R(t). In the latter, the inadequacy of a monoexponential hypothesis is also indicated and a semilogarithmic representation of the total emission curve S(t) given for reference. A summary of

![Figure 4. Perrin plot for 1,6-diphenyl-1,3,5-hexatriene in paraffin oil. Steady state emission anisotropies (r) were measured as described in the text and are displayed directly in Fig. 1. The zero-point emission anisotropy r0 was taken as 0.062 (see (6, 16) and text). The vertical error bars represent standard errors in (r), the horizontal bars refer to uncertainty in the absolute viscosities (see text). The mean lifetime (7) was constant in the temperature range investigated and was not included in the scale of the abscissa.](http://www.jbc.org/)

![Figure 3. Experimental and reconvolved fluorescence decay curves for 10−5 M DPH in egg lecithin vesicles at a phospholipid:probe mole ratio of 10:1. Other experimental details as in Fig. 2. (a), best single exponential parameters obtained were x = 0.770 ns−1, r = 7.77 ns. (b), best double exponential fit obtained with x1 = 0.188 ns−1, r1 = 3.31 ns, x2 = 0.645 ns−1, r2 = 8.33 ns.](http://www.jbc.org/)

| T   | Fractional amplitudes | τ   | (r) |
|-----|-----------------------|-----|-----|
| 3°  | 0.235 3.35 8.27       |     |     |
|     | 0.765 8.85 8.29       |     |     |
| 9.5 | 0.234 3.41 8.06       |     |     |
|     | 0.766 8.62 8.03       |     |     |
| 16  | 0.220 3.31 7.81       |     |     |
|     | 0.774 3.33 7.81       |     |     |
|     | 0.755 3.32 7.81       |     |     |
|     | 0.765 8.35 7.81       |     |     |
| 23.5| 0.224 2.84 7.43       |     |     |
|     | 0.776 7.90 7.45       |     |     |
|     | 0.770 7.94 7.45       |     |     |
| 31  | 0.235 2.73 6.98       |     |     |
|     | 0.765 7.46 6.99       |     |     |
|     | 0.769 7.46 6.99       |     |     |
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Table II

Decay parameters for the emission anisotropy \( r(t) \) of 1,6-diphenyl-1,3,5-hexatriene in paraffin oil

The results of double exponential decay analyses of duplicate experiments at each temperature are given. The double exponential decays are defined by:

\[
E(t) = \sum \beta_i \exp\left[-t/\phi_i\right]
\]

and displayed in Fig. 7a. The zero point emission anisotropy \( r_0 \) is equal to the sum of the partial emission anisotropies:

\[
r_0 = \sum \beta_i
\]

and the \( \phi \) values are rotational correlation times.

| \( T \) | \( r_0 \) | \( \beta \) | \( \phi \) | \( \phi \) |
|-------|-------|-------|-------|-------|
| 9°    | 0.366 | 0.046 | 2.8   | 24.4
| 0.370 | 0.320 |       |       |       |
| 9.5   | 0.389 | 0.079 | 1.7   | 15.0
| 0.366 | 0.319 | 5.4   | 16.3
| 16    | 0.364 | 0.088 | 2.3   | 10.3
| 0.359 | 0.276 | 4.6   | 13.0
| 23.5  | 0.357 | 0.060 | 1.1   | 5.9
| 0.345 | 0.207 | 3.6   | 8.6
| 31    | 0.352 | 0.104 | 1.5   | 0.9
| 0.361 | 0.248 | 1.1   | 3.9

the parameters defining the biexponential impulse responses for duplicate experiments at each temperature is given in Table II. The zero point emission anisotropy showed no trend with temperature and had a value \( r_0 = 0.362 \pm 0.013 \) which compares excellently with the literature steady state value of 0.362 measured in a "rigid" medium, propylene glycol at -50° (8, 16).

A simple model for DPH in which the emission and/or absorption oscillator lies along the long axis of a prolate ellipsoid (35) is ruled out by these data since, in agreement with the nonlinearity of the Perrin plot determined in this study (Fig. 4), the decay of the emission anisotropy is clearly not monoexponential. An essentially biexponential decay is predicted when these oscillators lie at some small angle to the long axis of such an ellipsoid (25). However, the change in the parameters of the apparently biexponential emission anisotropy decays as a function of temperature are inconsistent with such a model. Although somewhat elongated, DPH is distinctly planar and also devoid of mirror symmetry in this plane in or close to which the emission/absorption oscillators lie. Even assuming that the Brownian rotational model still holds for such a fluorophore in a solvent whose molecules are of similar dimensions, the emission anisotropy decay can be expected to have three exponential components (26, 51, 52). At least two of the relaxation time constants will be quite similar and therefore difficult if not impossible to resolve. In addition, the parameters of such a decay are complex functions of the geometry and size of the rotating unit as well as of the temperature and viscosity of the isotropic solvent. Indications of such complexity can be detected in the data as a consistent decrease in the ratio of the longer to shorter of the two correlation times: as the temperature increases from 3 to 31° this ratio decreases from about 7 to about 3 (Table II).

On analysis according to Equation 15, neither mono- nor biexponential decay models gave an adequate fit to the majority of the observed decays of the emission anisotropy of DPH in the vesicle system. However, addition of a constant term to the biexponential model resulted in a satisfactory representation of the data at all temperatures. The value of this constant term, whose significance will be discussed below, decreased from about 0.075 at 3° to about 0.017 at 31°. At the latter temperature it was actually no longer possible to differentiate between a biexponential decay law with and one without an added constant. Both models were equally acceptable for that data and only the evidence from lower temperatures provides a rationale for accepting the former model. One of the 16°
difference data curves $D(t)$ is displayed in Fig. 6a together with the impulse response $d(t)$ obtained from the responses recovered for $r(t)$ and $s(t)$ as described in Equation 15, convolved with the excitation profile $E(t)$. The equivalent comparison with $R(t)$ is made in Fig. 6b which also shows the best but unacceptable mono- and biexponential emission anisotropy fits obtained in the same way, along with a semilogarithmic representation of $S(t)$ for reference. The emission anisotropy decay parameters recovered from the duplicate data sets collected at each temperature are presented in Table III.

The mean value of the zero point emission anisotropy determined in the vesicle system, $r_0 = 0.337 \pm 0.017$ is somewhat lower than that found in the reference oil, but again no definite trend with temperature can be discerned. The difference in $r_0$ value between reference and vesicle systems may reflect the summation of small depolarizing processes in the latter, i.e., randomization of the polarization planes of excitation and emission due to scattering and birefringence in the vesicle suspension as also optical activity of asymmetric bilayer components (53). These effects are not linear in $r$ (53), so the whole course of the emission anisotropy decay will be changed. However, since the maximum error (that in $r_0$) is small, the errors in relative partial anisotropies $\beta$ and correlation times $\phi$ due to this will be second order at most.

There is a slight trend towards shorter rotational correlation times for DPH in the vesicle system as the temperature increases and, as in the reference oil, the ratio of longer to shorter correlation times decreases somewhat. However, the similarity does not carry over to the pre-exponential factors whose ratio shows at most a small trend in the oil but changes by a factor of about 10 in the vesicle system. The relative values of the partial emission anisotropies associated with the longer and shorter rotational correlation times change from about 3 or 4 at the lowest temperature to about 0.5 to 0.2 at the highest.

Attempts were also made to analyze the vesicle data in terms of the associative model described by Equation 14. Adequate fits to the difference curves $D(t)$ could be obtained in several of the associative models tested. However, in no case were the emission anisotropy decay parameters recovered physically realistic. As an example the data displayed in Fig. 6 could be adequately fitted to an emission anisotropy decay described by $r_1(t) = 0.294 \exp[-t/4.08]$ and $r_2(t) = 0.187 \exp[-(t/5.67) + 0.032]$ associated, respectively, with the shorter and longer components of the total emission decay. It was also fitted equally well by $r_1(t) = 0.110 \exp[t/5.47] + 0.677$ and $r_2(t) = 0.233 \exp[-t/1.40]$ and a total of six other associative models containing three components. In all cases, the pre-exponential factors of one of the emission anisotropy decays were impossibly large (e.g., in the above $r_{1m} = 0.800$ or 0.787) and/or negative correlation times were obtained (e.g., in the second example above, $\phi_1 = -5.47$ ns).

The effects of rotation of the spherical vesicle as a whole and of lateral diffusion of the excited probe around the curved vesicle interior are both included in the correlation times determined here which represent harmonic addition of the appropriate correlation times for these motions to the correlation times for rotation of the probe in absence of these additional depolarizing processes (54). The Brownian rotational correlation time for the spherical vesicles under consideration:

$$\phi_{\text{rot}} = \frac{\eta V}{kT}$$

where $V$ is the volume of the vesicle, $\eta$ the viscosity of the suspending medium, and $k$ the Boltzmann constant, is on the order of $1$ to $2 \mu$s. The equivalent correlation time correspond-
be seen, in Fig. 7, a and b, to be equivalent within the error DPH and similar fluorophores in oriented bilayer systems

average per vesicle (40) under the conditions utilized in the experiments described here, compared with a critical transfer distance (57) of about 18 Å (58).

significant effect, since the mean fluorophore separation is about 90 Å or more for the two or three probes found on here. Neither will resonance energy transfer have had any effect leading to correlation times of 100 ns or more will not have appreciably influenced the results obtained that might be expected for any given emission anisotropy value, i.e. within about 0.005 to 0.01 of each other at all except the very earliest times. It is worthwhile pointing out that no attempt should be made to compare the (convolved) experimental emission anisotropy decay curves R(t) for the reference oil and vesicle systems directly, Figs. 5b and 6b respectively, both because the excitation pulse widths E(t) differ considerably and, no less importantly, because the total emission impulse responses s(t) convolved into these data are far from similar. This does not apply to the impulse responses r(t) obtained within the framework of the models so far considered and the apparent quantitative differences between these decay laws in the reference oil and vesicle systems are qualitatively well demonstrated in Fig. 7, where it is readily seen that an initially faster rate of depolarization in the vesicle system at any given temperature is compensated to some extent by the negligible depolarization rate at later times. The latter represents the appearance of a constant term in the decay law, i.e. a term with an apparently infinite decay time as indicated in Table III.

TABLE III

Emission anisotropy decay parameters of 1,6-diphenyl-1,3,5-

hexatriene in sonicated single-bilayer egg lecithin vesicles

The data were analyzed in terms of three component decays defined by:

\[ r(t) = \sum \beta \exp(-t/\phi_i) \]

in which one of the rotational correlation times \( \phi \) is held equal to infinity (corresponding to setting the term in which it appears to a constant) and \( r_0 \), the zero point emission anisotropy is equal to \( \sum \beta \). The decay laws corresponding to these parameters are visualized in Fig. 7b.

| \( T \) | \( r_0 \) | \( \beta \) | \( \phi \) |
|-------|-------|-------|-------|
| 3°    | 0.344 | 0.068 | 1.4   |
|       |       | 0.203 | 7.7   |
|       |       | 0.073 | \( \infty \) |
| 9.5   | 0.349 | 0.065 | 1.1   |
|       |       | 0.211 | 7.2   |
|       |       | 0.075 | \( \infty \) |
| 16    | 0.350 | 0.162 | 2.1   |
|       |       | 0.144 | 8.1   |
|       |       | 0.044 | \( \infty \) |
| 23.5  | 0.347 | 0.091 | 0.7   |
|       |       | 0.216 | 4.7   |
|       |       | 0.040 | \( \infty \) |
| 31    | 0.327 | 0.141 | 1.4   |
|       |       | 0.157 | 4.6   |
|       |       | 0.020 | \( \infty \) |
|       | 0.307 | 0.184 | 1.0   |
|       |       | 0.192 | 5.6   |
|       |       | 0.021 | \( \infty \) |
|       | 0.326 | 0.206 | 1.1   |
|       |       | 0.105 | 4.8   |
|       |       | 0.016 | \( \infty \) |
|       |       | 0.260 | 1.4   |
|       |       | 0.018 | \( \infty \) |

where \( R \) is the radius of the spherical vesicle and \( D \) the lateral diffusion coefficient (55). \( D \) may be as high as about 2 \( \times 10^{-4} \) cm²/sec (56) which sets a lower limit of about 100 ns on \( \phi_{\text{trans}} \). Any motion leading to correlation times of 100 ns or more will not have appreciably influenced the results obtained here. Neither will resonance energy transfer have had any significant effect, since the mean fluorophore separation is about 90 Å or more for the two or three probes found on average per vesicle (40) under the conditions utilized in the experiments described here, compared with a critical transfer distance (57) of about 18 Å (58).

Although the final duplicate parameters recovered for \( r(t) \) seem rather variable in both reference oil (Table II) and vesicles (Table III), the corresponding impulse responses can be seen, in Fig. 7, a and b, to be equivalent within the error of to lateral diffusion of the probe is given by:

\[ \phi_{\text{trans}} = R^2/6D \]  

(17)
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(12, 31, 32) and interpreted for planar egg lecithin multilayers in terms of such a cage restricting the possible range of rotational reorientation (31). These studies on oriented systems show that, if the constant term in the emission anisotropy decay law is in fact due to immobilization of some of the fluorophores, this population must be at least partially oriented with respect to the bilayer. In the light of possible stacking of part of the linear aliphatic side chains of the bilayer phospholipids near their head groups, such an effect would not be unexpected. The much more dramatic orientational anisotropy observed for DPH in dipalmitoyllecithin bilayers below the gel-liquid crystalline transition temperature (12) and the extremely high value of the constant term, about 0.25, in the emission anisotropy decay of DPH in dimyristoyllecithin vesicles below its transition temperature reported recently from this laboratory (43), strongly support the present observations although, as in those cases, a definitive interpretation of the results in terms of immobilization or restricted range of motion is not possible. In view of this uncertainty, it would be premature to attempt an analysis of the rotational motions of the probe in the bilayer system according to the detailed model proposed recently for fluorescence depolarization in planar arrays of oriented fluorophores (59). However, it may be pertinent to note that the complex rotational relaxation of spin labeled probes in membranes reflected in the details of their ESR spectra have also been widely interpreted in terms of models including restricted anisotropic motions (60-64). Considering by way of example the wobbly or restricted tumbling or random walk model (61, 64), the curve half-saturation for the volume corresponding to such restricted motion of DPH in egg lecithin vesicles on the nanosecond time scale changes from ~55 to ~70° in the temperature range 3-31°C (65). This compares with the equivalent angle of about 30° reported previously for DPH in dimyristoyllecithin vesicles below the transition temperature (43) and about 15 and 20° for the subnanosecond motions of DPH in the reference oil and egg lecithin vesicles respectively calculated utilizing the same model.

**DISCUSSION**

The concept of a fluid bilayer lipid membrane has been supported by studies conducted on a variety of model membrane systems as well as with true biological membranes (66). A number of different physical techniques have been employed in this connection and, among others, steady state fluorescence depolarization measurements have provided useful information (67, 68).

In general, fluorescence depolarization data obtained with embedded aromatic fluorophores such as diphenylhexatriene have been interpreted in terms of a "microviscosity" for the hydrocarbon interior of the bilayer by comparison with depolarizations observed for the probe in a reference hydrocarbon oil of known bulk (macroscopic) viscosity. The nanosecond time-dependent depolarization data reported here for DPH in paraffin oil and in egg lecithin vesicles suggest a more complicated picture than that implied by this interpretation. It is quite certain that the fluorescence depolarizations observed in both reference and bilayer systems reflect the rates of rotation of the fluorophore. It is also true that these rates depend on the viscous opposition of the solvent to rotation of the probe. However, it is necessary to define in this connection precisely what is meant by a term such as "microviscosity." The bulk viscosities of homogeneous reference solvents can, of course, be determined and the steady state fluorescence depolarization of a dissolved probe may then be correlated with this viscosity scale. In terms of such a calibration, the "microviscosity" inferred from the depolarization observed for the probe embedded in the interior of the lipid bilayer, actually represents the apparent macroviscosity of a microscopic volume element which is unobtainable by direct measurement. A more physically realistic definition of microviscosity would have to take into account the local viscous opposition of the solvent to rotations of the probe in its microenvironment. This viscous opposition may differ, not only for different rotational modes of the probe (anisotropic probe) but also vary as a function of the direction of a given rotational mode in the coordinates of the medium (anisotropic solvent). It may or may not be simply related to the bulk viscosity.

The time-dependent fluorescence depolarization data presented in this communication show that the rotational motion of DPH dissolved in a reference paraffin oil or embedded in the interior of egg lecithin single bilayer vesicles is complex. Moreover, as indicated by comparing the emission anisotropy decays depicted in Fig. 7 for these systems, the rotational characteristics of DPH are not only quantitatively but also qualitatively different in the two media. The most striking difference observed is that, whereas in the homogeneous reference oil the emission anisotropy tends to zero indicating that complete randomization of orientations is attained at long times, it decays not to zero but to a finite constant level (on the nanosecond time scale) in the vesicle system.

Two extreme models may be advanced to interpret the complex rotational behavior observed for DPH in the bilayer environment. In one, a rotationally homogeneous population of probes in an anisotropic fluid membrane interior is assumed. The probes tumble on the nanosecond time scale over a range restricted by the confines of a cage formed by partial radial alignment of the fatty acid chains in the spherical bilayer vesicle. In the other extreme, a microheterogeneous population of probe sites in which DPH rotates at different rates is assumed. In a fraction of the sites, the probe is completely immobilized. It would hardly be surprising if the observed complexity reflected the details of both models in part. The data reported here also indicate that the decay of the total fluorescence intensity of DPH in the vesicle system cannot adequately be described as a single first order process. On the other hand, they are consistent with a double exponential decay of the excited state population, which may indicate that the probe occupies two different sites in the membrane interior. However, it was not found possible to associate these postulated sites with physically realistic emission anisotropy decay laws, so that a simple two-state model for heterogeneity of both emission and rotation appears to be ruled out. In connection with this it should be emphasized that, although it is tempting to attribute a double exponential excited state decay law to two sites for the probe, a homogeneous probe population undergoing a reversible excited state reaction may also exhibit biexponential, or even more complex, decay behavior.

In view of the results presented here, no matter what the precise interpretation of them may be, inferences drawn as to the "microviscosity" (i.e. apparent bulk viscosity of a microscopic volume element) of membrane bilayer interiors by comparison of steady state depolarizations of DPH in these and reference media should be viewed with caution. Furthermore, the problem of defining true local viscosities opposing the rotation of this probe in the bilayer interior, and for that
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matter even in the isotropic reference oil, remains. However, it is stressed in conclusion that time-resolved measurements of the rotational relaxation of this fluorophore in the lipid bilayer system support the concept of a fluid bilayer interior. At the same time, they also reflect the structurally anisotropic and/or inhomogeneous nature of the bilayer environment. Extension of time-resolved measurements to other kinds of hydrophobic probes, particularly ones which, like the recently characterized natural fluorescent polyene membrane component parinaric acid (69, 70), might be expected to exert a minimal perturbing effect on its bilayer environment, may eventually allow a detailed understanding of these complex motions. The potential of the time-resolved method should be enhanced still further by application to oriented bilayer systems (71).

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